Examination of the effect of weaning stress on the physiological and molecular regulation of immune function in circulating bovine leukocytes

Aran O'Loughlin B.Sc.

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy

October 2011

This research was conducted at the Animal and Bioscience Research Department and the Livestock Systems Research Department, Teagasc, Animal and Grassland Research and Innovation Centre, Grange, Dunsany, Co. Meath, Ireland and the National University of Ireland Maynooth, Co. Kildare, Ireland.

Head of Department:

Professor Kay Ohlendieck Dip.Biol., M.Sc., Ph.D.
Muscle Biology, Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland.

Supervisors:

Professor Seán Doyle B.Sc., Ph.D.
Department of Biology and National Institute for Cellular Biotechnology, National University of Ireland Maynooth, Co. Kildare, Ireland.

Bernadette Earley B.Sc., H. Dip. In Ed., Ph.D.
Animal and Bioscience Research Department, Teagasc, Animal and Grassland Research and Innovation Centre, Grange, Dunsany, Co. Meath, Ireland

Mark McGee B.Sc., Ph.D.
Livestock Systems Research Department, Teagasc, Animal and Grassland Research and Innovation Centre, Grange, Dunsany, Co. Meath, Ireland

Table of Contents

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.

_____

Aran O'Loughlin

Acknowledgements

Upon writing this, I am happy to say that after three and a half years, this thesis if officially finished. Throughout the course of the work embodied in this thesis, many people have contributed in one shape or form. I wish to thank all those who have contributed to this thesis, although I will keep these acknowledgements brief.

I would like to thank Teagasc and the Walsh Fellowship scheme for providing me with the support, both monetarily and in terms of facilities and opportunities, to carry out this work. Thank you to my supervisors, Bernadette Earley, Sean Doyle and Mark McGee. Each of you contributed in your own way to the work embodied in this thesis. Bernie, your critical analysis and quick review ensured my work never stagnated. Sean, your guideance and encouragement helped me to complete this thesis. Mark, you always approached each chapter from a different perspective than my own and helped me to develop a more global view of my research. It is also necessary to acknowledge the skilled technical support that I received from some of the laboratory technicians at Grange. Margaret Murray, you always bent over backwards to help me and I sincerely appreciate it. Joe Larkin, thank you for all the help and the humour along the way. Thank you also to Matthew McCabe for teaching me all the basics of molecular biology. I also want to thank the farm staff, in particular Eddie Mulligan, for their excellent animal husbandry skills throughout the three animal trials we ran over the course of this thesis.

I wish to thank the researchers at Grange, Eddie O'Riordan, Padraig O'Kiely, Aidan Moloney, Paul Crosson, Richard Dewhurst, Kieran Meade, Orla Keane, Sinead Waters, Chris Creevey and David Lynn for their constructive criticisms and advice. In

particular, thank you to David Lynn for the huge amount of critical input into the bioinformatic analysis of chapter 5.

To all my fellow students, both past and present, thank you for your friendship and support. I won't name each of you but I received a huge amount of help from many of you throughout this work.

To the lads, thanks for keeping me grounded. Your continual refusal to address me as "Dr" and instead use the term "nurse" may demonstrate a complete lack of knowledge of what it is that I have spent the last few years doing, but you have all provided me with some much needed breaks away from the research.

To my parents, Dermot and Mary, and my sister, Ruth, thank you for always supporting me. Your heavy investment in my education over the past 26 years has been the greatest gift of all. Thank you for instilling me with the confidence and encouragement necessary to succeed. I don't think you understand how much I appreciate all the time you have spent trying to make me happy but I want you to know that you have been successful.

And last, but certainly not least, Sarah, you are by far the best thing to have come out of doing a PhD. I would have done one sooner had I known I'd meet you! The completion of this thesis was in no small part down to your help, love and support and we can now look forward putting our theses (it's the plural of thesis, I checked!) on the book shelf and getting on with the rest of our life together.

Submitted Publications and Conference Proceedings

Submitted publications

O'LOUGHLIN, A., McGEE, M., WATERS, S., DOYLE, S. & EARLEY, B. (2011) Examination of the bovine leukocyte environment using immunogenetic biomarkers to assess immunocompetence following exposure to weaning stress. *BMC Veterinary Research,* 7**,** 45.

O'LOUGHLIN, A., LYNN, D.J., McGEE, M., DOYLE, S., McCABE, M. & EARLEY, B. (2011) Transcriptomic analysis of the stress response to weaning at housing in bovine leukocytes using RNA-seq technology. *BMC Genomics*, currently in review.

O'LOUGHLIN, A., McGEE, M., DOYLE, S. & EARLEY, B. (2011) Assessment of haematological distribution, the acute phase protein response, the stress hormone, cortisol and the neutrophil chemokine, CXCL8, as biomarkers of weaning stress at housing in the bovine. In preparation for submission to *Journal of Animal Science*.

Conference proceedings

O'LOUGHLIN, A., McGEE, M., DOYLE, S. & EARLEY, B. (2009). Impact of weaning strategy on haematological variables of beef calves. *Proceedings of the Association for Veterinary Teaching and Research Work (AVTRW).*

O'LOUGHLIN, A., McGEE, M., DOYLE, S. & EARLEY, B. (2009). Impact of weaning strategy on haematological variables of beef cows. *Proceedings of the Association for Veterinary Teaching and Research Work (AVTRW).*

O'LOUGHLIN, A., McGEE, M., DOYLE, S. & EARLEY, B. (2011). Examination of the responsiveness of the leukocyte environment in beef calves to weaning stress using immunogenetic biomarkers. *Proceedings of the Agricultural Research Forum*

O'LOUGHLIN, A., McGEE, M., DOYLE, S. & EARLEY, B. (2011). Examination of the bovine leukocyte environment using immunogenetic biomarkers to assess immunocompetence following exposure to weaning stress. *62nd Annual Meeting of the European Federation of Animal Science (EAAP).*

O'LOUGHLIN, A., LYNN, D.J., McGEE, M., DOYLE, S., McCABE, M. & EARLEY, B. (2011). RNA-seq analysis of bovine leukocytes following weaning stress in beef calves. *Proceedings of the 4th International Symposium on Animal Functional Genomics (ISAFG).*

List of Abbreviations

ACD	Acid citrate dextrose
ACTH	Adrenocorticotropin hormone
ADP	Adenosine diphosphate
AGP	α_1 acid glycoprotein
AMP	Adenosine monophosphate
ANS	Autonomic nervous system
AP-1	Activation protein 1
APC	Antigen presenting cell
APP	Acute phase protein
APR	Acute phase response
ATP	Adenosine triphosphate
AVP	Arginine-vasopressin
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BTA	Benzene-1,3,5-triacetic acid
BVD	Bovine viral diarrhoea
˚C	Degrees Celsius
cAMP	Cyclic adenosine monophosphate
CBP	cAMP response element binding protein
CD	Cluster of differentiation
CD62L	L-selectin
cDNA	Complementary DNA
Cq	Quantification cycle
CRF	Corticotrophin-releasing factor
CRH	Corticotrophin-releasing hormone

CNS	Central nervous system
d	Day
DAMP	Damage-associated molecular pattern
ddNTP	Dideoxynucleotide triphosphate
DEG	Differentially expressed gene
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DM	Dry matter
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EU	European Union
fg	Femtogram
g (× g)	Gram (gravity)
GA	Genome analyzer
GAP	GTPase-accelerating protein
GAS	General Adaptation Syndrome
GC	Glucocorticoid
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GO	Gene ontology
GPCR	G-protein-coupled receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP	Guanosine-5’-triphosphate

h	Hour
HCT	Haematocrit
HPA	Hypothalamic-pituitary-adrenal axis
HSP	Heat shock protein
IFN	Interferon
IBR	Infectious bovine rhinotracheitis
Ig	Immunoglobulin
IL	Interleukin
Kg	Kilogram
K_3EDTA	Tripotassium Ethylenediamine tetra-acetate
L	Litre
LPS	Lipopolysaccharide
Lsmeans	Least squares means
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MFI	Mean fluorescence intensity
mg	Milligram
mGR	Membrane-bound glucocorticoid receptor
MHC	Major histocompatibility complex
min	Minute
mL	Millilitre
MMP	Matrix metalloproteinase
mo	Month
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NaOH	Sodium hydroxide

NFκB	Nuclear factor kappa B
ng	Nanogram
nGRE	Negative glucocorticoid response element
NGS	Next-generation sequencing
NK	Natural killer cell
N:L ratio	Neutrophil:lymphocyte ratio
nm	Nanometer
NSB	None-specific binding
NTC	No template control
OD	Optical density
PRR	Pattern recognition receptor
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
pg	Picogram
pNpp	P-nitrophenyl phosphate
POMC	Pro-opiomelanocortin
Ppm	Parts per million
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RGS	Regulators of G-protein signalling
RNA	Ribonucleic acid
Rpm	Revolutions per minute
ROS	Reactive oxygen species
RT	Reverse transcriptase
SAA	Serum amyloid A

SAM	Sympathetic-adrenomedullary
SAS	Statistical Analysis Software
s.d.	Standard deviation
s.e.	Standard error
sec	Second
s.e.m.	Standard error of the mean
SNP	Single nucleotide polymorphism
SOCS-3	Suppressor of cytokine signalling 3
SRC-1	Steroid receptor coactivator 1
ssDNA	Single-stranded DNA
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOC	Total organic carbon
µg	Microgram
µL	Microlitre
UK	United Kingdom
USA	United States of America
V	Volt
vs	Versus
v/v	Volume per volume
w/v	Weight per volume

List of Tables

List of Figures

Abstract

Weaning is a multifaceted stressor and may involve numerous husbandry practices, including the abrupt separation of the calf from its dam, a nutritional adjustment to a non-milk diet and social reorganisation and, additionally, is often associated with other husbandry practices, such as housing and transport. Stress-induced disease susceptibility in livestock animals has documented economic and societal consequences and the sense of urgency to understand and intervene in these is becoming increasingly important. Available published research has examined the physiological and behavioural responses to weaning stress in calves, but no study has yet characterised the molecular response. This Ph.D. project aimed to obtain a greater understanding of the impacts of weaning on both the molecular and physiological responses in order to elucidate the immune mechanisms responsible for stress dependent immune suppression in calves post weaning. A series of studies were conducted to investigate i) the effect of weaning stress as an accumulative stressor on haematological and immunological variables to assess whether combining weaning with housing elicits a greater response than housing alone, ii) to (a) characterise, in male and female calves habituated to housing, the immune response to weaning stress at the physiological and molecular levels, and (b) assess the difference between calves weaned and housed in the presence of the dam and those weaned and housed away from the dam, and iii) the effect of accumulative stressors on the global transcriptomic response in blood leukocytes to weaning stress. The results of this thesis demonstrate that stress can have accumulative effects and by combining housing with weaning, an increase in neutrophil number and decrease in

lymphocyte number was found. Concerning management at weaning, it is evident that calves, particularly intact male calves, may benefit from a weaning strategy where the calves are allowed contact with the dam but prevented from suckling for a number of days before total separation occurs. Weaning calves away from the dam also resulted in a much stronger stress response, resulting in an increase in the expression of inflammatory cytokines, the glucocorticoid receptor, TLR4, CD62L and Fas, than weaning calves next to the dam. Additionally, a far more potent inflammatory response, at the transcriptomic level, was reported to occur in weaned and housed calves compared with housed calves, indicating that stress activates the innate immune system to increase surveillance. An important finding of this thesis was that simultaneously weaning and housing of calves produces a perturbation to the homeostasis of the leukocyte transcriptome which was still present 7 days following weaning. Thus the identification of novel biomarkers and regulatory gene networks that are stress activated provides a mechanistic framework to characterise the multifaceted nature of weaning stress adaptation in beef calves.

Chapter 1

Introduction

Background and Review of the Literature

1.1. Introduction

In Ireland, spring born calves are typically weaned at the end of the grazing season in autumn, typically between 6 and 9 months of age, at which time they are also housed (Drennan and McGee, 2009). In beef production systems, weaning is classed as a necessary husbandry practice that serves to increase herd performance and profitability through improved reproductive performance of the cows (Rasby, 2007). The weaning process involves separating the calf from its mother, resulting in numerous stressful events including dietary change, housing and social reorganisation. Weaning of beef calves has been previously shown to be a stressful event in the calf's lifetime (Hickey *et al*., 2003b; Blanco *et* al., 2009; Lynch *et al*., 2010a; 2010b). Efforts are being made to understand and minimise the impact of weaning stress on cattle as this has been implicated in a reduction of animal health and welfare in various production systems (Carroll *et al*., 2009; Babcock *et al*., 2010; Broom, 2011; Hubbard and Scott, 2011).

Health forms an integral component of animal welfare in high quality beef production systems and must be carefully monitored if acceptable animal welfare is to be achieved (Lawrence, 2008; Broom, 2011). Stress has been documented to negatively regulate the immune system, thus leaving an animal susceptible to disease (Sapolsky, 2000; 2004; Pruett, 2003; Reiche *et al*., 2004; Sapolsky, 2004; Glaser and Kiecolt-Glaser, 2005; Saul *et al*., 2005; Lynch *et al*., 2010a). Thus far, research measuring stress-related immune function in cattle has focused on the physiological response to a number of husbandry management practices including castration (Fisher *et al*., 1997a; Earley and Crowe, 2002; Ting *et al*., 2003a; 2003b; 2010; Pang *et al*. 2009a; 2009b; 2010; Marti *et al*., 2010), housing (Fisher *et al*., 1997b; 1997c; Hickey *et al*., 2003a; Gupta *et al*., 2007a), transport (Earley and O'Riordan 2006a; Earley *et al*., 2006b; Gupta *et al*., 2007b;

Buckham Sporer *et al*., 2007; 2008a; 2008b; Arthington *et al*., 2008; Earley and Murray 2010), and weaning (Hickey *et al*., 2003b; Arthington *et al*., 2005; Blanco *et al*., 2009; Carroll *et al*., 2009; Lynch *et al*., 2010a; 2010b).

In Ireland, bovine respiratory disease (BRD) and bovine viral diarrhoea (BVD) annually cost the economy an estimated €30 million and €102 million, respectively (Gunn and Stott, 1998; animalhealthireland.ie, 2011). This equates to anything from €30 to €114 per head at the farm level. As weaning is considered a stressful husbandry event that is inevitably undertaken on every suckler farm in Ireland, it is necessary to further examine the molecular and physiological mechanisms altered in response to abrupt weaning in order to understand the consequences they may have on both animal welfare and disease susceptibility. It is becoming increasingly clear that stress–immune interactions play a major role in the health and welfare of cattle. However, neither the molecular mechanisms involved nor the role of the neuroendocrine system in modulating the stress–immune interactions are yet clear.

1.2. Animal welfare and the implications of stress

1.2.1. Introduction

The issue of animal welfare has rapidly gained significant attention with many consumers no longer willing to purchase animal products at the expense of poor welfare standards (Broom, 2011). Therefore, an increasing emphasis has been placed on animal welfare over the past 50 years with a great degree of research focusing on what constitutes good welfare standards, particularly in relation to livestock. While no agreed definition or assessment of such a definition exists for the term ‘animal welfare’ (Barnard, 2007), it generally refers to a state of harmony between an individual and its

environment whereby welfare is achieved when the animal does not feel any long lasting negative emotions and they are allowed to achieve positive emotions (Dawkins, 1990) while maintaining a high standard of health. Responses to poor treatment and suffering are measureable in animals. Thus, animal welfare is measureable and is therefore a scientific concept, although a difficulty currently exists in identifying a universal method for assessing animal welfare (Fraser, 2008; Broom, 2011).

In 1965, the British government established the Brambell Committee to enquire into the welfare of intensively farmed livestock. The Brambell Report garnered international attention and is generally regarded as one of the earliest and most influential reports on animal welfare, credited with introducing the issue of animal welfare to the general public and highlighting the need for acceptable welfare standards across the EU. This led to the adoption of the European Convention for the Protection of Animals kept for Farming Purposes by the Council of Europe in 1976 (Council of Europe, 1976), effectively setting minimum welfare standards to avoid unnecessary suffering and insufficient housing or care for farmed animals. Outlining animal welfare standards can be difficult, particularly given their frequent anthropomorphic nature which focuses less on a complete understanding of what the animal actually feels and more on how a human caregiver thinks they might feel in the animals circumstances (Serpell, 2003; Barnard, 2007). Thus, the definition and understanding of animal welfare in the context of animal suffering has frequently changed in recent times. A number of diverse groups have interests in farm animal welfare including consumer groups seeking to ensure ethically produced products, producer groups who aim to balance profit with animal health and welfare, extremist animal rights groups who seek total abolition of any form of animal use and national governments, tasked with regulating animal legislation in response to pressure from the aforementioned groups. Therefore, sensible and well-

informed legislation based on a scientific understanding of animal behaviour and physiology is necessary to continually improve upon animal welfare, particularly concerning farmed livestock (Barnard, 2007).

In 1997, the concept of animal sentience was formally written into basic law of the European Union (EU). The legally-binding protocol recognises livestock as sentient beings (Webster, 2001) capable of a host of emotions ranging from fear, frustration, and stress to pleasure and happiness (Désiré *et al.*, 2002). According to Broom (2011), a sentient being is able to 1) evaluate the actions of others in relation to itself, 2) remember its own actions and their consequences, 3) assess risk, 4) have some feelings and 5) possess a degree of awareness. Despite initial welfare standards outlining production systems free from cruelty and suffering, a number of groups, such as the UK Farm Animal Welfare Council (1993; 2009), now call for policies to move beyond this and provide for the animals needs, allowing an acceptable quality of life. The concept of Five Freedoms as a guide to the minimum requirements to achieve best animal welfare practices was first proposed in the Brambell report but has since been refined by the Farm Animal Welfare Council and adopted internationally (Mench, 2008). The Five Freedoms are:

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

Collectively, these 5 freedoms constitute the basic requirements of an animal to express its normative behaviour while avoiding overtly stressful practices that can negatively influence health, and thus, welfare. More recently, between 2005 and 2010, the EU funded Welfare Quality® project has focussed on the integration of animal welfare into the food quality chain, addressing public concerns and aiming to improve welfare and the transparency of food quality. The aims of that project were 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 and focused on three main species and their products: cattle (beef and dairy), pigs, and poultry (broiler chickens and laying hens). The main principles and criteria that underpin the Welfare Quality® assessment system are presented in Table 1.1.

Confusion about the definition of animal welfare can sometimes arise among people who are not familiar with the area (Broom, 2011). Principally, health is a key part of animal welfare and cannot be viewed as a separate variable. However, more likely to be misinterpreted is the dignity of an animal. Dignity of an individual is a human concept that may be applied to animals but no evidence suggests that other species have a similar concept. While it may be used as an argument to treat animals well, it has nothing to do with animal welfare (Broom, 2011). In this sense, the killing of animals for human consumption is an ethical issue rather than a welfare one (Fraser and Weary, 2003; Fraser, 2008). Feelings are a brain construct involving perceptual awareness (Jensen, 2002). This biological mechanism has evolved to reinforce learning (Broom, 1998) and is integrally linked with health in the definition of welfare. Based on the commonly held assumption that animals possess a degree of cognitive ability, which therefore results in the ability to assess their environment and elicit feelings, it seems that stress should form an integral component of the assessment of welfare. While

reduced productivity and increased morbidity/mortality are useful indicators of poor animal welfare, scientific evidence through the evaluation of behaviour, physiology and immune competence may provide a more adept evaluation of animal welfare. The nature of stress, which relies on perception of a stimulus to alter physiological processes that allow the animal to effectively deal with its environment, means that the animal's cognitive evaluation of its own environment results in physiological changes that can be measured in order to assess animal welfare. Amadori *et al*. (1997) proposed the measurement of immunological functions in place of measurements of behaviour and neuroendocrine hormones for assessing welfare in cattle as immunological parameters are less susceptible to bias or timing of sample collection. However, normal endogenous systems are characterized by tempered oscillations over time, rather than by large changes (Amaral *et al*., 2004), and this sometimes presents a layer of difficulty in isolating treatment effects from normal biological fluctuations, meaning that suitable biomarkers must first be rigorously assessed to distinguish abnormal responses from normal physiological fluctuations.

1.2.2. Stress

The first comprehensive biological theory of stress, known as the General Adaptation Syndrome (GAS), described three universal stages of coping with an external stressor: 1) the initial alarm reaction, effectively the fight-or-flight response, 2) an adaptation stage associated with resistance to the external stressor, and 3) an exhaustion stage ending with the death of the animal if the external stressor did not cease (Seyle, 1946). Seyle (1973) went on to explain that the GAS was a relatively uniform, non-specific physiological response to a stressor. The GAS theory, now known to be incomplete, posited that the physiological stress response is the exact same, regardless of the type of stressor encountered. However, Seyle did separate negative and positive stress into two

terms, “distress” and “eustress”, respectively (Seyle, 1975). Due to the ambiguity of the term “stress”, the term “allostasis” was introduced by Sterling and Eyer (1988) as a means of referring to the active process the body uses to respond to daily events and to maintain homeostasis. Allostasis, which refers to achieving stability through change, is a more complete theory of stress and does not replace homeostasis, instead referring to the physiological changes that help the animal to better cope with a given situation (McEwen, 2008) (Figure 1.1).

Table 1.1. List of the criteria that underpin the Welfare Quality® assessment system

1) Animals should not suffer from prolonged hunger, i.e. they should have a sufficient and appropriate diet.
2) Animals should not suffer from 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.

Source: www.welfarequality.net

A number of definitions for “stress” exist. These definitions can be formed on opinion, behavioural observation or physiological processes, leading to some confusion as to what stress actually is. This confusion in defining stress derives partly from the differing uses of the term “stress” to frequently describe events that are not always comparable, an indication of the complex nature of stress. For instance, a

microbiologist can refer to a cell as being “stressed” in response to a change in pH, a process that occurs without any self-consciousness, whereas an animal behaviourist could assign a similar term to an animal that appears to have altered its behaviour based on a conscious response to its environment (e.g. appearance of a predator).

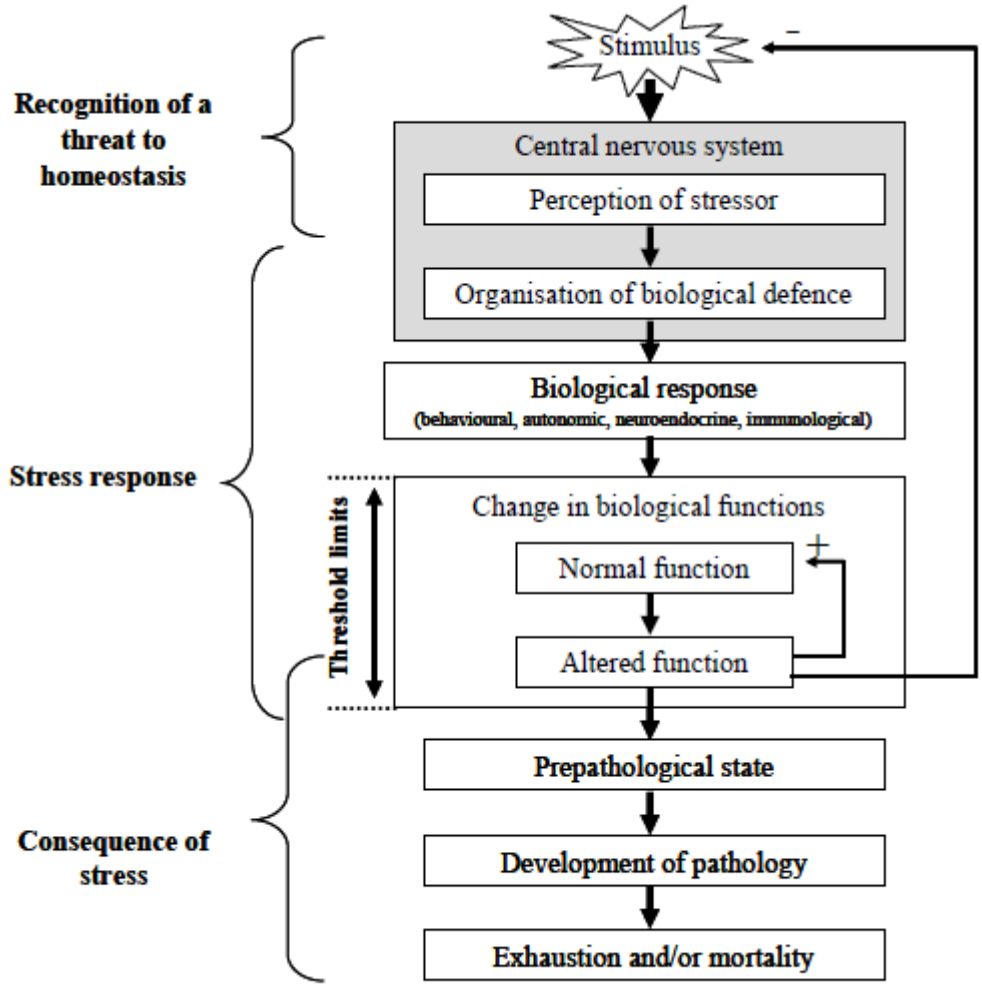

Figure 1.1. The biological stress response

A stressor results in a series of events intended to prepare the animal to cope with adverse stimuli and return biological systems to a state of homeostasis. If this allostatic process is successful, normal biological functioning is resumed. However, an inability to eliminate a stressor and return to homeostasis results in biological alterations in function that leave the animal in a prepathological, or distressed state, and thus susceptible to developing illness and, in extreme cases, death (Adapted from Moberg and Mench, 2000; Lynch, 2011).

The main definition of stress (Dhabhar and McEwen, 1997) states that stress is a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight-or-flight systems in the body (stress response). Therefore, stress will be viewed as a biological response to either a perceived or real threat to the health and welfare of an animal and it is not the aim to examine the emotions associated with husbandry induced (e.g. weaning) stress. However, animals, including cattle, perceive fear and are susceptible to both physical and psychological stress in a manner similar to that of humans, as reflected by the acceptance of farm animals as sentient beings by the EU (Dawkins, 1990; Amadori *et al*., 1997; Appleby, 1999; 2002; Webster, 1995; 2001; Désiré *et al*., 2002; Barnard, 2007; Boissy *et al*., 2007; Broom, 2011; Enriquez *et al*., 2011; Hubbard and Scott, 2011). Some have taken the view that a stressor is inherently destructive in terms of animal health (Broom, 2011). However, this is not strictly the case and concerns distress rather than stress. Distress refers to a negative state in which stress induced coping processes fail to return an animal to physiological homeostasis, typically occurring over an extended period of time (Carstens and Moberg, 2000) (Figure 1.2). While stress results in a physiological response to allow an animal to cope with a particular stimulus, prolonged, severe or multiple stressors can result in distress, severely reducing animal health and welfare (Moberg, 2000).

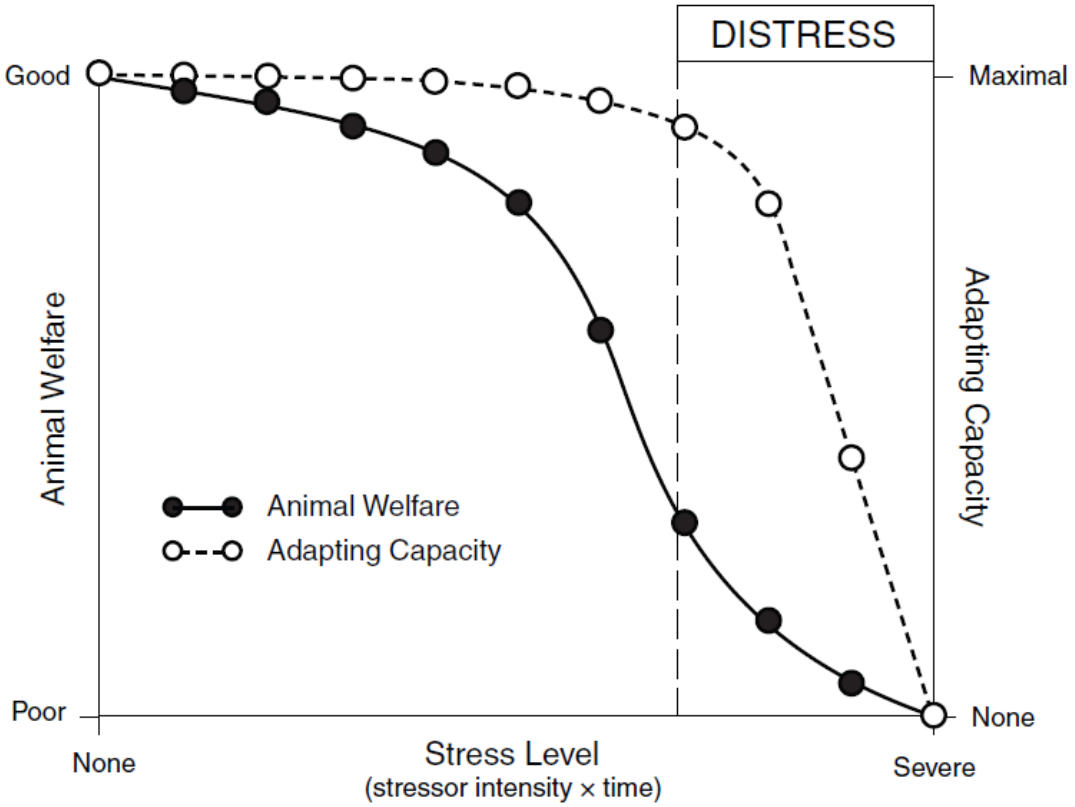

Figure 1.2. Representation of the relationship between stress and distress and the effect of animal adaptation on animal welfare.

Exposure to a stressor results in a physiological response intended to return an animal to homeostasis. If a stressor remains, an animal may appear to cope despite a decline in the welfare of the animal. The point at which welfare reaches a maladaptive state depends on the animal and the nature of the stressor, but once this point is reached, the animal's ability to cope with the stressor rapidly declines and can result in disease. This is known as distress (NRC, 1992).

1.2.3. Stress and disease susceptibility

The suggestion that stress is linked to an increased susceptibility to disease is not new. In 1878, Louis Pasteur demonstrated that chickens exposed to cold stress were far more likely to die when infected with anthrax than chickens that were not exposed to thermal stress but also infected with anthrax (Kelley, 1980). Typically, stress-induced immune suppression allows a primary viral or mycoplasma infection to establish itself in an animal where these micro-organisms further compromise the immune defences of the

host, facilitating a secondary infection by bacteria a number of days later (Aich *et al*., 2009; Griffin *et al*., 2010; Glass *et al*., 2011). In cattle, these pathogens are typically referred to collectively as BRD and BVD (Table 1.2).

In the **classic** understanding of stress-induced immune suppression (Iriwin *et al*., 1990; Kort, 1994; Agarwal and Marshall, 1998; Wu *et al*., 2000; Sapolsky, 2000; 2004), stress hormones such as glucocorticoids and catecholamines play a key role in the host following exposure to stress, suppressing immunity through anti-inflammatory mechanisms (Burton *et al*., 2005). The trafficking of neutrophils, macrophages, monocytes, dendritic cells, natural killer (NK) cells as well as T and B lymphocytes is altered in cattle (Hickey *et al*., 2003; Burton *et al*., 2005; Buckham *et al*., 2007; 2008; Lynch *et al*., 2011). In addition, lymphocytes, macrophages and NK effector cell functions are inhibited while the molecular expression of pro-inflammatory cytokines and chemokines is down-regulated, leading to the increased likelihood of developing illness (Padgett and Glaser, 2003).

However, it now appears that under many circumstances, exposure to stress can actually enhance the immune systems capability to fight infection (Dhabhar and McEwen, 1997; Black, 2002; Dhabhar, 2002; Dugan *et al*., 2007; Dhabhar, 2009). Mice exposed to a psychological stressor prior to challenge with either lipopolysaccharide (LPS) or *E. coli* were reported to have significantly lower mortality rates than unstressed controls (Dugan *et al*., 2007). While 58 % of unstressed controls died following LPS challenge, mortality among those exposed to a stressor was only 8 %. Following challenge with *E. coli*, mortality in unstressed controls was 67 % while in mice exposed to a predator as a stressor, only 17 % died. Thus, the psycho-physiological stress response is one of the most important survival mechanisms by which animals interact with their environment

and section 1.3 gives an overview of the physiological stress response and its intricately linked interactions with the immune system.

Table 1.2. Pathogens commonly implicated in bovine respiratory disease (BRD)

Viruses	Mycoplasma	Bacteria
Bovine respiratory syncytial virus (BRSV)	*Mycoplasma dispar*	*Mannheimia haemolytica*
Bovine parainfluenza virus type 3 (PIV3)	*Mycoplasma bovis*	*Histophilus somni*
Bovine viral diarrhoea virus (BVDV)	*Mycoplasma hyorhinis*	*Pasteurella multocida*
Respiratory bovine corona virus (RBCV)		*Staphylococcus aureus*
Bovine adenovirus		*Streptococcus pneumoniae*
Bovine herpes virus type 1 (BHV-1) leading to infectious bovine rhinotracheitis virus (IBRV)		*Actinomyces pyogenes*

Angen *et al*., 1999; Fulton, 2009; Griffin *et al*., 2010

1.3. The immune response to stress

1.3.1. Introduction

Stress can have a profound effect on both the cellular distribution and biological function of the immune system (Kort, 1994; Dhabhar and McEwen, 1997; Agarwal and Marshall, 1998; Wu *et al*., 2000; Sapolsky, 2000; 2004; Dhabhar, 2002; Haddad *et al*., 2002; Reiche *et al*., 2004; Glaser and Kiecolt Glaser, 2005; Saul *et al*., 2005; Dhabhar, 2009). The brain serves a highly sensitive, immunoregulatory role via a network of neurotransmitters, peptide hormones and cytokines that link together the nervous, endocrine and immune systems (Haddad *et al*., 2002). Two pathways are principally implicated in stress-induced immune alterations: the sympathetic-adrenal medullary

(SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis. The physiological response to a stressor involves the nervous, endocrine and immune systems interacting in a highly complex manner, making it difficult to elucidate accurate characterisations of the immune response to stress. However, a causal relationship between stress and alterations in immune function has long been associated with pathology in a diverse range of species (Irwin *et al*., 1990).

1.3.2. The immune system

Traditionally, immunology has been segregated into two distinct categories, innate and adaptive, or acquired, immunity. However, recent advances have demonstrated that a highly important, synergistic relationship exists between the two (Shanker, 2010). The innate immune system elicits a protective inflammatory response within minutes of the recognition of a pathogen through the use of three primary mechanisms: pattern recognition receptors (PRRs), damage-associated molecular patterns (DAMPs), and through the identification of 'missing self molecules' such as major histocompatability complex (MHC) class I and class II (Stuart *et al*., 2010). Despite the emphasis typically placed on the adaptive immune response to infection, the innate immune system clears the vast majority of pathogens encountered on a daily basis (Chaplin, 2010). It is only in rare circumstances, where the innate system fails to clear an infection, that the adaptive immune response is activated. However, it is important that the activation of the innate immune system occurs as without activation of the innate immune response, the adaptive immune response can not be primed for pathogen clearance, although recent evidence suggests that the converse is also true (Shanker, 2010). Therefore, stress related immune alterations deal principally with the innate immune system (Elenkov and Chrousos, 1999; Charmandari *et al*., 2004; Charmandari *et al*., 2005).

The innate immune system is responsible for the detection and clearance of foreign antigens and is heavily reliant upon a host of leukocyte subsets in order to function effectively. Principally, this is driven by the rapid innate immune synthesis and secretion of inflammatory mediators which result in immune cells infiltrating to sites of infection. Leukocytes are the main effectors of the innate immune system and are charged with efficient surveillance and defence against infection (Medzhitov and Janeway, 1997). Macrophages, neutrophils, lymphocytes, eosinophils and basophils play a primary role in this process, with a further role for platelets in immune surveillance and the early innate immune response. However, numerous studies have chiefly identified neutrophils and lymphocytes as the key targets of bovine stress, both in terms of cellular distribution and function at weaning (Hickey *et al*., 2003; Blanco *et al*., 2003; Lynch *et al*., 2011) and transport (Buckham Sporer *et al*, 2007; Riondato *et al*., 2008). Active leukocyte trafficking between the periphery and tissues/body compartments is important to maintain immune surveillance and ensures a rapid, appropriate and effective response can be mounted in the event of pathogenic detection (Engler *et al*., 2004).

1.3.3. Sympathetic-adrenal medullary (SAM) axis

Upon the perception of a stressor by the cerebral cortex, neurological impulses alert the hypothalamus and stimulate the activation the autonomic nervous system (ANS). This results in the rapid synthesis of the catecholamines, epinephrine and norepinephrine, occurring within seconds of the activation of the SAM axis (Sapolsky *et al*., 2000). Some 80 % of catecholamine secretion from the adrenal medulla, a key organ of the SAM axis, is in the form of epinephrine (Goldfien, 2001) while norepinephrine is released predominantly by sympathetic nerve fibres in direct contact with target tissues (Padgett and Glaser, 2003).

Catecholamines play a major role in the modulation of immune functions such as cytokine production, cell proliferation, cell trafficking and cytolytic activity (Saunders and Straub, 2002; Riondato *et al.*, 2008). Links have been identified that directly connect the sympathetic nervous system to the immune system by noradrenergic sympathetic nerve fibres which run from the central nervous system to primary and secondary lymphoid organs where they secrete norepinephrine (Stevens-Felten, 1997). Meanwhile, epinephrine released from the adrenal medulla enters peripheral circulation and binds to specific adrenergic receptors abundantly present on immune cells (Madden, 2003).

Many key cells of the immune system, including dendritic cells, lymphocytes, neutrophils and macrophages express adrenergic receptors on their surface (Madden, 2003; Abraham *et al*., 2004; Odore *et al*., 2004; LaBranche *et al*., 2010). Part of a large receptor family of seven-transmembrane domain receptors, better known as G-protein-coupled receptors (GPCR), adrenergic receptors can be separated into two active groups, α and β adrenergic receptors. Found in the plasma membrane of immune cells, the β2-adrenergic receptor is one of the most important immune GPCR, stimulating the G-protein complex to allow communication with the cytoplasm when bound by a catecholamine. This triggers a cascade resulting in the synthesis of cyclic adenosine monophosphate (cAMP) which can suppress the expression of a number of genes of immunological origin (Vallejo, 1994; Gardner, 2001; Padgett and Glaser, 2003).

Elenkov *et al*. (1996) found an *in vitro* increase in IL-10 synthesis along with a suppression of IL-12 following treatment of peripheral blood leukocytes with catecholamines. This indicates a shift in the cytokine balance from an inflammatory

Th1 profile towards a more specific humoral Th2 profile (Padgett and Glaser, 2003). While some subsequent investigations have validated this work (Fleshner *et al*., 1996; Franchimont *et al*., 2000; Salicrú *et al*., 2007), a number of further studies failed to identify any indication of this shift (Braun *et al*., 1997; MacPhee *et al*., 2000; Pruett and Fan, 2001). Sanders *et al*. (1997) reported that Th1 cells preferentially express β2-adrenergic receptors which suppressed the production of IgG_{2a} *in vitro*, but this was not the case for Th2 cells.

Norepinephrine mediates the rapid alterations in leukocyte numbers following the onset of exercise related stress (Hoffman-Goetz *et al*., 1994). This includes an increase in neutrophil and monocyte concentrations. Additionally, a brief induction of NK cells, resulting in their demargination via stimulation by catecholamines results in NK cell numbers briefly increasing followed by a significant reduction post exercise stress (Shephard and Shek, 1999).

1.3.4. Hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis, also known as the hypothalamic-pituitary-adrenocortical axis, is activated by the failure of the SAM axis to resolve a stressor (Figure 1.3). Hypothalamic initiation occurs through a series of different stimuli, both internal and external stressors, including activation by the limbic system in the case of psychological stress (Herman *et al*., 2005). Upon activation, corticotropin-releasing hormone (CRH, also referred to as corticotropin-releasing factor (CRF)) along with small quantities of arginine-vasopressin (AVP) are released from neurons in the paraventricular nucleus of the hypothalamus into the hypophyseal portal blood system, thus initiating a cascade of events intended to resolve a homeostatic environment (Ulrich-Lai and Herman, 2009). Reaching the anterior pituitary, CRH, acting synergistically with AVP, stimulates the

immediate synthesis of adrenocorticotropic hormone (ACTH) from the precursor molecule, pro-opiomelanocortin (POMC). Once ACTH enters peripheral circulation, it acts upon the outer adrenal cortex of the adrenal gland, resulting in the potent secretion of glucocorticoids (GC) from the zona fasciculata which act upon a diverse range of cells, tissues and organs throughout the body, including those of the immune system (Chrousos *et al*., 2004). In cattle, the predominant glucocorticoid is cortisol (Mormede *et al*., 2007), with only 10 % of cortisol found in its unbound state while 80 % is bound by corticosterone binding protein and the remaining 10 % is bound by albumin (Gayrard *et* al., 1996).

Glucocorticoids exert a rapid negative feedback on the secretion of CRH from the hypothalamus and POMC derived peptides, primarily ACTH, from the pituitary. This provides a rapid suppression of both CRH and ACTH secretion, preventing prolonged exposure of tissues to the anti-inflammatory actions of GCs which can result in immunosuppressive activity if not properly regulated (Elenkov and Chrousos, 2002).

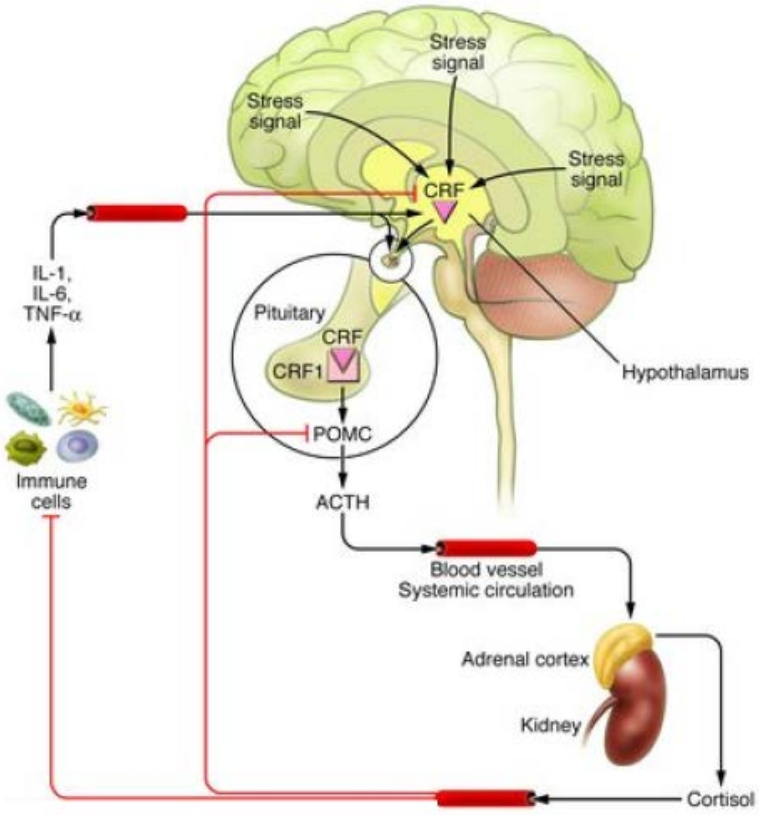

Figure 1.3. The Hypothalamic-Pituitary-Adrenal (HPA) Axis.

Upon perception of a stressor, the hypothalamus secretes CRH and AVP which act in unison upon the anterior pituitary. This promotes the secretion of ACTH into general circulation, resulting in the release of cortisol which acts upon cells of immunological origin. In turn, these cells secrete cytokines, many of which are also known as immunotransmitters as they share many properties with neurotransmitters (Haddad and Land, 2002), resulting in a secondary feedback system to the hypothalamus and pituitary by the immune system (See section 1.3.7) (Adapted from Slominski, 2007).

1.3.5. Regulation of the innate immune response by glucocorticoids

As final effectors of the HPA axis, lipophilic glucocorticoids are readily capable of migrating through the plasma membrane of cells via passive diffusion. Following a strong ultradian rhythm (Lefcourt *et al.*, 1993; Droste *et al.*, 2008), their role in both the regulation of normal homeostatic activities as well as their modulation of the immune system under conditions of stress cannot be understated. Basal secretion of GC is of vital importance to the normal function of most tissues, serving a plethora of functions

including gluconeogenesis (McMahon *et al*., 1988), the regulation of growth (Sartin *et al*., 1998), cardiovascular output (Brotman *et al*., 2007), maintenance of homeostasis (von Borell, 2001) and the regulation of the stress response and immune function (Webster Marketon and Glaser, 2008). Despite peaking between ten minutes and one hour following the initiation of the stress response (Sapolsky *et al*., 2000; Droste *et al*., 2008), glucocorticoids can have far reaching physiological consequences. Even brief alterations to GC concentrations can result in a variety of physiological conditions (Turnbull and Rivier, 1999). In order to stimulate alterations in immune function, GC must bind to one of two specific receptors within the cytoplasm of the target cell of immunological origin. The first, mineralocorticoid receptor (MR; affinity 0.5 nM), preferentially bind glucocorticoids when the hormone is available at normal physiological levels and is responsible for numerous physiological processes (Pascual-Le Tallec and Lombes, 2005). During a stress response, when circulating GC levels surge, MR become saturated, leading to the low specificity binding of glucocorticoids to the glucocorticoid receptor (GR; affinity 5.0 nM) (De Kloet *et al*., 1998; Sorrells and Sapolsky, 2007). Glucocorticoids have a much lower affinity (~10 fold) for GR than MR, ensuring large scale binding to GR only when GC hormonal levels are increased, such as during a major stress response. On ligand binding to GR, heat shock protein 90 (HSP90) and other proteins dissociate from the oligomeric complex that constitutes the inactive GR, allowing the GR to form homodimers and translocate to the nucleus where they can bind to glucocorticoid response elements (GRE), resulting in transactivation of gene expression (La Baer and Yamamoto, 1994).

Many genes of the immune system contain GRE in their promoter regions, allowing GR to directly induce transcription (Figure 1.4). The GRE is a 15 bp sequence encompassing two half-sites separated by three base pairs that can consist of any

arrangement of nucleotides. The palindromic GRE sequence is 5'-AGAACA-*nnn*-TGTTCT-3' (Luisi *et al*, 1991). The binding of the GR to the GRE can also allow other transcription factors to bind to previously unavailable regions of DNA as a result of the induction of chromatin remodelling (Deroo and Archer, 2001). GR can also bind to a 'negative' glucocorticoid response element (nGRE) to inhibit transcription. This process involves the binding of three GR molecules instead of the typical formation of a GR homodimer. GR binds to the nGRE to suppress activation through transactivating factors (Subramaniam *et al*, 1997). One key gene inhibited by the GR-nGRE complex is POMC (Bamberger *et al*, 1996) reducing local synthesis of ACTH.

However, GR also results in the transrepression of a number of inflammatory cytokines. Glucocorticoids indirectly regulate expression of these cytokines through binding to a number of transcription factors, inhibiting subsequent transcription (Auphan *et al*, 1995). Many inflammatory genes that do not have GRE in their promoter regions contain sites for activation protein (AP) -1 and NFκB. This explains how cytokines without GRE in their promoters can still be suppressed by glucocorticoids as the glucocorticoid receptor binds to the transcription factors AP-1 and NFκB, preventing their transmigration to the nucleus, thus suppressing transcription (Gupta and Lalchhandama, 2002; De Bosscher *et al*., 2000, 2003; Padgett and Glaser, 2003).

Currently, several other mechanisms are suspected by which GCs regulate gene expression of the immune system. Two separate studies by Scheinman *et al*. (1995) and Auphan *et al*. (1995) presented work indicating that GCs activate the transcription of IκBα, inhibiting NFκB activity by sequestering it in an inactive state in the cytoplasm. Therefore, many genes responsible for the proliferation of cytokines are abruptly turned off or prevented from activation. However, Adcock *et al*. (1999) demonstrated that

IκBα was not always required to inhibit NFκB, implicating other mechanisms in the GC regulation of gene expression.

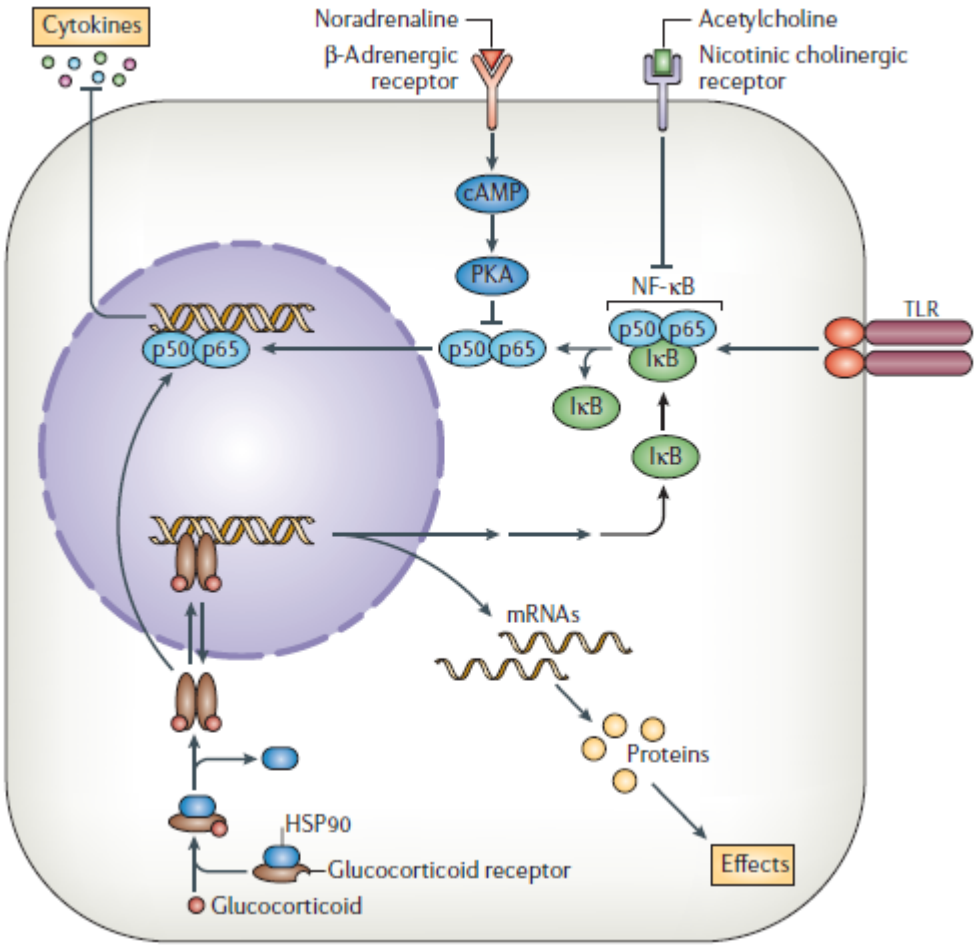

Figure 1.4. Molecular regulation of cytokine gene expression by glucocorticoids and catecholamines.

Glucocorticoids bind to glucocorticoid receptors in the cytoplasm of the cell, resulting in the dissociation of HSP90 and the formation of a glucocorticoid-bound glucocorticoid receptor homodimer. This glucocorticoid homodimer translocates to the nucleus of the cell where it acts as a transcription factor, binding to GRE and increasing the transcription of a number of genes. One such gene is IκBα which sequesters NFκB in an inactive state, preventing its activation of pro-inflammatory cytokines. Norepinephrine (noradrenaline) binds with the β-adrenergic receptor to induce activation of cAMP while acetylcholine binds to the nicotinic cholinergic receptor, both of which lead to the inhibition of NFκB (Adapted from Sternberg, 2006).

Reichardt *et al.* (2001) proposed that the activated GR binds directly to NFκB, preventing its transmigration to the nucleus, whereas De Bosscher *et al.* (2000)

demonstrated that competition between the GR and NFκB for cofactors such as cAMP response element binding protein (CBP) and steroid receptor coactivator (SRC) -1 suppress NFκB activity. It is likely that more than one mechanism plays an active role in the inhibition of NFκB, and these mechanisms may differ by cell type and stressor (Padgett and Glaser, 2003; De Bosscher *et al*., 2003) (Figure 1.5).

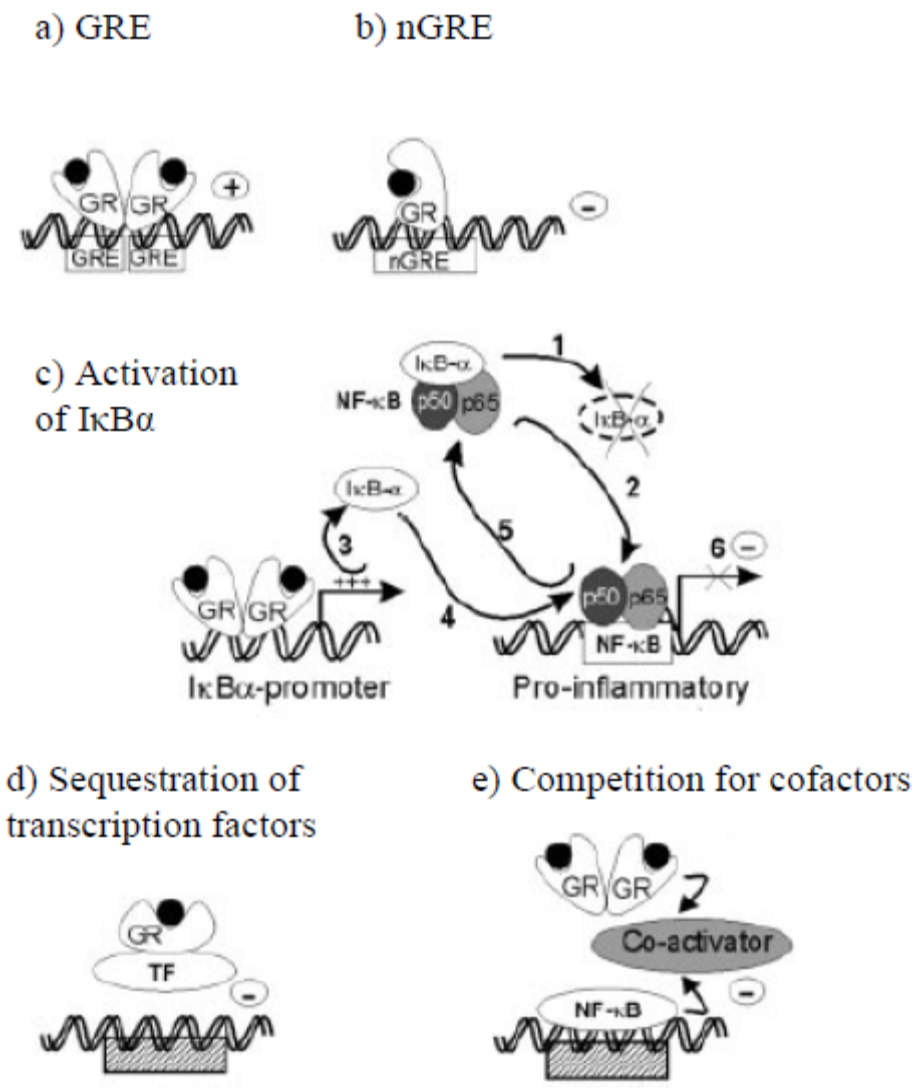

Figure 1.5. Mechanisms by which glucocorticoids activate (a) and suppress (b-e) gene expression.

A number of proposed mechanisms for glucocorticoid action exist, as described in the text above (Modified from De Bosscher *et al*., 2003).

1.3.6. Non-genomic effects of glucocorticoids

The effects of GC cannot be fully explained by the genomic mechanisms described above, primarily because GC elicit highly rapid effects upon secretion (Mulholland *et al*., 2006). Circulating GC levels will peak between 10 and 60 minutes following a stressor, which demonstrates the long-term, far reaching effects they have on the physiological system (Sapolsky *et al*, 2000; Droste *et al*, 2008). Significant alterations at the regulatory protein level do not occur for 30 minutes following the activation of the GR. Given the need for several hours before these proteins have significant effects at a physiological level, it seems likely that other, more rapid mechanisms of action also exist (Buttgereit and Scheffold, 2002).

One such mechanism proposes that the cytosolic glucocorticoid receptor can activate two distinct pathways. Following GC binding to the glucocorticoid receptor, the GR is released from the HSP90 complex and alters transcriptional regulation. However, Src, a kinase from the MAPK signalling system, is also released upon GC binding, thus providing a mechanism by which glucocorticoids can stimulate intracellular signalling (Croxtall *et al*., 2000).

Another explanation for the rapid effects that glucocorticoids produce is that cells of the immune system have membrane-bound glucocorticoid receptors (mGR). Buttgereit and Scheffold (2002) employed high-sensitivity immunofluorescent staining to peripheral blood mononuclear cells, identifying mGR on 25% of monocytes and B lymphocytes. T lymphocytes were found to be negative for mGR. This indicates that mGR is present on at least a few different populations of cells of immunological origin, albeit at very low concentrations. They are present at higher levels under inflammatory conditions, suggesting that they may provide a negative feedback mechanism. Membrane-bound

glucocorticoid receptor expression appears to be up-regulated in the presence of high levels of glucocorticoids, preventing an excessive immune response by initiating apoptosis of specific cell types (Bartholome *et al*, 2004; Stahn *et al*, 2008). While these mechanisms appear to play a role in the rapid GC effects, it is important to note that the GC regulation of the transcriptome appears to be responsible for the majority of the longer term glucocorticoid alterations to immunity.

During the inflammatory response, when GC concentrations are elevated in circulation, pro-inflammatory cytokines production is suppressed but their corresponding cytokine receptors are generally highly abundant (Sorrells and Sapolsky, 2007). At low GC concentrations, these cytokines are highly abundant but their cytokine receptors are not (Figure 1.6). This means that cytokine signalling remains low at both abnormally high and low concentrations of GC. Optimum cytokine signalling occurs at mid-range concentrations of GC (Munck and Naray-Fejes-Toth, 1992; Sorrells and Sapolsky, 2007) which may help the optimisation of the inflammatory response (Wiegers and Reul, 1998).

Glucocorticoids suppress inflammation at high doses involving chronic stress or when introduced following the onset of inflammation (Goujon *et al*., 1995; Connor *et al*., 2005; Curtin *et al*., 2009). However, GC appear to enhance inflammation under certain conditions including low doses, acute stress, and GC exposure prior to inflammation (Sorrells and Sapolsky, 2007), suggesting that GC can also play a role in the induction of pro-inflammatory measures. Neutrophil survival and proliferation are increased by GC (Cox, 1995; Tempelman *et al*., 2002; Weber *et al*., 2006; Buckham Sporer *et al*., 2008a) while the depletion of lymphocytes frequently identified during the stress response reflects extravasation to sites of inflammation rather than cell death (Dhabhar

and McEwen, 1997). Furthermore, a number of studies have concluded that acute stress can enhance the immune response to external insult by means of an inflammatory response (Bierhaus *et al*., 2003; O'Connor *et al*., 2003; Deinzer *et al*., 2004). The pro-inflammatory cytokine IL-1β was found to increase following acute stress in both rats (O'Connor *et al*., 2003) and humans (Deinzer *et al*., 2004), indicating not only the importance of IL-1β in a stress model, but also the cross-species conservation of function. Using peripheral blood mononuclear cells (PBMC), Bierhaus *et al*. (2003) identified an increase in the expression of NFκB target genes following exposure to psychosocial stress in humans. These authors also reported increased activity levels of the transcription factor, NFκB, but found no increase in NFκB gene expression.

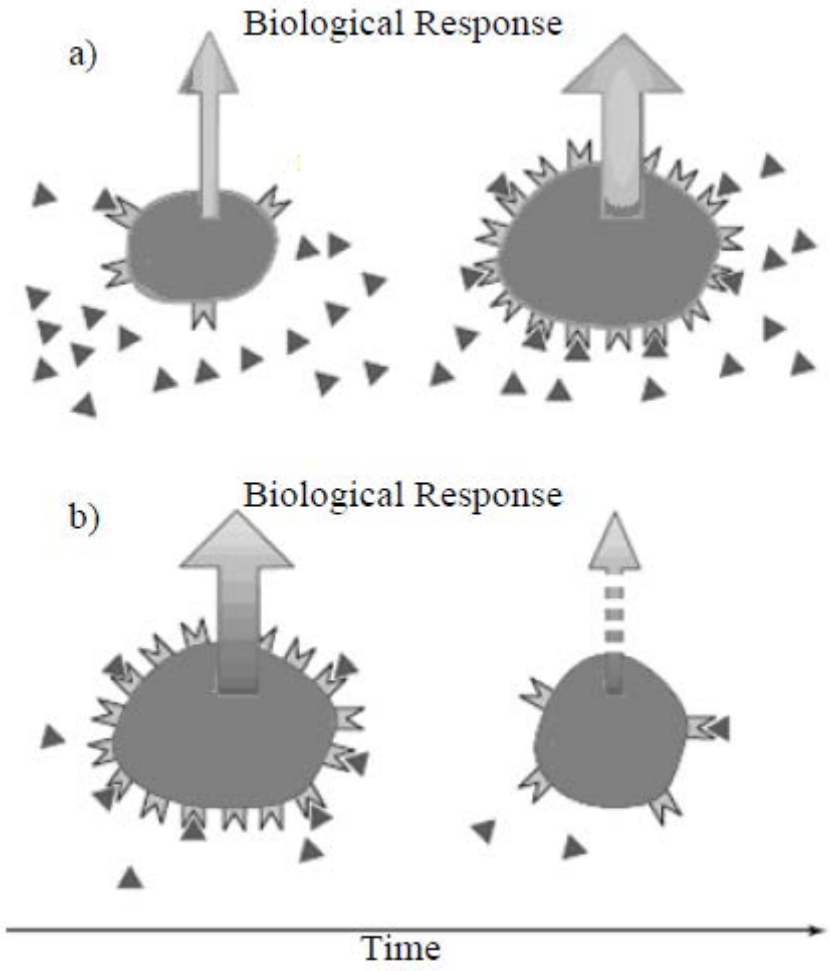

Figure 1.6. Glucocorticoid optimisation of cytokine response.

When cortisol is not present (a), cytokine concentrations are high but cytokine receptors are low. Over time, the cytokine receptor is up-regulated, resulting in a strong biological response with a slow onset time and delayed termination. Conversely, when cortisol is present (b), cytokine concentrations are low but their complementary receptors are so abundantly available that a rapid biological response occurs. This response becomes rate limiting as both cytokine concentrations and their receptors reduce in numbers and terminate the response (Adapted from Wiegers and Reul, 1998).

1.3.7. Cytokine regulation of the HPA axis

The actions of glucocorticoids, catecholamines, opiates and other peptides provide a mechanism for the regulation of the immune system by the neuroendocrine system. However, these cannot be viewed in isolation as the relationship between the immune

and neuroendocrine systems is a complex, bidirectional one that requires important regulatory input from cells of immunological origin (Maier and Watkins, 1998; Turnbull and Rivier, 1999; Black, 2002; Guyon *et al*., 2008). It has been suggested that during periods of stress, pathology is caused by the HPA axis responding inappropriately to soluble polypeptides secreted by the immune system (Rivier, 1994). These cytokines are rapidly induced in response to infection or inflammation and are involved in immunoregulation, tissue repair, haemopoiesis, growth, differentiation and function in a number of cell types (Chesnokova and Melmed, 2002; Holloway *et al*., 2002; Safieh-Garabedian *et al*., 2002; Rostene *et al*., 2011) (Figure 1.7). Under normal physiological conditions, most cytokines are expressed at very low concentrations. As mediators of both intercellular and intracellular communication (Oppenheim, 2001; Holloway *et al*., 2002), they are a highly redundant, pleiotropic group that alter transcriptional levels of a range of target genes (Turnbull and Rivier, 1999), complementing neuroendocrine hormones with a second mode of action via the indirect modulation of immunity (Glaser and Kiecolt-Glaser, 2005).

Despite having distinct cellular receptors, many cytokines share common biological activities allowing for a functional redundancy within the immune system (Turnbull and Rivier, 1995). The cytokine network is a highly complex system that involves multiple interactions between specific cytokines that can alter their response to or production of other cytokines. Modifications to the biological effect of one cytokine can occur through interactions with a second cytokine. This means that the removal of a single cytokine will generally not result in a massively deleterious effect to the immune system (Haddad, 2002).

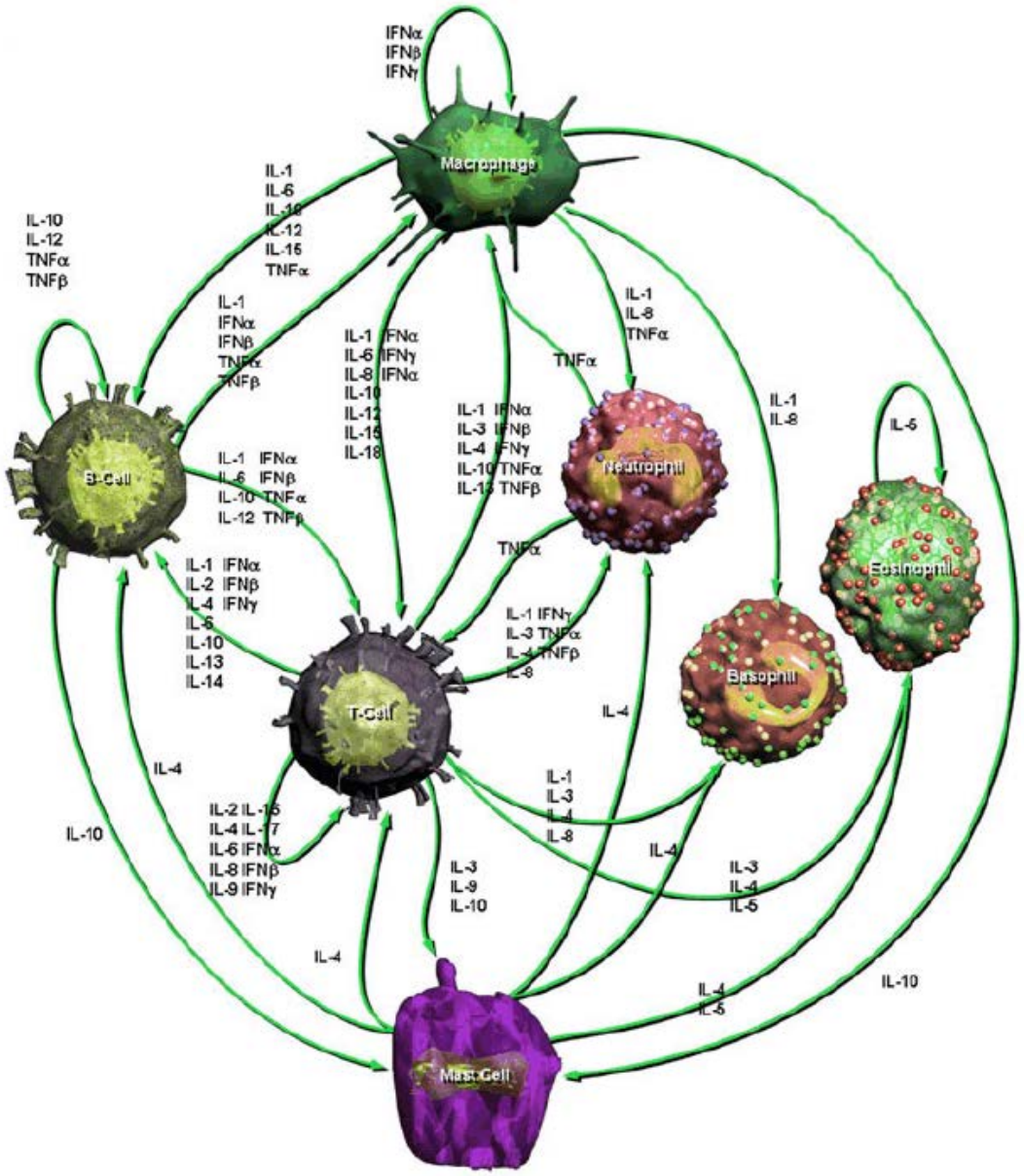

Figure 1.7. The cytokine network.

Cytokines are secreted by a number of cells of the immune system. In addition to auto-stimulation of a cell, cytokines can influence the physiology of other cell types, either inducing or abating cytokine secretion. The pleiotropic effects of cytokines mean that more than one cytokine can frequently have a similar functions.

A number of cytokines, including interleukin (IL) -1, IL-6 and tumour necrosis factor alpha (TNFα), are known as immunotransmitters because of their ability to act upon the nervous system, serving autocrine, paracrine and endocrine functions by regulating the secretion of hormones and other cytokines as well as the proliferation and

differentiation of cells (Haddad and Land, 2002b; Rostene *et al*., 2011). Additionally, a number of cytokines (IL-1, IL-2, IL-6, TNFα, interferon (IFN)) act on the hypothalamus to alter the release of anterior pituitary hormones, resulting in the activation of the HPA axis while simultaneously suppressing other axes involved in growth and reproduction (Bumiller *et al*., 1999; Safieh-Garabedian *et al*., 2002; Pruett, 2003). While many cytokines are capable of activating and otherwise regulating the HPA axis, most research has focused on IL-1, IL-2, IL-6, TNFα (Turnbull *et al*., 1995; Turnbull and Rivier, 1999) and IFN-α (Dunn, 2000) thus far (Bumiller *et al*., 1999). The expression of a number of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α and IFN-γ is inhibited or reduced following exposure to GC (Black, 2002) while the expression of anti-inflammatory cytokines like IL-4, IL-10 and TGF-β is increased (Sorrells and Sapolsky, 2007).

There are a number of truncated, soluble cytokine receptors which can bind a host of cytokines including IL-1, IL-6 and TNF-α, providing an additional regulatory level to cytokine signalling (Fernandez-Botran, 1991). While the binding of IL-1 or TNF-α to their soluble receptor results in antagonistic effects by reducing the amount freely available to bind to the membrane-bound receptors, the converse is true for IL-6 (Heaney and Golde, 1996). IL-6 bound soluble receptors can interact with gp130 to alter the cellular response (McLoughlin *et al*., 2004). Therefore, the biological activity of cytokines is determined by the concentration of the individual cytokine, the concentration of cytokines which alter its activity, the presence of complementary membrane-bound receptors and finally, the presence of soluble receptors (Turnbull and Rivier, 1999).

Of significant consequence to the elaborate interaction of the HPA axis and immune system is the cytokine IL-1β (Dunn, 2000; 2007; Elenkov *et al*., 2000; Chesnokova and Melmed, 2002; Haddad *et al*., 2002). IL-1β is released systemically following activation of an immune response which indicates that it may act as an immunotransmitter by alerting the brain to immune activity (Haddad *et al*., 2002). Cunningham *et al*. (1992) identified an intense, homogeneously distributed signal for type I IL-1 receptor in the hypothalamus and anterior pituitary using *in situ* histochemical techniques. However, these authors failed to find an autoradiographic signal above background noise on the adrenal gland, signifying that IL-1β acts upon the hypothalamus and possibly the pituitary in its activation of the HPA axis, but not the adrenal gland. In addition to direct IL-1 access to the brain, stimulation through peripheral vagal afferents occurs. Noradrenergic neurons have terminals in the hypothalamus and are known to regulate the secretion of CRH. They also contain IL-1 receptors, allowing for rapid IL-1β feedback (Haddad *et al*., 2002). Paraganglia cells in the parasympathetic ganglia express IL-1 receptors, rapidly signalling the presence of peripheral inflammation to the brain through the afferent fibres of the vagus nerve (Watkins and Maier, 1999). This indicates numerous signalling pathways through which IL-1β regulates hypothalamic inflammatory signalling.

IL-1 feedback stimulation has been shown to be inhibited by glucocorticoids (Plagemann *et al*., 1998). However, pro-inflammatory cytokines such as IL-1 can alter the function of the GR by reducing its ability to translocate to the nucleus, restricting the functionality of this transcription factor (Pariante *et al*., 1999). Based on these findings, it appears that a cytokine driven inflammatory response likely results in GR resistance, demonstrating another pathway by which the immune system can regulate the response of the HPA axis to stress (Elenkov *et al*., 2000; Haddad *et al*, 2002)

While IL-1 plays an important role in the immune systems feedback to the hypothalamus, it does not do so in isolation. IL-6 is induced by IL-1β (Mastorakos *et al*., 1994) and plays a vital role as an immunotransmitter, acting synergistically with IL-1β to activate the HPA axis, primarily at the pituitary (Zhou *et al*., 1996). IL-6 is a member of the gp130 cytokine family, a group of cytokines that work together with CRH to induce the secretion of POMC from the pituitary (Chesnokova and Melmed, 2002). Bethin *et al*. (2000) demonstrated that gp130 receptor cytokines do not require CRH to activate the HPA axis and are in fact required for the secretion of CRH during later phases of inflammation (Vallieres and Rivest, 1999).

By stimulating the HPA axis, cytokines antagonise their pro-inflammatory actions in the periphery (Chesnokova and Melmed, 2002). This is due to two main pathways. The first is their induction of the HPA axis to continue secretion of CRH, ACTH and GC as an end product. Second, and perhaps more importantly, is that exposure to pro-inflammatory cytokines over a long period of time results in an increase of the β-isoform of GR which blocks the activation of the α-isoform, resulting in glucocorticoid resistance (Webster *et al*., 2001). The lack of responsiveness of the HPA axis to glucocorticoids during prolonged periods of inflammation protects the organism from the damaging effects of cytokines by continuing to produce suppressive glucocorticoids (Chesnokova and Melmed, 2002). The balance between a number of systems, hormones and peptides is required in order to return to a homeostatic state. In order to prevent the excessive activation of the HPA axis, cytokine function must be regulated and suppressed. Early in the inflammatory response, gp130 cytokines, in addition to IL-1, induce the up-regulation of the gene suppressor of cytokine signalling (SOCS)-3 in both the hypothalamus and pituitary (Auernhammer and Melmed, 2001). SOCS-3

prevents gp130 stimulation of cells by inhibiting both JAK-STAT signalling as well as the cytokine driven secretion of ACTH from the pituitary.

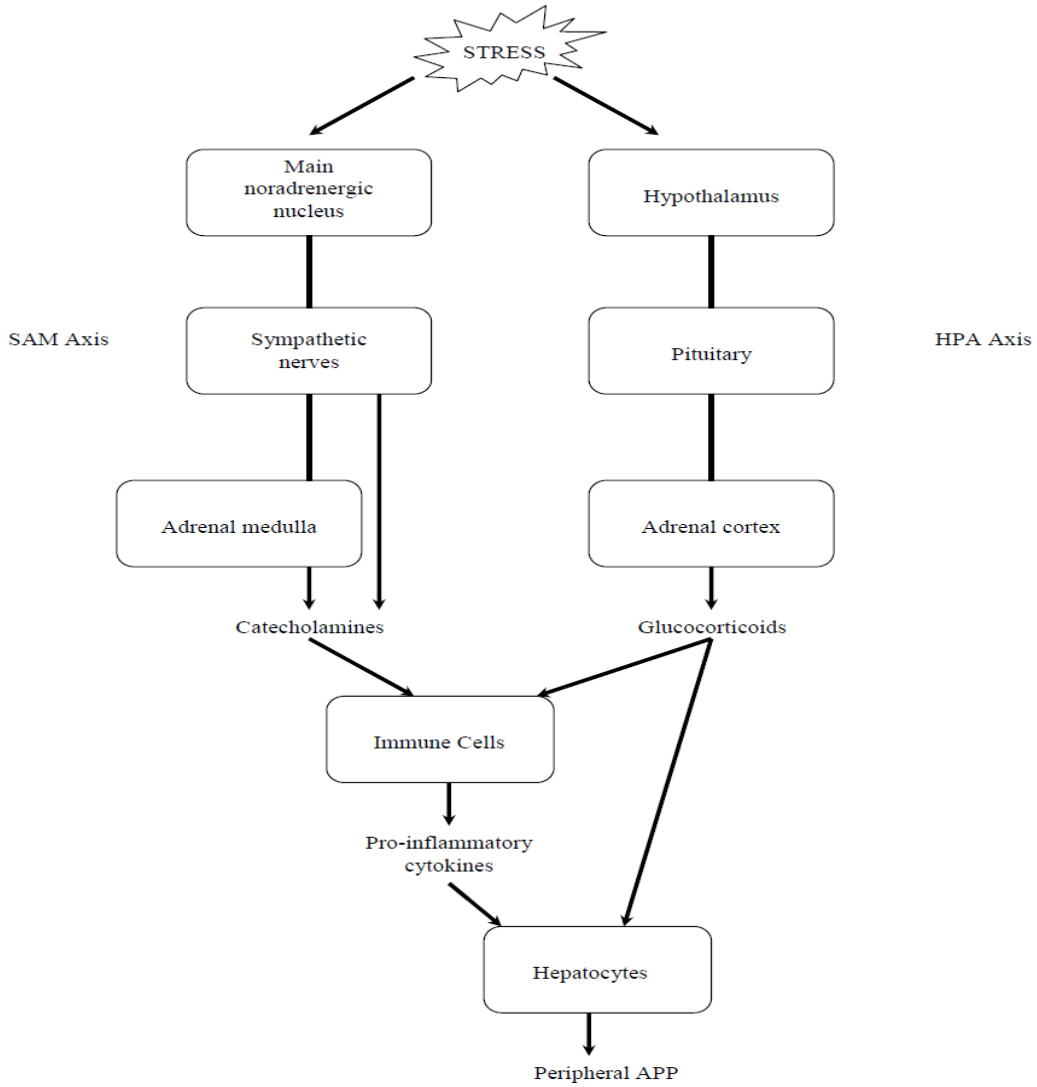

Figure 1.8. The acute phase protein (APP) response to stress.
Stress activates both the SAM and HPA axes, leading to the catecholamine and glucocorticoid induction of cytokines from cells of the innate immune system in addition to direct action of glucocorticoids on acute phase protein synthesis from hepatocytes (Adapted from Murata, 2007).

1.3.8. Acute phase protein response to stress

Acute phase proteins (APP) are important innate immune components, serving to restore homeostasis following infection (Murata *et al*., 2004). The acute phase response (APR) consists of positive and negative proteins based on their response to immune

challenge. These proteins are predominantly synthesised by hepatocytes and released into general circulation where they alter numerous physiological processes, including pro-inflammatory cytokine secretion, and minimise damage arising from the inflammatory response to infection (Murata *et al*., 2004; Nikunen *et al*., 2007). Stress hormones stimulate hepatocytes, enhancing the cellular synthesis of APP, particularly in the presence of the cytokines IL-1, IL-6 and TNF-α (Kurash *et al*., 2004) (Figure 1.8).

1.4. Biomarkers of stress

1.4.1. Introduction

By definition, a biomarker is a biometric measurement that can be used to monitor and assess the biological state of an animal, particularly in relation to disease (Aronson, 2005; LaBaer, 2005; Rifai and Gerszten, 2006). This allows the early and indirect detection of a given physiological state, and may also allow the measurement of severity. Because any change in biological state is accompanied by a large number of tempered physiological alterations (Amaral *et al*., 2004), a good biomarker is one that can be measured quickly and with ease, assessing a measurable process at the molecular, cellular, hormonal or organ level (Aronson, 2005). The selection of a biomarker relies heavily on its intended use, so it is necessary to thoroughly validate new biomarkers in order to ascertain whether the biomarker can accurately, sensitively and repeatedly reflect the process under examination (LaBaer, 2005).

Well known biomarkers include cholesterol levels and blood pressure which serve as biomarkers for heart disease and stroke in humans. While blood pressure also serves as a biomarker of stress in humans, it does not have a great clinical relevance to the study of stress in cattle, and traditional biomarkers of livestock stress typically involve

monitoring HPA activation via measurement of cortisol, catecholamines and other stress hormones as well as behavioural modifications. However, it has been suggested that alterations in immune state may be better functional biomarkers for stress in cattle than behaviour or adrenocorticol hormones (Amadori *et al*., 1997).

1.4.2. Biomarkers of stress in cattle

1.4.2.1.1. Leukocyte distribution

Leukocyte subsets, which are predominantly responsible for the surveillance and clearance of pathogens, are targets of stress hormones derived from the HPA axis. This makes them highly suitable as biomarkers of stress in cattle, with particular emphasis on neutrophils, lymphocytes and the neutrophil:lymphocyte (N:L) ratio (Hickey *et al*., 2003a; 2003b; Yagi *et al*., 2004; Gupta *et al*., 2005; Pang *et al*., 2006; Buckham Sporer *et al*., 2007; 2008a; Blanco *et al*., 2009; Earley and Murray, 2010; Lynch *et al*., 2010a; 2010b). A concurrent increase in total circulating neutrophil number and decrease in total circulating lymphocyte number in the periphery following a stressor is one of the more visible biomarkers of the stress response in cattle, resulting in a two-fold increase in the N:L ratio. Other leukocyte subsets, including monocytes, are unreliable biomarkers of stress given their relative distributional variation and apparent lack of sensitivity to stress hormones in cattle (Jones and Allison, 2007). It is important to note that while it is uncommon to see alterations in monocyte distribution, the functional activity of these cells may be greatly altered by stress. Table 1.3 details the normal distributional range of bovine leukocytes.

Table 1.3. Normal distributional values of bovine blood constituents

Variable	Range	Average	
Erythron			
Erythrocytes ($\times 10^6/\mu L$)	5.0-10.0	7.0	
Haemoglobin (g/dL)	8.0-15.0	11.0	
Haematocrit (%)	24.0-46.0	35.0	
Mean Corpuscular volume (MCV) (fL)	40.0-60.0	52.0	
Mean Corpuscular Haemoglobin Conc. (MCHC) (pg)	11.0-17.0	14.0	
Leukocytes (µL)			**Percent** (%)
Total Leukocytes	4,000-12,000	8,000	
Neutrophils	600-4,000	2,000	15-45
Lymphocytes	2,500-7,500	4,500	45-75
Monocytes	25-840	400	2-7
Eosinophils	0-2,400	700	0-20
Basophils	0-200	5	0-2
Platelets ($\times 10^3/\mu L$)	100-800		

Adapted from Jones and Allison, 2007

1.4.2.1.2. Neutrophil structure and function

The neutrophil, characterised by a polymorphic, segmented nucleus, cytoplasmic granules and large glycogen stores, is the first line of cellular defence against pathogens (Paape *et al*., 2003). The segmented nucleus is an important feature of the neutrophil as it allows rapid transendothelial migration via a mechanism that arranges the nuclear lobes into a straight line. This enables the neutrophil to arrive at sites of infection before macrophages, which have a larger, horseshoe shaped nucleus (Paape *et al*., 1979; Wagner and Roth, 2000). The bovine neutrophil contains three main types of bactericidal granules. The primary granules, also known as azurophilic granules, produce peroxidase, an important antibacterial property. In the presence of hydrogen peroxide, peroxidase adopts a potent bactericidal activity (Bujak and Root, 1974). Very low concentrations of lysozyme, found in the azurophilic granules of other species, are present in bovine azurophilic granules (Rausch and Moore, 1975; Paape *et al*., 2003). Secondary granules outnumber the primary granules in mature neutrophils, but the tertiary granules are the predominant granule in cattle and, like the secondary granule, are peroxidase-negative. These tertiary granules contain powerful, oxygen-independent

bactericidal proteins called β-defensins. These β-defensins inhibit both Gram-positive and –negative bacteria in addition to fungi and viruses. Certain inflammatory mediators, such as platelet-activating factor (PAF) and CXCL8 (formally IL-8), cause the secretion of lactoferrin from neutrophil secondary and tertiary granules into the phagosomes and extracellular environment (Swain *et al*., 2000). The high affinity of lactoferrin for iron results in the sequestration of iron, preventing its availability to Gram-negative bacteria. This technique in itself may not be enough to prevent bacterial uptake of iron as bacteria contain a number of iron-binding molecules known as siderophores that efficiently transport iron to the pathogen (Nathan, 2006). To counter these siderophores, neutrophils secrete lipocalin-2 which binds bacterial siderophores and prevents them from returning iron to the bacteria (Flo *et al*., 2004).

Neutrophils become activated upon receptor binding by cytokines, complement components and immunoglobulins. This results in the activation of the oxidative burst reaction, where neutrophils release large amounts of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Myeloperoxidase, located in the azurophilic granules, is released into phagosomes containing pathogens and acts to form hypochlorite from H_2O_2 and Cl^-, aiding in the destruction of pathogens (Paape *et al*., 2003; Janeway *et al*., 2005).

Oxygen radicals and proteases, released by neutrophils in the process of inflammation, serve to clear infection but are also cytotoxic to host tissues (Ledbetter *et al*., 2001). While neutrophils play an essential role in the clearance of pathogens by the immune system, prolonged exposure to activated neutrophils can result in severe tissue damage via prolonged inflammation (Boaventura *et al*., 2010). In order to regulate this tissue damage, rapid phagocytosis of apoptotic neutrophils by macrophages is required, reducing systemic tissue damage from inflammation (Chin *et al*., 2000). Sladek and

Rysanek (2001) demonstrated that neutrophil apoptosis plays a primary role in the resolution of inflammation in cattle. During this resolution of inflammation, neutrophils do not re-circulate as occurs with lymphocytes, rather they undergo apoptosis (Janeway *et al.*, 2005). Apoptosis, also known as programmed cell death, is morphologically characterised by chromatin condensation, nuclear fragmentation, cell shrinkage and blebbing of the plasma membrane (Reed, 2000) and is a protective mechanism that plays an essential role in the resolution of the inflammatory response (Haslett, 1999). The small, membrane-bound bodies that result from this process can be rapidly cleared by phagocytic cells such as the macrophage. Numerous pro-inflammatory cytokines, including IL-1β, IL-2, IL-6, CXCL8, IL-15 and IFN-γ slow the apoptotic rate, extending neutrophil survival (Akgul *et al.*, 2001; Paape *et al.*, 2003). Pro-inflammatory TNF-α has the opposite effect, inducing apoptosis in bovine neutrophils (Van Oostveldt *et al.*, 2002). However, the induction of apoptosis by TNF-α can be halted by exposure to lipopolysaccharide (LPS) (Hachiya *et al.*, 1995), indicating a role for TNF-α in removing activated neutrophils from sites of inflammation only after bacterial clearance.

1.4.2.1.3. Neutrophil response to stress

The bovine neutrophil is a direct target for stress hormones, as evidenced by its normal physiological expression of both glucocorticoid (Chang *et al.*, 2004) and functional adrenergic receptors (LaBranche *et al.*, 2010). Neutrophilia has been frequently reported in response to either endogenous GC by means of a stress response to weaning (Hickey *et al.*, 2003; Lynch *et al.*, 2010a; 2010b), transport (Buckham Sporer *et al.*, 2007; 2008a; Lomborg *et al.*, 2008; Riondato *et al.*, 2008), castration (Fisher *et al.*, 1997a; Ting *et al.*, 2003a; Pang *et al.*, 2009b) or exogenous GC following experimental challenge (Burton *et al.*, 2005; Weber *et al.*, 2006) (Table 1.4). This response can be

attributed to a series of physiological alterations in bovine neutrophil function including the release of a large number of reserve and immature neutrophils from the bone marrow (Paape *et al*., 2003; Jones and Allison, 2007), a reduction in the neutrophil apoptotic rate (Chang *et al*., 2004; Madsen-Bouterse *et al*., 2006; Weber *et al*., 2006; Buckham Sporer *et al*., 2007), an increase in neutrophil chemotaxis (Anderson *et al*., 1999) and a potential decrease in surface marker CD62L expression, reducing neutrophil margination along endothelial walls (Weber *et al*., 2004; Burton *et al*., 2005; Lynch *et al*., 2011). Pang *et al*. (2009) reported no alterations to surface expression of CD62L following either band or burdizzo castration, or following hydrocortisone infusion. Regardless, CD62L may not be as necessary for margination of leukocytes as was originally believed as it has been demonstrated that platelets can re-establish leukocyte trafficking in CD62L deficient mice (Diacovo *et al*., 1998).

The GR appears to be responsible for the vast majority of these GC induced effects on neutrophils (Chang *et al*., 2004; Weber *et al*., 2006). However, the GC bound GR also appears to play a role in enhancing phagocytosis of apoptotic neutrophils by macrophages which suggests that GC binding to the GR of macrophages plays a role in resolving the inflammatory process (Liu *et al*., 1999). Additionally, two of the above studies identified increased neutrophil expression of genes involved in tissue remodelling and wound healing following *in vitro* dexamethasone treatment (Burton *et al*., 2005; Weber *et al*., 2006) with a subsequent transport stress study identifying the same effect *in vivo* (Buckham Sporer *et al*., 2007).

Despite numerous studies indicating that GC is the main effector of neutrophilia following a stress response in cattle (Chang *et al*., 2004; Burton *et al*., 2005; Madsen-Bouterse *et al*., 2006; Weber *et al*., 2006; Buckham Sporer *et al*., 2007; Jones and

Allison, 2007; Lynch *et al*., 2010a; 2010b), it is also likely that catecholamines play a role via the adrenergic receptor (Stevenson *et al*., 2001; Engler *et al*., 2004). Engler *et al*. (2004) demonstrated that by removing the adrenal gland, and thus the secretion of GC, neutrophila still occurred in rats. However, this response was blocked by the exogenous adrenergic receptor antagonists phentolamine, propranolol and nadolol and indicated that either α- or β- adrengergic receptors could alter the distribution of neutrophils.

Further evidence of a role for stress in neutrophil function hails from the induction of a number of neutrophil chemoattractants, primarily CXCL8. This chemokine has been found to be up-regulated in response to transport (Buckham Sporer *et al*., 2007) and castration (Pang *et al*., 2009a) in cattle, and weaning in pigs (Pie *et al*., 2004). Apart from serving as a potent neutrophil chemokine, CXCL8 induces the release of alkaline phosphatase from secondary granules and the production of reactive oxygen species during chemotaxis, enhancing the bactericidal activity of the neutrophil (Galligan and Coomber, 2000). Transcriptomic analysis of transported bulls identified a reprogramming of neutrophil gene expression for greater bactericidal activity (Buckham Sporer *et al*., 2007; 2008a) although bactericidal activity has been shown to be unaffected by weaning (Lynch *et al*., 2010a). Another cytokine induced by weaning stress, TNF-α (Carroll *et al*., 2009), works synergistically with the complement component C5a at sites of inflammation to increase the rate of neutrophil phagocytosis (Rainard *et al*., 2000). This suggests that stress enhances neutrophil function by not only increasing circulating neutrophils, but also through enhanced phagocytosis and chemotaxis as evidence by increased chemokine expression. Nevertheless, not all studies have reported this effect with Lynch *et al*. (2010a) identifying a decrease in the phagocytic capacity of peripheral neutrophils following weaning. This is potentially

due to the measurement of peripheral neutrophils rather than activated neutrophils at the site of infection. It has also been reported that endogenous glucocorticoids do not adversely impact upon oxidative burst activity in bovine neutrophils, even at supraphysiological concentrations (Hoeben *et al.*, 1998; Pang *et al.*, 2009; Lynch *et al.*, 2010a). In fact, the oxidative burst activity of bovine neutrophils is actually enhanced by exposure to IFN-γ and TNF-α, both of which may be involved in a typical stress response (Hoeben *et al.*, 1998).

1.4.2.1.4. Lymphocyte function

Lymphocytes are typically considered to be part of the adaptive immune response. Common lymphoid progenitors differentiate into 4 major populations of mature lymphocytes: B cells, T cells, NK cells, and NK-T cells (Chaplin, 2010). B cells play a vital role in humoral immunity by producing antibodies against foreign antigens and operating as antigen-presenting cells (APC). This process occurs in unison with the work of T cells which are responsible for cell-mediated immunity. T cells possess T cell receptors (TCR) on their surface membrane which enables the identification of peptide antigens presented by either class I or class II major histocompatibility complex (MHC) proteins on infected cells. These T cell can be further subdivided into $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are associated with MHC class II molecules and function in the activation of the humoral response, stimulating B cells while $CD8^+$ cells interact with MHC class I molecules, reacting with cytotoxic effects upon identification of infected cells (Singer *et al.*, 2008).

Table 1.4. Distributional alterations in bovine leukocytes following husbandry stressors

Leukocyte	Stress	Response	Reference
Total leukocytes	Weaning	↑	Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b
		↔	Hickey *et al*., 2003b
		↓	Phillips *et al*., 1989
	Transportation	↑	Yagi *et al*., 2004, Buckham Sporer *et al*., 2007; Riondato *et al*., 2008
	Castration	↑	Fisher *et al*., 1997a; Earley and Crowe, 2002, Ting *et al*., 2003a; Pang *et al*., 2006; 2009b
	Dexamethasone challenge	↑	Menge and Dean-Nystrom, 2008
Neutrophils	Weaning	↑	Hickey *et al*., 2003b, Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b
	Transportation	↑	Yagi *et al*., 2004; Earley and O'Riordan, 2006; Earley *et al*., 2006; Buckham Sporer *et al*., 2007; Gupta *et al*., 2007b; Lomborg *et al*., 2008; Riondato *et al*., 2008
	Castration	↑	Fisher *et al*., 1997a; Ting *et al*., 2003a; Pang *et al*., 2009b
	Dexamethasone challenge	↑	Burton *et al*., 2005; Weber, 2006; Menge and Dean-Nystrom, 2008
Lymphocytes	Weaning	↓	Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b
	Transportation	↓	Earley and O'Riordan, 2006; Earley *et al*., 2006; Buckham Sporer *et al*., 2007; Gupta *et al*., 2007b
	Castration	↓	Ting *et al*., 2003
Monocytes	Weaning	↔	Lynch *et al*., 2010a; 2010b
	Transportation	↔	Riondato *et al*., 2008
	Castration	↑	Ting *et al*., 2003
Eosinophils	Weaning	↔	Lynch *et al*., 2010a
		↑	Lynch *et al*., 2010b
	Transportation	↑	Gupta *et al*., 2007
		↓	Riondato *et al*., 2008
	Castration	↔	Ting *et al*., 2003

↑ = increase, ↓ = decrease, ↔ = no significant change

1.4.2.1.5. Lymphocyte response to stress

Stress can alter lymphocyte function and distribution in a number of ways, as identified by numerous bovine stress studies (Van Kampen and Mallard, 1997; Dixit *et al*., 2001; Odore *et al*., 2004; 2011; Lynch *et al*., 2010a) (Table 1.4). Similar to neutrophils, bovine lymphocytes express both glucocorticoid (Preisler *et al*., 2000; Odore *et al*., 2004) and adrenergic (Abraham *et al*., 2004; Odore *et al*., 2004) receptors which act to regulate lymphocyte activity based on neuroendocrine signalling. Glucocorticoids have been shown to result in significant reductions in circulating lymphocyte number (Hickey *et al*., 2003; Lynch *et al*., 2010a). However, it also seems that catecholamines may contribute to the reduction in circulating lymphocytes (Hickey *et al*., 2003; Engler *et al*., 2004). Contrary to being immunosuppressive, the depletion of lymphocytes from peripheral circulation actually reflects increased extravasation to susceptible tissues rather than apoptosis or necrosis, indicating an increased capacity for immune surveillance (Dhabhar and McEwen, 1997; Kehrli *et al*., 1999; Viswanathan and Dhabhar, 2005).

The proportion of $CD4^+$ and $CD8^+$ T cells in peripheral circulation has been shown to decrease in calves following weaning (Lynch *et al*., 2010a) or 14 hours of truck transportation (Riondato *et al*., 2008). Additionally, lymphocytes presenting MHC class II molecules increased considerably following either weaning (Lynch *et al*., 2010a) or transportation (Riondato *et al*., 2008). This is likely due to GC action as demonstrated by a pharmacological challenge of Holstein bulls with dexamethasone, resulting in an increase in MCH class II presenting lymphocytes (Saama *et al*., 2004).

Additionally, lymphocytes possess receptors for ACTH, the pituitary derived hormone responsible for GC secretion from the adrenal gland (Dixit *et al*., 2001). Stefano *et al*.

(1996) demonstrated that ACTH can regulate the immune system through direct effects on cellular targets independent of its capacity to stimulate GC secretion from the adrenal gland. In fact, lymphocytes can actually synthesise ACTH, particularly in response to stress, and this autocrine action may be an important part of this process (Smith and Blalock, 1981; Clarke *et al.*, 1993; Dixit *et al.*, 2001). This has most clearly been highlighted following transportation stress, whereby ACTH secretion from non-stimulated lymphocytes increased in cows transported for 14 hours (Dixit *et al.*, 2001). Lymphocyte derived ACTH remained elevated in cows left on the truck for 24 hours following transportation while cows that were off-loaded had baseline ACTH concentrations following 24 hours of rest. This suggests that lymphocyte derived ACTH may not follow the cyclical nature of pituitary derived ACTH and may be a more reliable biomarker of stress.

1.4.2.1.6. Platelet functioning beyond haemostasis

Haemostasis is an important platelet mediated innate immune component primarily intended to arrest the bleeding process (Verhamme and Hoylaerts, 2009). Nevertheless, recent evidence suggests that in addition to preventing excessive bleeding, platelets may function with other innate immune cells to induce haemostasis in capillary beds and veins in order to exclude infection, preventing propagation of blood borne pathogens and sacrificing damaged tissues to ensure host survival (Weyrich and Zimmerman, 2004; Alcock and Brainard, 2008; Delvaeye and Conway, 2009; Ivanov and Gritsenko, 2009; Smyth *et al.*, 2009; Yeaman, 2010), indicating the tight link between platelets and inflammation (Verhamme and Hoylaerts, 2009). This process is achieved through the expression of adhesive and immune receptors on the platelet surface, along with the secretion of a large number of cytokines, chemokines and other inflammatory mediators

(Von Hundelshausen and Weber, 2007; Von Hundelshausen *et al*., 2009; Gleissner *et al*., 2008).

In response to tissue damage, collagen is released into vessels, acting as a potent agonist of platelets and other leukocytes (Crowley *et al*., 2007). Collagen peptide precursors have also been shown to reduce in concentration following exposure to the stress hormone cortisol (Kahan *et al*., 2009). IL-1, which can be synthesised by activated platelets in response to stress, rapidly activating the innate immune system (Lindemann *et al*., 2001), has been shown to up-regulate the expression of collagenase genes such as matrix metalloproteinases (MMP), responsible for the degradation of collagen peptide bonds (Burrage *et al*., 2006). It is likely that the glucocorticoid induced reduction in the expression of vessel wall collagens, along with the platelet and other leukocyte-derived, cytokine-induced secretion of collagenases, aids in the rapid migration of leukocytes through the extracellular matrix (ECM) to sites of inflammation (Barreiro *et al*., 2007). Platelets can also induce epithelial cells to secrete the potent neutrophil chemoattractants CXCL7 and CXCL8 (Brandt *et al*., 2000; Gleissner *et al*., 2008). Platelet-induced epithelial secretions of CXCL8 are typically accompanied by the monocyte chemotactic, CCL2 (Henn *et al*., 1998). However, CXCL5, typically produced in response to IL-1 (McLoughlin *et al*., 2004; Craig *et al*., 2009), is produced concomitantly with CXCL8 and is also known as a neutrophil chemoattractant and activator which demonstrates the important role of platelets in neutrophil activation and chemotaxis. CXCL8 is one of the primary mediators of inflammation, typically associated with the inflammatory response to bacterial infection (Hirao *et al*., 2000). Increased mRNA levels of this important neutrophil chemokine have been reported in the bovine, coinciding with an initial surge in neutrophil number following transport stress (Buckham Sporer *et al*., 2007). CXCL8 is primarily secreted by macrophages at

sites of inflammation (Waugh and Wilson, 2008) although activated platelets can also secrete this chemokine (Gleissner *et al*., 2008).

These chemokines are part of a small family of CXC chemokines (Craig *et al*., 2009) which share two common GPCR, CXCR1 and CXCR2, and have highly similar ligand functionalities (Cascieri and Springer, 2000). It is likely that CXCL7 may reduce signalling of several of these chemokines as following the induction of CXCL7, it down-regulates CXCR2 in neutrophils, resulting in a rapid negative feedback mechanism (Petersen *et al*., 1994; Gleissner *et al*., 2008).

The integrin α2β1 is a cell surface receptor available in high concentrations on platelets and other leukocytes and is mainly involved in firm adhesion of platelets to damaged endothelium (Pugh *et al*., 2010; Nuyttens *et al*., 2011), although it has also been implicated in the "outside-in" activation of platelets (Samaha and Kahn, 2006), further serving a role in neutrophil locomotion in extravascular tissue (Werr *et al*., 2000).

1.4.2.1.7. Signalling by G-protein-coupled receptors

G-protein-coupled receptors (Figure 1.9) are one of the most essential components involved in the transference of extracellular stimuli into intracellular signals, being responsible for the regulation of the physiological response to hormones, neurotransmitters, cytokines, chemokines and ions (Kroeze *et al*., 2003; Rosenbaum *et al*., 2009). In its inactive state, the Gα subunit of the GPCR is guanosine diphosphate (GDP) bound and forms a further tight bond with the Gβγ heterodimer (Evanko *et al*., 2001). Upon ligand binding of the GPCR, conformational changes of the Gα subunit promote the release of GDP and the binding of guanosine-5'-triphosphate (GTP) by acting as a guanine nucleotide exchange factor (GEF) (Wall *et al*., 1998). This process

initiates signalling cascades by both the GTP-bound Gα subunit and the free Gβγ heterodimer. Signalling is arrested by guanosine triphosphatase (GTPase) activity, resulting in the hydrolysis of GTP to GDP (McCudden *et al*., 2005). A family of GTPase-accelerating proteins (GAPs), known as the regulators of G-protein signalling (RGS) proteins, have been identified as catalyzing rapid GTP hydrolysis, resulting in a desensitisation of the heterotrimeric GPCR pathway (Neubig and Siderovski, 2002) and are also implicated in the rapid activation and deactivation of GPCR signalling (Saitoh *et al*., 1999).

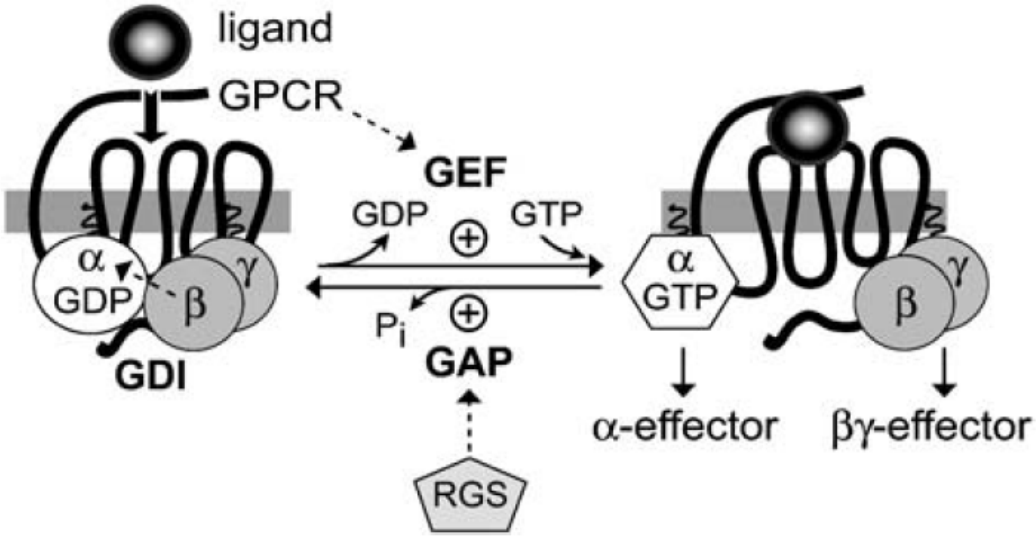

Figure 1.9. A schematic representation of G-protein-coupled receptor signalling. GPCR signalling is initiated by guanine nucleotide exchange factors (GEF) on ligand binding, inducing a conformational change of the Gα subunit allowing the exchange of GTP for GDP. The Gβγ heterodimer dissociates from the Gα subunit and both can signal separate effectors (McCudden *et al*., 2005).

1.4.2.2. Hormones of the neuroendocrine system

The HPA axis is the primary axis involved in the stress response and secretes a number of hormones that have been used as biomarkers. The pituitary derived stress hormone ACTH, which is responsible for glucocorticoid secretion from the adrenal gland, has

also been shown to have direct input on a number of immune cell targets (Stefano *et al*., 1996). ACTH has been shown to increase during transport stress, highlighting its potential use as a biomarker in future studies of stress (Dixit *et al*., 2001; Knights and Smith, 2007). Cortisol is perhaps the best known biomarker of stress and can be rapidly measured (Sapolsky, 2000). However, it is relatively variable between individual animals as well as between breed, gender and age, following a strong ultradian rhythm (Lefcourt *et al*., 1993) and highly frequent sampling times (~15 minutes) are required to accurately quantify a cortisol response. However, numerous bovine studies have successfully used cortisol measurements as an adjunct to other measurements in order to assess welfare in cattle, including studies examining weaning (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b), transport (Yagi *et al*., 2004; Aich *et al*., 2007; Buckham Sporer *et al*., 2007; 2008a; Gupta *et al*., 2007b), castration (Pang *et al*., 2006) and regrouping (Gupta *et al*., 2007a). The ratio of cortisol to dehydroepiandrosterone (DHEA) is also sometimes used as a biomarker of stress in weaning (Lynch *et al*., 2010b) and transport (Buckham Sporer *et al*., 2008b) studies. DHEA has been implicated in the stress response (Zinder and Dar, 1999) as a balance to the assumed immunosuppressive effects of cortisol (Saccò *et al*., 2002; Bauer, 2005). A reduction in DHEA concentration has been shown to occur with an increase in cortisol concentration in a number of studies (Buckham Sporer *et al*., 2008). Catecholamines are also used to assess the activation of the HPA stress axis in livestock (Griffin, 1989) with both epinephrine and norepinephrine exhibiting increases in response to weaning in cattle (Hickey *et al*., 2003b).

1.4.2.3. Acute phase proteins

Acute phase proteins are used in the assessment of stress in cattle due to their response to glucocorticoids, although contradictory results have been produced regarding the

APR. Abrupt weaning, social disruption and transport stress have been shown to induce an increases in the APP serum amyloid A (SAA), fibrinogen, haptoglobin and ceruloplasmin in cattle (Arthington *et al*., 2005; Hickey *et al*., 2003b; Murata, 2007; Qiu *et al*., 2007; Lomborg *et al*., 2008; Aich *et al*., 2009; Carroll *et al*., 2009; Lynch *et al*., 2010b). In another study, haptoglobin and fibrinogen were reported to decrease following 9 hours of transport (Buckham Sporer *et al*., 2008b) while Arthington *et al*. (2003) demonstrated that haptoglobin decreased while SAA, fibrinogen and ceruloplasmin increased following transport and comingling. Lomborg *et al*. (2008) found SAA increased following transport and isolation, but haptoglobin only increased slightly, and only in 5 of the 8 animals. These results suggest that while an APR appears to be detectable following exposure to stress in cattle, the direction of change is not always consistent, although they tend to increase. Therefore, caution must be adhered to when using APP as biomarkers and it is necessary to use them in unison with other, well characterised biomarkers.

1.4.2.4. The proteome

Proteomics is at the forefront of biomarker discovery with proteins having a massive potential to serve as biomarkers for stress as well as numerous disease states (Thadikkaran *et al*., 2005; Rifai and Gerszten, 2006). Effectively, this allows detection of the actual transcribed message, eliminating the ambiguity that can occur at the transcriptomic level where transcripts can be degraded or modified prior to translation and alternate splicing can result in multiple protein products. The plasma proteome, easily accessible through the collection of blood, surprisingly represents the expression of almost all of the protein species from tissues throughout the body (Anderson *et al*., 2004). While proteomics has yet to be aggressively applied to the detection of biomarkers in cattle, several proteomic based approaches have been used to identify

biomarkers of weaning stress in cattle (Herzog, 2007), bovine spongiform encephalopathy (BSE) from urine samples (Simon *et al*., 2008), distinguish between subclinical mycobacterial infections (Seth *et al*., 2009), identify proteomic biomarkers for BRD fatality when challenged with BHV-1 and *Mannheimia haemolytica* following transport (Aich *et al*., 2009) and also for muscle growth in cattle (Ikegami *et al*., 2008; Keady *et al*., 2011). Unfortunately, proteomic technology lags behind that of genomics, lacking both the sensitivity and the large dynamic range that is required to accurately identify and characterise proteomic biomarkers, although this is rapidly changing (Diamandis and van der Merwe, 2005; Koomen *et al*., 2005; Rifai and Gerszten, 2006). Therefore, the search for biomarkers has focused on genomic studies, principally using transcriptomics to identify genes that are responsible for the regulation of physiological processes of interest.

1.4.2.5. The transcriptome

Understanding the transcriptome can provide a great deal of insight into the functioning of an organism. Given recent advances in genome sequencing technologies (Marioni *et al*., 2008), the field of transcriptomics currently provides unparalleled potential to understand key physiological processes and identify biomarkers. Numerous genes that play a role in the stress response have already been identified (Tempelman *et al*., 2002; Chang *et al*., 2004; Weber *et al*., 2004; 2006; Madsen-Bouterse *et al*., 2006; Buckham Sporer *et al*., 2007; 2008a). Future work will contribute to the identification of multiple genes which can be incorporated into pathway analysis tools or multivariate analysis to detect gene signatures for disease. One of the shortcomings of using the transcriptome for biomarker discovery is that the presence of a transcript does not necessarily imply the presence of its respective protein (Vogel *et al*., 2010). Nevertheless, so long as a gene biomarker presence is sensitive and accurate, it does not necessarily have to

translate to a protein level (Aronson, 2005). This is because the increased expression of a gene may be in response to a physiological process of interest, even if the transcript itself does not have a physiological effect.

1.4.2.6. Other potential biomarkers of stress

A number of other hormones and neurotransmitters play a role in the stress response and may be functionally suited to biomarker utilisation in the bovine (Bergquist *et al*., 1994; Yang and Glaser, 2000; Neufeld-Cohen *et al*., 2010). Urocortin, a member of the CRH peptide family, binds with greater specificity to the type 2 CRH receptor and has been shown to play an integral role in stress recovery, with knockout mice showing increased anxiety levels 24 hours following a stressor when compared with stressed wild type mice (Weninger *et al*., 1999; Neufeld-Cohen *et al*., 2010). It has also been demonstrated that lymphocytes can synthesise dopamine, and that dopamine can inhibit lymphocyte function, indicating that dopamine may also alter immune function and serve in a negative feedback loop when it is secreted during a stress response (Bergquist *et al*., 1994). Meanwhile, stress has been shown to increase β-endorphins, promoting the response of T cells (Moynihan *et al*., 2000) while growth hormone may be suppressed (Chrousos, 2009).

1.5. Weaning stress in calves

1.5.1. Introduction

The weaning process is unique to mammals, characterised by a gradual shift from total nutritional dependence of offspring on their mother to nutritional independence. A steady decrease in the availability of milk from the mother, combined with a developing digestive system means that offspring increase their intake of solid food over a period of time. However, weaning covers more than just nutrition, and offspring are effectively

weaned from their social dependence on their mother as much as their nutritional dependence (Veissier and Neindre, 1989; Veissier *et al*., 1990; Lee, 1996). Domestic cattle form relatively strong mother-young bonds, with this bond solidifying between 1 and 4 days of birth (Stehulova *et al*., 2007). While it is difficult to identify the age of natural weaning in domestic cattle, it is known that there are species, breed and sex differences in this age (Reinhardt and Reinhardt, 1981; Gonyou and Stookey, 1987). It has been proposed that natural weaning typically occurs in ruminants once the offspring reaches a weight equivalent to four times its birth weight (Lee *et al*., 1991). One of the few studies to examine the age of weaning in cattle focused on *Bos indicus*, identifying weaning to occur between about 9 and 14 months of age (Reinhardt and Reinhardt, 1981). These authors found that the cows prevented their female offspring from suckling beginning around 9 months, but allowed their male calves suckle for a further 2 and a half months. However, they also noted that following weaning, the calf remains within close proximity to the cow for a number of months, gradually reducing their reliance on the cow for social direction, although female calves may maintain a stronger social bond with their mother throughout their life.

Weaning is a multi-factorial stressor, as demonstrated by the multi-component nature of the weaning process where calves are exposed to nutritional, physical and psychological stress (Budzynska *et al*., 2008; Weary *et al*., 2008; Lynch *et al*., 2010a). These stressors include early, abrupt weaning and separation of the calf from its dam, a new solid diet, social disruption and reorganisation, novel environment, handling and the accompanying husbandry practices that these bring including castration, dehorning, transportation and marketing.

While abrupt weaning involves permanent separation of the calf from its dam, several other weaning methods have been adopted aimed at reducing stress in calves at this time. Fenceline weaning involves moving calves to a field adjacent to their dams where they are only separated by a fence (Price *et al*., 2003; Rasby, 2007; Siegford *et al*., 2007; Enriquez *et al*., 2010). This prevents suckling but allows the animals visual, auditory and at times, nose to nose contact. Another method, progressive weaning, involves moving a small number of calves each day, initially to a field adjacent to their dams, followed by moving them to a pasture further away (Church and Hudson, 1999). This reduces weaning stress because only a small number of calves are weaned at any one time, preventing a group mentality of panic while also including some components of fenceline weaning practices. Another weaning method, using anti-suckling devices (nose-clips), allows the calf to remain with its dam while it is being nutritionally weaned (Boland *et al*., 2008; Burke *et al*., 2009; Enriquez *et al*., 2010). The nose-clip stops the calf from suckling, encouraging it to switch to a solid diet. While each method has been hailed as reducing the stress associated with abrupt weaning, Enriquez *et al*. (2010) found no overall reduction in stress came from either fenceline or nose-clip weaning methods with calves displaying increased behaviours associated with frustration. These authors found that this was because following nutritional weaning, the calves still had to be separated from their dams, resulting in a further traumatic event and extending the period of stressor exposure.

1.5.2. Behavioural response to weaning

Weaning results in a number of behavioural responses that have been used to assess welfare and may vary based on age and pre-weaning diet (Pollock *et al*., 1991). Church and Hudson (1999) found increased vocalisations from abruptly weaned calves versus those in a progressively weaned treatment. Price *et al*. (2003) reported that the

frequency of vocalisation in abruptly weaned calves is over 2000 times more than that of unweaned control calves. Both of these groups also found abruptly weaned calves spent significantly more time walking and less time eating than controls calves. These indicators of stress are at their most extreme during the first 2 days following weaning, and the gradual decrease in their magnitude may indicate adaptation to their new situation (Price *et al*., 2003; Boland *et al*., 2008). This is an important point as weaning is a unique husbandry stressor in that it is much longer lived than other events, such as castration and transportation, and has no defined end, requiring adaptation on behalf of the calf rather than mere tolerance of the situation as would occur with a stressor of defined length, such as transportation. Immediately following weaning, calves' increase the amount of time spent walking, although this decreases over the first 3 days following weaning (Lynch *et al*., 2011). These authors also reported a gradual increase in the time spent at both silage and concentrate meal troughs up to 7 days post weaning. Stehulova *et al*. (2007) found increased vocalisation, licking and locomotor activity of early weaned calves, corresponding to the length of time left with the cow prior to weaning (1, 4 or 7 days). Unique vocalisation signatures, both in frequency and duration, favour communication between specific cow-calf pairs, although vocalisation is relatively low when the cow is present (Thomas *et al*., 2001). It has also been suggested that the stress of weaning, coupled with social reorganisation, may impair learning and memory, making it difficult for calves to remember the outcome of fights or remember opponents, thus leading to prolonged aggression (Croney and Newberry, 2007; Newberry and Swanson, 2008).

1.5.3. Physiological response to weaning

Numerous studies have identified physiological indicators of stress following weaning of beef calves. These physiological mechanisms are in place to drive specific

behaviours in calves, thus ensuring the calf seeks out maternal care and attention (Enriquez *et al*., 2011). Beef calves, which are traditionally reared with their dam until weaning, form much stronger cow-calf bonds than dairy breeds (Le Neindre, 1989). It has been demonstrated that this bond can last periods of separation, with eight month old heifers showing a minimal reduction in social interactions with their dams following three weeks of separation, despite being nutritionally weaned (Veissier and Le Neindre, 1989). As the process of recognition and bonding is principally driven by hormone and neuroendocrinological mediators including dopamine, gonadal steroids, oxytocin, prolactin, vasopressin and other endogenous opioids, it is reasonable to predict that some degree of information on the cow-calf bond can be drawn from their measurements, indicating strength of the bond (Nelson and Panksepp, 1998). Oxytocin appears to have an important role in bond maintenance and has been associated with suckling in a number of mammalian species. Lupoli *et al*. (2001) found oxytocin was secreted in calves while they were suckling the dam but not when fed milk from a bucket, indicating that the presence of the dam is required for oxytocin release. Interestingly, it has been found that cows' milk contains α-lactophorin, a tetra-peptide with structural homology to endogenous opioids and a medium affinity to opioid receptors (Yoshikawa *et al*., 1986). This has led to speculation that the phase of calf attachment to the cow may be similar to that of opiate addiction and that weaning encompasses a component of withdrawal symptoms (Nelson and Panksepp, 1998).

Plasma concentrations of cortisol (Lay *et al*., 1998; Blanco *et al*., 2003; Hickey *et al*., 2003b; Loberg *et al*., 2008), epinephrine (Lefcourt and Elsasser, 1995) and norepinephrine (Lefcourt and Elsasser, 1995; Hickey *et al*., 2003b) have been shown to increase in beef calves following weaning. However, plasma cortisol has proved to be unreliable as several weaning studies failed to detect any significant alteration of this

glucocorticoid (Lynch *et al*. 2010a). It is possible this is due to breed differences as breed has been shown to have a significant effect on baseline cortisol levels in beef calves (Zavy *et al*., 1992), although the issue is more likely to do with the low frequency of blood sampling employed in these studies as frequent measurements are necessary to accurately assess cortisol concentrations.

The acute phase protein response to weaning of beef calves has not been clearly characterised and there appears to be differences in their response. Fibrinogen, haptoglobin, ceruloplasmin and SAA have been shown to be activated in response to weaning (Arthington *et al*., 2003; 2008; Blanco *et al*., 2009; Carroll *et al*., 2009; Lynch *et al*., 2010a). However, others have failed to find differences in plasma fibrinogen (Hickey *et al*., 2003b; Lynch *et al*., 2010a) and haptoglobin concentrations (Hickey *et al*., 2003b) following weaning of beef calves. Other physiological alterations of beef calves in response to weaning stress include increased heart rate (Loberg *et al*., 2008) and rectal body temperature (Lynch *et al*., 2010a; 2010b).

1.5.4. Leukocyte response to weaning

Total leukocyte number has been reported to increase following abrupt weaning in beef calves (Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b) (Table 1.4). However, Phillips *et al*. (1989) reported a decreased total leukocyte number while Hickey *et al*. (2003b) reported no difference following weaning. It has been suggested that the simultaneous increases and decreases of a number of leukocyte subpopulations can leave total leukocyte number relatively unchanged, although a slight increase is often identified as a result of neutrophilia (Jones and Allison, 2007). Increased neutrophil number, concurrently presenting with a decreased lymphocyte number and the resultant increase in the N:L ratio has also been frequently reported following weaning of beef calves (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b). Lynch *et al*.

(2010a) also demonstrated that the number of circulating neutrophils performing phagocytosis and positive for surface expression of CD62L significantly decreased following weaning although oxidative burst was unaffected. $CD4^+$ and $CD8^+$ lymphocytes decrease following abrupt weaning, although in different proportions, resulting in a transient increase in the $CD4^+$:$CD8^+$ ratio while there were no functional alterations in IFN-γ (Lynch *et al*., 2010a). MHC^+ lymphocytes also increased in response to either housing or abrupt weaning, although the increase was greater in abruptly weaned calves at housing.

1.6. Transcriptomics as a tool to understand weaning stress

1.6.1. Introduction

The study of protein-coding gene transcripts, also known as transcriptomics, provides unique and novel insight into biological functions by allowing the identification and quantification of expressed genes. Recent advances in the field of genomics have moved transcriptomics into a position where results can be garnered relatively cheaply and in a short period of time. The Bovine Genome Sequencing and Analysis Consortium released the sequence, annotation and comparative analysis of the bovine genome (7.1 fold coverage) which covers 29 autosomes and two sex chromosomes (Elsik *et al*., 2009). The bovine genome contains almost 3 billion base pairs and identifies the presence of a minimum of 22,000 protein-coding genes. Of these genes, an estimated 80 % have human orthologs. This has made the design of primers for use in RT-qPCR along with probes on microarrays a much easier and more reliable affair. However, it is perhaps the application of the sequenced bovine genome to such innovative technologies as the Illumina Genome Analyser II (GA_{II}) that has really accelerated progress within the transcriptomic field in recent years.

1.6.2. The transcriptome

Unlike the genome which remains fixed throughout an organisms lifetime, the transcriptome constantly changes in response to environmental cues with mRNA accounting for between 2-5 % of the transcribed genome (Frith *et al*., 2005). Despite this, the transcriptome is responsible for a greater level of complexity than the genome (Adams, 2008). A number of translational modifications can occur including alternative splicing of exons, RNA editing and alternative transcription initiation and termination sites, meaning a single gene can produce numerous mRNA transcripts, and thus proteins. This helps to explain why an estimated 22,000 genes result in over 300,000 protein products (Anderson *et al*., 2004; Gerszten *et al*., 2008).

Transcription involves the synthesis of a complementary RNA copy from a sequence of DNA (Figure 1.10). This process begins when helicase breaks the hydrogen bonds between the complementary nucleic acids of DNA, unwinding a small portion of the DNA double helix. RNA polymerase enzymes bind to the single-stranded DNA template and begin transcribing at specific promoter regions upstream of the sequence by pairing ribonucleotides to the DNA template, producing a complementary, antiparallel RNA strand in the 5'-to-3' direction. While RNA and DNA share the three nucleic acids guanine (G), cytosine (C) and adenine (A), the fourth nucleic acid, thymine (T), is substituted for uracil (U) in RNA. The hydrogen bonds connecting the newly formed RNA transcript to the DNA template are broken just behind the RNA polymerase enzyme, releasing an immature RNA transcript and allowing the DNA helix to reform. This is a rapid process, occurring at a rate of about 20 nucleotides per second. Additionally, multiple RNA transcripts can by synthesised simultaneously, with additional RNA molecules being started before the first RNA strand is complete.

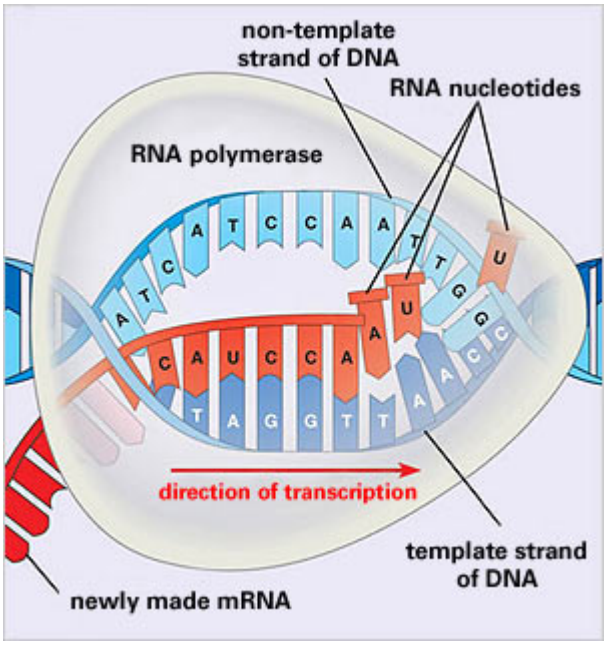

Figure 1.10. The initial step in transcription involves RNA polymerase synthesising RNA from a DNA template

The helicase activity of RNA polymerase breaks the hydrogen bonds of DNA, allowing RNA polymerase to produce a complementary RNA transcript by pairing ribonucleotides to the DNA template strand.

The most common RNA molecule studied in transcriptomics is mRNA, which is responsible for protein synthesis via translation, and forms part of the central dogma of molecular biology (DNA → RNA → Protein). The process of transcription described above is only the first step in producing an mRNA transcript. In order for a cell to assess if an mRNA fragment is intact and ready to be exported from the nucleus, it checks for the presence of capping. Once the RNA polymerase has produced approximately 25 nucleotides of RNA, the 5' end is modified by the addition of an mRNA 5'-methyl cap (Figure 1.11), allowing its recognition as an mRNA molecule. The transcripts that are synthesised by the RNA polymerase are effectively complementary copies of the DNA sequence of a gene. However, genes are made up of two distinct regions, intronic and exonic regions. Most genes contain a number of both introns and exons, but introns are non-coding, intervening sequences and it is only exons that contain a protein-coding sequence, generally accounting for only a small

portion of the length of the gene. Therefore, RNA splicing is required to remove introns from the newly synthesised pre-mRNA transcript. The spliceosome identifies splicing signals on pre-mRNA molecules, notably, the 5' splice site, the 3' splice site and the branch point in the intron sequence which forms the base of the excised lariat. This intron lariat is excised and is degraded in the nucleus while the exons on either side of the splice site are joined together. This proves a useful method in the production of different transcripts from the same gene, where two or more introns surrounding one or more exons are spliced out of the pre-mRNA molecule, resulting in an altered transcript. This process begins while the RNA molecule is still being elongated by RNA polymerase at the 3' end, but can continue after the transcript is complete. Several components of the spliceosome are carried on the phosphorylated tail of RNA polymerase and transfer directly to the nascent pre-mRNA, allowing the cell to keep track of introns and exons by delineating the intron-exon boundaries. Specific DNA sequences at the end of a gene are transcribed into RNA which the RNA polymerase recognises as a stop signal. This results in the cleavage of the RNA transcript, followed by the addition of approximately 200 A nucleotides to the cleaved 3' end by an enzyme known as poly-A polymerase, resulting in a mature mRNA transcript with introns spliced out, a 5' cap and a 3' polyadenylated end. The mature mRNA transcript is then exported from the nucleus and into the cyotsol via the nuclear pore complex where it is then translated into a protein (Alberts *et al*., 2002).

1.6.3. RT-qPCR

Quantitative Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) is used to measure gene expression as well as the detection of single nucleotide polymorphisms (SNP) and pathogens (Kubista *et al*., 2006). The development of RT-

qPCR in 1992 revolutionised molecular biology, allowing simultaneous and accurate detection of specific DNA sequences during amplification (Higuchi *et al.*, 1992).

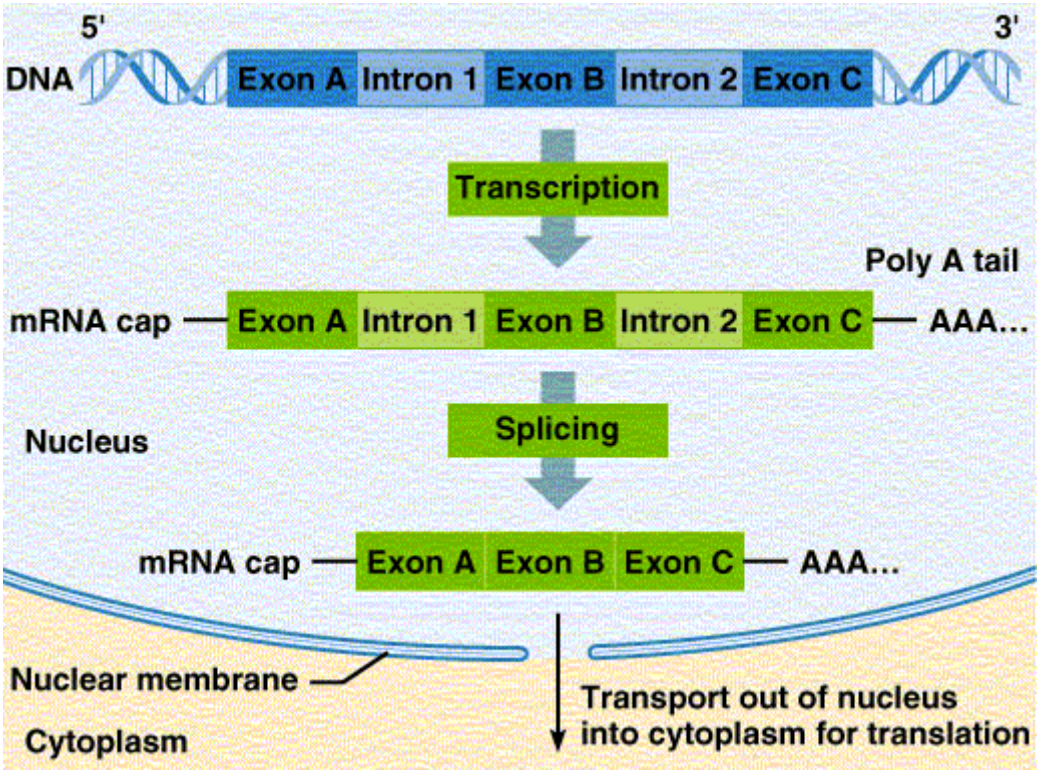

Figure 1.11. A number of steps are involved in the processing of pre-mRNA into a mature mRNA transcript.

RNA polymerase transcribes a pre-mRNA transcript containing both protein-coding exons and non-coding introns. An mRNA 5'-methyl cap is added to the 5' end of the transcript and the spliceosome then works to remove introns, and occasionally exons, before a poly A tail is added to the 3' end, resulting in a mature mRNA transcript ready for translation in the cytoplasm (Weaver, 2008).

1.6.3.1. Polymerase Chain Reaction

The basic principle of RT-qPCR is based on that of PCR, first developed by a group led by Kary Mullis (Saiki *et al.*, 1985) who was awarded the Nobel Prize for Chemistry in 1993. A PCR reaction can be performed on either a single or double-stranded DNA template. The reaction is prepared which includes a DNA sample, a forward and reverse oligonucleotide primer, dNTP mix, a heat-stable polymerase and magnesium

ions (Kubista *et al.*, 2006). The primers bind to either side of the DNA region to be amplified while the dNTP mix contains the four nucleotide triphosphates. A temperature cycling reaction is then performed on the sample. A high initial temperature 'melts' or denatures the strands of helical DNA, separating them from one another, following which the temperature is lowered to allow the primers to anneal the template DNA. The temperature is then raised to about 72 ˚C to allow the polymerase to extend the primers by incorporating the dNTP mix. The denaturation, annealing and elongation steps are repeated multiple times, effectively doubling the specific region of interest with each cycle. This allows detection of a specific gene or region from a sample containing the full genomic complement of genes.

1.6.3.2. RT-qPCR reaction

RT-qPCR is used to accurately quantify gene expression by first synthesising cDNA from mRNA samples. This method uses the principle of PCR but also adds a fluorescent reporter that binds to the product formed by amplification (Bustin, 2002). This fluorescent reporter generates a fluorescent signal that reflects the amount of product in the sample. A weak initial signal which is indistinguishable from background gives way to the exponential growth phase of the reaction after several PCR cycles. During the growth phase, the response curves reflect the initial amount of template cDNA (Kubista *et al.*, 2006). A threshold fluorescence signal level is set in each reaction and the number of amplification cycles required for the response curve of each sample to reach this threshold is known as a quantification cycle (Cq) value (Bustin *et al.*, 2010). The amplification response curves are expected to be parallel during the growth phase,thus the cycle threshold value allows the difference in starting cDNA template to be accurately quantified between samples (Deepak *et al.*, 2007) (Figure 1.12).

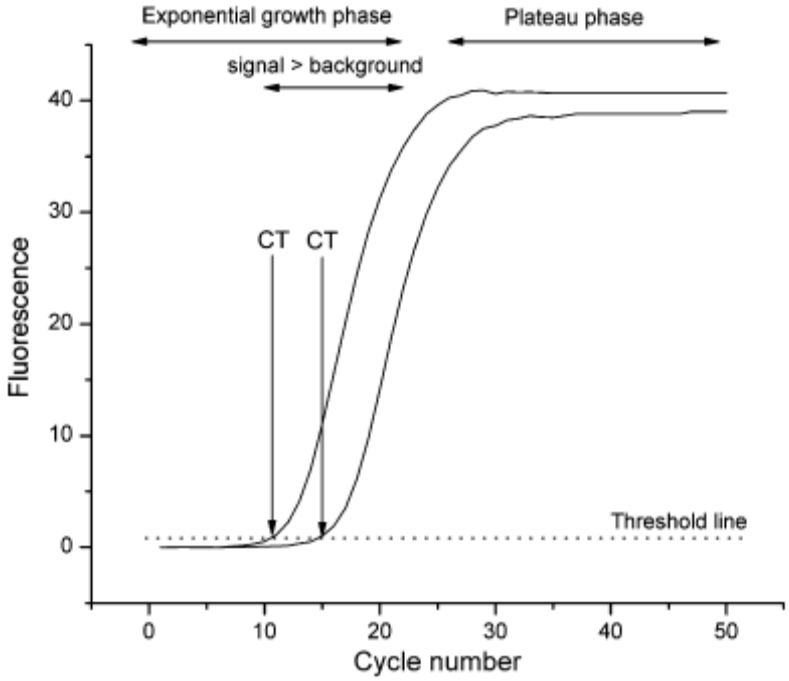

Figure 1.12. RT-qPCR response curves.

The response curves from two differentially expressed samples show how the Cq values are selected during the early exponential growth phase when the amplification curves are equal (Kubista *et al*., 2006).

1.6.4. Next generation RNA-seq

While RT-qPCR is a useful tool for examining differential gene expression in a small subset of selected genes, expression microarrays have long been the most widely used technology to examine global transcriptome expression (Marioni *et al*., 2008; Mortazavi *et al*., 2008; Wang *et al*., 2009). Microarray data, however, can be unreliable as a result of high background noise, cross-hybridization issues and dye-based detection. Further confounding the issue is the great degree of variation that exists between platforms and an inability to comprehensively include all possible RNA transcripts or identify alternative splicing or single nucleotide polymorphisms (SNP) in the same experiment (Mortazavi *et al*., 2008). However, time is required to assess the full utility of next-generation sequencing (NGS) technologies, and it will be necessary to identify their shortfallings.

The development of a new massively parallel technique for sequencing the transcriptome, known as RNA-seq, has opened up a number of novel opportunities to examine gene expression that were previously unavailable with conventional microarrays (Cloonan and Grimmond, 2008). RNA-seq provides a unique opportunity to deeply sequence the transcriptome of any species with single base resolution (Cloonan and Grimmond, 2008) and without the need for a known genome sequence, allowing unparalleled, highly accurate quantification of differential gene expression in addition to the identification of novel or unannotated genes, transcripts and alternative splicing events (Marioni *et al*., 2008; Mortazavi *et al*., 2008; Chen and Zheng, 2009; Wetterbom *et al*., 2010). It has rapidly become a more reliable and advantageous technology than microarrays (Marioni *et al*., 2008). To date, this technology has been employed in transcriptome sequencing and characterisation projects with little emphasis on differential gene detection between treatments, preferring to characterise gene expression and novel splicing isoforms in specific tissues (Mortazavi *et al*., 2008; Graveley, 2010; Wetterbom *et al*., 2010; Turro *et al*., 2011) (Table 1.5). Thus far, there have been few, if any, studies to attempt the examination of differential gene expression in the bovine using RNA-seq

Table 1.5. Recent research employing RNA-seq technology to examine the transcriptome.

Species	Description	Library details	Reference
Drosophila	Examined gene and transcript isoform expression differences between males and females heads to determine how the sex hierarchy generates somatic sex differences.	Genome Analyzer I 6 × 36 bp single end lanes 2 × biological replicates per treatment (male, female, *tra* mt)	Chang *et al*., 2011
Medicago sativa (Alfalfa)	Aimed to identify genes and polymorphisms and profile the transcriptome of alfalfa in order to contribute to the understanding of its use as a cellulosic ethanol feedstock.	Genome Analyzer II 6 × 75 bp single end lanes	Yang *et al*., 2011
Human, chimpanzee, macaque	Compared the transcriptomes of 3 males and 3 females from humans, chimpanzees and macaques to identify differing expression patterns via alternative splicing as well as copy number variation (CNV).	Genome Analyzer II 36 × 36 bp single end lanes	Blekhman *et al*., 2010
Bovine	Transcriptomic survey of 92 cattle with samples hailing from 15 different tissues and 5 distinct breeds.	Genome Analyzer IG 18 bp single end lanes	Harhay *et al*., 2010
Bovine	Comparison of gene expression between viable bovine embryos and those failing to transition to blastocyts. Twenty embryos from each group were pooled for analysis.	Genome Analyzer IIx 1 × 75 bp single end lane 1 × 81 bp paired end lane	Huang and Khatib, 2010
Chimpanzee	Sought to identify novel exons and transcribed regions in the chimpanzee brain and liver transcriptome and quantify known regions.	SOLiD platform 4 × 35 bp slides 2 × brain, 2 × liver	Wetterbom *et al*., 2010

1.6.4.1. Sequencing by synthesis on the Illumina Genome Analyzer II

RNA-seq involves the sequencing of small (18-150 bp) cDNA libraries prepared from fragmented mRNA. Universal adapters ligated onto the ends of each library bind to PCR primers that have been immobilized onto a glass slide, better known as a flowcell (Fedurco *et al*., 2006; Shendure *et al*., 2008). Originally developed by Manteia Predictive Medicine, this method involves attaching 5'-aminated DNA primers and templates onto an aminosilanized glass surface with the tricarboxylate reagent benzene-1,3,5-triacetic acid (BTA) (Fedurco *et al*., 2006). A cluster station is then used to hybridize the cDNA libraries to the flowcell. This process allows the generation of millions of clusters, with each cluster containing copies of a single molecule. The 3' end of the primer then extends to form a complement of the ssDNA library and the original template is removed. This single-strand then bends to allow the adapter at the end of the strand to hybridize to the adjacent primer. Polymerase extends the hybridized primer, forming a double-stranded bridge. This bridge is then denatured to produce two single-stranded templates. The above process is repeated a number of times in a process known as bridge amplification (Fuller *et al*., 2009). The reverse strands are then cleaved from each cluster and removed in a wash, leaving only the forward strands. The free 3' ends are blocked with dideoxynucleotide triphosphates (ddNTPs) in order to prevent any unwanted DNA priming. This process results in millions of clusters that produce easily detectable fluorescent signals during sequencing (Figure 1.13).

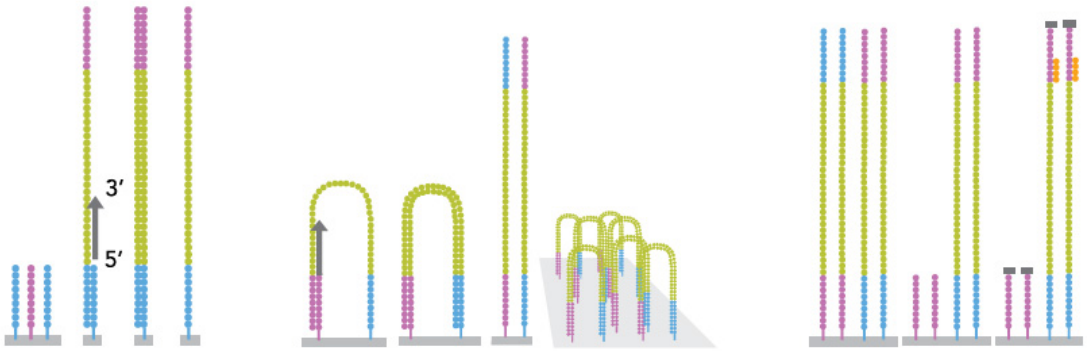

Figure 1.13. Clusters are generated by bridge amplification prior to sequencing. Prepared cDNA libraries are hybridized to primers immobilised on the flowcell. DNA polymerase extends the primer and the original template is denatured. Bridge amplification is used to generate clusters and the reverse strands are then removed by specific base cleavage prior to sequencing. Finally, the 3' ends are blocked with ddNTPs to prevent non-specific priming and the sequencing primer attaches to the adapter sequence (Source: www.illumina.com).

The flowcell is transferred from the cluster station to the GA_{II} for sequencing. Sequencing by synthesis is used to simultaneously sequence each cluster on a flowcell. A sequencing primer is initially added to the flowcell which binds to the adapter sequence at the 3' end of each transcript. The flowcell is then flooded with modified nucleotides and a proprietary Illumina polymerase. The nucleotides are engineered as reversible terminators with each containing a cleavable fluorophore at the base and a cap at the 3'-OH group with a small chemically reversible moiety (Ju *et al.*, 2006). Initial attempts at designing a cleavable chemical moiety linked to a fluorescent dye that could be used to cap the 3'-OH group failed, mostly because the 3' position is close to the amino acid residues in the active site of the polymerase, leaving the polymerase highly sensitive to modification, particularly in relation to large fluorophores (Pelletier *et al.*, 1994; Metzker, 2005; 2010). Instead, the sequencing technology uses a small, chemical reversible moiety to cap the 3'-OH group. The fluorophore is attached, via a cleavable linker, to the 5-position of the pyrimidines (T and C) and the 7-position of the purines (G and A) (Ju *et al.*, 2006). This means that the engineered nucleotides are still

recognised by DNA polymerase as substrates while also allowing a different fluorophore to be linked to each of the four bases, each producing a distinct fluorescent signal (Figure 1.14).

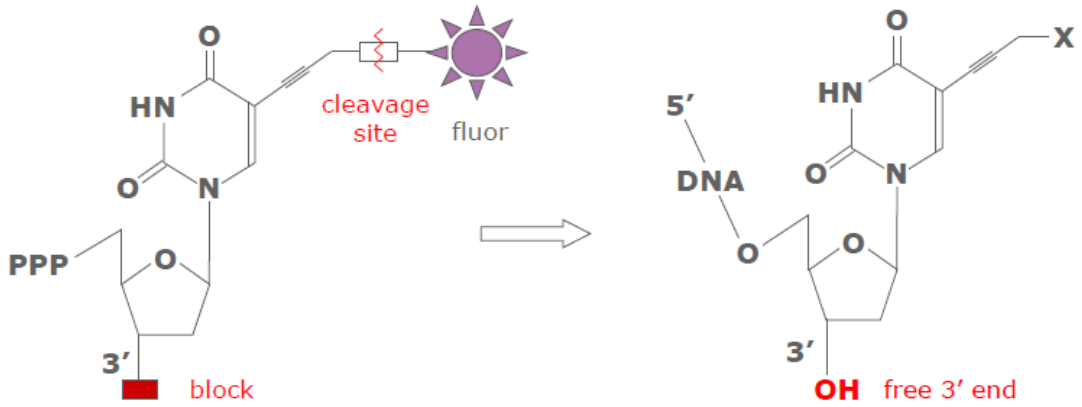

Figure 1.14. Chemically reversible terminator chemistry employed in RNA-seq. Each nucleotide has been molecularly engineered to be capped at the 3'-OH group with a chemically reversible moiety and a cleavable fluorophore at the base. Following signal detection, the fluorophore and moiety are cleaved, allowing the DNA polymerase to add an additional nucleotide (Ju *et al*., 2006).

This is used to identify the sequence of each cluster. The flowcell is flooded with a sequencing reagent containing DNA polymerase and the engineered dNTPs, and a single nucleotide is then incorporated to each strand by the polymerase. All unincorporated nucleotides are washed from the flowcell and a laser is used to detect fluorescence at four different wavelengths (Figure 1.15). A camera located above the flowcell takes a picture of the fluorescing flowcell and records the colour and location. Each flowcell contains eight lanes, each lane two columns and each column sixty tiles. The camera moves along each tile and takes a picture at every cycle. Once the signal has been detected, a Pd-catalyzed deallylation buffer simultaneously removes the fluorophore and the 3'OH group in less than 30 seconds (Ju *et al*., 2006). This allows the incorporation of a new nucleotide to the sequence and the step above is repeated.

The GA_{II} collates the information and assembles sequences for each cluster which can then be exported for analysis.

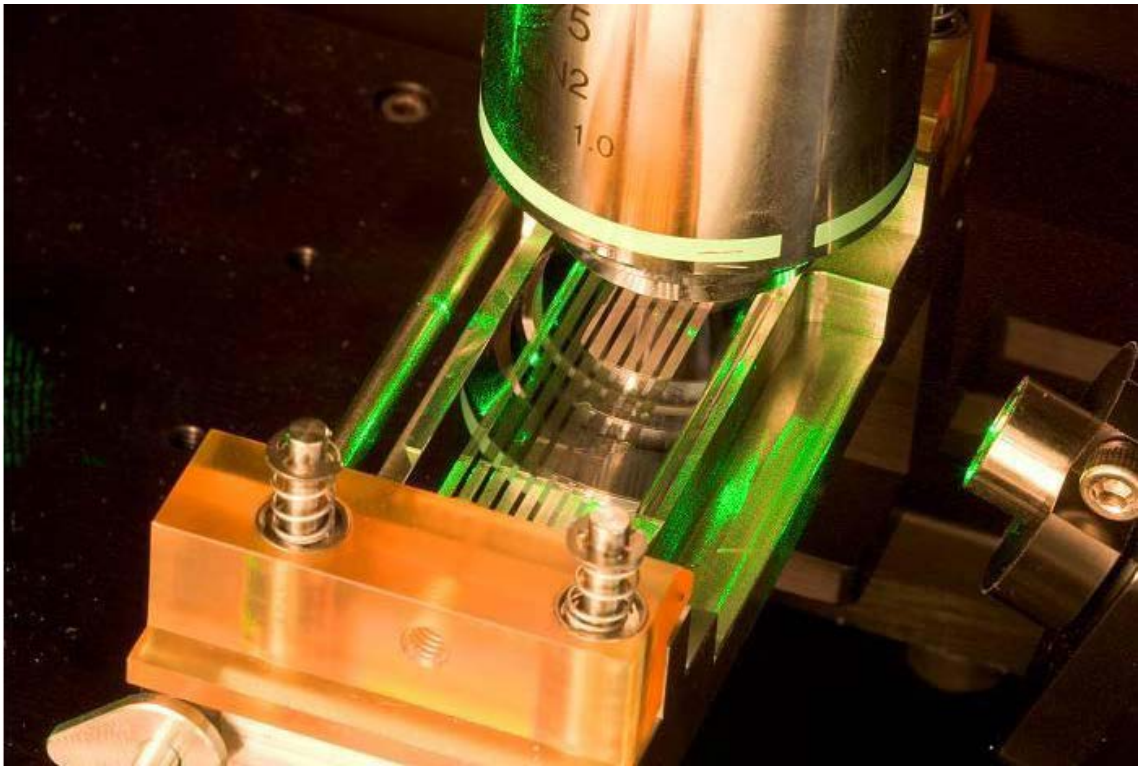

Figure 1.15. A laser directed through the prism at the base of the flowcell allows the detection of incorporated nucleotides.
Incorporated nucleotides are detected by their fluorescence wavelength following excitation with a laser on the Genome Analyzer II.

1.7. Hypotheses and objectives of thesis

The aim of this PhD thesis was to obtain a greater understanding of the impacts of weaning stress on the welfare of calves typical of the Irish beef industry. In particular, the project focussed on the impact of weaning of beef calves weaned between 7 and 8 months of age. The weaned beef calf is a convenient biological model to study fundamental questions related to the regulation of the leukocyte transcriptome in response to stress challenges. Our understanding of the regulation of functional genes responsive to stress signals is still nascent. In order to progress the scientific understanding of weaning stress in cattle, the molecular and physiological conditions of

this response were characterised. This will inevitably lead to improvements in animal health and welfare of domestic cattle. With the background knowledge discussed throughout chapter 1, the examination of the molecular and physiological effects of stress on the immune system will aid in our understanding of these processes. The overall hypothesis of this thesis was that weaning of beef calves would result in molecular and physiological alterations to cells of the immune system which could have long term repercussions for animal health. To test this hypothesis, three studies were undertaken with the following objectives:

1. to (i) investigate the haematological, acute phase and hormonal stress response to either housing beef calves with their dam or simultaneously housing and abruptly weaning beef calves, and (ii) examine the difference between housed calves and those housed and weaned (Chapter 3).

2. to (i) characterise, in male and female calves habituated to housing, the immune response to weaning stress at the physiological and molecular levels, and (ii) assess the difference between calves weaned and housed in the presence of the dam and those weaned and housed away from the dam (Chapter 4).

3. to (i) characterise the physiological and molecular mechanisms essential to the biological response to weaning at housing in beef calves using RNA-seq to examine global gene expression patterns, alternative splicing events and pathway analysis, and (ii) examine the molecular differences between the less extreme stress of housing and the stress of housing combined with abrupt weaning (Chapter 5).

These studies may contribute to our understanding of the stress response and can help to characterise the weaning response of beef calves. Furthermore, data drawn from these studies may prove useful in the future identification of biomarkers for use in bovine stress and weaning studies, and may provide cues as to the molecular events underlying stress induced diseases of beef calves.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Experimental subjects

Animals used in this study are described in Table 2.1.

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

Chapter	Breed	Number (Sex)	Mean age at weaning[5]	Mean weight at weaning[5]
Chapter 3	CH[1]×SM[2], CH×LM[3], LM×SM, LM×FR[4]	16 (male)	235 (27.5) days	294.3 (48) kg
Chapter 4	SM	14 (male) 14 (female)	214 (29.6) days	269.9 (58.3) kg
Chapter 5	SM	16 (male)	204 (34.9) days	250.7 (38.5) kg

[1]Charolais, [2]Simmental, [3]Limousin, [4]Friesian
[5]Mean (s.d.)

2.1.2. Reagents for cortisol measurements

2.1.2.1. Cortisol assay kit and reagents

Plasma cortisol was assayed using a competitive immunoassay (Correlate-EIA Cortisol, Assay Designs, Ann Harbour, MI, USA, catalogue number 901-701) which contained all the required reagents.

2.1.2.2. 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).

2.1.2.3. 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.

2.1.2.4. Cortisol EIA conjugate

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

2.1.2.5. Cortisol EIA antibody

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

2.1.2.6. 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).

2.1.2.7. 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).

2.1.2.8. 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).

2.1.3. Reagents for haematology measurements

2.1.3.1. ADVIA 2120 haematology analyser reagents

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

2.1.3.2. *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.

2.1.4. Reagents for acute phase protein assays

2.1.4.1. Haptoglobin

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

2.1.4.2. Haemaglobin (Reagent 1)

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

2.1.4.3. Chromogen/substrate (Reagent 2)

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

2.1.4.4. Serum Amyloid A

Serum Amyloid A was measured using a commercial kit containing all required reagents (Phase Range Multispecies SAA ELISA kit, Tridelta Development Ltd., Co. Kildare, Ireland).

2.1.5. Reagents for CXCL8 assay

CXCL8 was quantified from blood plasma using the Quantikine IL-8 Immunoassay (R&D Systems Europe, Ltd., Abingdon, UK) which contained a Capture Antibody, Detection Antibody, Standard and Streptavidin-HRP. Table 2.2 lists additional solutions.

Table 2.2. Additional Reagents required for CXCL8 assay

Reagent	Composition
Wash Buffer	0.05 % Tween 20 in PBS, pH 7.2 – 7.4
Block Buffer	1 % bovine serum albumin (BSA) in PBS with 0.05 % NaN_3
Reagent Diluent	0.1 % BSA, 0.05 % Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4
Substrate Solution	1:1 mix of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) (R&D Cat DY994)
Stop Solution	2 N H_2SO_4

2.1.6. Reagents for RNA extraction, preparation and quantification

2.1.6.1. Nanopure water

Nanopure water (Barnstead NANOpure Water purification Systems, Thermo Fisher Scientific Inc., Dublin, Ireland) was used where nuclease free water was not required. The water was deionised, passed through a total organic carbon (TOC) analyzer, treated with dual band (185 and 254 nm) UV light and passed through a 0.2 µm filter.

2.1.6.2. Diethyl pyrocarbonate (DEPC)

DEPC solution (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland, catalogue number D5758) was added to Nanopure water in order to deactivate RNase enzymes, left overnight at room temperature and autoclaved to hydrolyze the DEPC.

2.1.6.3. TRI reagent

TRI reagent (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland, catalogue number T9424) was used in the extraction of total RNA.

2.1.6.4. RNeasy Mini Kit

The RNeasy Mini Kit (Qiagen, Sussex, UK, catalogue number 74106) and reagents are listed in Table 2.3. Following extraction, total RNA was DNase treated and then placed in a spin column to remove contaminants.

Table 2.3. List of reagents in RNeasy Mini Kit

Reagent	Aliquot
Buffer RLT	350 µL
Ethanol (100 %)	250 µL
Buffer RPE	500 µL
Nuclease free water	30 µL
Buffer RW1	350 µL
DNase I	10 µL
Buffer RDD	70 µL

2.1.6.5. Agilent 2100 Bioanalyzer

In order to assess RNA quality prior to cDNA synthesis, each sample was run on the Agilent 2100 Bioanalyzer (Agilent Technologies Ireland Ltd., Cork, Ireland, catalogue number G2939AA) with an RNA 6000 Nano reagent kit (Agilent Technologies Ireland Ltd., Cork, Ireland; Cat no. 5067-1511) (Table 2.4). The reagents are stored at 4 °C and allowed to equilibrate to room temperature prior to use.

Table 2.4. List of reagents in RNA 6000 Nano Reagent kit

Reagents	RNA 6000 Nano gel Matrix (Red)
	RNA Nano dye concentrate (Blue)
	RNA 6000 Nano marker (Green)
	RNA 6000 ladder (Yellow)
	RNA Nano Chip
	Spin filters

2.1.6.6. cDNA synthesis

The High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies Ltd., Paisley, UK, catalogue number 4368814). Reagents are listed in Table 2.5. All reagents were stored at -20 °C.

Table 2.5. List of reagents in High Capacity cDNA Reverse Transcription Kit

Reagents	**Starting Concentration**	**Working Volume**
10 × RT buffer	1.0 mL	2 µL
10 × RT Random Primers	1.0 mL	2 µL
25 × dNTP Mix	100 mM	0.8 µL
Multiscribe Reverse Transcription	50 U/µl	1 µL
Nuclease-free water		4.2 µL

2.1.6.7. Fast SYBR Green Mastermix

Fast SYBR Green Mastermix (Applied Biosystems, Life Technologies, Paisley, UK, catalogue number 4385616), which contains a fluorescent marker, deoxynucelotides and a DNA polymerase, was used in the RT-qPCR reaction to detect fluorescence in the amplification stage of the reaction.

2.1.6.8. PCR reagents

PCR products were produced using the following reagents: 45 µL Platinum PCR Supermix (Applied Biosystems, Life Technologies, Paisley, UK), 2 µL of 20µM primer (Commericially Synthesised by Sigma-Aldrich Ireland Ltd., Wicklow, Ireland), 2 µL cDNA and 1 µL nuclease-free water.

2.1.7. RNA-seq library preparation

2.1.7.1. Dynabeads Oligo(dT)

Dynabeads Oligo (dT) (Bio Sciences Ltd., Dublin, Ireland, catalogue number 610-05) bind the poly A tails of mRNA. The kit reagents are listed in Table 2.6.

Table 2.6. Dynabead Oligo (dT) kit contents

Reagent	Content
Dynabeads Oligo (dT)	5 mg/mL Dynabeads, phosphate buffered saline (pH 7.5), 250 mM Tris-HCl (pH 7.5), 20 mM EDTA, 1 % (v/v) Tween, 0.02 % (w/v) NaN_3
Binding Buffer	17.1 mL H_2O, 0.4 mL 1 M Tris/0.1 M EDTA (pH 8.0), 2.5 mL 8 M LiCl
Washing Buffer	19.43 mL H_2O, 0.2 mL 1 M Tris/0.1 M EDTA (pH 8.0), 0.37 mL 8 M LiCl
Tris-HCl	20 mM Tris-HCl

2.1.7.2. Fragmentation reagent

Fragmentation Reagent was supplied by Ambion (Life Technologies Ltd., Paisley, UK, catalogue number AM8740) in a buffered zinc solution, along with a stop solution containing 200 mM EDTA (pH 8). Glycogen (5mg/mL) was supplied by Ambion (Life Technologies Ltd., Paisley, UK, catalogue number AM9510).

2.1.7.3. cDNA synthesis

All reagents for first and second strand cDNA synthesis were supplied by Bio Sciences Ltd. (Dublin, Ireland) are listed in Table 2.7.

Table 2.7. List of reagents used for first and second strand cDNA synthesis

Strand	Reagents	Concentration
First	Random Hexamer Primers	3 µg/µL
	5 × first strand buffer	-
	DTT	100 mM
	dNTP mix	10 mM
	RNaseOUT Recombinant ribonuclease inhibitor	40 U/µL
	SuperScriptII	-
Second	5 × second strand buffer	-
	dNTP mix	10 mM
	Ribonuclease H	2 U/µL
	E. coli DNA Polymerase I	10 U/µL

2.1.7.4. cDNA end repair

The reagents required for the end repair and blunting of the cDNA fragments by the addition of a single 'A' base are listed in Table 2.8. All reagents were supplied by Bio Sciences Ltd. (Dublin, Ireland) and New England Biolabs (NEB) Ltd., Hertfordshire, UK.

Table 2.8. Reagents required for end repair and the addition of a single 'A' base to cDNA fragements

	Reagent	Concentration	Supplier
End Repair	T4 DNA Ligase buffer with 10 mM ATP	10×	NEB
	dNTP mix	10 mM	Bio Sciences
	T4 DNA Polymerase	3 U/µL	NEB
	Klenow DNA Polymerase	5 U/µL	NEB
	T4 PNK	10 U/µL	NEB
'A' Base Addition	Klenow buffer	6 mL	NEB
	dATP	1 mM	Bio Sciences
	Klenow 3'-to-5' exo-	5 U/µL	NEB

2.1.7.5. Adapter ligation

The reagents used to ligate adapters to the prepared cDNA libraries were supplied by New England Biolabs ((NEB) Ltd., Hertfordshire, UK) and Illumina (Essex, UK). The reagents are listed in Table 2.9.

Table 2.9. Reagents required for ligation of Illumina sequencing adapters

Reagent	Concentration	Supplier
Quick DNA Ligase buffer	2 ×	NEB
Adaptor-Oligo mix	Proprietary Restricted	Illumina
Quick T4 DNA Ligase	10 ×	NEB

2.1.7.6. Gel purification of adapter ligated DNA templates

The reagents required for gel purification of the adapter ligated DNA templates were supplied by New England Biolabs ((NEB) Ltd., Hertfordshire, UK), Bio-Rad Laboratories (Hertfordshire, UK), Sigma-Aldrich Ireland Ltd. (Wicklow, Ireland) and WebScientific (Cheshire, UK) and are listed in Table 2.10.

Table 2.10. List of reagents required for gel purification of adapter ligated DNA templates

Reagent	Concentration	Supplier
Certified Low Range Ultra Agarose Gel	2 % (w/v)	Bio-Rad
Ethidium bromide	400 ng/mL	Sigma-Aldrich
DNA ladder and loading buffer	500 µg/mL	NEB
TAE buffer	10 ×	Sigma-Aldrich
GeneCatcher Disposable Gel Excision Tips	-	Web Scientific

2.1.7.7. PCR enrichment of purified adapter ligated DNA templates

The reagents used in the PCR enrichment of the gel purified adapter ligated DNA templates were supplied by New England Biolabs ((NEB) Ltd., Hertfordshire, UK), Bio Sciences Ltd. (Dublin, Ireland) and Illumina (Essex, UK) and are listed in Table 2.11.

Table 2.11. Reagents required for the PCR enrichment of purified adapter ligated DNA

Reagents	Concentration	Supplier
Cloned Phusion High-Fidelity Buffer	5 ×	NEB
Phusion polymerase	2 U/µL	NEB
PCR primer 1.1	25 µM	Illumina
PCR primer 2.1	25 µM	Illumina
dNTP mix	25 mM	Bio Sciences

2.1.7.8. PCR spin column to remove contaminating reagents

The QIAquick PCR spin column, QIAquick MinElute column and QIAquick Gel Extraction kits were used throughout the mRNA library preparation protocol and supplied by Qiagen Ltd. (Sussex, UK). Each kit shares three reagents but contain their own proprietary spin column. The contents of the kits are listed in Table 2.12. The PCR spin column purifies DNA over 100 bp, the MinElute purifies DNA over 70 bp and allows elution in small volumes and the Gel extraction column allows up to 10 µg of DNA over 70 bp to be purified from agarose gels.

Table 2.12. Kit contents for QIAquick PCR clean-up kits

Kit	Reagent	Composition
QIAquick PCR spin column, MinElute, Gel Extraction	Buffer PB	Guanidine hydrochloride and isopropanol
	Buffer PE	Sodium perchlorate, isopropanol, ethanol
	Buffer EB	10 mM Tris Cl (pH 8.5)
Gel Extraction	Buffer QC	1.0 M NaCl, 50 mM MOPS (pH 7.0), 15 % isopropanol (v/v)

2.1.8. Behavioural measures

2.1.8.1. IceTag pedometers

Standing, lying and movement behaviours were recorded by pedometers (IceRobotics, Midlothian, Scotland) attached to the front left leg of 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: (Bernadette Earley).

2.2.2. Experimental design

The experimental design and animal management for chapters 3 to 6 are described in the following sections.

2.2.2.1. Environmental measurements

Shed and ambient temperatures were monitored using data loggers (Testo 175 data loggers, Eurolec, Ireland).

2.2.2.2. Animal measurements

Calves were born at the Animal and Grassland Research and Innovation Centre and as part of standard husbandry and research management practices were accustomed to routine handling in the facilities and to stockpersons.

2.2.2.3. Rectal temperature measurements

Rectal body temperature was recorded while the calves were waiting in the holding chute just prior to blood sample collection using a digital thermometer (Jorgen Kruuse A/S; Model VT-801 BWC Lot No 0701, Marslev, Denmark).

2.2.2.4. Experimental design and animal management (Chapter 3)

Sixteen clinically healthy, spring-born, single-suckled, beef bull calves (Charolais × Simmental, Charolais × Limousin, Limousin × Simmental, Limousin × Friesian) were used in this study. Prior to housing, cows and their calves were rotationally grazed on a predominantly perennial ryegrass (*Lolium* perenne) based sward from early April until housing on the 9th November. All calves in this study were vaccinated 28 days prior to weaning according to Teagasc Grange animal management guidelines. The calves were immunized against bovine respiratory syncytial virus (BRSV) and infectious bovine rhinotracheitis (IBR) virus using *Rispoval-3* and *Rispoval-IBR* vaccines (Pfizer Animal Health, Co. Cork, Ireland), respectively. On the day (d) of weaning (d 0), calves were moved to a handling yard and assigned to one of two treatments: 1), Housed treatment (Control; n = 8; mean weight (s.d) 292.0 (36.5) kg; mean age (s.d.) 228 (22.1) days), these animals were housed with their dam, 2), Weaned treatment (Weaned; n = 8; mean weight (s.d.) 296.5 (59.5) kg; mean age (s.d.) 242 (32.8) days), these animals were abruptly separated from their dam and loose-housed with other abruptly weaned calves, introducing a social reorganisation component.

2.2.2.4.1. Sample collection (Chapter 3)

Calves were blood sampled via jugular venipuncture on d -4, 0, 1, 2, 3, 7, and 14 relative to weaning (d 0). For this procedure, they were led gently to a holding pen, with a squeeze chute facility and were blood sampled with minimal restraint. Blood sampling

was carried out by the same experienced operator on each occasion and the time taken to collect the blood samples was less than 60 s/calf. Blood samples were collected into 1 × 6 mL K_3Ethylenediaminetetraacetic acid (K_3EDTA) tube (Vacuette, Cruinn Diagnostics, Ireland) for haematological analysis and 1 × 8 mL lithium heparin tubes for cortisol, acute phase protein and CXCL8 analysis.

2.2.2.4.2. Statistical analysis (Chapter 3)

Haematological, acute phase protein, CXCL8 and cortisol data were tested for normality using PROC UNIVARIATE and the Shapiro-Wilk test and values that were not normally distributed were log transformed prior to statistical analyses. Haematological, physiological and rectal temperature data were analysed as repeated measures using the PROC MIXED procedure of SAS (Version 9.1, SAS Institute, Cary, NC). The first sample (d -4; sample 1) was used as the baseline covariate in the statistical analysis of the data. Animal was the experimental unit and was specified as a repeated measures effect, and the dependence within animal was modelled using an unstructured covariance structure. Data subject to transformation were used to calculate P-values. The corresponding least squares means (Lsmeans) and SE of the non-transformed data are presented to facilitate interpretation of results. Differences between treatments were determined using the Tukey-Kramer test for multiple comparisons. Lsmeans were considered significantly different at the $P < 0.05$ probability level.

2.2.2.5. Experimental design and animal management (Chapter 4)

Twenty-eight clinically healthy, spring-born, single-suckled, three-quarter bred Simmental beef calves were housed indoors in concrete slatted floor pens with their dams on day (d) -28 of the study. Prior to housing, cows and their calves were rotationally grazed on a predominantly perennial ryegrass (*Lolium* perenne) based sward

from early April until housing on the 10th October. Calves were also immunized at housing against bovine respiratory syncytial virus (BRSV) and infectious bovine rhinotracheitis (IBR) virus using *Rispoval-3* and *Rispoval-IBR* vaccines, respectively. Pens were equipped with automatic drinkers and the calves were offered a new diet of grass silage (mean dry matter digestibility (DMD) (s.d.) 719 (16.3) g/kg; mean crude protein (CP) concentration (s.d.) 145 (11.8) g/kg dry matter (DM)) and had free access to concentrates (931 g/kg barley, 50 g/kg molasses and 15 g/kg minerals and vitamins (mean DMD (s.d.) 825 (7.9) g/kg, mean CP (s.d.) 108 (9.2) g/kg DM, mean neutral detergent fibre (NDF) (s.d.) 188 (19.6) g/kg DM)) per animal daily. Feedstuff analysis was determined as described by Owens *et al*. (2009).

The calves were weaned on the 13th November. At weaning (d 0), the cows were removed and the calves were regrouped and assigned to new pens with concrete slatted floors (mean space allowance of 2.9 m^2/head/calf) mimicking social reorganisation, and allocated to one of four treatments: 1), females weaned beside the dam (n = 7; mean weight (s.d.) 267.3 (45.1); mean age (s.d.) 227 (17.9) d), 2), females weaned away from dam (n = 7; mean weight (s.d.) 274.1 (35.3) kg; mean age (s.d.) 224 (25.1) d), 3), males weaned beside the dam (n = 7; mean weight (s.d.) 270.6 (69.8) kg; mean age (s.d.) 197 (38.1) d), or 4), males weaned away from dam (n = 7; mean weight (s.d.) 267.9 (82.8) kg; mean age (s.d.) 209 (37.3) d). Treatments 1 and 3 were assigned to pens adjacent to their dam. A metal grid fence prevented suckling whereas visual and auditory contact was allowed (Figure 2.1). Treatments 2 and 4 were assigned to pens sufficiently distant from their dam to prevent any visual or auditory contact. Haematology analysis was preformed at each time point and RT-qPCR analysis was carried out for D 0, 1, 3 and 7.

2.2.2.5.1. Sample collection (Chapter 4)

Calves were blood sampled via jugular venipuncture on d -3, 0, 1, 2, 3, 7, and 11 relative to weaning (d 0). For this procedure, they were led gently to a holding pen, with a squeeze chute facility and were blood sampled with minimal restraint. Blood sampling was carried out by the same experienced operator on each occasion and the time taken to collect the blood samples was less than 60 s/calf. Blood samples were collected into 1 × 6 mL K_3Ethylenediaminetetraacetic acid (K_3EDTA) tube (Vacuette, Cruinn Diagnostics, Ireland) for haematological analysis and into 5 × 9 mL acid citrate dextrose (ACD) tubes (Vacuette, Cruinn Diagnostics, Ireland) for leukocyte isolations. ICE Tag Pedometers were randomly attached to the front left leg of five animals from each treatment at weaning on d 0.

Figure 2.1. Calves weaned next to the dam.

Calves weaned next to the dam were prevented from suckling while still allowed physical contact with the dam by a mesh wire fence as seen above.

2.2.2.5.2. Statistical analysis (Chapter 4)

All data were checked for adherence to a normal distribution (PROC UNIVARIATE, SAS v 9.1). The natural logarithm transformation was used to normalize the distributions of neutrophil and eosinophil numbers. Interleukin (IL) -1β, IL-2, IL-4, CXCL8, interferon (IFN) -γ, tumour necrosis factor (TNF) α, lymphotoxin, toll-like receptor (TLR) 4, glucocorticoid receptor (GR) α, nuclear factor (NF) κB1, NFκB2, Fas, p21, CD62L, haptoglobin, bactericidal/permeability increasing protein (BPI)) were $\log_2$ transformed (GenEx Software v.5.2.2.8 (2010) (MultiD Analyses AB, Göteborg, Sweden)) prior to statistical analysis. Haematological data, behavioural data, relative gene expression and rectal temperature data were analysed as a 2 × 2 factorial with repeated measures using the PROC MIXED procedure of SAS (Version 9.1, SAS Institute, Cary, NC). The effects of treatment, sampling time, gender and all possible interactions were listed in the model statement. Animal was the experimental unit and was specified as a repeated measures effect, and the dependence within animal was modelled using an unstructured covariance structure. For haematological data and rectal temperature measurements, the first sample (d -3; sample 1) was used as the baseline covariate in the statistical analysis of the data. Data subject to transformation were used to calculate P-values. The corresponding least squares means (Lsmeans) and SE of the non-transformed data are presented to facilitate interpretation of results. Differences between means were tested using the PDIFF option in PROC MIXED of SAS. Means were considered significantly different at the $P < 0.05$ probability level.

2.2.2.6. Experimental design and animal management (Chapter 5)

Sixteen clinically healthy, spring-born, single-suckled, intact male continental beef calves were used in this study. Calves were rotationally grazed together with their dams on a predominantly perennial ryegrass (*Lolium perenne*) based sward from April until

weaning on 20 October, 2009. All calves in this study were vaccinated 28 days prior to weaning according to Teagasc Grange animal management guidelines. The calves were immunized against bovine respiratory syncytial virus (BRSV) and infectious bovine rhinotracheitis (IBR) virus using *Rispoval-3* and *Rispoval-IBR* vaccines (Pfizer Animal Health, Co. Cork, Ireland), respectively. On the day (d) of weaning (d 0), calves were moved to a handling yard and assigned to one of two treatments: 1), Housed treatment (Control; n = 8; mean weight (s.d) 251.0 (47.8) kg; mean age (s.d.) 199 (34.4) days), these animals were housed with their dam, 2), Weaned treatment (Weaned; n = 8; mean weight (s.d.) 250.4 (29.1) kg; mean age (s.d.) 209 (35.4) days), these animals were abruptly separated from their dam and loose-housed with other abruptly weaned calves, introducing a social reorganisation component. Pens were equipped with automatic drinkers and pen size was adjusted based on animal weight to allow the same space allowance in each pen (mean space allowance of 2.9 m^2/head/calf). Calves were offered *ad libitum* grass silage and 1 kg/head/day concentrate feed throughout the study.

2.2.2.6.1. Sample collection (Chapter 5)

On d -4, 0, 1, 2, 3 and 7 relative to weaning (d 0), blood samples were collected via jugular venipuncture into 6 mL K_3Ethylenediaminetetraacetic acid (K_3EDTA) tubes for haematological analysis, 8 mL lithium heparin tubes for cortisol and haptoglobin analysis and 9 mL acid citrate dextrose (ACD) tubes (Vacuette, Cruinn Diagnostics, Ireland) for leukocyte isolations. For this procedure, the calves were led gently to a squeeze chute facility in a holding pen and were blood sampled with minimal restraint. Blood sampling was carried out by the same experienced operator on each occasion and the time taken to collect the blood samples was less than 60 s/calf.

2.2.2.6.2. Statistical analysis (Chapter 5)

Haematological, haptoglobin and cortisol data were tested for normality using PROC UNIVARIATE and the Shapiro-Wilk test and values that were not normally distributed were log transformed prior to statistical analyses. Haematological, physiological and rectal temperature data were analysed as repeated measures using the PROC MIXED procedure of SAS (Version 9.1, SAS Institute, Cary, NC). The first sample (d -4; sample 1) was used as the baseline covariate in the statistical analysis of the data. Animal was the experimental unit and was specified as a repeated measures effect, and the dependence within animal was modelled using an unstructured covariance structure. Data subject to transformation were used to calculate P-values. The corresponding least squares means (Lsmeans) and SE of the non-transformed data are presented to facilitate interpretation of results. Differences between treatments were determined using the Tukey-Kramer test for multiple comparisons. Lsmeans were considered significantly different at the $P < 0.05$ probability level. For details on the analysis of RNA-seq data, see section 2.2.7.

2.2.3. Physiological measurements

2.2.3.1. Cortisol

Cortisol was quantified from blood plasma using a competitive immunoassay on an automated Dynex DSX Four-Plate Automated ELISA Processing System (Dynex Technologies Ltd., West Susses, UK). In brief, a100 µL aliquot of assay buffer was added to the non-specific binding (NSB) and the Blank only (Bo) (0 pg/mL Standard) wells. A 100 µL aliquot of each of 7 standards (10,000 pg/mL – 156 pg/mL cortisol) was added to the plate. For each sample, 100 µL of plasma was added to the plate along with 50 µL of assay buffer to the NSB wells. To each well, 50 µL of conjugate was

added with the exception of the Total Activity (TA) and Bo wells. A further 50 µL of antibody was added to all wells except for the TA, Bo and NSB wells. The plates were sealed and incubated at room temperature on a plate shaker for 2 hours (h) at 500 rpm. The content of each well was emptied following incubation and 400 µL of wash solution was used to rinse each well 3 times. Five microlitres of conjugate was added to the TA wells while 200 µL of P-nitrophenyl phosphate (pNpp) substrate was added to all wells and left to incubate at room temperature for 1 h without shaking. To stop the reaction, 50 µL of Stop Solution was added to all wells and the plate was immediately read using a plate reader (TECAN SUNRISE Absorbance Reader, TECAN Austria GmbH, Austria) at 405 nm optical density (OD). The plate was blanked against the Bo wells.

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

2.2.3.2. Haematology

Leukocyte, red blood cells and associated constituents were determined using an ADVIA 2120 automatic haematology analyser (ADVIA 2120 Haematology System, Siemens, UK). Using flow cytometry, the ADVIA 2120 identifies different cell types according to their light scattering properties as they pass through laser beams that detect size and density. The cell types counted were total leukocytes, neutrophils,

lymphocytes, monocytes, eosinophils, red blood cells (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (HGB), mean corpuscular haemoglobin concentration (MCHC) and platelet number. A neutrophil:lymphocyte (N:L) ratio was calculated based on total neutrophil and lymphocyte counts.

2.2.3.3. Haptoglobin

A colorimetric assay (Phase Range Haptoglobin Assay kit (2nd Generation), Tridelta Development Ltd., Co. Kildare, Ireland) run on an automatic clinical analyser (Olympus AU 400, Tokyo, Japan) was used to quantify the concentration of haptoglobin from blood plasma according to manufacturer's instructions. This is performed by allowing haptoglobin to bind to haemoglobin in the sample. At low pH, the peroxidise activity of haemoglobin bound to the haptoglobin is preserved, with the peroxidise activity of haemoglobin being directly proportional to the amount of haptoglobin available in the sample. An intra assay CV of 6.3% and inter assay CV of 4.1% was calculated.

2.2.3.4. Serum Amyloid A

SAA was measured from blood plasma with a commercial multispecies SAA ELISA kit (Phase Range SAA ELISA kit, Tridelta Development Ltd., Co. Kildare, Ireland) according to manufacturer's instructions. The plasma samples were first diluted 1:500 in 1X diluent buffer. A calibration curve was then prepared by diluting the SAA calibrator five times, leaving a sixth tube as a blank (diluent buffer only). To each well of the assay, 50 µL of Anti-SAA/HRP conjugate was added. The calibrators, control and samples were then added to each well in duplicate (50 µL per well) and the side of the plate gently tapped to mix. The plate was then covered and left to incubate at 37 ˚ for 1 hour. Following incubation, the plate contents were then decanted and the plate

washed four times with the diluted wash buffer. A 100 µL aliquot of TMB substrate was then added to each well and the plate was covered and left to incubate at room temperature for 15 min. The reaction was then arrested by adding 100 µL of stop solution and gently tapping the sides of the plate to mix. The absorbance of each well was then read at 450 nm and concentrations were calculated using GraphPad Prism v3.03 (GraphPad Software, California, USA). An intra assay CV of 5% was calculated.

2.2.3.5. Interleukin-8 (CXCL8) Immunoassay

Plasma CXCL8 was quantified using the Quantikine IL-8 Immunoassay (R&D Systems Europe, Ltd., Abingdon, UK). The Capture Antibody was thawed and 56 µL was added to 10 mL PBS, mixed gently, and poured into a reservoir. To each well of the ELISA 96-well plate, 100 µL of Capture Antibody was added, ensuring each well was properly coated. The plate was then covered with parafilm and left to incubate at room temperature overnight. After incubation overnight, a wash buffer was prepared by added 500 µL 0.05% Tween 20 to 1 L PBS. The contents of the plate were then emptied and the plate was filled with the wash buffer. This was buffer was removed and the plate washed a further three times before drying with paper. In order to block the plate reaction, 300 µL blocking reagent was added to each well of the ELISA plate and left to incubate in the dark for 1 h at room temperature. Standards were prepared in 8 tubes ranging from 200 pg/mL to 15.63 pg/mL. To tubes 2 to 8, 300 µL Reagent Diluant was added to out serial dilutions. After 1 h, the ELISA plate was washed as described above. The standards and samples were then added to the ELISA plate. The plate was covered in parafilm and left to incubate for 2 h in the dark at room temperature, following which, the plate was washed as described above. In order to detect antibody binding to IL-8, 56 µL Detection Antibody was added to 10 mL of Reagent Diluent and 100µL of this solution was added to each well. The plate was

covered with parafilm and left to incubate for 2 h in the dark at room temperature, following which it was washed as described above. The HRP-Streptavidin was diluted by adding 50 µL to 10 mL Reagent Diluent and 100 µL was added to each well. The plate was then covered in parafilm and left to incubate in the dark for 20 min at room temperature, following which it was washed as described above. To each well of the ELISA plate, 100 µL TMB substrate was added and left to incubate in the dark at room temperature until a blue colour developed, typically 20 min. To stop this reaction, 50 µL Stop Solution (H_2SO_4) was added and the plate gently tapped to mix. The ELISA plate was then read at 450 and 560 nm and concentrations were calculated using GraphPad Prism v3.03 (GraphPad Software, California, USA). An intra assay CV of 7.8% and inter assay CV of 11.6% was calculated.

2.2.4. Leukocyte collection

2.2.4.1. Hypotonic lysis solution preparation

The hypotonic lysis solution was prepared from 3.0 g Na_2HPO_4 and 0.64 g NaH_2PO_4 in 2 litres (L) of Milli-Q water (10 mM Na_2HPO_4, 2 mM NaH_2PO_4). This was initially made up in 1.8 L of water, at which point in time the pH was adjusted to 7.2 with 2N HCl and 0.5N NaOH. Water was then added to reach the final working volume of 2 L. Diethylpyrocarbonate (DEPC) was then added to the solution at a ratio of 1.0 mL DEPC to 1 L solution. The Lysis solution was capped and shaken vigorously and stored overnight at room temperature. The DEPC was then inactivated by autoclaving the solution and storing at 4 ^{o}C.

2.2.4.2. Hypertonic restore solution preparation

The hypertonic restore solution was prepared from 3.0 g Na_2HPO_4, 0.64 g NaH_2PO_4 and 54 g NaCl in 2 L of Milli-Q water (10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 461 mM NaCl). This was initially made up in 1.8 L of water, at which point in time the pH was adjusted to 7.2 with 2N HCl and 0.5N NaOH. Water was then added to reach the final working volume of 2 L. Diethylpyrocarbonate (DEPC) was then added to the solution at a ratio of 1.0 mL DEPC to 1 L solution. The restore solution was capped and shaken vigorously and stored overnight at room temperature. The DEPC was then inactivated by autoclaving the solution and storing at 4 ^{o}C.

2.2.4.3. Sample preparation

Throughout this thesis, a total leukocyte population was used in the molecular analysis of weaning stress. Blood was collected from each animal into five 9 mL tubes coated in acid citrate dextrose (ACD) anti-coagulant and gently inverted several times. The ACD blood tubes were transferred to the lab within 20 minutes of blood collection where they were pooled into one sterile 50 mL tube per animals and placed on ice.

2.2.4.4. Leukocyte isolation

Three 50 mL tubes were assigned to each animal and 24 mL of cold Hypotonic lysis solution was added to each 50 mL tube. An automatic pipette was then used to add 12 mL of blood to each tube containing lysis solution and gently inverted several times (Figure 2.2). These tubes were left to sit for 90 seconds before the addition of 12 mL of cold Hypertonic restore solution. They were then gently inverted several times and place in a centrifuge at 2000 × g for 5 min at 4 ^{o}C. The lysed red blood cells were aspirated away from the pellet which was then resuspended in 10 mL of cold PBS. The tubes were then filled to the 40 mL mark with cold PBS and centrifuged at 2000 × g for

5 min at 4 ^{o}C. The supernatant was removed and the pellet re-suspended in cold PBS for a second time and re-centrifuged. The supernatant wash was again removed and 1 mL of TRI reagent was added to each leukocyte pellet. The TRI reagent containing the suspended leukocyte pellets from each animal was pooled into a 15 mL tube, left at room temperature for 5 minutes to ensure the TRI reagent penetrated the suspended pellets, and stored at -80 ^{o}C.

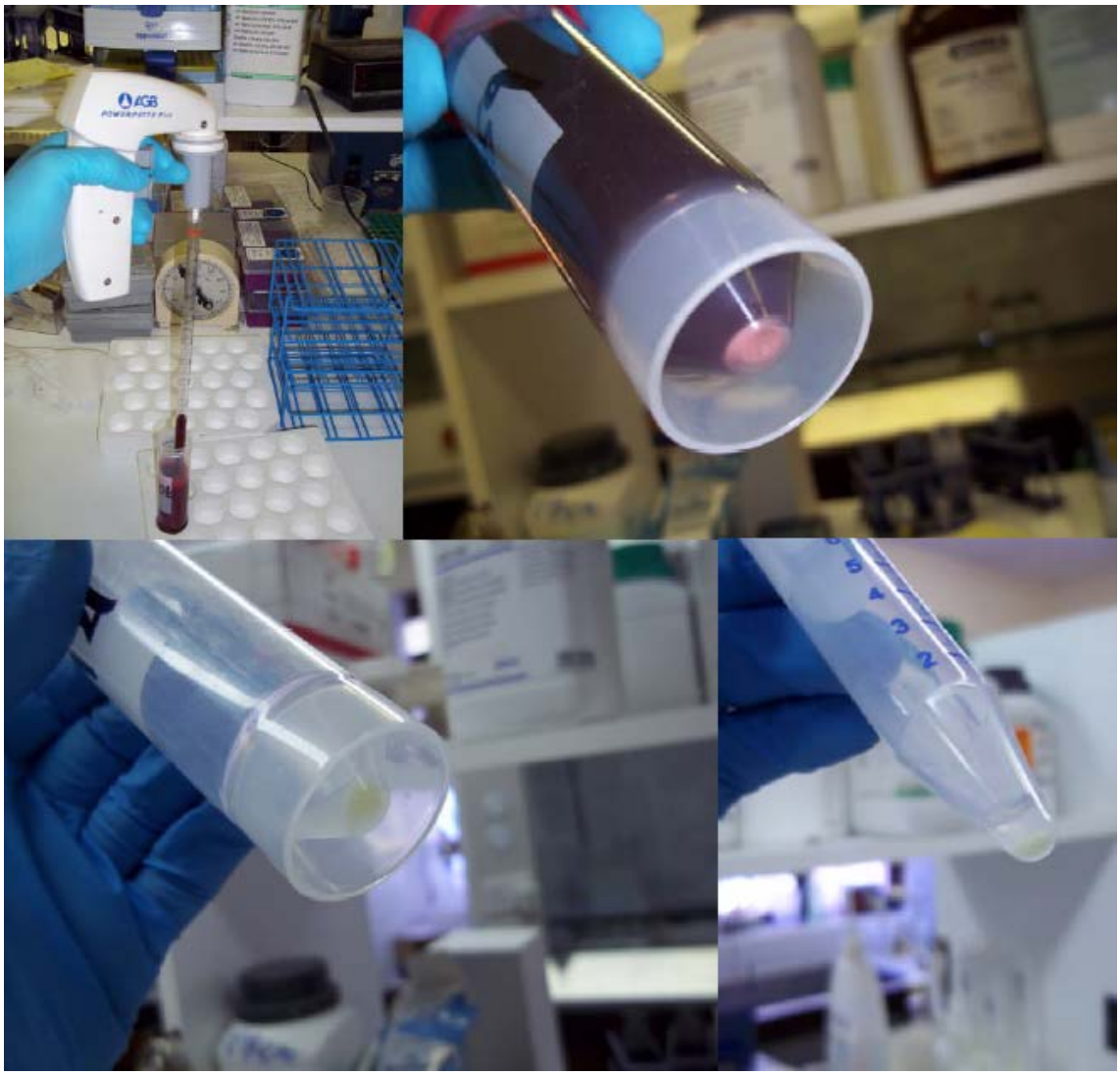

Figure 2.2. Isolation of leukocytes from whole blood.
Whole blood was added to a hypotonic solution to lyse red blood cells. A hypertonic solution was then added to stop this process and the sample was placed in the centrifuge to collect a leukocyte pellet. The leukocytes were then washed using PBS.

2.2.5.1. RNA extraction

The frozen leukocytes were removed from the -80 ^{o}C freezer and left on ice to thaw in the TRI reagent. Once thawed, the entire contents were transferred to a glass tube for homogenisation. After homogenising for 30 sec, the samples were then left to sit at room temperature for 5 min. Each sample was then divided into three 1 mL aliquots and placed into 1.5 mL Eppendorf tubes. Immediately following this, 200 µL of chloroform was added to each sample, shaken for 15 sec and left to sit at room temperature for 2 min. The samples were then centrifuged at 12,000 × g for 15 min at 4^{o}C. Following centrifugation, the supernatant was removed from each sample and transferred to sterile tubes. Isopropanol was then added to each sample at a ratio of 0.6 isopropanol to supernatant (~360 µL). This was placed on the vortex for 10 sec and centrifuged at 12,000 × g for 10 min at 4 ^{o}C. The supernatant was carefully removed to ensure the RNA pellet was not disturbed. A brief spin collected residual supernatant and this was removed. The pellet was then washed with 1 mL of 75 % ethanol and spun at 7,500 × g for 5 min at 4 ^{o}C. The ethanol supernatant was carefully removed and the pellet briefly left to air dry. Once sufficiently dry, 20 µL of nuclease-free water was added to each pellet. The re-suspended RNA was then pooled into one sample per animal.

2.2.5.2. RNA quantitation

In order to identify RNA quantities, the NanoDrop 1000 Spectrophotometer was used. Approximately 2.0 µL of sample was added to the fibre optic cable on the spectrophotometer which is capable of estimating RNA concentration based on the absorbance of light at a wavelength of 260 nm. Less light passing through the sample indicates a greater RNA concentration. This is calculated based on the average extinction coefficient for RNA which is 0.027 $(\mu g/ml)^{-1}$ cm^{-1} with the result reported in

ng/µL (Figure 2.3). The spectrophotometer was also used as an indicator of protein contamination in the sample as the 260/280 ratio is a reliable marker of this contamination. Both of these values were recorded for each sample.

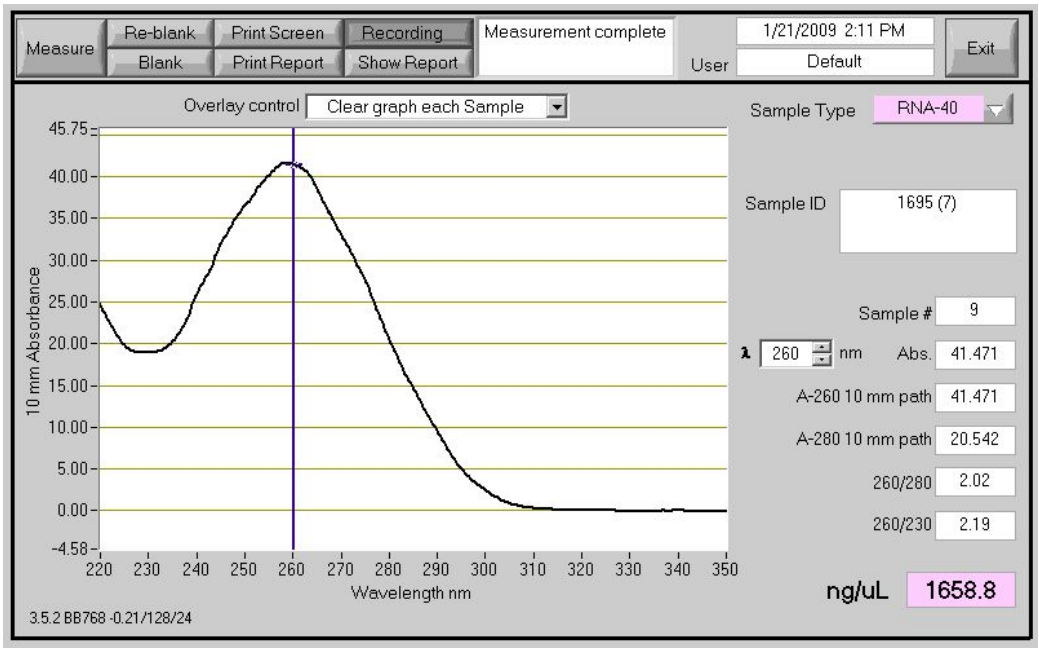

Figure 2.3. NanoDrop 1000 Spectrophotometer output.
A distinct peak at 260 nm allows an estimation of the quantity of RNA in a sample.

2.2.5.3. RNA qualitation

Each RNA sample was assessed for quality using the Agilent 2100 Bioanalyzer. Using concentrations identified with the NanoDrop 1000, samples were diluted with nuclease-free water to concentrations of 250 ng/µL. The reagents were allowed to equilibrate to room temperature for 30 min prior to use. In order to prepare the gel for use, 550 µL of RNA 6000 Nano gel matrix was pipetted into a spin filter and centrifuged at 1500 × g for 10 min at room temperature. The RNA 6000 Nano dye concentrate was then placed on the vortex for 10 sec, briefly centrifuged, and then 1 µL was aliquoted into 65 µL of filtered gel. This solution was vortexed, briefly inspected to ensure proper gel-dye mixing, and then centrifuged at 13,000 × g for 10 min at room temperature. A RNA 6000 Nano chip was then placed onto the chip priming station. A 9 µL aliquot of the

gel-dye mix was then added to the well marked with a 'G' surrounded by a circle. The priming station was closed and the plunger depressed until it was held by the clip. After 30 sec, the clip was released and returned to starting point (1 mL position). The priming station was then opened and 9 µL of gel-dye mix was added to each of the two wells marked 'G' surrounded by a square. Prior to loading on the chip, the RNA samples and ladder were heat denatured at 70 ˚C for 2 minutes. To each well marked 1-12 and ladder, 5 µL RNA 6000 Nano marker was added. Following this, 1 µL of sample was added to each well, and 1 µL of ladder to the ladder well. The chip was loaded onto an IKA vortexer for 1 min at 2400 rpm. While the vortex was running, 350 µL of RNaseZap was placed in the Agilent 2100 Bioanalyzer to ensure sterile electrodes. This was then washed with 350 µL of nuclease-free water. The chip was then placed into the Agilent 2100 Bioanalyzer for analysis. The bioanalyzer produces an electropherogram based on transcript size (Figure 2.4). The 28s/18s ratio and the RNA Integrity Number (RIN) were recorded. The RIN number is the most reliable indicator of RNA quality and samples were deemed acceptable above a RIN of 8.0.

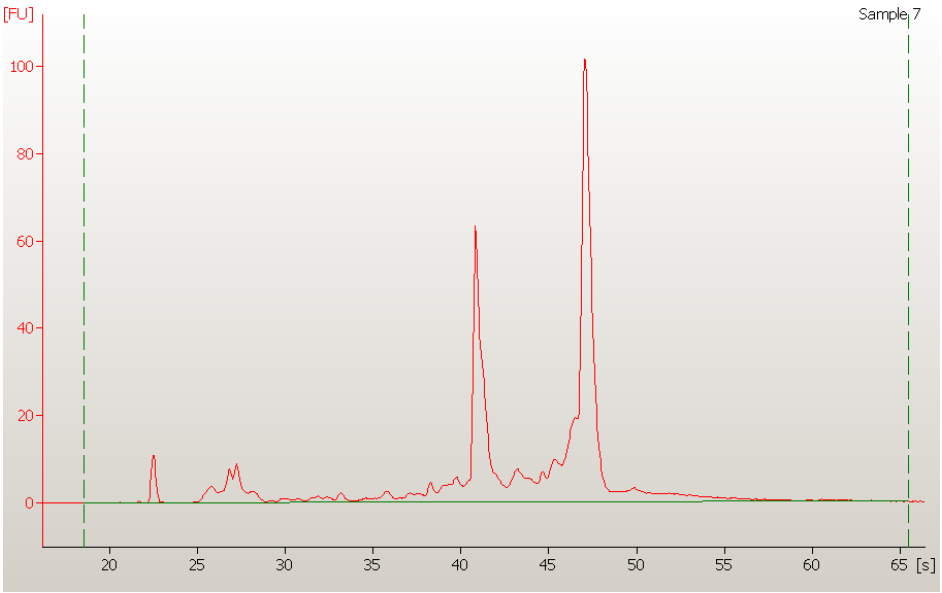

Figure 2.4. Electropherogram of a total RNA sample with a RIN of 10.0 on the Agilent 2100 Bioanalyzer.

The 18s and 28s ribosomal peaks stand out clearly from background and allow an estimate of RNA quality based on the speed of ribosomal degradation. As the 28s molecule is twice the size of the 18s molecule, it should give twice the fluorescence intensity at the same concentration, indicating degradation at 28s/18s ratio < 2.

2.2.5.4. DNase digestion and RNA cleanup

DNase digestion and RNA cleanup were performed using the Qiagen RNeasy Mini kit. To prepare the DNase I stock solution, an RNase-free syringe was used to inject 550 µL of RNase-free water into the lyophilized DNase I and gently mixed by inverting the tube until the lyophilized DNase I went into solution.

A 20 µg aliquot of total RNA was taken from each sample based on the NanoDrop 1000 results. These aliquots were then topped up to 100 µL with Nuclease-free water. To each sample, 350 µL of buffer RLT was added and mixed well by pipetting. Following this, 250 µL of 100 % ethanol was added and again mixed well by pipetting. The entire sample (700 µL) was then transferred to an RNeasy Mini spin column and centrifuged at 8,000 × g for 20 sec at room temperature. A further 350 µL of Buffer RW1 was then added to the spin column to wash the spin column membrane, centrifuged at 8,000 × g for 20 sec at room temperature and the flow-through discarded. To prepare the DNase I stock solution for use, 10 µL was added to 70 µL Buffer RDD, gently mixed, and briefly centrifuged to collect residual liquid from the sides of the tube. All 80 µL of this DNase I incubation mix were then added to the spin column membrane and allowed to sit at 25 ^{o}C for 15 min. Following on-column DNase digestion, 350 µL Buffer RW1 was added to the spin column and centrifuged at 8,000 × g for 20 sec at room temperature, after which the flow-through was discarded. This was followed by 500 µL of Buffer RPE being added to the spin column membrane, centrifuged at 8,000 × g for 20 sec at room temperature and the flow-through discarded. This step was repeated a second time, with the only difference being that the sample was centrifuged for 2 min to

ensure the spin column membrane was dried, preventing ethanol was not carried over into the elution step. The spin column was transferred to a fresh collection tube and and centrifuged at 8,000 × g for 2 min at room temperature to ensure that there was absolutely no ethanol carry over to the final step. The spin column was then transferred to a fresh collection tube and 20 µL of nuclease-free water was added directly to the spin column membrane. This was left to sit for 1 min and then placed in the centrifuge 8,000 × g for 80 sec at room temperature. This final step RNA elution step was repeated with an additional 20 µL of nuclease-free water, giving a final working volume of 20 µL. The concentration of cleaned up total RNA was recorded using the NanoDrop 1000 Spectrophotometer and a percent recovery was estimated based on input and output concentrations.

2.2.5.5. cDNA synthesis

The synthesis of cDNA was performed using the Applied Biosystems High Capacity cDNA Reverse Transcription kit. A 10 µL solution consisting of 1 µg cleaned up total RNA and nuclease-free water was prepared for each sample in PCR safe tubes. A cDNA master mix was then prepared. This contained 2.0 µL 10 × RT Buffer, 0.8 µL 25X dNTP Mix (100mM), 2.0 µL 10 × RT Random Primers, 1.0 µL MultiScribe Reverse Transcriptase and 4.2 µL nuclease-free water per reaction. The MultiScribe Reverse Transcriptase was added to the master mix last as this is heat sensitive enzyme. A total of 10 µL of master mix was added to each PCR safe tube containing total RNA, and cDNA synthesis was performed by placing each tube into the Mastercycler Thermal Cycler under the following conditions:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 ˚C	37 ˚C	85 ˚C	4 ˚C
Time	10 min	120 min	5 min	∞

The main cDNA stock was then stored at -80 ˚C while neat and 1:5 aliquots were prepared and stored at -20 ˚C.

2.2.5.6. RT-qPCR (Chapter 4)

2.2.5.6.1. Primer design

Primers were designed for each candidate gene using the web-based Primer3 software (Rozen and Skaletsky, 2000). Bovine gene sequences were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/nuccore/) and imported to Primer3. Primers were designed to cross exon junctions, have amplicon sizes between 100-200 bp, melting temperatures between 59-61 ˚C and a GC % between 45-55 %. To ensure each primer had a specific binding site, Basic Local Alignment Search Tool (BLAST) was used to check each forward and reverse primer (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were then commercially synthesised (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) (Table 2.13), lyophilized prior to shipment, and reconstituted prior to use based on manufacturers specifications to 100 µM concentrations.

2.2.5.6.2. RT-qPCR reactions

A total of 112 RT-qPCR reactions (28 animals at 4 time points) were performed in triplicate on the Applied Biosystems 7500 FAST RT-PCR equipment v.2.0.1 for each gene of interest (GOI). In brief, one µL of cDNA was added to individual wells of a 96 well PCR plate. A 19 µL master mix which included 10 µL of Fast SYBR Green I master mix (Applied Biosystems, Ireland), 8 µL of nuclease-free water and 0.5 µL each of forward and reverse primers at concentrations individually optimised for each primer set was then added to each well containing cDNA. The following RT-qPCR cycle

conditions were applied: 95 ^{o}C for 20 s followed by 40 cycles of 95 ^{o}C for 3 s and 60 ^{o}C for 30 s, finishing with amplicon dissociation at 95 ^{o}C for 15 s, 60 ^{o}C for 1 min increasing 0.5 ^{o}C per cycle until 95 ^{o}C was reached for 15 s followed by 60 ^{o}C for 15 s. A pooled cDNA sample was included in triplicate on each plate to serve as an interplate calibrator. Additionally, a no template control (NTC) was also included in triplicate on each plate. A quantification cycle (Cq) value was reported for each reaction, where this value represents the number of cycles at a given threshold level of fluorescence and can be used to calculate a relative quantity of input template present at the start of the PCR reaction.

2.2.5.6.3. RT-qPCR optimisation and efficiency calculation

In order to assess optimum concentrations of use for each primer, 5 µM, 10 µM and 20 µM aliquots were made by combining the forward and reverse primer for each gene and making dilutions with nuclease-free water. RT-qPCR was then performed and the dissociation curves were examined for a single melting curve and the absence of primer dimer. The lowest acceptable concentration for each adapter was chosen. Once an optimum primer concentration was selected, the efficiency of each primer reaction was assessed. This was performed through the use of a serial dilution of pooled cDNA samples, starting with neat cDNA and working up to a 1 in 32 dilution. The slope of the linear curve of Cq values, plotted against the log dilution was used to calculate the efficiency of each reaction using the equation: $-1 + 10^{(-1/Slope)}$. Primers with a PCR efficiency of between 90 and 110 % were selected for use.

Table 2.13. Primers for RT-qPCR candidate genes for Chapter 4 were designed using Primer3 software based on bovine sequences obtained from the NCBI database.

Gene		Sequence 5' → 3'	Amplicon Size	NCBI Accession
IL1β	F:	CAGTGCCTACGCACATGTCT	167 bp	NM 174093
	R:	CCAGGGATTTTTGCTCTCTG		
IL2	F:	GTGAAGTCATTGCTGCTGGA	101 bp	NM_180997
	R:	GGCGCGTAAAAGTCAAATGT		
IL4	F:	CTGCCCCAAAGAACACAACT	165 bp	EU276069
	R:	TCGTCTTGGCTTCATTCACA		
IL8	F:	TGGGCCACACTGTGAAAATT	92 bp	Pang *et al*., 2009a
	R:	CCTTCTGCACCCACTTTTCC		
IFNγ	F:	TTCAGAGCCAAATTGTCTCC	205 bp	Neuvians *et al*., 2004
	R:	AGTTCATTTATGGCTTTGCGC		
TNFα	F:	TGGAGGGAGAAGGGATTCTT	140 bp	AF011926
	R:	CCAGGAACTCGCTGAAACTC		
Lymphotoxin	F:	GCTGCATCCCTAAGAACAGC	141 bp	BC149732
	R:	CATCCGGCTCAAAAATCAGT		
TLR4	F:	TGGTAAACCCCAGAGTCCAG	164 bp	NM_174198
	R:	GCACAATGCTTGGTACATGG		
NFkB1	F:	GCACCACTTATGACGGGACT	148 bp	NM_001076409
	R:	TCCTCATCCCAGGAGTCATC		
NFkB2	F:	ATCTGAGCATTGTGCGACTG	131 bp	NM_001102101
	R:	CTTCAGGTTTGAGGCTCCAG		
GRα	F:	CCATTTCTGTTCACGGTGTG	132 bp	AY238475
	R:	CTGAACCGACAGGAATTGGT		
Fas	F:	AGTTGGGGAGATGAATGCTG	171 bp	NM_174662
	R:	CCTGTGGATAGGCATGTGTG		
Haptoglobin	F:	TGGTCTCCCAGCATAACCTC	185 bp	BC109668
	R:	AGGGTGGAGAACCACCTTCT		
CD62L	F:	CCGATTGCTGGACTTACCAT	194 bp	NM_174182
	R:	CCAAGTCCACACCCCTTCTA		
p21	F:	TCCAAGGACTTTTTCCATTTG	75 bp	Buckham *et al*.,2008a
	R:	TCTGACTCCTTCAGCTGTTAT		
BPI	F:	TTCAGAAATGATCCAAACAT	81 bp	Buckham *et al*., 2007
	R:	GCCCTTGGAAGAAACAATTC		
ACTB	F:	ACTTGCGCAGAAAACGAGAT	123 bp	BT030480
	R:	CACCTTCACCGTTCCAGTTT		
SDHA	F:	AACTGCGACTCAACATGCAG	132 bp	NM_001034034
	R:	TGTCGAACGTCTTCAGATGC		
GAPDH	F:	GGGTCATCATCTCTGCACCT	176 bp	DQ402990
	R:	GGTCATAAGTCCCTCCACGA		

2.2.5.6.4. PCR product verification

To validate the sequence of each primer used in the RT-qPCR analysis, PCR products were generated for size confirmation and sequencing. A master mix containing 45 µL Platinum PCR Supermix, 2 µL of 20 µM primer, 2 µL cDNA and 1 µL nuclease-free water was prepared in a PCR safe tube. This was place into the Mastercycler Thermal Cycler under the following conditions.

	Step 1	**Step 2**	**Step 3**	**Step 4**	**Step 5**	**Step 6**
Temperature	95 ˚C	95 ˚C	60 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	2 min	30 sec	30 sec	20 sec	5 min	∞

Repeat: 38 cycles

The PCR products were then stored at -20 ˚C until analysis. A 2 % agarose gel was prepared using 1X TAE buffer. Each PCR product was removed from the freezer and a 20 µL aliquot removed, to which 5 µL of dye was then added. This was then loaded onto the agarose gel. Each gel was examined to ensure a single product and an amplicon similar in size to the Primer3 design. Once size was confirmed, the PCR products were then outsourced for Sanger Sequencing to confirm their sequence identity (Biochemistry DNA Sequencing Facility, University of Cambridge).

2.2.5.6.5. Reference gene selection

Five high abundant genes were examined for their suitability as reference genes for use in this study. These genes were beta-actin (*ACTB*), succinate dehydrogenase complex subunit A (*SDHA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*) and 14-3-3 protein zeta/delta (*YWHAZ*). Reference genes were selected using geNorm software v3.5 (Vandesompele *et al.*, 2002) which determines the average stability of each gene based on its expression across all samples and returns an

average stability value, M, for each individual gene along with a suitable collection of genes for use in differential gene expression analysis. Based on the average pairwise variation, V, three genes (beta-actin (*ACTB*), succinate dehydrogenase complex subunit A (*SDHA*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)), were found to have an average stability value of M = 0.24 and were used for subsequent analysis.

2.2.5.6.6. RT-qPCR data analysis

Raw Cq values were exported from the Applied Biosystems 7500 FAST RT-PCR equipment into GenEx software v.5.2.2.8 (MultiD Analyses AB, Göteborg, Sweden) and used to perform the following analysis. Outliers were removed from replicate wells using a modified Grubbs test (Miller and Miller, 2000) at a $P < 0.05$ confidence interval for any replicate differing from the replicate mean by a standard deviation of more than 0.25 cycles using the following equation where x is the value of the suspected outlier, X_{group} is the group mean, N is the number of measurements in the group and SD_0 is the cutoff standard deviation, in this case 0.25:

$$Z = \frac{|x - \bar{X}_{group}|}{SD\,(group)}$$

$$G = \frac{N-1}{\sqrt{N}} \sqrt{\frac{t^2_{\alpha/(2N),N-2}}{N-2+t^2_{\alpha/(2N),N-2}}}$$

If Z is greater than G, or the SD is greater than SD_0, then the value is considered an outlier. As four plates per gene were required, adjustments were made to mean centre the interplate calibrators (IPC) prior to normalisation with the following equation where m is the number of interplate calibrators in run m and n is the total number of interplate calibrators:

$$Cq_{norm} = Cq_{GOI} - \frac{1}{m}\sum_{j=1}^{m}\left(Cq_{IPC_j} - \frac{1}{n}\sum_{i=1}^{n} Cq_{IPC_i}\right)$$

Cq values were then adjusted based on efficiencies calculated in section 2.2.5.6.3 and the following equation where Cq_E is the uncorrected Cq value and E is the calculated efficiency:

$$Cq_{E=100\%} = Cq_E \frac{\log(1+E)}{\log(2)}$$

A mean value was then calculated within each set of Cq values, after which the expression of each gene of interest (GOI) was normalised to the expression of the reference genes (RG) using the following equation:

$$Cq_{norm} = Cq_{GOI} - \frac{1}{n}\sum_{i=1}^{n} Cq_{RG_i}$$

Finally, GenEx was used to calculate relative quantities by making all values relative to the most highly expressed value, as demonstrated by the following equation:

$$N_{Rel} = 2^{(Cq_{min} - Cq)}$$

2.2.6. RNA-seq library preparation (Chapter 5)

Leukocytes were isolated from whole blood and total RNA extracted as described in Sections 2.2.4.2 and 2.2.5.1. In order to prepare 48 libraries (6 animals from 2 treatments on d 0, 1, 2 and 7) for sequencing on the Illumina Genome Analyzer II (GA_{II}), the following library preparation was carried out.

2.2.6.1. Reagent preparation

Binding (1X) and washing (1X) buffers were prepared prior to mRNA purification of total RNA. To prepare a 20.0 mL working solution of Binding Buffer, 400 µL 1 M Tris/0.1 M EDTA was added to 17.1 mL of nuclease-free water. A 2.5 mL aliquot of 8 M LiCl was then added, giving a binding buffer with 20 mM Tris-HCl, 1 M LiCl and 2 mM EDTA. Washing buffer was prepared by adding 200 µL 1 M Tris/0.1 M EDTA

and 370 µL 8 M LiCl to 19.43 mL of nuclease-free water, resulting in a 10 mM Tris-HCl, 0.15 M LiCl, 1 mM EDTA washing buffer.

2.2.6.2. mRNA purification from total RNA

A 10 µg aliquot of total RNA was placed in a 2.0 mL RNase free non-sticky Dolphin Microcentrifuge tube and diluted to 50 µL with nuclease-free water. Each sample was placed in a heat bath at 65˚C for 5 min in order to disrupt the secondary RNA structures and then placed immediately on ice. A 100 µL aliquot of Dynabeads Oligo(dT) was then placed into a 2.0 mL RNase free non-sticky Dolphin microcentrifuge tube. A 100 µL aliquot of beads will bind up to 1 µg of poly A^+ RNA. These beads were then washed twice with 100 µL of binding buffer where they were briefly vortexed each time and then placed on a magnetic rack for 30 sec before removal of the supernatant in order to wash storage buffer from the beads. The beads were then resuspended in 50 µL Binding Buffer and the 50 µL of heated total RNA was added, mixed well and placed on a Rotator for 5 min at room temperature. This tube was then placed on the magnetic rack and approximately 100 µL of supernatant containing mRNA depleted RNA was removed and stored. The mRNA bound beads were then washed with 100 µL washing buffer, placed on the magnetic rack and the supernatant removed. This step was repeated a second time, followed by the addition of 20 µL 10 mM Tris-HCl (pH 7.5). The mRNA bound beads were heated at 80˚C for 2 min in order to elute the mRNA and then immediately placed on the magnetic rack. The supernatant containing mRNA was then transferred to a tube containing 80 µL binding buffer, heated at 65 ˚C for 5 min and then placed on ice. To prepare the beads for the second round of mRNA binding and reduce rRNA contamination, the beads were washed twice with 100 µL of washing buffer as described above. The 100 µL of first round selected mRNA was then added to the beads and placed on a rotator for 5 min at room temperature. Approximately 100

µL of mRNA depleted RNA was removed and added to the aliquot earlier removed and stored at -80˚C. The mRNA bound beads were then washed twice with 100 µL washing buffer and following the second wash, 10 µL of 10 mM Tris-HCl was added and mixed well. The beads were then heated at 80 ˚C for 2 min and immediately transferred to a magnetic rack. Approximately 9 µL of supernatant containing mRNA was collected and placed into a 200 µL thin wall PCR tube.

2.2.6.3. mRNA fragmentation

Zinc mediated fragmentation was employed to fragment mRNA from this study in order to prepare libraries for sequencing. To the 9 µL of isolated mRNA was added 1 µL 10X Fragmentation Reagent. This reaction was incubated in the Mastercycler Thermal Cycler at 70 ˚C for exactly 5 min. The reaction was then halted by the addition of 1 µL Stop Buffer and placed on ice. The fragmented contents of the tube were then transferred to a 1.5 mL microcentrifuge tube and 1 µL 3 M NaOAc (pH 5.2), 2 µL Glycogen (5 µg/µL) and 30 µL 100 % Ethanol were added. This was incubated at -80 ˚C for 30 min, followed by centrifugation at 14,000 × g for 25 min at 4 ˚C in a microcentrifuge. The supernatant was carefully removed as the glycogen carrier pellet was very loose, followed by the addition of 500 µL of 80 % Ethanol to wash the pellet. The tube was then spun at 14,000 × g for 5 min at 4 ˚C. The Ethanol supernatant was removed and the pellet allowed to air-dry. The fragmented mRNA pellet was then resuspended in 10.5 µL of nuclease-free water.

2.2.6.4. cDNA synthesis

In order to synthesise the first strand of cDNA, the 10.5 µL of fragmented mRNA transferred to a 200 µL thin wall PCR tube, along with 1 µL Random Hexamer Primers (3 µg/µL). This reaction was incubated in the Mastercycler Thermal Cycler at 65 ˚C for

5 min and then placed on ice. A master mix was then prepared, consisting of 4 µL 5 × first strand buffer, 2 µL 100 mM DTT, 1 µL 10 mM dNTP mix 0.5 µL RNaseOUT (40 U/µL). To each mRNA/Hexamer mix, 7.5 µL of master mix was added and mixed well by pipetting. The tube was then incubated at 25 ˚C for 2 min. Following this incubation, 1 µL SuperScriptII was added to each sample. The samples were then placed into the Mastercycler Thermal Cycler under the following conditions:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 ˚C	42 ˚C	70 ˚C	4 ˚C
Time	10 min	50 min	15 min	∞

To synthesise the second strand of cDNA, 51 µL of nuclease-free water was added to the first strand synthesis and then placed on ice. This first strand synthesis was removed from the ice and 20 µL 5 × Second Strand buffer and 3 µL 10 mM dNTP mix was added and mixed well. This reaction was then allowed to incubate on ice for 5 min. Once well chilled, 1 µL of RNaseH (2 U/µL) and 5 µL DNA Pol I (10 U/µL) were added, mixed well and placed in the Mastercycler Thermal Cycler for 150 min.

The double-stranded cDNA was then purified with a QIAquick PCR spin column. Briefly, this involved adding 500 µL Buffer PB to each PCR reaction and mixing well. The sample was then transferred to a QIAquick PCR spin column and centrifuged at 10,000 × g for 60 sec at room temperature. The flow-through was discarded and 750 µL of Buffer PE was added to the spin column to wash the sample. This was left for 1 min and then spun at 10,000 × g for 60 sec. The flow-through was discarded and the column was respun for an additional 1 min to ensure residual buffer PE was removed. The column was placed into a clean tube and 30µL of EB solution was added directly to membrane and left to stand for 1 min prior to centrifugation at 10,000 × g for 60 sec. The flow-through containing purified, double-stranded cDNA was then saved for library preparation.

2.2.6.5. Preparation of cDNA fragments for adaptor ligation

Following cDNA synthesis, the cDNA fragments had to be prepared for adapter ligation. This was accomplished by blunting the ends of each DNA fragment through a combination of T4 DNA Polymerase and *E.* coli DNA Polymerase I Klenow Fragment with the 3'-to-5' exonuclease activity of the enzymes removing 3'-overhangs and the polymerase activity filling in 5'-overhangs. A reaction mix was prepared which included 30 µL elucted cDNA, 45 µL nuclease-free water, 10 µL T4 DNA Ligase buffer with 10 mM ATP, 4 µL 10 mM dNTP mix, 5 µL T4 DNA Polymerase (3 U/µL), 1 µL Klenow DNA Polymerase (5 U/µL) and 5 µL T4 Polynucleotide Kinase (PNK) (10 U/µL). This was incubated at 20 ˚C for 30 min and purified with a QIAquick PCR spin column as described at the end of the previous section, and eluted in 32 µL of EB solution.

Following the blunting of the DNA fragments, a single 'A' base was then added to the 3' end of the blunt phosphorylated DNA fragments using the polymerase activity of Klenow (3'-to-5' exo minus). This prepared the end of the DNA fragments for ligation to the adapters which have a single 3'-T overhang. A reaction mix was prepared which contained the 32 µL eluted DNA along with 5 µL Klenow buffer, 10 µL 1 mM dATP and 3 µL Klenow 3'-to-5' exo- (5 U/µL). This reaction was incubated at 37 ˚C for 30 min, following which the DNA was purified using a QIAquick MinElute column. Briefly, 250 µL Buffer PB was added to each sample and mixed well. This was then added to the MinElute spin column and spun at 17,900 × g for 1 min at room temperature. The flow-through was discarded and 750 µL wash Buffer PE was added to the column and centrifuged at 17,900 × g for 1 min. The flow-through was discarded and the spin column respun for 1 min. The column was placed into a fresh tube and 23

µL of EB solution was added directly to the membrane, left to sit for 1 min, and then spun at 17,900 × g for 1 min.

Adaptors were ligated to the 'A' overhang of the DNA fragments in a reaction that consisted of the 23 µL eluted DNA, 25 µL 2X Quick DNA Ligase buffer, 1 µL Adaptor-Oligo mix and 1 µL Quick T4 DNA Ligase. This reaction was left to incubate at 22 ˚C for 15 min and then purified with a QIAquick MinElute spin column as described above. However, the column was spun at full speed twice following removal of Buffer PE to ensure absolutely no ethanol carry over and the DNA was eluted in 10 µL of EB solution.

2.2.6.6. Gel purification of adapter ligated DNA templates

A 2 % agarose gel was prepared using Certified Low Range Ultra Agarose in 1 × TAE buffer. As this agarose is difficult to melt, it was microwaved for 15 min, with vigorous swirling every 3 min to ensure the agarose was evenly dispersed in the TAE, with 1 µL ethidium bromide per 25 mL of gel added to the cooling gel. A DNA ladder was prepared by adding 2 µL of dye to 8 µL of DNA ladder. To prepare adapter ligated DNA templates, 8 µL of dye was added to the 10 µL DNA template. These libraries were loaded onto the gel, with a ladder loaded into the wells on either side and a space between each set of ladder surrounded samples to prevent cross contamination. The gels were run at 120 V for 70 min. The gels were then transferred to a Dark Reader Transilluminator and a band corresponding to the 250 bp band on the ladder was excised using a GeneCatcher Disposable Gel Excision Tip for each sample. The QIAquick Gel Extraction kit was then used to purify DNA out of these gel slices. Briefly, 600 µL of Buffer QC was added to a tube containing the gel slice. This was then incubated at 50 ˚C for 10 min, with the sample being vortexed every 3 min. Once

the gel was dissolved in the Buffer QC, 200 µg of isopropanol was added and thoroughly mixed. The sample was transferred to a QIAquick spin column and spun at 10,000 × g for 1 min at room temperature. The flow-through was discarded and 500 µL of Buffer QC was added to the column to remove all traces of agarose, allowed to sit for 1 min, and then centrifuged 10,000 × g for 1 min. A 750 µL wash, Buffer PE, was added to the spin column and left to sit for 5 min prior to centrifugation at 10,000 × g for 1 min. The flow-through was then discarded, the spin column moved to a fresh tube and respun. The spin column was again transferred to a fresh tube, 30 µL of EB solution added to the membrane, left to sit for 1 min and centrifuged at 10,000 × g for 1 min.

2.2.6.7. PCR enrichment of purified, adapter ligated DNA templates

A PCR master mix was prepared in order to amplify the purified, adapter ligated libraries. This master mix consisted of 10 µL 5 × cloned Phusion High-Fidelity Buffer, 1 µL PCR primer 1.1, 1 µL PCR primer 2.1, 0.5 µL 25 mM dNTP mix, 0.5 µL Phusion DNA polymerase and 7 µL nuclease-free water per reaction. This master mix was well mixed and 20 µL was then transferred into each thin walled PCR tube. The 30 µL gel purified adapter ligated material was then added to make a final reaction volume of 50 µL. This reaction was then placed into the Mastercycler Thermal Cycler to amplify the library under the following conditions:

	Step 1	**Step 2**	**Step 3**	**Step 4**	**Step 5**	**Step 6**
Temperature	98 ˚C	98 ˚C	65 ˚C	72 ˚C	72 ˚C	4 ˚C
Time	30 sec	10 sec	30 sec	30 sec	5 min	∞

Repeat: 15 cycles

The DNA libraries were then purified with a QIAquick PCR spin column as described above.

2.2.6.8. Quantitation of RNA-seq libraries prior to sequencing

Before sequencing, each sample was assessed by two methods, gel assessment to examine library size and integrity, and quantification assessment with the Qubit Fluorometer. To check that the correct library size had been selected, 5 µL of library was mixed with 1 µL of dye and loaded next to a ladder on a 2 % agarose gel. This was run at 120 V for 60 min and examined on a Bio-Rad PharosFX Plus Molecular Imager (Figure 2.5).

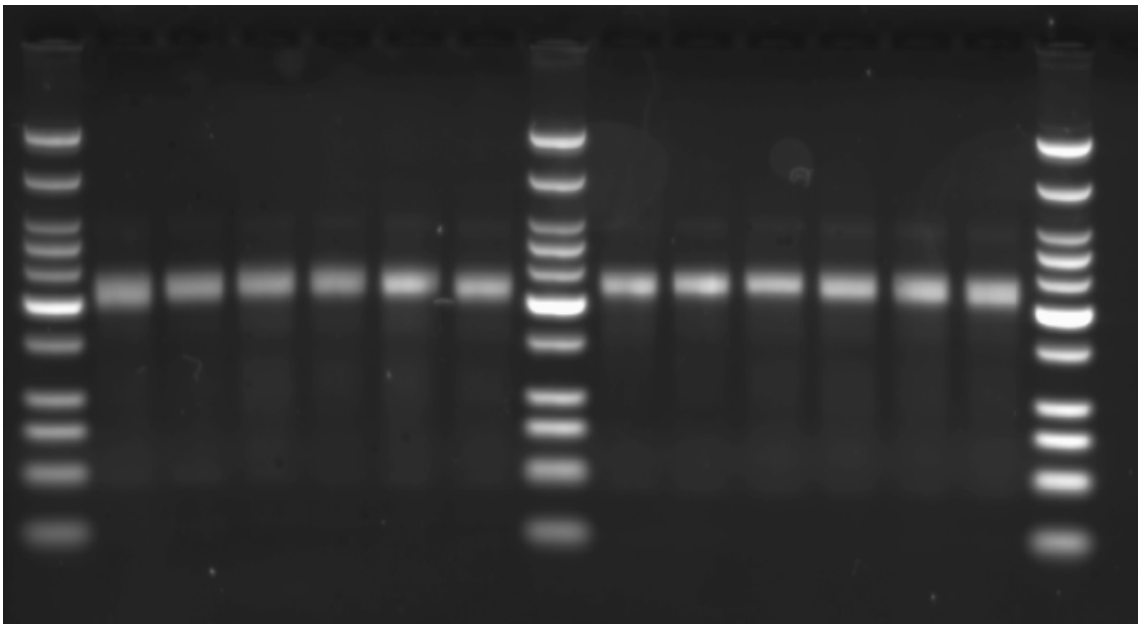

Figure 2.5. Libraries prepared for sequencing on the Illumina GA_{II}.

The Qubit Fluorometer was calibrated each time prior to use. One thin walled, 500 µL PCR tubes was labelled per sample plus 2 tubes for the calibration standards. A 1:200 dilution of Qubit dsDNA HS reagent in Qubit dsDNA HS buffer was prepared, allowing 200 µL per sample. From this Qubit working solution, 190 µL was added to each of the tubes labelled as standards. Qubit Standard 1 and 2 were then added to their respective tubes and vortexed for 3 sec, ensuring no bubbles remained following removal from the vortex. To prepare samples for measurement, 199 µL of Qubit working solution was

added to each labelled tube and 1 µL of sample was then added to their respective tube. These were then placed on the vortex and examined to ensure there were no bubbles following removal. All tubes were then allowed to sit at room temperature for 2 min. The Qubit was then turned on and dsDNA HS Assay was selected. The option to read new standards was selected and once prompted, the first standard was inserted into the Qubit. When prompted, the second standard was inserted into the Quibit. Once calibrated, the Qubit was set to read DNA at a dilution of 1:200 and each sample was then run one at a time. The results were then converted from ng/mL to ng/µL.

2.2.6.9. Sequencing of libraries

All prepared RNA-seq libraries were brought to the Conway Institute, University College Dublin, where they were sequenced on an Illumina Genome Analzer II (Figure 2.6) using 36 bp, single read, version 4 kits, according to the manufacturers' instructions.

2.2.7. RNA-seq bioinformatic analysis

A suite of bioinformatic tools were utilised in the analysis of data output from the GA_{II} following RNA-seq, converting raw reads into read counts, allowing a calculation of differential expression and the identification of over-represented Gene Ontology (GO) terms and pathways (Figure 2.6).

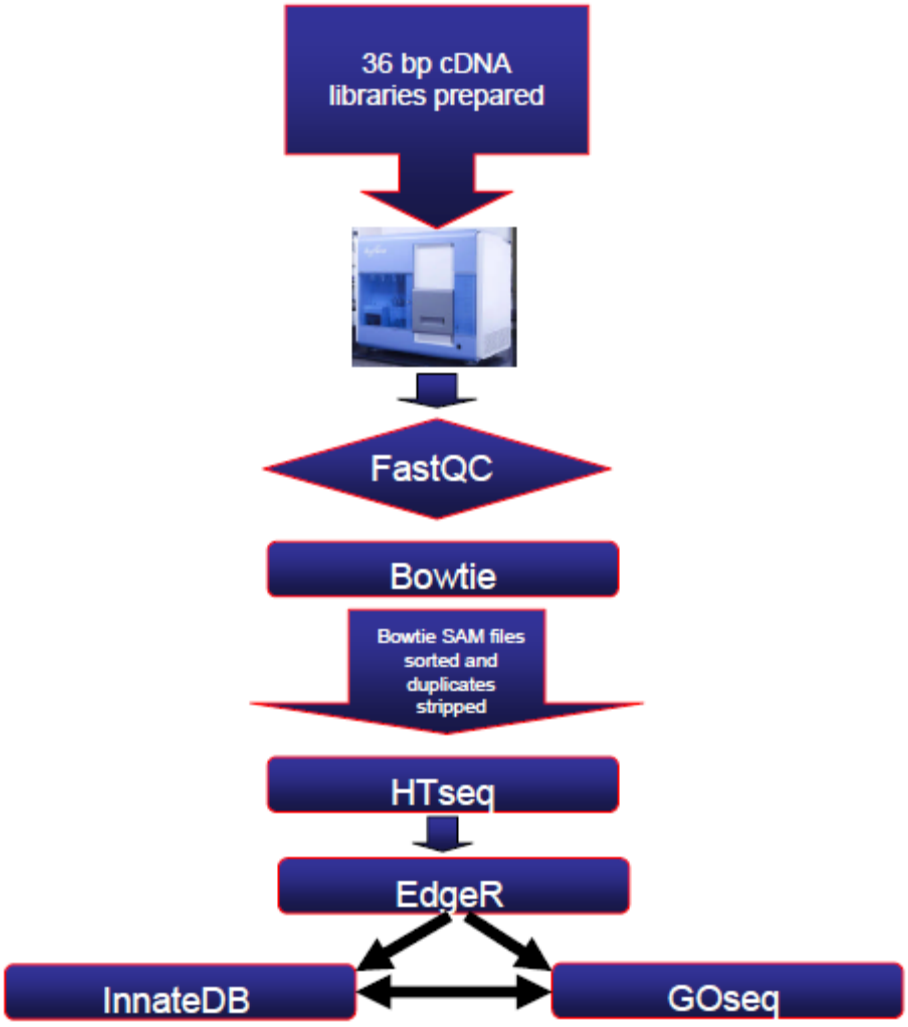

Figure 2.6. Bioinformatic pipeline used in the analysis of RNA-seq data

2.2.7.1. Alignment of sequencing reads to the bovine genome and generation of gene counts

FastQC (version 0.9.1) (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) was used to assess the quality of reads from each lane of each flowcell based on a number of variables including per base sequence quality, per sequence quality score, per base N count, sequence length distribution and over-represented sequences. These quality control checks were performed for each sample in order to assess if there were any sequencing or library preparation issues (Figure 2.7).

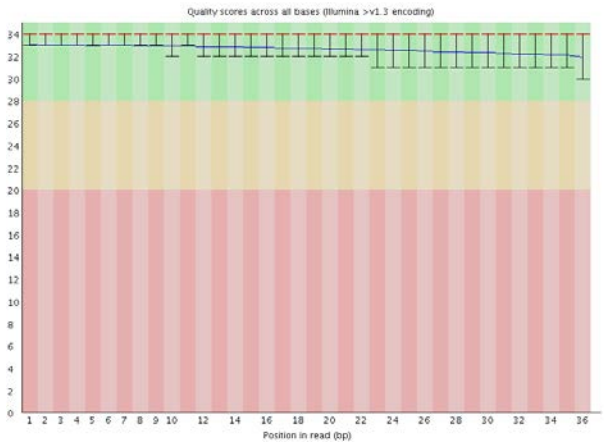

Figure 2.7. High quality "Per Base Sequence Quality" Report from FastQC. The "Per Base Sequence Quality" report is one of the quickest assessments of the quality of raw reads. A trailing off of quality is typically found in later reads (~28 bp).

Bowtie (version 0.12.7), the ultrafast, memory-efficient, short read aligner was used to align the fastq output reads from the Genome Analyzer to version 4.0 of the bovine genome (Btau4) (Langmead *et al.*, 2009). The Bowtie script used allowed for up to 1 mismatch between a read and the reference genome and any read that had more than one reportable alignment to the reference genome was suppressed to avoid ambiguity in location. All retained reads were output in SAM format for further analysis. Using Strip_Sam_Duplicates, a script developed in house (Chris Creevey, personal communication), multiple reads that aligned to the exact same position in the genome were removed due to potential PCR bias introduced during library preparation. A single read with the highest PHRED quality score was retained at each of these locations. HTseq (v0.4.6p2) (http://www-huber.embl.de/users/anders/HTSeq) was used to convert aligned reads into counts per gene using the union model (Figure 2.8) and the Ensembl v61 annotation of the bovine genome (ftp://ftp.ensembl.org/pub/release-61/fasta/bos_taurus/dna/). In order to search for evidence of alternative splicing, HTseq was also used to assign counts to each annotated exon.

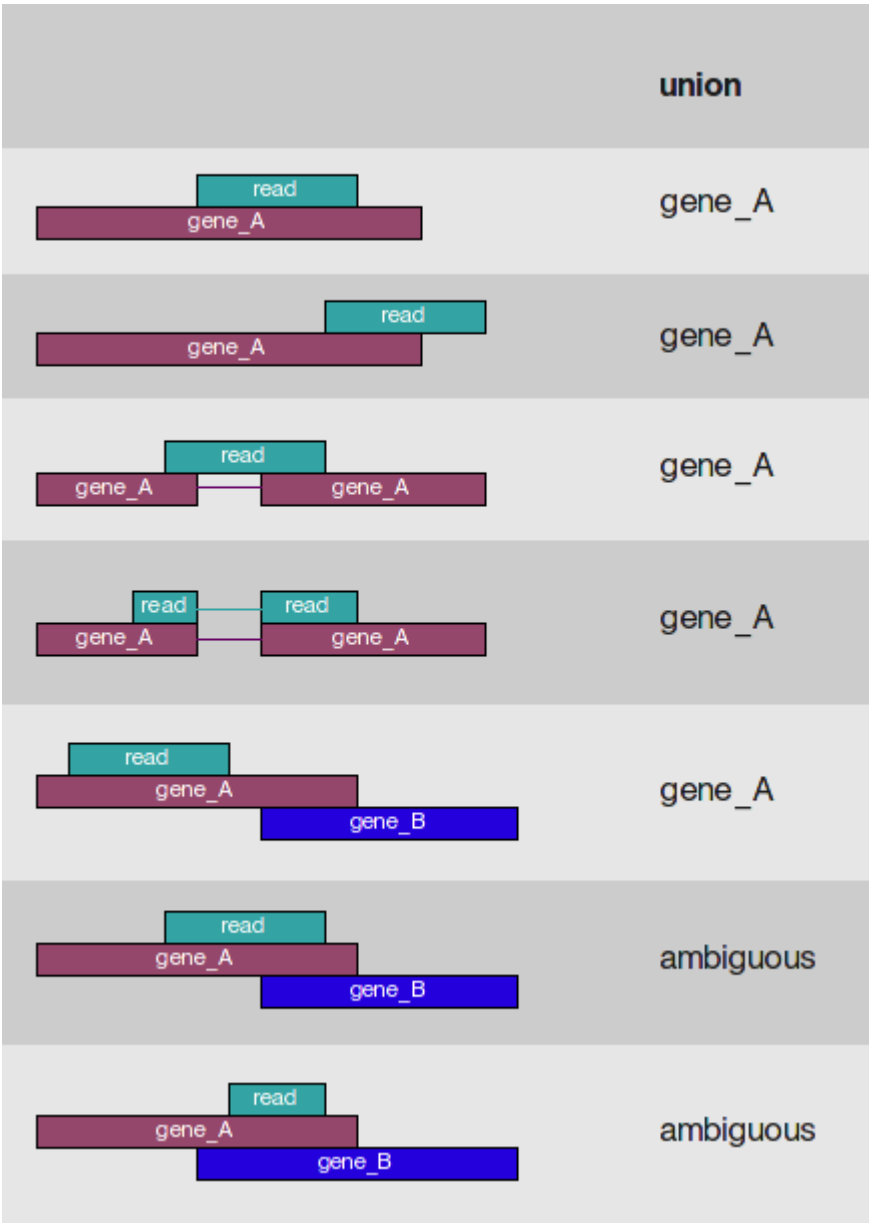

Figure 2.8. HTseq union model used in the generation of read counts.

(http://www-huber.embl.de/users/anders/HTSeq/doc/count.html#count)

2.2.7.2. Identification of differentially expressed genes

The R (version 2.11.0) Bioconductor package EdgeR (version 1.6.12) (Robinson *et al.*, 2010) was used to identify differentially expressed genes, and separately, exons based on read counts assigned by HTseq. EdgeR models data as a negative binomial distribution to account for biological and technical variation using a generalisation of the Poisson distribution model. Prior to assessing differential expression, data were first

normalised across libraries using the trimmed mean of M-values normalization method (Robinson and Oshlack, 2010; Roberts *et al*., 2011). Genes and exons were considered differentially expressed with a Benjamini-Hochberg false discovery rate (FDR)-corrected P-value < 0.05 and a fold change ≥ 2 (Benjamini and Hochberg, 1995).

2.2.7.3. Identification of over-represented GO terms and pathways

Differential expression in RNA-seq data is calculated based on the number of reads aligning to a gene, thus there is greater statistical power to detect longer genes as significantly differentially expressed than shorter genes (Oshlack and Wakefield, 2009). If a set of genes associated with Gene Ontology (GO) categories (The Gene Ontology Consortium, 2000) are a non-random set with a preponderance of long or short genes, as it often the case, then transcript length will result in a bias of the GO analysis (Young *et al*., 2010). Therefore, it is necessary to correct for this bias in RNA-seq data when identifying over-represented GO terms as there will be greater statistical power to detect GO categories with a prevalence of longer genes as over-represented.

Differentially expressed genes (DEG) were analysed using the R package GOseq (version 1.1.7) which corrects for the inherent gene length bias present in RNA-seq data (Young *et al*., 2010). The software package quantifies the likelihood of a gene being differentially expressed as a function of transcript length which is incorporated into the final statistical test. GOseq was used to identify GO terms which were significantly more represented than expected by chance. GO terms were considered statistically significant at an FDR < 0.1.

The InnateDB pathway analysis tool (Lynn *et al*., 2008; 2010) was used to identify significantly over-represented biological pathways using the hypergeometric test.

InnateDB incorporates pathway annotations from several of the major publicly available pathway databases including the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the NCI-Nature Pathway Interaction Database (PID) (http://pid.nci.nih.gov), Integrating Network Objects with Hierarchies (INOH) pathway database (http://www.inoh.org/), NetPath (http://www.netpath.org) and Reactome databases. Due to the minimal amount of pathway annotation available for the bovine genome, bovine genes were converted to their human Ensembl orthologs prior to analysis. Pathways were considered significantly differentially expressed with an FDR < 0.1. A potential concern was that the pathway analysis of RNA-seq data may be subject to the same gene length related biases affecting the GO analysis. To determine whether this was the case, GOseq was also used (using imported InnateDB pathway annotations, March 15, 2011 release) to identify over-represented pathways. The same pathways were identified by InnateDB and GOseq and as such InnateDB, an interactive web-based platform, was utilised.

2.2.7.4. oPOSSUM analysis to detect transcription factors likely to contribute to differential gene expression

A further analysis was performed to identify transcription factors that may be involved in regulating gene expression during the weaning stress response. Significantly differentially expressed human orthologs from each comparison were divided into two groups for analysis, up-regulated and down-regulated genes. These groups of genes were then analysed using the publicly available web-based system, oPOSSUM (Ho Sui *et al*., 2005) to detect over-represented transcription factor binding sites (TFBS) in the promoter regions of each set of genes. To detect common TFBS, the approach was taken to examine 5,000 base pairs upstream and downstream of each transcription start site (TSS). Transcription factors were matched based on their corresponding binding

site and over-represented transcription factors were considered to be significant at a Z-score ≥ 10 and a Fisher score ≤ 0.01.

Chapter 3

Assessment of haematological distribution, the acute phase protein response, the stress hormone, cortisol and the neutrophil chemokine, CXCL8, as biomarkers of weaning stress at housing in the bovine

3.1. Introduction

Weaning has been documented to cause a physiological stress response in beef calves, altering blood metabolites (Lynch *et al*., 2010b), leukocyte cellular distribution patterns (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b), acute phase proteins (Arthington *et al*., 2005; Lynch *et al*., 2010b) and hormones of the neuroendocrine system (Lefcourt and Elsasser, 1995; Hickey *et al*., 2003b). Weaning is a multifaceted stressor and may involve numerous husbandry practices, including the abrupt separation of the calf from its dam, a nutritional adjustment to a non-milk diet and social reorganisation and, additionally, is often associated with other husbandry practices, such as housing (Lynch *et al*., 2010a; Enriquez *et al*., 2011). Neutrophilia is one of the most frequently reported biomarkers of stress in cattle following transport (Buckham Sporer *et al*., 2007, 2008a; Gupta *et al*., 2007b), weaning (Hickey *et al*., 2003b; Gupta *et al*., 2007a; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b) and housing (Hickey *et al*., 2003a; Gupta *et al*., 2007a). Gene expression of the potent neutrophil chemokine, CXCL8, was also found to be up-regulated following transport (Buckham Sporer *et al*., 2008a) and castration (Pang *et al*., 2009a), and may account for distributional alterations in circulating neutrophils, serving to increase immune surveillance. Stress is also reported to induce an acute phase response and a number of acute phase proteins (APP), including haptoglobin and serum amyloid A (SAA), have been proposed as biomarkers of stress in cattle (Alsemgeest *et al*., 1995; Arthington *et al*., 2003; Hickey *et al*., 2003b) with increased concentrations of APP demonstrated in calves following transportation (Murata and Miyamoto, 1993), abrupt weaning (Hickey *et al*., 2003b) and housing (Alsemgeest *et al*., 1995). Housing has been reported to alter the neutrophil and lymphocyte immunophenotype of calves, along with the acute phase response (Lynch *et al*., 2010a), with a more pronounced stress response occurring in calves weaned at housing compared with those housed with their dams (Lynch *et al*.,

2010a; 2010b). Therefore, separating these husbandry practices appears to be an acceptable strategy to reduce the accumulative effects of stress during the weaning procedure.

While stress is typically suspected of suppressing immune function (Reiche *et al*., 2004; Glaser and Kiecolt Glaser, 2005; Saul *et al*., 2005; Babcock *et al*., 2010), there is evidence to suggest that a stress response, triggered by weaning or transport, may in fact activate an immune response in animals that prepares them to respond to a disease challenge (Black, 2002; Buckham Sporer *et al*., 2007; Dhabhar, 2009). Therefore, the objective of this study was to further examine the response to weaning and housing in order to assess the APP response to stress while investigating the role that plasma CXCL8 plays in the immune response to weaning. By identifying the participation of inflammatory mediators in the stress response, this thesis will contribute to a better understanding of the immune status of weaned beef calves and aid in future targeted gene expression studies of stress in cattle. The experimental design for this chapter is described, in detail, in Chapter 2 (section 2.2.2.4). In brief, calves were divided into two treatments, 1) control; calves were removed from pasture and housed with their dams, 2) weaned; calves were removed from pasture, abruptly weaned and housed with cohorts.

3.2. Physiological response of calves weaned at housing

3.2.1. Rectal body temperature

There was no treatment × sampling time (P > 0.05) interaction for rectal body temperature, although rectal temperature did increase (P < 0.01) in both treatments following either housing or weaning at housing, with a higher temperature reported in weaned calves on d 2 that control calves (P < 0.01) (Table 3.1). Rectal temperature remained elevated from baseline to d 14.

Table 3.1. Effect of weaning induced stress at housing on rectal body temperature in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	14	T	S	T×S
Rectal Temperature (˚C)	C	38.0± 0.21	38.8^{b}± 0.21	38.7^{bx}± 0.07	38.8^{b}± 0.12	38.6^{b}± 0.09	38.7^{a}± 0.11	*	*	NS
	W	38.1± 0.21	39.1^{c}± 0.21	39.0^{cy}± 0.07	39.0^{b}± 0.12	38.8^{b}± 0.09	38.8^{a}± 0.11			

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.
^{a,b,c}Within rows, Lsmeans differ from pre-weaning baseline by *P* < 0.05, *P* < 0.01 and *P* < 0.001, respectively. ^{x,y}Between rows, Lsmeans differ by *P* < 0.05.

3.2.2. Leukocyte response to weaning at housing

There was no effect (P > 0.05) of treatment, sampling time or their interaction on lymphocyte, monocyte or basophil number (Table 3.2). For total leukocyte number, there was an interaction of treatment × sampling time (P < 0.01) whereby total leukocyte number was greater (P < 0.05) in weaned calves than control calves on day (d) 1 and d 2. Similarly, there was a treatment × sampling time interaction (P < 0.05) for neutrophil number. In weaned calves, neutrophils increased on d 1 (P < 0.05), d 2 (P < 0.001) and d 3 (P < 0.01) relative to baseline, resulting in higher levels compared to control calves on d 1, whereas in control calves, neutrophil number did not increase (P > 0.05) from baseline until d 3 (P < 0.05) (Figure 3.1). Despite no significant main

effects, lymphocyte number decreased ($P < 0.05$) from baseline in weaned calves on d 14. As a reflection of alterations to total neutrophil number, there was a treatment × sampling time interaction ($P < 0.001$) for the N:L ratio where the N:L ratio was elevated ($P < 0.01$) from baseline in weaned animals at sampling on d 1, d 2 and d 3, whereas an increase in control calves was not evident until d 3. There was an effect of treatment for eosinophil number where, on d 2, eosinophil number was greater ($P < 0.05$) in weaned calves than control calves.

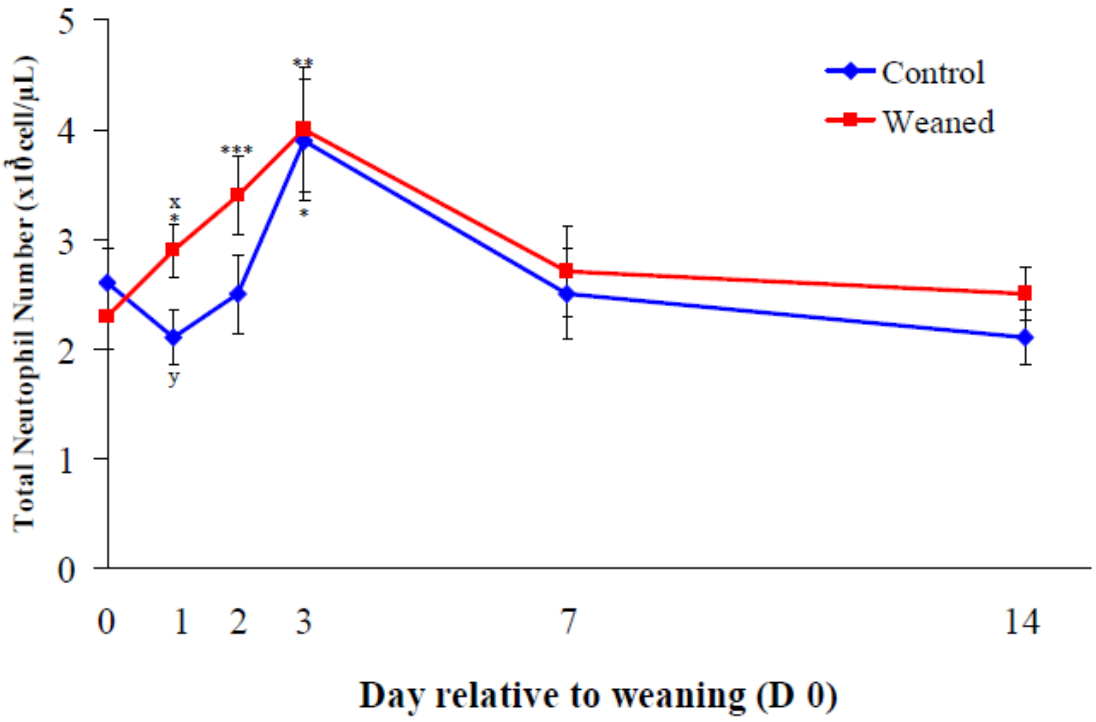

Figure 3.1. The neutrophil response to housing and weaning stress. Total neutrophil number increased immediately following abrupt weaning at housing while there is a delayed neutrophilia in housed calves on d 3. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ Lsmeans differ relative to baseline (D 0). X, Y Lsmeans differ between treatments ($P < 0.05$).

Table 3.2. Effect of weaning induced stress at housing on total leukocyte, neutrophil and lymphocyte number, neutrophil:lymphocyte (N:L) ratio, monocyte, basophil and eosinophil number in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	14	T	S	T×S
Total Leukocytes	C	8.7 ± 0.79	7.9^{x} ± 0.49	8.6^{x} ± 0.52	10.8^{a} ± 0.67	8.6 ± 0.72	8.3 ± 0.50	**NS**	*******	******
(×10^3 cells/µL)	W	10.1 ± 0.79	9.6^{y} ± 0.49	10.5^{y} ± 0.52	11.2 ± 0.67	9.7 ± 0.72	8.2 ± 0.50			
Neutrophils	C	2.6 ± 0.31	2.1^{x} ± 0.24	2.5 ± 0.36	3.9^{a} ± 0.56	2.5 ± 0.41	2.1 ± 0.24	**NS**	**NS**	*****
(×10^3 cells/µL)	W	2.3 ± 0.31	2.9^{ay} ± 0.24	3.4^{c} ± 0.36	4.0^{b} ± 0.56	2.7 ± 0.41	2.5 ± 0.24			
Lymphocytes	C	6.5 ± 0.47	6.1 ± 0.36	6.7 ± 0.37	6.9 ± 0.39	6.2 ± 0.35	5.9 ± 0.30	**NS**	**NS**	**NS**
(×10^3 cells/µL)	W	7.0 ± 0.47	6.4 ± 0.36	6.4 ± 0.37	7.1 ± 0.39	6.8 ± 0.35	5.8^{a} ± 0.30			
N:L	C	0.39 ± 0.041	0.36 ± 0.033	0.39 ± 0.040	0.62^{b}± 0.096	0.42 ± 0.044	0.35 ± 0.046	**NS**	*****	*******
Ratio	W	0.36 ± 0.041	0.46^{b}± 0.033	0.49^{c}± 0.040	0.55^{b}± 0.096	0.39 ± 0.044	0.46^{a}± 0.046			
Monocytes	C	0.52 ± 0.079	0.53 ± 0.051	0.43 ± 0.035	0.58 ± 0.044	0.51 ± 0.081	0.55 ± 0.049	**NS**	**NS**	**NS**
(×10^3 cells/µL)	W	0.46 ± 0.079	0.42 ± 0.051	0.39 ± 0.035	0.51 ± 0.044	0.48 ± 0.081	0.42 ± 0.049			
Basophils	C	0.11 ± 0.015	0.11 ± 0.011	0.12 ± 0.012	0.14 ± 0.013	0.13 ± 0.012	0.11 ± 0.011	**NS**	**NS**	**NS**
(×10^3 cells/µL)	W	0.13 ± 0.015	0.10 ± 0.011	0.11 ± 0.012	0.12 ± 0.013	0.12 ± 0.012	0.10 ± 0.011			
Eosinophils	C	0.16 ± 0.072	0.15 ± 0.074	0.18^{x}± 0.107	0.16 ± 0.113	0.16 ± 0.055	0.15 ± 0.038	*****	**NS**	**NS**
(×10^3 cells/µL)	W	0.33 ± 0.072	0.32 ± 0.074	0.49^{y}± 0.107	0.47 ± 0.113	0.26 ± 0.055	0.17 ± 0.038			

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

^{a,b,c}Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. ^{x,y}Between rows, Lsmeans differ by $P < 0.05$.

3.2.3. Platelet number, mean platelet volume, red blood cell number, haemoglobin concentration, haematocrit percentage, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration response to weaning stress at housing

There was no effect ($P > 0.05$) of treatment, sampling time or their interaction on mean corpuscular haemoglobin (Table 3.3). Platelet number increased from baseline in control calves on d 3 ($P < 0.01$), d 7 ($P < 0.001$) and d 14 ($P < 0.01$). A treatment × sampling time interaction ($P < 0.05$) for mean platelet volume (MPV) meant that MPV was greater ($P < 0.05$) in control calves on d 7 than weaned calves and the inverse was true for d 14. RBC was lower than baseline ($P < 0.01$) on d 1 and d 14 in weaned calves and on d 7 and d 14 in control calves. Haemoglobin concentration (HGB) followed a similar trend and was lower than baseline ($P < 0.01$) in weaned calves on d 1, d 7 and d 14, and was lower than baseline ($P < 0.05$) in control calves on d 14. Haematocrit (HCT) % also decreased from baseline on d 1 and d 14 in weaned calves ($P < 0.01$) and on d 1, d 7 and d 14 in control calves ($P < 0.05$). Likewise, mean corpuscular volume (MCV) decreased in weaned calves ($P < 0.001$) on d 1 and d 14 and in control calves ($P < 0.05$) on d 1 and d 7. Mean corpuscular haemoglobin concentration (MCHC) was higher ($P < 0.05$) than baseline in weaned calves on d 14.

Table 3.3. Effect of weaning induced stress at housing on platelet number, mean platelet volume (MPV), red blood cell (RBC) number, haemoglobin (HGB) concentration, haematocrit (HCT) %, mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	14	T	S	T×S
Platelets	C	421.4 ± 64.1	528.4 ± 60.3	609.5 ± 76.1	713.1^{b}± 27.0	840.9^{c}± 48.0	731.3^{b}± 50.1	**NS**	*	**NS**
($\times 10^3$ cells/µL)	W	540.6 ± 64.1	653.1 ± 60.3	529.2 ± 76.1	648.4 ± 27.0	674.3 ± 48.0	618.1 ± 50.1			
MPV (fL)	C	12.5 ± 1.88	17.7 ± 2.10	13.3 ± 1.97	10.6 ± 1.25	14.3^{x} ± 1.10	9.7^{x} ± 1.14	**NS**	*	*
	W	12.6 ± 1.88	12.2 ± 2.10	12.9 ± 1.97	11.1 ± 1.25	10.5^{y} ± 1.10	13.6^{y} ± 1.14			
RBC	C	11.4 ± 0.21	10.9 ± 0.14	11.1 ± 0.23	10.9 ± 0.26	10.7^{b} ± 0.25	10.7^{a} ± 0.30	**NS**	*	**NS**
($\times 10^6$ cells/µL)	W	11.5 ± 0.21	10.8^{b} ± 0.14	11.3 ± 0.23	11.2 ± 0.26	11.1 ± 0.25	10.1^{c} ± 0.30			
HGB (g/dL)	C	13.3 ± 0.28	12.9 ± 0.17	13.1 ± 0.24	12.9 ± 0.25	12.9 ± 0.22	12.8^{a} ± 0.34	**NS**	*	**NS**
	W	13.3 ± 0.28	12.5^{b} ± 0.17	13.1 ± 0.24	12.9 ± 0.25	12.6^{b} ± 0.22	11.8^{c} ± 0.34			
HCT (%)	C	35.7 ± 0.75	33.7^{a} ± 0.44	34.6 ± 0.82	34.6 ± 0.84	33.8^{a} ± 0.64	33.7^{a} ± 1.03	**NS**	*	**NS**
	W	35.8 ± 0.75	33.1^{b} ± 0.44	34.8 ± 0.82	34.6 ± 0.84	34.4 ± 0.64	30.9^{c} ± 1.03			
MCV (fL)	C	31.3 ± 0.13	30.8^{b} ± 0.09	31.1 ± 0.17	31.3 ± 0.14	31.5^{a} ± 0.15	31.3 ± 0.15	**NS**	***	**
	W	31.6 ± 0.13	30.9^{c} ± 0.09	31.4 ± 0.17	31.5 ± 0.14	31.6 ± 0.15	30.9^{c} ± 0.15			
MCH (pg)	C	11.7 ± 0.13	11.8 ± 0.16	11.8 ± 0.17	11.7 ± 0.16	12.0 ± 0.24	11.8 ± 0.18	**NS**	**NS**	**NS**
	W	11.8 ± 0.13	11.6 ± 0.16	11.9 ± 0.17	11.7 ± 0.16	11.6 ± 0.24	11.9 ± 0.18			
MCHC (g/dL)	C	37.4 ± 0.34	38.2 ± 0.35	37.9 ± 0.41	37.4 ± 0.48	38.2 ± 0.57	37.7 ± 0.51	**NS**	*	**NS**
	W	37.3 ± 0.34	37.7 ± 0.35	37.9 ± 0.41	37.3 ± 0.48	36.7 ± 0.57	38.4^{a} ± 0.51			

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

^{a,b,c}Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. ^{x,y}Between rows, Lsmeans differ by $P < 0.05$.

3.2.4. Acute phase protein response to weaning stress at housing

There was no significant effect of treatment or treatment × sampling time ($P > 0.05$) interaction for either haptoglobin or serum amyloid A (SAA) (Table 3.4). However, significant effects of time ($P < 0.01$) were identified for both acute phase proteins (APP). Haptoglobin was significantly elevated ($P < 0.01$) from baseline in weaned calves on d 2 and d 7, with no alterations in control calves. A similar elevation was identified in SAA concentration on d 2 ($P < 0.05$) while SAA was lower than baseline values ($P < 0.01$) in control calves on d 7 and d 14.

3.2.5. Cortisol response to weaning stress at housing

There was a treatment × sampling time interaction ($P < 0.05$) for cortisol where circulating cortisol concentration increased from baseline in weaned animals on d 2 ($P < 0.05$) and d 3 ($P < 0.01$) and remained elevated at sampling throughout the rest of the study ($P < 0.05$). A significant increase ($P < 0.05$) in cortisol concentration occurred in control calves on d 7 (Table 3.5).

3.2.6. Plasma CXCL8 response to weaning stress at housing

A treatment × sampling time ($P < 0.01$) interaction was identified for circulating plasma CXCL8 concentrations where CXCL8 concentration increased ($P < 0.001$) in both treatments following either housing or weaning at housing but was higher ($P < 0.05$) in control calves than weaned calves on d 1 and d 3 (Figure 3.2).

Table 3.4. Effect of weaning induced stress at housing on the acute phase proteins haptoglobin and serum amyloid A (SAA) in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	14	T	S	T×S
Haptoglobin	C	0.57 ± 0.089	0.52 ± 0.043	0.66 ± 0.072	0.79 ± 0.104	0.74 ± 0.115	0.67 ± 0.038	NS	**	*
(mg/mL)	W	0.54 ± 0.089	0.46 ± 0.043	0.72^{b}± 0.072	0.64 ± 0.104	0.78^{a}± 0.115	0.64 ± 0.038			
SAA	C	104.3 ± 16.0	101.8 ± 15.5	98.9 ± 15.9	116.3 ± 13.8	53.7^{b} ± 16.5	46.4^{a} ± 11.2	NS	**	NS
(µg/mL)	W	62.1 ± 17.5	89.4 ± 16.9	119.5^{a}± 17.4	105.8 ± 15.0	62.5 ± 18.1	32.8 ± 12.3			

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

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

Table 3.5. Effect of weaning induced stress at housing on circulating serum cortisol concentrations. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	14	T	S	T×S
Cortisol	C	4.8 ± 1.09	5.4 ± 1.13	5.1 ± 1.07	5.4 ± 1.05	7.2[a] ± 0.75	3.7 ± 0.95	**NS**	**NS**	*****
(ng/mL)	W	3.4 ± 1.09	4.2 ± 1.13	5.1[a]± 1.07	5.6[b]± 1.05	5.2[a] ± 0.75	5.1[a] ± 0.95			

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

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

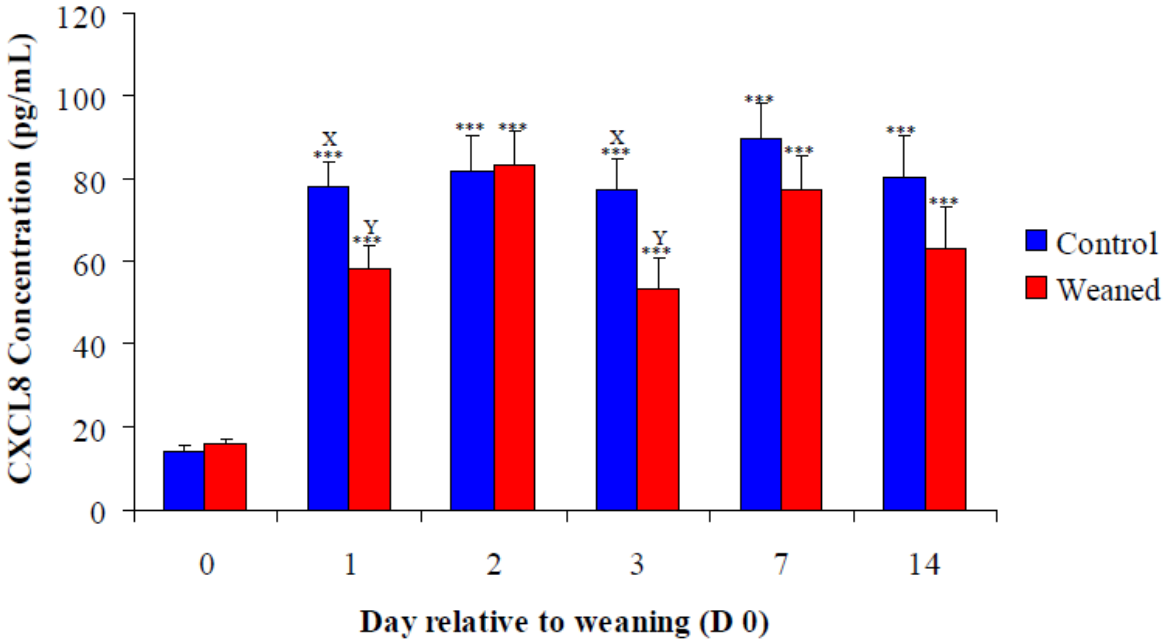

Figure 3.2. The circulating plasma CXCL8 response to housing and weaning stress. Following either housing or weaning, CXCL8 concentration increased significantly and remained elevated at sampling throughout the course of the study. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ Lsmeans differ relative to baseline (d 0). X, Y Lsmeans differ between treatments ($P < 0.05$).

3.3. Chapter Summary, Discussion and Conclusions

3.3.1. Chapter Summary

Because weaning typically combines a number of physical and psychological stressors, it is important to fully characterise the weaning process in order to assess the health and animal welfare implications that weaning can have when combined with housing and social reorganisation. This will allow the adaptation of herd management practices that will help to reduce stress in livestock and potentially have beneficial consequences for animal health at weaning.

There was an interaction of treatment × sampling time ($P < 0.01$) for total leukocyte number whereby it was greater ($P < 0.05$) in weaned calves than control calves on day (d) 1 and d 2. Additionally, a treatment × sampling time interaction ($P < 0.05$) for neutrophil number and the N:L ratio where neutrophils increased in weaned calves on d 1 ($P < 0.05$), d 2 ($P < 0.001$) and d 3 ($P < 0.01$) relative to baseline, resulting in higher levels compared to control calves on d 1 ($P < 0.05$). Significant effects of time ($P < 0.01$) were identified for both APP. Haptoglobin and SAA were significantly elevated ($P < 0.05$) from baseline in weaned calves on d 2. There was a treatment × sampling time interaction ($P < 0.05$) for cortisol where circulating cortisol concentration increased from baseline in weaned animals on d 2 ($P < 0.05$) and d 3 ($P < 0.01$) and remained elevated at sampling throughout the rest of the study ($P < 0.05$). A treatment × sampling time ($P > 0.01$) interaction was identified for circulating plasma CXCL8 concentrations where CXCL8 concentration increased ($P < 0.001$) in control and weaned calves but was higher ($P < 0.05$) in control calves than weaned calves on d 1 and d 3.

3.3.2. Discussion

A slight increase in rectal temperature was identified in the days immediately following weaning. While physical examinations of the calves indicated no other sign of clinical infection, an increase in rectal temperature is typically associated with early stage infection. It appears that stress can drive this increase by itself, thereby mimicking clinical infection, although to conclusively prove this will require future studies to collect nasal swabs in order to eliminate viral or bacterial infection as the culprit.

A number of alterations to the blood leukogram were identified over the course of this study. Weaning and housing have both been previously shown to increase total leukocyte number (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a) as a result of fluctuations in a number of leukocyte subpopulations, particularly neutrophils (Jones and Allison, 2007). Neutrophilia has been reported in response to abrupt weaning (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b), transportation (Earley and O'Riordan, 2006; Buckham Sporer *et al*., 2007; Gupta *et al*., 2007b; Lomborg *et al*., 2008) and castration (Pang *et al*., 2009b), making it a suitable biomarker to detect acute stress in cattle. The increase in neutrophil number that was identified in weaned animals over the first three days following weaning would suggest that these animals were acutely stressed but had adapted to their new environment by d 7. However, it is also clear that these animals underwent a more intense stress response than those calves housed with their dam as neutrophil number only increased in the control group on d 3. Housing is regarded as a mild stressor (Gupta *et al*., 2007a) and may not elicit as rapid a stress response as weaning. Rather, the calf may become frustrated after several days of being restricted to an enclosed environment, reflected in the increased neutrophil and total leukocyte number in control calves on d 3. It is likely

that separating housing and weaning can reduce these cumulative stressors (Lynch *et al*., 2010b).

While lymphocytes are generally regarded as being an adjunct to neutrophils in the detection of the bovine stress response, no major alterations to lymphocyte distribution were recorded during this study. This is in contrast with a number of studies that found lymphocyte number decreased in response to weaning and returned to baseline levels with 7 days (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b). The N:L ratio has proved to be a highly sensitive measurement for assessing the relationship between neutrophil and lymphocyte number as it picks up even slight changes in total neutrophil and lymphocyte numbers (Zahorec *et al*., 2001). While not significant, there was a numerical decrease in the lymphocyte number following weaning. Coupled with the reported neutrophilia, this meant that the N:L ratio increased from baseline on the first three days following weaning, while also increasing in the control animals on d 3.

A number of other variables were also examined for their potential role as biomarkers of weaning stress. No change was found in monocyte, basophil or eosinophil numbers following weaning, a finding that concurs with previous research (Lynch *et al*., 2010a). While monocyte function is known to respond to stress hormones, distributional changes in monocytes are rare, particularly given their highly variable animal to animal numbers, making the reliable detection of fluctuations difficult (Jones and Allison, 2007). However, it may be necessary to monitor monocyte number in studies of stress as a large alteration to monocyte number is an indication of clinical pathology and can serve as an early warning sign of animals that should be removed from the study. Basophils are present in such small numbers that it is extremely difficult to detect significant changes to this population (Brun-Hansen *et al*., 2006; Jones and Allison,

2007). These cells are not known targets of stress hormones but should also be monitored as part of the assessment of health throughout stress studies. However, as basophilic function somewhat overlaps with eosinophils, it may be more practical to monitor eosinophil numbers as they have been reported to respond to stress (Jones and Allison, 2007). That said, no alterations in eosinophil number were reported in the current study and eosinophil number does not appear to be reliably sensitive enough to detect weaning stress in cattle.

It is unclear whether platelet number is a suitable biomarker of stress from the current study as there were transitory changes, although often not significant. Platelet number may fluctuate too much to be used a biomarker of stress but may be a useful indicator of the health of an animal. MPV was also measured over the course of the study as the mean size of the platelets provides a degree of insight into platelet turnover. A higher MPV indicates increased platelet turnover as younger platelets are generally larger (Park *et al*., 2002). There was a degree of variability in the MPV, but platelet number is perhaps a better indicator of changes going on within the platelet population. The measurements of red blood cells and their haemoglobin capacity has been deemed a useful tool to monitor the health of cattle (Jones and Allison, 2007; Lynch *et al*., 2010c) and was undertaken for that purpose in this study. Throughout the study, a number of mild fluctuations occurred within these variables, but all of these were within the normal physiological range (See Chapter 1, Table 1.3).

CXCL8 is an important inflammatory mediator, predominantly responsible for the chemotaxis of neutrophils, although it also functions in T cell migration (Hirao *et al*., 2000; Elenkov *et al*., 2005; Keller *et al*., 2005). This chemokine is secreted both at sites of inflammation as well as in general circulation by macrophages and endothelial cells

in response to a host of stimuli (Waugh and Wilson, 2008; Gleissner *et al*., 2008). No indication of clinical infection, based on physical examination and assessment of leukocytes, was found in any of the calves during or immediately following this study, indicating the observed increase in CXCL8 was as a result of the stress hormone induced stimulation of the immune system. Most tissues and cell types of the immune system contain receptors for catecholamines and glucocorticoids, well known neuroendocrine effectors of the stress response (Preisler *et al*., 2000; Abraham *et al*., 2004; Chang *et al*., 2004; Odore *et al*., 2004; Pascual-Le Tallec and Lombes, 2005; LaBranche *et al*., 2010), providing direct immune targets for the HPA axis following activation. A surprising result of this study was the elevation of plasma CXCL8 for a full fourteen days following exposure to a standard husbandry stressor. Additionally, CXCL8 increased in control calves in a manner similar to weaned calves, suggesting that this potent chemokine can be non-specifically activated to respond to numerous stressors. Despite a statistically significant difference between weaned and control animals on day 1 and 3, it is likely that this was not a biologically significant result and the small variation was down to intra assay and animal variation. In the short term, the increased concentration of CXCL8 likely served to enhance leukocyte migration and surveillance of the tissues most at risk from infection. However, prolonged exposure to inflammatory cytokines can paradoxically result in deleterious effects, producing severe tissue damage, particularly in soft tissues such as the lung (Eruslanov *et al*., 2005; Buckham Sporer *et al*., 2007; Mitchell *et al*., 2008). It is not clear if the promotion of chemotaxis by increased concentrations of CXCL8 remained salubrious, but it is likely that it may have contributed to tissue damage and increased the susceptibility of the calves to disease. The increase in CXCL8 is likely due, at least in part, to an increase in the potent inflammatory inducers, IL-1 and TNFα, via their receptors and signalling through the NFKB/MAPK signalling pathways.

This study was not designed to identify when, and to what extent, cortisol levels peak and the first plasma samples were not collected until 24 hrs following either weaning or housing. Cortisol generally peaks early in the stress response (10 – 60 min) (Sapolsky *et al.*, 2000; Droste *et al.*, 2008). Therefore, more frequent sampling, in the range of every 15-20 min, would be required in order for it to be accurately used as a biomarker of stress. Additionally, the strong ultradian rhythm of cortisol concentrations (Lefcourt *et al*., 1993; Droste *et al*., 2008) means it is sensitive to the time of sampling, although this was partially circumvented by sampling the animals at the same time each day. While cortisol concentrations were clearly elevated following weaning, it is difficult to draw conclusions based on the sampling schedule used in this study. However, cortisol concentrations were clearly elevated following weaning. These results would indicate that infrequent sampling of cortisol is not a reliable biomarker of stress, although it is still a useful complement to other, more reliable biomarkers.

The acute phase protein response to glucocorticoids, along with their relative ease to measure, has meant that they are frequently selected as biomarkers of stress in cattle (Arthington *et al*., 2003;2005; Hickey *et al*., 2003b; Qui *et al*., 2007; Carroll *et al*., 2009; Lynch *et al*., 2010b). Haptoglobin and SAA were both monitored over the course of this study as they are both known to increase in response to inflammation (Murata *et al*., 2004; Nikunen *et al*., 2007), with haptoglobin reported to remain elevated at sampling 35 d following weaning (Lynch *et al*., 2010b). Haptoglobin and SAA increased in response to weaning in the current study, although the increase did not occur until day 2. This is in line with a number of other weaning (Hickey *et al*., 2003b; Aich *et al*., 2009; Lynch *et al*., 2010b; Kim *et al*., 2011) and transport (Lomborg *et al*., 2008) studies and indicates that those stressors result in an inflammatory activation of

the immune system. However, there was a large, non-significant difference in baseline SAA concentrations, with a high standard error indicating a great degree of variability in this measurement. These two APP may be more appropriate to monitor for signs of infection rather than as biomarkers of stress, although haptoglobin may be sufficient as an adjunct to other stress markers, showing a low, but significant, increase following weaning. In future, it may be beneficial to look at using additional APP as biomarkers of stress, in particular, α_1 acid glycoprotein (AGP). Neutrophils have been shown to produce AGP, which in turn dampens neutrophil degranulation (Miranda-Ribera *et al*., 2010). Additionally, the longevity of monocytes is increased in response to AGP (Ceciliani *et al*., 2007) while CXCL8 has been demonstrated to induce AGP secretion from neutrophils *in vitro*.

3.3.3. Conclusions

Weaning is an accumulative stressor with calves that are weaned at housing showing a stronger physiological stress response than those that were housed with their dams. Weaning at housing is a robust model that resulted in the classic neutrophilia response. However, this study identified plasma CXCL8 as a highly suitable biomarker for stress in cattle and its induction suggests that a number of other cytokines may be involved in the bovine stress response. This cytokine is likely part of the underlying mechanism by which previously reported alterations to leukocyte subsets occurs. It also seems to be a highly sensitive biomarker, detectable over the long term, and its increase in both control and weaned calves indicates it may not be suitable for detecting the magnitude of a stressor, but rather the presence or absence of a stress response. As CXCL8 remained elevated a full 14 d following weaning, it seems that it is a far better measure of stress than either neuroendocrine hormones (cortisol) or leukocyte counts (neutrophils). When coupled with the increase in CXCL8, the fact that haptoglobin and

total neutrophil number were also elevated suggests that weaning stress triggers an inflammatory response in calves that may be present a week following weaning. This response was not identified in control calves and is perhaps one of the clearest indicators from the study regarding the benefits of reducing accumulative stressors, such as weaning at housing. It may be of particular interest to examine the gene expression of CXCL8 and other inflammatory mediators to assess their molecular response to weaning stress and further elucidate mechanisms by which immune function is altered. However, it is also clear that stress results in the activation of an inflammatory immune response in calves, likely aiding in the initial elimination of pathogens. As weaning clearly requires at least 14 days of habituation before a return to homeostasis, it is likely damage incurred as a result of this inflammatory response contributes to the increased disease susceptibility frequently reported at weaning. It is necessary to isolate weaning from the housing process to examine the response to weaning without the accumulative effect of housing. This will also aid in identifying the magnitude of stress associated with different weaning strategies and it may be beneficial to examine the differential response of calves abruptly separated from their dam versus those weaned next to their dam. Additionally, through the employment of molecular techniques in future studies, it may be possible to elucidate the basic mechanisms responsible for the physiological stress response, thereby allowing a thorough characterisation of the calves stress response to weaning and subsequent conclusions on the efficacy of acute stress to enhance the immune system at a time that it may be required. By identifying the participation of inflammatory mediators in the stress response, this work will contribute to a better understanding of the immune status of weaned beef calves and aid in future targeted gene expression studies of stress in cattle.

Chapter 4

Examination of the bovine leukocyte environment using immunogenetic biomarkers to assess immunocompetence following exposure to weaning stress

4.1. Introduction

Stress has been well documented to negatively impact the immune system in both humans and animals (Kort, 1994; Agarwal and Marshall, 1998; Wu *et al*., 2000; Sapolsky, 2000; 2004; Reiche *et al*., 2004; Glaser and Kiecolt Glaser, 2005; Saul *et al*., 2005). In cattle, research measuring stress-related immune function has focused on a number of husbandry management practices including castration (Fisher *et al*., 1997a; Earley and Crowe, 2002; Ting *et al*., 2003a; 2003b; 2010; Pang *et al*. 2009a; 2009b; 2010; Marti *et al*., 2010), housing (Fisher *et al*., 1997b; 1997c; Hickey *et al*., 2003a; Gupta *et al*., 2007a), transport (Earley and O'Riordan 2006a; Earley *et al*., 2006b; Gupta *et al*., 2007b; Buckham Sporer *et al*., 2007; 2008a; 2008b; Arthington *et al*., 2008; Earley and Murray 2010), and weaning (Hickey *et al*., 2003b; Arthington *et al*., 2005; Blanco *et al*., 2009; Carroll *et al*., 2009; Lynch *et al*., 2010a; 2010b). Weaning involves separation of the calf from its dam, resulting in a breaking of the maternal-offspring bond and removal of milk from its diet. Additionally, the calf may be exposed to other stressors including social reorganisation, housing, transport and novel handling. Thus, the weaning procedure can be an acute stressful event in young calves combining social, physical, nutritional and psychological stresses (Lynch *et al*, 2010a; 2010b). Hickey *et al*. (2003) found alterations in immune function and hormonal mediators of stress still present at 7 days following abrupt weaning whereby the cows were suddenly removed from the calves. Compared with abrupt weaning, practices such as progressive weaning or fenceline weaning have reduced the frequency of behavioural distress, heart-rate and neutrophil: lymphocyte (N:L) ratio in calves (Church and Hudson, 1999; Price *et al*., 2003; Rasby, 2007). Similarly, Lynch *et al*. (2010a) reported that deferring housing at the time of weaning resulted in a less marked stress response in beef calves

compared with the traditional practice of weaning and simultaneous housing indoors at the end of the grazing season (Chapter 3).

In view of these findings, the present study was designed to remove the effect of housing stress and adaptation to a new diet at the time of weaning by housing the cows and calves 28 days prior to weaning, thus allowing them time to adjust to their new environment before this event. This provided the opportunity to examine both the effect of breaking the maternal-offspring bond and the effect of social reorganisation by using a fence-line weaning system (Church and Hudson, 1999; Price *et al*., 2003; Rasby, 2007; Enríquez *et al*., 2010) modified for a housing environment to detect if animals weaned in the presence of their dam would be less susceptible to weaning induced stress compared with those weaned and penned away from their dam. Both male and female animals were used in order to establish if physiological and molecular differences exist in their responses to stress as the literature is equivocal on this point. The experimental design is described in more detail in chapter 2 (2.2.2.5).

To the authors' knowledge, no research has examined the molecular mechanisms underlying weaning stress, particularly with a focus on immunogenetic markers of stress. Through the use of real-time RT-qPCR, the expression of a number of cytokines and other key immune biomarkers of stress may be used to characterise the molecular response of the calf to stress. Although bovine leukocytes are well-characterised in relation to stress (Burton *et al*., 2005), recent molecular studies have focused on specific cell populations (Buckham Sporer *et al*., 2007; 2008a) leaving leukocytes and their cytokine networks largely uncharacterised at the molecular level (Stordeur *et al*., 2003). Examining combined leukocyte populations provides a broader picture of immunological interactions than can be garnered from focusing on particular cell types.

The study hypothesis was that calves that were weaned and penned away from their dams would be more stressed than calves that were weaned and penned adjacent to their dams. The objectives of this study were to characterise, in male and female calves, the immune response to weaning stress at the physiological and molecular levels, and to assess the difference between calves weaned and penned in the presence of the dam and weaned and penned away from the dam. Additionally, the behavioural responses of calves were also characterized.

4.2. Behavioural and physiological response of male and female calves weaned next to the dam versus those weaned away from the dam

4.2.1. Environmental measures

The temperature of the housing environment ranged from 3.9^{o}C to 17.4^{o}C with a mean (s.d.) temperature of 9.8^{o}C (2.2). The ambient temperature ranged from 2.8^{o}C to 26.0 ^{o}C with a mean (s.d.) temperature of 9.2 ^{o}C (3.4).

4.2.2. Rectal body temperature

Rectal body temperature was higher ($P < 0.01$) for away calves than near calves (Table 4.1). There was a gender × time ($P < 0.01$) interaction whereby rectal temperature was lower than baseline on d 2, 3 and 11, particularly in female animals.

4.2.3. Standing, lying and activity behaviour of male and female calves following weaning

In response to weaning, there was a gender × location ($P < 0.05$) and gender × time ($P < 0.001$) interaction for standing behaviour (Table 4.2). Following weaning, male calves weaned next to the dam spent less time standing ($P < 0.01$) and more time lying than male and female calves weaned away from the dam and male calves spent less time standing ($P < 0.05$) and more time lying than female calves on the day of weaning (d 0) and on d 4, 5 , 7, 8, 9 and 11. There was a location × time interaction ($P < 0.001$) for activity levels whereby calves weaned away from the dam had greater activity levels ($P < 0.001$) than those weaned next to the dam on the day of weaning (d 0). There was a gender × time interaction ($P < 0.001$) for activity levels with female calves displaying greater activity than male calves on d 1, 4, 5, 7, 9 and 11 ($P < 0.05$). A significant location × gender × time interaction ($P < 0.001$) existed where no difference existed

between male and female calves weaned next to the dam on d 0 whereas female calves weaned away from the dam spent more time active ($P < 0.001$) than either male calves weaned away from the dam or male and female calves weaned next to the dam.

4.2.4. Leukocyte population

There was no effect of gender, location and time or their interaction on total leukocyte or monocyte number (Table 4.3). There was a location × time interaction ($P < 0.01$) but no effect ($P > 0.05$) of gender for total neutrophil number (Table 4.3; Figure 4.1). Following weaning, neutrophil number increased in all calves ($P < 0.001$) but the increase was greater ($P < 0.001$) in calves weaned away from the dam than those weaned beside the dam. Neutrophil number returned to baseline by d 11. Lymphocyte number decreased ($P < 0.05$) from baseline on d 2 and remained lower throughout the study. There was a gender × location interaction ($P < 0.01$) for lymphocyte number, whereby away males had lower ($P < 0.05$) lymphocyte numbers than near males and the opposite ($P < 0.01$) occurred with females. There was a location × time ($P < 0.01$) interaction for N:L ratio (Table 4.3). Post-weaning N:L ratio increased ($P < 0.01$) from baseline and did not return to pre-weaning levels until d 11, but on d 1, the increase was greater ($P < 0.01$) in away calves than in near calves. There was no effect ($P > 0.05$) of gender on N:L ratio. A gender × time effect ($P < 0.01$) existed for eosinophil number (Table 4.3) whereby eosinophil number in males decreased ($P < 0.01$) from baseline on d 1 and decreased ($P < 0.01$) in females on d 7. See Appendix 1 for breakdown of Lsmeans by treatment and time.

Table 4.1. Effect of stress on rectal body temperature (˚C) in weaned beef calves. The values are expressed as least square means (Lsmeans) ± s.e.

	Gender		s.e.	**Location**		s.e	**Time**						s.e.	***P*-Values**			
Variable	Male	Female		Near	Away		0	1	2	3	7	11		G	L	T	I
Rectal temperature (˚C)	38.7	38.6	0.05	38.5	38.7	0.05	38.8	38.7	38.6[b]	38.6[b]	38.7	38.4[c]	0.05	NS	**	*	**[1]

G = gender, L = location, T = time, I = interaction, NS = not significant ($P > 0.05$).
* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
[1]Time × Gender interaction
[a,b,c]Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 4.2. Effect of weaning induced stress on the percentage (%) of time spent standing, lying and active in beef calves.

	Gender	s.e.	Location	s.e.	Time†	s.e.	*P*-Values

Table 4.3. Effect of weaning induced stress on total leukocyte, neutrophil and lymphocyte number, neutrophil:lymphocyte (N:L) ratio, eosinophil and monocyte number in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

Variable	Male	Female		Near	Away		0	1	2	3	4	5	6	7	11		G	L	T	I
Standing (%)	41.4	46.9	1.88	42.2	46.1	1.88	68.4	51.1[c]	38.7[c]	38.1[c]	46.0[c]	42.8[c]	44.4[c]	36.4[c]	32.1[c]	3.1	*	NS	***	[1,2]
Lying (%)	56.3	49.9	2.0	55.1	51.1	2.0	24.8	43.9[c]	58.9[c]	59.9[c]	51.3[c]	54.8[c]	53.4[c]	61.9[c]	66.6[c]	3.3	*	NS	***	[3]
Active (%)	2.3	3.2	0.23	2.6	2.8	0.23	6.7	4.9[c]	2.3[c]	1.9[c]	2.6[c]	2.3[c]	2.2[c]	1.7[c]	1.4[c]	0.20	**	NS	***	[4,5,6]

G = gender, L = location, T = time, I = interaction, NS = not significant ($P > 0.05$). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

† Day 8, 9 and 10 are not listed in the table as there were no significant differences between day 7 and day 11.

[1]Gender × Location (P < 0.05), 47.7 vs 46.0 and 36.7 vs 46.1 for near vs away female and males, respectively; 36.7 vs 47.7 for male and female calves next to the dam.

[2]Gender × Time (P < 0.001), 65.1 vs 71.8, 53.3 vs 48.9, 37.8 vs 39.7, 33.3 vs 42.8, 42.2 vs 59.9, 39.9 vs 45.8, 38.4 vs 50.4, 38.9 vs 48.8, 39.5 vs 46.0, 41.4 vs 48.6, 35.9 vs 28.1 for male and female calves at time 0 to 11, respectively.

[3]Gender × Time (P < 0.01), 29.38 vs 19.7, 41.9 vs 46.0, 59.9 vs 57.9, 65.0 vs 54.8, 55.8 vs 46.9, 58.1 vs 51.6, 59.9 vs 56.1, 59.3 vs 48.6, 58.8 vs 51.7, 56.7 vs 48.6, 62.6 vs 70.5 for male and female calves at time 0 to 11, respectively.

[4]Location × Time (P < 0.001), 5.4 vs 8.1, 4.7 vs 5.1, 2.3 vs 2.3, 2.0 vs 1.9, 2.7 vs 2.5, 2.5 vs 2.2, 2.3 vs 2.1, 1.8 vs 1.7, 2.2 vs 2.1, 2.0 vs 1.9, 2.4 vs 2.3, 1.4 vs 1.3 for near and away calves at time 0 to 11, respectively.

[5]Gender × Time (P < 0.001), 4.9 vs 8.5, 4.7 vs 5.1, 2.2 vs 2.4, 1.6 vs 2.4, 2.0 vs 3.2, 2.0 vs 2.6, 1.8 vs 2.6, 1.4 vs 2.1, 1.7 vs 2.6, 1.7 vs 2.2, 1.9 vs 2.8, 1.4 vs 1.3 for male and female calves at time 0 to 11, respectively.

[6]Location × Gender × Time (P < 0.001), 5.5 vs 5.2 vs 11.4 vs 4.8 for near female and male calves and away female and male calves on day 0.

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

	Gender		Location		s.e	Time						s.e.	*P*-Values			
Variable	Male	Female	Near	Away		0	1	2	3	7	11		G	L	T	I
Total Leukocytes ($\times 10^3$ cells/µL)	9.9	9.7	9.4	10.2	0.40	9.8	10.5	9.8	9.7	10.5	8.8	0.41	NS	NS	NS	NS
Neutrophils ($\times 10^3$ cells/µL)	3.1	2.6	2.5	3.2	0.29	2.3	3.5^{c}	2.9^{b}	2.9^{b}	3.5^{c}	2.2	0.27	NS	NS	***	**[1]
Lymphocytes ($\times 10^3$ cells/µL)	7.1	6.7	6.8	6.9	0.13	7.2	7.2	6.8^{b}	6.8^{b}	6.9^{a}	6.5^{c}	0.13	*	NS	*	**[2]
N:L Ratio	0.46	0.38	0.39	0.45	0.04	0.33	0.49^{c}	0.43^{b}	0.42^{b}	0.52^{c}	0.33	0.04	NS	NS	**	**[3]
Eosinophils ($\times 10^3$ cells/µL)	0.12	0.13	0.12	0.13	0.01	0.13	0.10^{a}	0.11	0.15	0.13	0.10^{b}	0.01	NS	NS	NS	**[4]
Monocytes ($\times 10^3$ cells/µL)	0.59	0.57	0.55	0.61	0.03	0.60	0.54	0.62	0.58	0.58	0.57	0.03	NS	NS	NS	NS

G = gender, L = location, T = time, I = interaction, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[1]Location × Time interaction, 1.9 vs 2.8, 2.6 vs 4.4, 2.5 vs 3.2, 2.7 vs 3.1, 3.6 vs 3.4, 2.1 vs 2.3 for near and away calves at time 0, 1, 2, 3, 7 and 11, respectively.

[2]Gender × Location interaction, 6.9 vs 6.4 and 6.7 vs 7.5 for near and away male and female, respectively; away male 6.4 vs away female 7.5.

[3]Location × Time interaction, 0.27 vs 0.38, 0.38 vs 0.59, 0.40 vs 0.45, 0.41 vs 0.42, 0.54 vs 0.48, 0.33 vs 0.33 for near and away calves at time 0, 1, 2, 3, 7 and 11, respectively.

[4]Gender × Time interaction, 0.12 vs 0.15, 0.12 vs 0.08, 0.14 vs 0.10, 0.17 vs 0.12, 0.13 vs 0.14, 0.08 vs 0.12 for male and female calves at time 0, 1, 2, 3, 7 and 11, respectively.

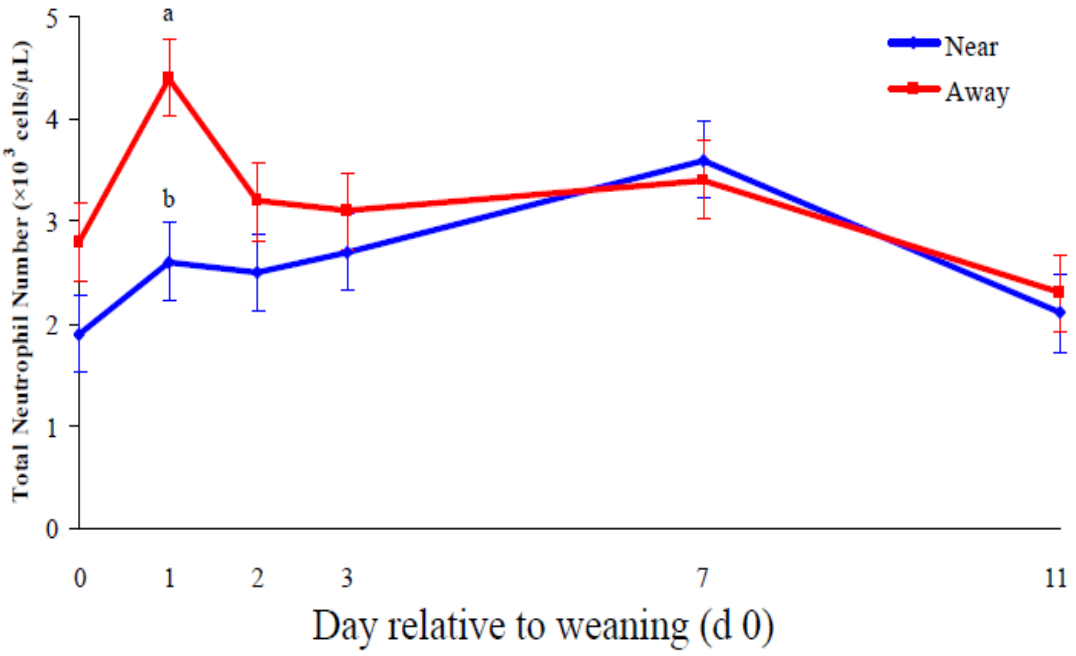

Figure 4.1. Alterations in total neutrophil number between calves weaned next to the dam and those weaned away from the dam. Neutrophil number for calves weaned away from the dam was significantly higher on day 1 than for calves weaned next to the dam, indicating an effect of location. [a, b]Lsmeans differ between location by $P < 0.05$.

4.2.5. Red blood cell number, haemoglobin concentration, haematocrit percentage, mean corpuscular haemoglobin concentration and platelet number

There was no effect ($P > 0.05$) of gender, location and time or their interaction on HGB concentration (Table 4.4). RBC ($P < 0.001$) and HCT % ($P < 0.01$) decreased from baseline on d 11 post weaning. There was a location × time × gender interaction ($P < 0.05$) for MCHC. MCHC increased ($P < 0.05$) from baseline on d 1 in away calves and on d 2 in female calves, while increasing ($P < 0.001$) from baseline substantially in all animals on d 11. It was also higher ($P < 0.05$) in away animals on d 2. There was a gender × time interaction ($P < 0.01$) for platelet number, whereby males had higher ($P < 0.01$) platelet numbers than females in the first 3 days following weaning, before decreasing ($P < 0.001$) from baseline in all calves on d 11. However, there was no effect ($P > 0.05$) of location on platelet number. See Appendix 2 for breakdown of Lsmeans by treatment and time.

Table 4.4. Effect of weaning induced stress on red blood cell (RBC) number, haemoglobin (HGB) concentration, haematocrit (HCT) %, mean cell haemoglobin concentration (MCHC) and platelet number in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

	Gender		**Location**		s.e	**Time**						s.e.	***P*-Values**			
Variable	Male	Female	Near	Away		0	1	2	3	7	11		G	L	T	I
RBC ($\times 10^6$ cells/µL)	10.2	9.5	9.8	9.9	0.29	10.6	10.6	10.2	10.7	10.7	6.6[c]	0.24	NS	NS	***	NS
HGB (g/dL)	12.4	12.3	12.4	12.3	0.10	12.4	12.5	12.4	12.6	12.2	12.2	0.10	NS	NS	NS	NS
HCT (%)	31.6	30.9	31.5	31.0	0.67	33.7	33.5	32.4[a]	33.9	33.1	21.1[c]	0.62	NS	NS	**	NS
MCHC (g/dL)	41.5	40.9	40.8	41.6	0.59	36.9	37.4	38.2	36.9	36.9	60.7[c]	0.93	NS	NS	***	*[1]
Platelet ($\times 10^3$ cells/µL)	872.1	741.1	819.0	794.1	31.6	815.9	952.5[c]	953.2[c]	882.8	739.5[a]	495.6[c]	31.8	**	NS	**	**[2]

G = gender, L = location, T = time, I = interaction, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[1]Gender × Location × Time interaction, near male 57.9 vs away male 68.1 at time 7

[2]Gender × Time interaction, 857.9 vs 773.8, 1043.4 vs 861.7, 1037.9 vs 868.5, 1013.9 vs 751.7, 787.8 vs 691.2, 491.6 vs 499.7 for male and female calves at time 0, 1, 2, 3, 7 and 11, respectively.

4.3. Molecular response of calves weaned next to the dam versus those weaned away from the dam

4.3.1. Reference gene selection

Five reference genes (β-actin (*ACTB*), succinate dehydrogenase complex subunit A (*SDHA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygense activation protein zeta polypeptide (YWHAZ) and β2-microglobulin (B2M)) were examined for use in this study. Based on the average pairwise variation, V, geNorm returned three genes, *ACTB*, *SDHA* and *GAPDH* with an average stability value of M = 0.24 and these were used for subsequent analysis (Figure 4.2).

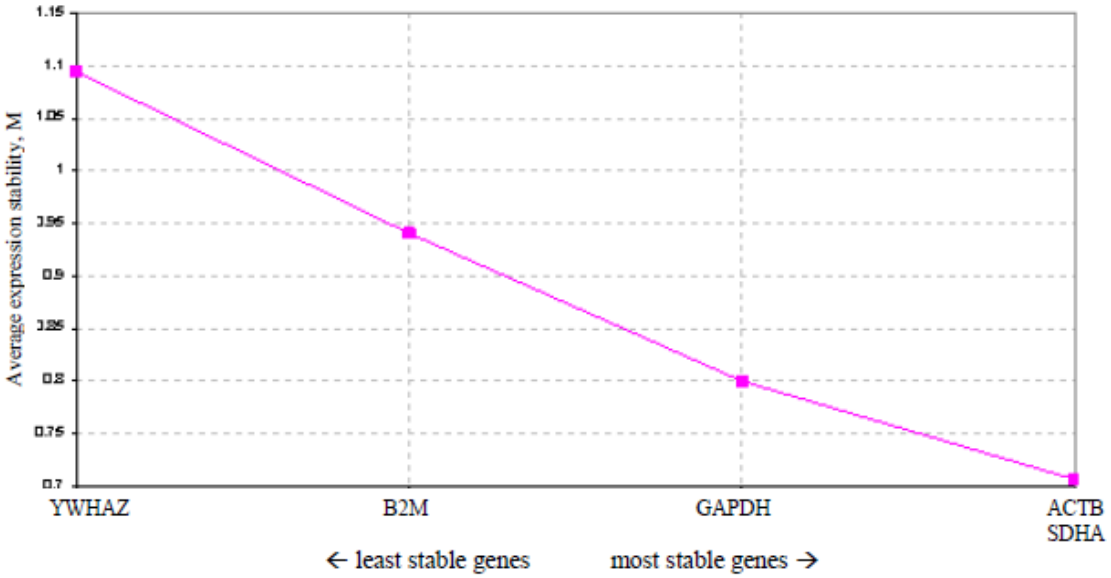

Figure 4.2. Average expression stability values for the 5 reference genes.
ACTB, *SDHA* and *GAPDH* were found to have an average stability value, M, of 0.24.

4.3.2. Cytokine gene expression

There was no effect (P > 0.05) of gender, location and time or their interaction on expression of IL-4 and lymphotoxin, and no effect (P > 0.05) of location on IL-1β, IL-2, CXCL8, IFN-γ and TNFα (Table 4.5).

Expression of IL-1β increased over 2-fold ($P < 0.001$) from baseline on d 1 following weaning and remained increased on d 3 and 7 (Table 4.5; Figure 4.4). There was a gender × time interaction ($P < 0.05$) for expression of IL-2 (Table 4.5), whereby an increase ($P < 0.05$) from baseline occurred in females on d 1 but not in males. Female calves had greater ($P < 0.05$) expression of CXCL8 versus male calves. Expression of CXCL8 increased 2-fold ($P < 0.001$) from baseline on d 1 and remained elevated ($P < 0.05$) on d 3 (Table 4.5; Figure 4.3). There was a gender × time interaction ($P < 0.05$) for IFN-γ (Table 4.5; Figure 4.5) and TNFα (Table 4.5) expression. IFN-γ expression increased over 3-fold ($P < 0.001$) from baseline in all animals on d 1 and remained elevated ($P < 0.01$) on d 3 and d 7. IFN-γ expression was significantly ($P < 0.001$) higher in female calves on d 1 versus male calves. TNFα expression increased ($P < 0.001$) from pre-weaning baseline on d 1 and remained elevated ($P < 0.01$) on d 3 and d 7. However, expression levels of TNFα were significantly greater in females on d 1 ($P < 0.01$) and d 7 ($P < 0.05$) compared with males. See Appendix 3 for breakdown of Lsmeans by treatment and time.

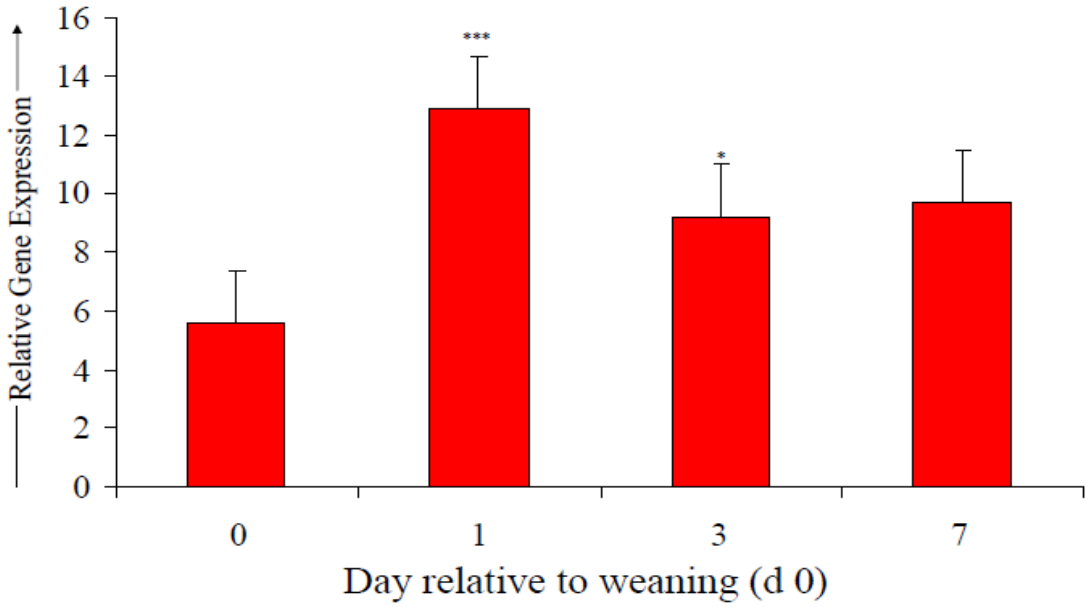

Figure 4.3. The effect of weaning induced stress on the relative gene expression of CXCL8. The potent neutrophil chemokine, CXCL8, increased in expression following weaning in all calves. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

Table 4.5. Effect of weaning induced stress on the relative gene expression of IL-1ß, IL-2, IL-4, CXCL8, IFN-γ, TNFα and lymphotoxin in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

	Gender		**Location**		s.e.	**Time**				s.e.	***P*-Values**			
Variable	Male	Female	Near	Away		0	1	3	7		G	L	T	I
IL-1β	17.7	16.2	16.6	17.3	1.3	8.7	18.5[c]	18.5[c]	21.9[c]	1.7	NS	NS	***	NS
IL-2	9.9	10.8	11.8	8.9	1.9	9.6	12.6	10.1	9.2	1.8	NS	NS	NS	*[1]
IL-4	11.8	11.9	13.6	10.1	1.8	11.5	15.3	10.5	10.2	1.8	NS	NS	NS	NS
CXCL8	7.1	11.5	8.9	9.7	1.3	5.6	12.9[c]	9.2[a]	9.7	1.8	*	NS	**	NS
IFN-γ	19.4	36.8	29.3	26.8	5.8	14.2	41.4[c]	30.5[b]	26.2[b]	5.7	*	NS	***	*[2]
TNFα	3.0	3.7	3.5	3.3	0.22	2.4	3.8[c]	3.4[b]	3.8[c]	0.29	*	NS	***	*[3]
Lymphotoxin	78.6	104.1	100.3	82.4	11.9	96.1	100.6	82.1	86.5	10.2	NS	NS	NS	NS

G = gender, L = location, T = time, I = interaction, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[1]Gender × Time interaction, 9.3 vs 9.8, 10.9 vs 14.4, 11.8 vs 8.4, 7.6 vs 10.8 for male and female calves at time 0, 1, 3 and 7, respectively.

[2]Gender × Time interaction, 9.0 vs 19.4, 22.2 vs 60.6, 26.7 vs 34.3, 19.6 vs 31.7 for male and female calves at time 0, 1, 3 and 7, respectively.

[3]Gender × Time interaction, 2.4 vs 2.4, 3.0 vs 4.6, 3.5 vs 3.2, 3.1 vs 4.4 for male and female calves at time 0, 1, 3 and 7, respectively.

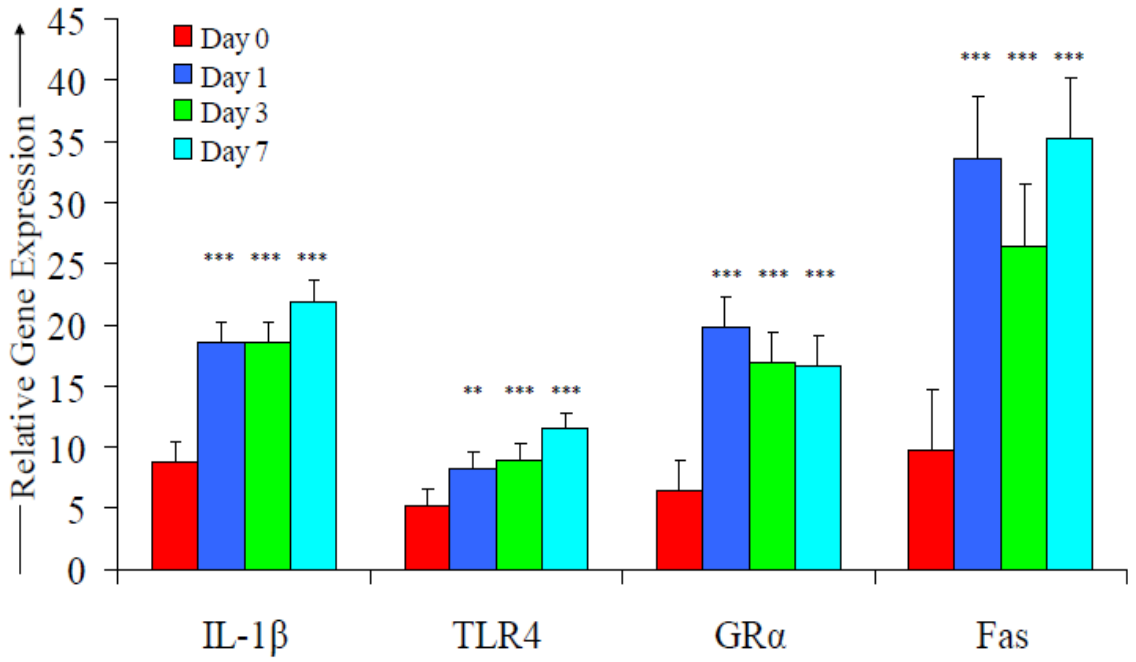

Figure 4.4. The effect of weaning induced stress on the relative gene expression of IL-1β, TLR4, GRα and Fas. These profiles demonstrate a clear effect of weaning stress on the expression of a number of genes. However, no effect of location was detected.

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ Lsmeans differ relative to pre-weaning baseline.

4.3.3. Immunological biomarker gene expression

There was no effect (P > 0.05) of gender, location and time or their interaction on expression of NFκB2, p21 and haptoglobin, and no effect (P > 0.05) of gender and location or their interaction on TLR4, GRα, CD62L and Fas (Table 4.6; Figure 4.4). There was a significant effect of time on expression of TLR4, GRα, CD62L and Fas whereby expression increased on d 1 and remained elevated through to d 7 (P < 0.001). The magnitude of this expression on d 7 was over 1.5-fold for CD62L, over 2-fold for TLR4, and over 3-fold for GRα and Fas. Expression of NFκB1 increased (P < 0.05) from baseline on d 1 and d 7 post weaning. Expression of BPI (Table 4.6) was lower (P < 0.05) on d 3 and d 7, post weaning. See Appendix 4 for breakdown of Lsmeans by treatment and time.

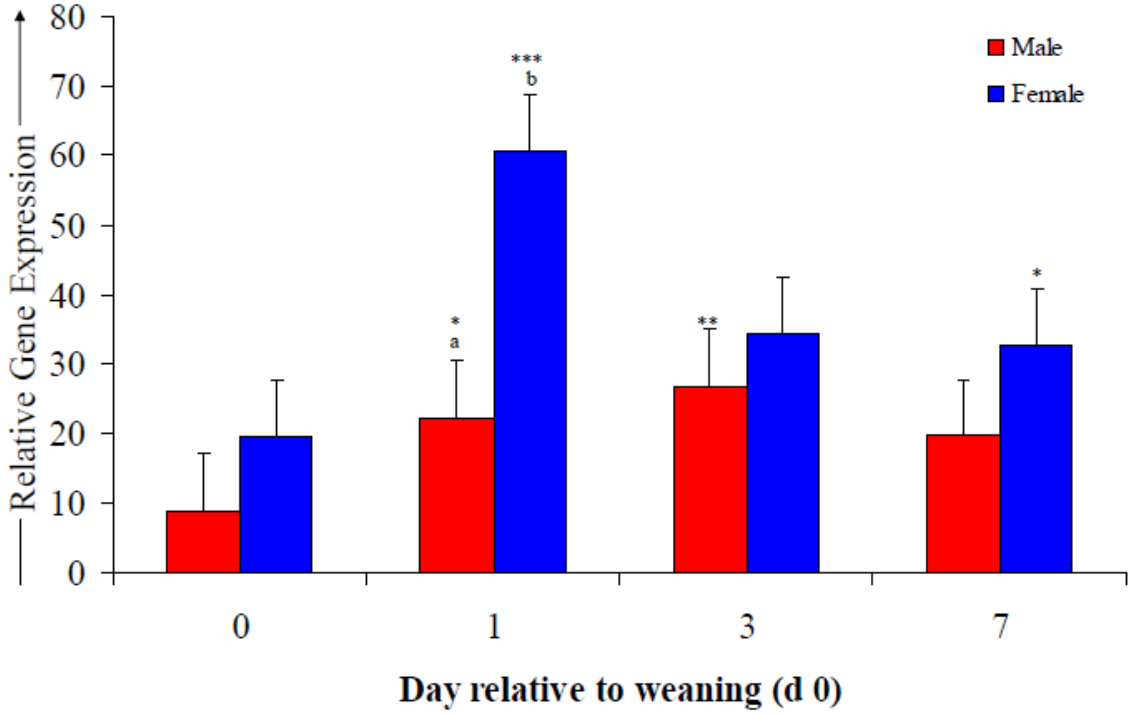

Figure 4.5. The effect of weaning induced stress on gender response in the relative gene expression of IFN-γ. A clear effect of gender can be seen whereby female calves had increased expression of IFN-γ versus male calves following weaning.

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Lsmeans differ relative to pre-weaning baseline. [a, b]Lsmeans differ between gender by $P < 0.05$.

Table 4.6. Effect of weaning induced stress on the relative gene expression of toll-like receptor (TLR) 4, glucocorticoid receptor (GR)α, nuclear factor kappa B (NFκB), Fas, p21, CD62l, haptoglobin and bactericidal/permeability increasing protein (BPI) in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

	Gender		**Location**		s.e.	**Time**				s.e.	***P*-Values**			
Variable	Male	Female	Near	Away		0	1	3	7		G	L	T	I
TLR4	9.4	7.5	8.0	8.9	1.4	5.2	8.3[b]	8.9[c]	11.5[c]	1.3	NS	NS	***	NS
GRα	13.4	16.5	16.4	13.5	2.1	6.4	19.8[c]	16.9[c]	16.6[c]	2.5	NS	NS	***	NS
NFκB1	2.9	2.9	2.8	3.0	0.27	2.5	3.1[b]	2.9	3.1[b]	0.25	NS	NS	*	NS
NFκB2	2.9	2.8	2.9	2.7	0.22	2.8	2.9	2.7	2.8	0.24	NS	NS	NS	NS
Fas	27.9	24.5	25.7	26.8	4.8	9.7	33.6[c]	26.4[c]	35.2[c]	5.1	NS	NS	***	NS
p21	5.8	5.5	5.4	5.9	0.70	6.2	4.9	5.7	5.6	0.71	NS	NS	NS	NS
CD62L	33.7	35.5	34.4	34.8	5.1	26.0	34.8[b]	37.5[c]	40.1[c]	4.3	NS	NS	***	NS
Haptoglobin	14.4	16.6	13.7	17.3	3.9	12.8	16.3	17.7	15.2	3.5	NS	NS	NS	NS
BPI	14.8	15.6	16.5	13.9	2.5	18.3	16.7	14.8[a]	11.1[c]	2.1	NS	NS	***	NS

G = gender, L = location, T = time, I = interaction, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

4.4. Chapter Summary, Discussion and Conclusions

4.4.1. Chapter Summary

This study utilised a model that was designed with the objective of isolating the maternal-offspring separation and social reorganisation occurring at weaning time, in order to characterise the weaning response without the associated stress resulting from simultaneous housing and novel handling. Management factors that serve to increase the stress load on young animals result in decreased feed intake and partitioning of nutrients away from growth and immune dysfunction (Grimble, 1994). If left unchecked, these management factors can result in chronic stress and suppression of the immune system (Dhabhar, 2002). For this study, RT-qPCR analyses together with haematological profiles were examined to compare gene expression in blood leukocytes of weaned calves over a period of 7 days.

The following is a summarisation of the main findings of this chapter. There was a gender × time ($P < 0.01$) interaction for rectal temperature whereby it was lower than baseline on d 2, 3 and 11 in all calves, but lower in female than male calves. Following weaning, male calves weaned next to the dam spent less time standing and more time lying than male and female calves weaned away from the dam and male calves spent less time standing and more time lying than female calves on the day of weaning (d 0) and on d 4, 5 , 7, 8, 9 and 11. There were a number of significant effects ($P < 0.001$) of both gender and location on behavioural observations of standing, lying and activity levels with female calves and calves weaned away from the dam being the most active immediately following weaning. There was a location × time interaction ($P < 0.01$) for total neutrophil number where, following weaning, neutrophil number increased in all calves ($P < 0.001$) but the increase was greater ($P < 0.001$) in calves weaned away from

the dam than those weaned beside the dam with neutrophil number returning to baseline by d 11. Post-weaning, there was a gender × location interaction ($P < 0.01$) for lymphocyte number, whereby away males had lower ($P < 0.05$) lymphocyte numbers than near males and the opposite ($P < 0.01$) occurred with females. A location × time ($P < 0.01$) interaction for N:L ratio occurred where post-weaning N:L ratio increased ($P < 0.01$) from baseline and did not return to pre-weaning levels until d 11, but on d 1, the increase was greater ($P < 0.01$) in away calves than in near calves. A decrease from baseline occurred of RBC ($P < 0.001$) and HCT % ($P < 0.01$) on d 11 post weaning. There was a location × time × gender interaction ($P < 0.05$) for MCHC. MCHC increased ($P < 0.05$) from baseline on d 1 in away calves and on d 2 in female calves, while increasing ($P < 0.001$) from baseline substantially in all animals on d 11. Following weaning, there was a gender × time interaction ($P < 0.01$) for platelet number, whereby males had higher ($P < 0.01$) platelet numbers than females in the first 3 days following weaning, before decreasing ($P < 0.001$) from baseline in all calves on d 11.

Expression of IL-1β increased over 2-fold ($P < 0.001$) from baseline on d 1 following weaning and remained increased on d 3 and 7. Post-weaning, there was a gender × time interaction ($P < 0.05$) for expression of IL-2 whereby an increase ($P < 0.05$) from baseline occurred in females on d 1 but not in males. Female calves had greater ($P < 0.05$) expression of CXCL8 versus male calves. Expression of CXCL8 increased 2-fold ($P < 0.001$) from baseline on d 1 and remained elevated ($P < 0.05$) on d 3. There was a gender × time interaction ($P < 0.05$) for IFN-γ and TNFα expression. IFN-γ expression increased over 3-fold ($P < 0.001$) from baseline in all animals on d 1 and remained elevated ($P < 0.01$) on d 3 and d 7. IFN-γ expression levels were significantly ($P < 0.001$) higher in female calves on d 1 versus male calves. TNFα expression increased

($P < 0.001$) from pre-weaning baseline on d 1 and remained elevated ($P < 0.01$) on d 3 and d 7. However, expression levels of TNFα were significantly greater in females on d 1 ($P < 0.01$) and d 7 ($P < 0.05$) compared with males. There was a significant effect of time on expression of TLR4, GRα, CD62L and Fas whereby expression increased on d 1 and remained elevated through to d 7 ($P < 0.001$).

4.4.2. Chapter Discussion

Immediately following weaning, calves spent less than 25 % of their time resting which is in agreement with other studies (Haley *et al*., 2005; Boland *et al*., 2008; Lynch *et al*., 2011). The elevated activity observed immediately following weaning slowly decreased over the course of the study. The increased activity is likely an attempt to search for the dam following weaning, particularly given the fact that calves that were weaned away from the dam spent significantly more time searching for the dam than those weaned in pens adjacent to the dam. In agreement with other studies (Enriquez *et al*., 2010; Lynch *et al*., 2011), resting behaviour increased significantly with calves spending from 40 % to 70 % of their time lying following the day of abrupt weaning through to day 11 of the study.

Total leukocyte number did not change throughout the study which may be due to the simultaneous fluxes in individual cell types that often occurs during the physiological process to maintain homeostasis (Jones and Allison, 2007). In chapter 3 of this thesis, and keeping in line with a similar model by Lynch *et al*. (2010a), an increased leukocyte number in animals weaned at housing was found, indicating that the failure to identify increased leukocyte number in the current study suggests a decreased stress load by separating housing and weaning into two distinct stages.

The significant increase in neutrophil number observed within 24 hours followed by a return to baseline by day 11 in this study is in agreement with the findings of Hickey *et al*. (2003b), Blanco *et al*. (2009) and Lynch *et al*. (2010a; 2010b) who reported increased neutrophil percentage following weaning. In the present study, neutrophilia was greater in away animals, particularly males, suggesting the lack of contact with the dam following weaning may increase the stress load in calves. Hickey *et al*. (2003b) reached similar conclusions, finding increased neutrophil number in bull calves following abrupt weaning and suggesting that male calves experience a greater difficulty dealing with the stress of weaning than female calves as plasma noradrenaline also remained elevated 7 days following weaning in males. An initial surge in the endogenous glucocorticoid, cortisol, following weaning increased the number of circulating neutrophils via the decreased expression of the surface marker CD62L, preventing margination and subsequent migration from the vasculature (Tempelman *et al*., 2002) along with a later burst of mature cells from the bone marrow (Jones and Allison, 2007).

In the present study, lymphocyte number decreased on day 2, a finding that concurs with a number of previous weaning studies (Hickey *et al*., 2003b; Blanco *et al*. 2009; Lynch *et al*., 2010a). This reduction in lymphocyte number may be attributed to the trafficking of lymphocytes from general circulation into tissues and organs at risk of infection (Dhabhar, 2009). Differences in lymphocyte distribution resulting from gender may not be surprising as it has previously been reported that gender differences exist in response to stress (Arthington *et al*., 2003). However, a pen effect may also exist by which the pen and not the gender of the animals may account for the differences identified between male and female calves. It is possible that gender × location × time interactions might be due to a pen effect rather than a gender effect. To

control for this, in the present study, all animal pens, housing the male and female calves, were located adjacent to each other and were of similar dimension and orientation in the housing shed. Further research involving the use of a number of pens of both genders may be required to elucidate the extent of gender differences.

The N:L ratio is often viewed as a sensitive measurement of the relationship between neutrophils and lymphocytes, accounting for bidirectional alterations in cellular numbers and acts as an accurate indicator of stress (Zahorec *et al*., 2001). The increase in N:L ratio during the first 24 h was more profound in the away calves than the near calves, which suggests that calves experienced a heightened stress response when they were penned away from their dam. By day 11, N:L ratio had returned to baseline levels, indicating the animals had become adapted to their penning environment. Hickey *et al*. (2003b) and Blanco *et al*. (2009) reported that N:L ratio returned to pre-weaning baseline by day 7 post-weaning. While monocytes are generally regarded as an accurate biomarker of stress (Saul *et al*., 2005; Dhabhar, 2009), this is not the case in the bovine due to the relative variability of monocyte distribution in cattle and their lack of sensitivity to stress hormones (Jones and Allison, 2007). This is verified by the findings of this study in which no major changes were detected in monocyte number although it is important to note that monocyte biological function remains important.

Dhabhar *et al*. (1995) examined the effect of stress-induced changes in leukocyte number and concluded that stress-induced alterations in cellular populations were rapidly reversed (minutes to hours) following elimination of the stressor. Given the aforementioned results, this suggests that weaning results in a relatively short lived (less than 11 days) acute stressor and reestablishment of cellular homeostasis by 11 days post weaning indicates an adjustment by the calves to post weaning conditions.

Despite no change in haemoglobin concentrations throughout the study, significant changes in RBC number, mean corpuscular haemoglobin concentration and haematocrit percentage were evident. On day 11, haematocrit percentage decreased, coinciding with a large increase in MCHC. This hyperchromasia suggests an increased concentration of haemoglobin in the red blood cells of the animals. These results are somewhat more extreme than observed in other similar studies (Blanco *et al*., 2009). Prasse *et al*. (2003) suggested a mild anaemia can occur in young, milk fed cattle as a result of a transient iron deficiency, although that work was carried out in young dairy calves.

In the present study, the relative gene expression of a number of pro- (IL-1β, IL-2, CXCL8, IFN-γ, TNF-α and Lymphotoxin) and anti- (IL-4) inflammatory cytokines were measured in order to ascertain if an inflammatory stress response was evident following weaning. Plasma CXCL8, a potent neutrophil chemoattractant, has previously been found to increase in response to weaning stress (Chapter 3). Therefore, it was expected that mRNA expression would increase following weaning in the current study. The over 2-fold increase in CXCL8 expression on day 1 may be involved in the reported neutrophilia. CXCL8 is expressed early in the inflammatory response by macrophages at the site of inflammation (Hirao *et al*., 2000) suggesting transcript abundance may have already peaked by the initial post-weaning sampling time at 24 hours. Buckham Sporer *et al*. (2007) found increased expression of CXCL8 at 4.5 hours following the onset of transport and this had returned to baseline levels by 24 hours. It is probable that if more frequent blood samples were collected in the first 24 h post weaning that greater concentrations of CXCL8 mRNA may have been identified.

A relationship exists between the pro-inflammatory antiviral cytokine IFN-γ and the anti-inflammatory mediator IL-4 whereby the secretion of one will dictate T helper (Th)

cell differentiation and abrogate expression of the other (Morinobu and Kumagai, 1998). Transcript abundance of IFN-γ increased considerably following weaning in the current study and may have aided in the promotion of a cell-mediated inflammatory response while the expression of IL-4 did not increase significantly. Morinobu and Kumagai (1998) found that increased expression of IFN-γ promotes cell-mediated immunity whereas increased IL-4 promotes the humoral immune response. Therefore, the IFN-γ secretion by Th1 cells in this study skewed the immune response by increasing innate cell-mediated immunity, activating neutrophils and macrophages (Boehm *et al*., 1997), stimulating CD4+ cell differentiation towards Th1 and inhibiting the Th2 secretion of IL-4 (Gajewski and Fitch, 1988). The increase in IFN-γ is identified in the study is likely related to NK cells (Boysen and storset, 2009).

The increased expression of IFN-γ following exposure to weaning as a stressor is similar to that seen following an adrenocorticotropic hormone (ACTH) challenge using twelve Brahman heifers (Burdick *et al*., 2010). These authors reported that the gene expression of IFN-γ increased 16-fold following ACTH challenge which resulted in an increase in the concentration of endogenous cortisol. Carroll *et al*. (2009) identified increased serum concentration of IFN-γ in weaned beef calves three hours after endotoxin challenge which coincided with the reduction in cortisol concentration from its peak immediately following challenge. However, several recent studies have suggested that the anti-inflammatory effects of glucocorticoids result in decreased expression of pro-inflammatory cytokines, particularly IFN-γ (Meltzer *et al*., 2004; Connor *et al*., 2005; Curtin *et al*., 2009). These studies were based on murine models and involved sampling immediately following exposure to a brief physical or psychological stressor (shaking, swimming and electric shock, respectively). In another study, in which LPS was administered beginning 4 days following the use of

inescapable tail shock as a stressor, Johnson *et al*. (2002) reported that animals exposed to the stressor had a more potent inflammatory cytokine response within the first hour of LPS challenge than non-stressed controls which is in agreement with the findings of the present study and with those of Burdick *et al*. (2010) and Carroll *et al*. (2009). It was proposed by Goujon *et al*. (1995) that the *in vivo* pro-inflammatory cytokine response to LPS is inhibited by increased concentrations of glucocorticoids if administered during or immediately following stress, thus resulting in the types of suppression seen in certain studies (Meltzer *et al*., 2004; Connor *et al*., 2005; Curtin *et al*., 2009). This theory is further supported by the findings of Carroll *et al*., (2009) in which the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ did not begin to increase until 30 to 120 minutes following the cortisol surge. *In vitro*, studies have shown that long periods of treatment with glucocorticoids are required to suppress IFN-γ signalling beyond several minutes to hours (Hu *et al*., 2003). The findings of Johnson *et al*. (2002), Goujon *et al*. (1995) and Carroll *et al*. (2009) suggest that the increased expression of IFN-γ may be the result of a heightened immune responsiveness following weaning. An earlier sampling time between 1 and 12 h following maternal separation may have resulted in the increased detection of the anti-inflammatory cytokine IL-4.

The expression of a number of adhesion molecules and chemokines is up-regulated by IFN-γ, often synergistically working with IL-1β and TNF-α, to increase margination of lymphocytes and macrophages (Boehm *et al*., 1997). The increased expression of IL-1β on days 1, 3 and 7 coincides with the expression of IFN-γ and the reported neutrophilia. The concentration of TNF-α remains elevated over a similar time course to IFN-γ in this study, which is in accordance with research indicating that IFN-γ production is stimulated by TNF-α in addition to auto-stimulation (Boehm *et al*., 1997; Schroder *et*

al., 2004). Bailey *et al*. (2009) found TNF-α was up-regulated in a murine social stress model, indicating the key role TNF-α plays in stress-induced inflammation. It has also been demonstrated that endogenous cortisol, resembling concentrations present during the physiological stress response, result in markedly increased gene expression of TNF-α in cattle (Burdick *et al*., 2010). IL-1β can be produced by neutrophils whilst also being chemotactic for neutrophils, resulting in neutrophila (Dinarello, 2009). Furthermore, neutrophils release IL-1 and proteases during apoptosis.

Expression of TLR4 was up-regulated in all calves following weaning, increasing to an over 2-fold increase on day 7, indicating its potential use as a novel biomarker of weaning stress in the bovine. While TLR4 is well established as a modulator of both innate and adaptive immunity (Gan and Li, 2006), it has also recently been identified as playing a role in non-infectious inflammatory disease (Seki *et al*., 2007), with activation of TLR4 increasing the expression of a number of pro-inflammatory cytokines (Shahrara *et al*., 2006). Zhang *et al*. (2008a) demonstrated that TLR4 can be activated by a chronic restraint stressor in mice. These authors were the first to identify stress-induced alterations in TLR4 gene expression, showing a 3-fold increase versus non-stressed controls (Yi *et al*., 2008; Zhang *et al*., 2008a; 2008b). Detectable levels of stress, based on TLR4 expression, remain at 7 days post weaning, suggesting the adjustment period to weaning induced stress may be longer than the anticipated 7 days.

The mRNA expression of GRα was more than 3-fold increased in all animals on day 1 following maternal separation, validating the work of Burdick *et al*. (2010) which found a peak in endogenous cortisol resulted in the up-regulation of GRα throughout the 4 hour challenge. Two bovine studies found significant down-regulation of GRα within 6 hours of dexamethasone administration (Weber *et al*., 2006) or the surge of endogenous

cortisol that occurs during parturition (Burton *et al*., 2005), but the reliance of these studies on exogenous glucocorticoids or the strikingly different physiological environment of parturition mean they cannot be directly compared with the current study. Buckham Sporer *et al*. (2007; 2008a) reported no significant change in expression of GRα following 9 hours of truck transportation. However, a trend existed whereby GRα expression tended to increase immediately following transportation, an increase that remained at sampling 4.5 h later (Buckham Sporer *et al*., 2007; 2008a). It is reported that glucocorticoids trigger a number of anti-inflammatory genes and increase neutrophil lifespan by acting on GRα (Weber *et al*., 2006). In turn, the suppression of GRα by abundant glucocorticoid levels is part of the hypothalamic-pituitary-adrenal axis' negative feedback system, preventing unregulated, systemic glucocorticoid induced damage by the immune system (Bamberger *et al*., 1996). The increase observed in this study and in the study of Buckham Sporer *et al*. (2007) may be due to the reported neutrophilia. GRα is abundantly expressed in neutrophils and they are extremely sensitive to glucocorticoids (Burton *et al*., 2005). It would be reasonable to assume that despite an acute down-regulation of GRα, the increased number of total neutrophils in circulation may have resulted in an increased expression of GRα, despite a decrease in expression on a per cell basis.

It is generally accepted that glucocorticoids can inhibit the inflammatory signalling pathway involving NFκB through a number of methods including increasing the transcription of IκBα (Adcock *et al*., 1999) or by binding directly to NFκB (Padgett and Glaser, 2003), although no detectable alterations in the expression of NFκB were measured in this study.

The pro-apoptotic gene, Fas, codes for a transmembrane death receptor protein (CD95/APO-1) which is present on a number of cells (Nagata and Golstein, 1995). Additionally, the cell cycle regulator p21, induced in response to DNA damage, has been shown to be involved in apoptosis with activation following T lymphocyte induced Fas signaling (Hingorani *et al*., 2000; Coqueret, 2003). Fas increased in expression over 3-fold within 24 hours of weaning contrary to recent work that found expression of Fas to be slightly down-regulated by stress in cattle transported for 9 hours (Buckham Sporer *et al*., 2007), and also in an *in vitro* bovine neutrophil study (Chang *et al*., 2004). The expression of p21 was shown to be over 800-fold increased 4.5 hours into transport induced stress (Buckham Sporer *et al*., 2008a), although no alterations in p21 were identified in this study. Nonetheless, Kono *et al*. (2001) demonstrated that apoptosis was actually induced in T cells that were co-cultured with monocytes from stressed patients. There was no indication of apoptosis in T cells cultured with monocytes from unstressed controls. Additionally, both Fas and p21 were found to be up-regulated (~2-fold) following 2 days of restraint stress in murine models (Yin *et al*., 2000; Yin *et al*., 2006). This acceleration in the rate of cellular apoptosis indicates an immunological attempt to restore homeostasis (Jones and Allison, 2007). Increased expression of p21 may be a short lived event and was not found immediately following transport stress by Buckham Sporer *et al*. (2008a) despite being highly expressed hours earlier.

The cell adhesion molecule, CD62L, plays an important role in the margination of neutrophils to sites of infection and inflammation (Kansas, 1996). Glucocorticoid induced neutrophilia, similar to what is seen in this study, is generally attributed to the down-regulation of CD62L, causing the de-margination of neutrophils from blood vessels back into circulation (Weber *et al*., 2004). However, despite significant neutrophilia, Buckham Sporer *et al.* (2007) found no alterations in CD62L gene

expression in neutrophils over the course of 9 hours of transportation and subsequent monitoring. Significant pharmacological doses of glucocorticoids, as used by Tempelman *et al*. (2002) and Weber *et al*. (2004) may be required to induce this suppression. Lynch *et al*. (2010a), who reported that total leukocyte and neutrophil number increased in calves on day 2 after weaning, also found percentage phagocytic neutrophils and mean fluorescence intensity of CD62L positive neutrophils decreased in weaned calves compared with baseline, whereas they were unchanged in control. The increase seen in the current study on days 1, 3 and 7 could be due to an increase in the release of mature, CD62L expressing neutrophils from bone marrow, as this has been demonstrated to occur following glucocorticoid exposure (Weber *et al*., 2004; Jones and Allison, 2007).

4.4.3. Chapter Conclusions

The profoundly increased neutrophil number and N:L ratio, reduced lymphocyte number, alterations to the erythron and an increased expression of pro-inflammatory cytokines leads to the conclusion that weaning stress results in a number of modifications to the immune system which have seemingly enhancive characteristics and serve the purpose of clearing infection at a time of increased exposure to novel pathogens. While stress was traditionally understood to suppress the immune system and result in numerous pathologies (Irwin *et al*., 1990; Sapolsky, 2004), more recent work suggests that acute stress can actually have salubrious consequences for the immune system, resulting in an enhanced response to pathogenic infection (Dhabhar, 2009), whereas chronic stress suppresses the immune system with resulting changes in immunopathology (Dhabhar and McEwen, 1997; Dhabhar, 2002; Dhabhar, 2009).

This study has established that a number of robust biomarkers for weaning stress exist. The use of more traditional measurements, including total neutrophil number, lymphocyte number and N:L ratio, combined with the highly sensitive immunogenetic markers, IL-1β, CXCL8, IFN-γ, TLR4, GRα and Fas, provide a framework for investigation in future bovine stress studies.

Further work is required to characterise the leukocyte response to stress at weaning and may serve to elucidate key regulatory genes. From this study, it is clear that weaning induced alterations in gene expression may extend beyond the 7 days measured and it is necessary to establish at what point in time this returns to baseline levels. It would be of interest to investigate the effect of combining husbandry management stressors (e.g. weaning, housing, castration, transport) on immunomodulation as an increase in the magnitude of stressors may overwhelm the immune system and result in the kind of chronic or extreme stress that could potentially suppress immunity.

Concerning management at weaning, it is evident that calves, particularly intact male calves, may benefit from a weaning strategy where the calves are allowed contact with the dam but prevented from suckling for a number of days before total separation occurs. However, unless other stressors, such as castration or transportation accompany this event, weaning calves without contact with their dams may have no negative impact on their health and welfare.

Chapter 5

Transcriptomic analysis of the stress response to weaning at housing in bovine leukocytes using RNA-seq technology

5.1. Introduction

Weaning is a stressful event in the calf's lifetime with alterations in behaviour (Veissier and Le Neindre, 1989; Price *et al*., 2003; Haley *et al*., 2005; Enriquez *et al*., 2010; Chapter 4), hormonal mediators of stress (Hickey *et al*., 2003; Blanco *et al*., 2009; Chapter 3), and immune function (Arthington *et al*., 2005; 2008; Lynch *et al*., 2010; O'Loughlin *et al*., 2011; Enriquez *et al*., 2011) documented post-weaning. 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 increase susceptibility to disease (Mackenzie *et al*., 1997; Ensminger *et al*., 1997; Carroll *et al*., 2007; 2009; Babcock *et al*., 2010; Broom, 2011; Hubbard and Scott, 2011).

Weaning, together with movement of beef calves from a pasture environment to a housing environment, has been shown to negatively affect total leukocyte, neutrophil and lymphocyte counts, lymphocyte immunophenotypes and the functional activity of neutrophils in beef calves (Lynch *et al*., 2010a). These authors also reported a neutrophil and lymphocyte immunophenotypic response to housing of calves, indicating that housing can elicit a stress response, even when separated from weaning. More recently, O'Loughlin *et al*. (2011) reported that the expression of pro-inflammatory cytokine genes in leukocytes, including IL-1β, CXCL8, IFN-γ and TNFα, were up-regulated, up to 7 days post weaning, in calves that were housed together with their dams for 28 days prior to separation. The molecular mechanisms involved and the role of glucocorticoids in modulating the immune response to weaning at housing, however, remain to be elucidated.

Few studies in any species have sought to characterise the stress response and identify the regulatory input by cells of the immune system, particularly at the transcriptomic level. RNA-seq is a relatively new technique that provides a unique opportunity to deeply sequence the transcriptome of any species with single base resolution and without the need for a known reference genome sequence, allowing unparalleled, highly accurate quantification of differential gene expression, in addition to the identification of novel or unannotated genes, transcripts and alternative splicing events. Furthermore, RNA-seq generates absolute gene expression measurements, providing greater resolution and accuracy than microarrays (Marioni *et al*., 2008). Recently, Huang and Khatib (2010) published the first bovine study to use RNA-seq, focusing on the transcriptomic landscape of embryos and concluded that this technology was highly suitable for future gene expression studies in the bovine, a conclusion in line with other groups (Rinaldi *et al*., 2010).

In this study, a global gene expression approach was used to investigate the response to weaning at housing and to elucidate the key regulatory genes and pathways in beef calves. Thus, capturing subtle transcriptomic changes could provide insight into the molecular mechanisms that underlie the physiological response to stress. In order to carry out this study, 16 male calves were selected along with their dams and divided into one of two treatments, 1) Control, on day 0, these calves were removed from pasture and housed in an unfamiliar environment on slatted floors with their dams, 2) Weaned, on day 0, these calves were removed from pasture, abruptly separated from their dams, socially reorganised and housed on slatted floors in an unfamiliar environment. The experimental design is described in more detail in chapter 2 (2.2.2.6).

5.2. Physiological response of calves to weaning stress at housing

5.2.1. Physiological response to weaning stress at housing on neutrophil and lymphocyte percentage, platelet and monocyte number and mean corpuscular haemoglobin concentration

There was a treatment × sampling time ($P < 0.001$) interaction for total leukocyte number on d 3 where leukocyte number was greater in control calves compared with baseline with no change in weaned calves relative to baseline (Table 5.1). There was a treatment × sampling time interaction ($P < 0.001$) for neutrophil percentage whereby on d 1 it increased in weaned calves before returning to pre-weaning baseline, whereas control calves differed from baseline on d 3 (Figure 5.1A). This resulted in a higher neutrophil percentage in weaned calves compared with control calves on day 1. There was a treatment × sampling time interaction ($P < 0.001$) for lymphocyte percentage, which was converse to that of neutrophils (Figure 5.1B). This relationship was reflected in the N:L ratio, which increased from pre-weaning baseline in weaned calves on d 1 and was higher than control calves at this time, while it also increased from baseline in weaned calves on d 3. There was a treatment × sampling time interaction for monocyte percentage ($P < 0.05$) and MCHC ($P < 0.05$) where monocytes were lower than baseline in weaned calves on d 7 and MCHC increased from baseline on d 1 in weaned and control calves, and on d 3 in weaned calves (Table 5.2). Platelet number decreased from baseline on d 1 in weaned calves and was also lower than control calves at this sampling time-point (Figure 5.1C).

Table 5.1. Effect of stress on total leukocyte, neutrophil and lymphocyte percentage, neutrophil:lymphocyte (N:L) ratio, eosinophil and monocyte percentage in calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days post weaning					*P*-values		
Variable		0	1	2	3	7	T	S	T × S
Total Leukocytes	C	13.3 ± 0.97	13.8 ± 1.44	13.9 ± 0.71	15.6^{bx} ± 1.08	13.9^{x} ± 0.61	NS	NS	*
($\times 10^3$ cells/µL)	W	12.2 ± 0.91	13.2 ± 1.34	12.1 ± 0.66	12.2^{y} ± 1.01	11.3^{y} ± 0.57			
Neutrophils, %	C	23.7 ± 2.63	24.9^{x} ± 2.30	28.9 ± 2.92	34.9^{b} ± 3.12	28.9 ± 2.29	NS	NS	***
	W	30.9 ± 2.45	43.4^{cy} ± 2.14	29.7 ± 2.72	30.1 ± 2.90	26.3 ± 2.14			
Lymphocytes, %	C	63.7 ± 2.87	63.9^{x} ± 2.17	58.3 ± 2.44	53.3^{b} ± 2.59	59.0 ± 2.50	NS	NS	***
	W	58.4 ± 2.67	44.9^{cy} ± 2.02	58.8 ± 2.27	57.8 ± 2.41	64.1 ± 2.33			
N:L Ratio	C	0.38 ± 0.076	0.41^{x} ± 0.061	0.52 ± 0.079	0.69^{b} ± 0.098	0.51 ± 0.056	NS	NS	***
	W	0.57 ± 0.071	0.99^{cy} ± 0.057	0.52 ± 0.074	0.54 ± 0.091	0.42 ± 0.052			
Eosinophils, %	C	2.7 ± 0.46	1.9^{a} ± 0.23	3.4 ± 0.86	3.7 ± 0.89	3.5 ± 1.18	NS	NS	NS
	W	1.6 ± 0.43	1.9 ± 0.21	2.3 ± 0.79	1.9 ± 0.83	1.8 ± 1.09			
Monocytes, %	C	6.6 ± 0.36	7.1 ± 0.44	7.2 ± 0.39	6.1 ± 0.63	5.9 ± 0.45	NS	NS	*
	W	7.5 ± 0.33	6.9 ± 0.41	6.9 ± 0.36	7.5 ± 0.59	5.7^{b} ± 0.42			

C = Control, W = Weaned, T = treatment, S = sampling time, T × S = treatment × sampling time interaction, NS = not significant ($P > 0.05$).
* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
[a,b,c]Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

Table 5.2. Effect of stress on red blood cell (RBC) number, haematocrit (HCT) percentage, haemoglobin (HGB), mean corpuscular haemoglobin concentration (MCHC) and platelet number in calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days post weaning					*P*-values		
Variable		0	1	2	3	7	T	S	T × S
RBC	C	11.9 ± 0.21	11.8 ± 0.19	12.3^{b} ± 0.24	12.1 ± 0.27	12.1 ± 0.28	NS	NS	NS
($\times 10^6$ cells/µL)	W	11.6 ± 0.22	11.6 ± 0.21	11.8 ± 0.26	11.8 ± 0.29	11.8 ± 0.29			
HCT, %	C	34.6 ± 0.68	34.4 ± 0.69	35.9^{b} ± 0.84	35.5 ± 0.85	35.3 ± 0.81	NS	*	NS
	W	33.5 ± 0.64	33.4 ± 0.65	34.1 ± 0.78	34.5 ± 0.79	34.2 ± 0.76			
HGB (g/dL)	C	13.0 ± 0.19	13.1 ± 0.25	13.5^{b} ± 0.28	13.4 ± 0.29	13.4 ± 0.29	NS	*	NS
	W	12.7 ± 0.19	12.8 ± 0.23	12.9 ± 0.26	12.9 ± 0.28	12.9 ± 0.27			
MCHC (g/dL)	C	37.7 ± 0.28	38.1^{b} ± 0.24	37.7 ± 0.22	37.7 ± 0.23	37.9 ± 0.29	NS	NS	*
	W	37.9 ± 0.26	38.5^{c} ± 0.22	37.8 ±0.21	37.5^{a} ± 0.21	37.7 ± 0.27			
Platelet number	C	640.1 ± 63.06	697.2^{x} ± 60.73	700.4 ± 55.59	684.8 ± 57.03	665.2 ± 49.91	NS	*	NS
($\times 10^3$ cells/µL)	W	604.5 ± 58.79	469.4^{ay} ± 56.62	596.4 ± 51.83	562.3 ± 53.17	536.3 ± 46.54			

C = Control, W = Weaned, T = treatment, S = sampling time, T × S = treatment × sampling time interaction, NS = not significant ($P > 0.05$).
* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
[a,b,c]Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

5.2.2. Physiological response to weaning stress at housing on cortisol and haptoglobin

There was a treatment × sampling time interaction ($P < 0.05$) for serum cortisol concentration whereby on d 1 concentration was greater in weaned calves compared with control calves but not subsequently ($P > 0.05$) (Table 5.3). Plasma concentration of haptoglobin decreased ($P < 0.001$) from baseline on d 2 in control calves and on d 3 in weaned calves and subsequently remained lower ($P < 0.001$) than baseline (Figure 5.1D).

Table 5.3. Effect of stress on circulating cortisol and haptoglobin concentrations in control and weaned calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days post weaning					*P*-values		
Variable		0	1	2	3	7	T	S	T × S
Cortisol	C	6.1 ± 1.17	4.3^{x} ± 1.36	8.5 ± 1.81	5.7 ± 1.31	9.7 ± 2.29	NS	*	*
(ng/mL)	W	6.3 ± 1.09	8.4^{y} ± 1.27	8.4 ± 1.68	8.4 ± 1.23	6.5 ± 2.14			
Haptoglobin	C	1.04 ± 0.052	1.07 ± 0.068	0.52^{b} ± 0.184	0.30^{c} ± 0.122	0.29^{c} ± 0.076	NS	***	NS
(mg/mL)	W	1.02 ± 0.049	1.14 ± 0.063	1.03 ± 0.173	0.49^{b} ± 0.114	0.39^{c} ± 0.071			

C = Control, W = Weaned, T = treatment, S = sampling time, T × S = treatment × sampling time interaction, NS = not significant ($P > 0.05$).
* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
[a,b,c]Within rows, Lsmeans differ from pre-weaning baseline by $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

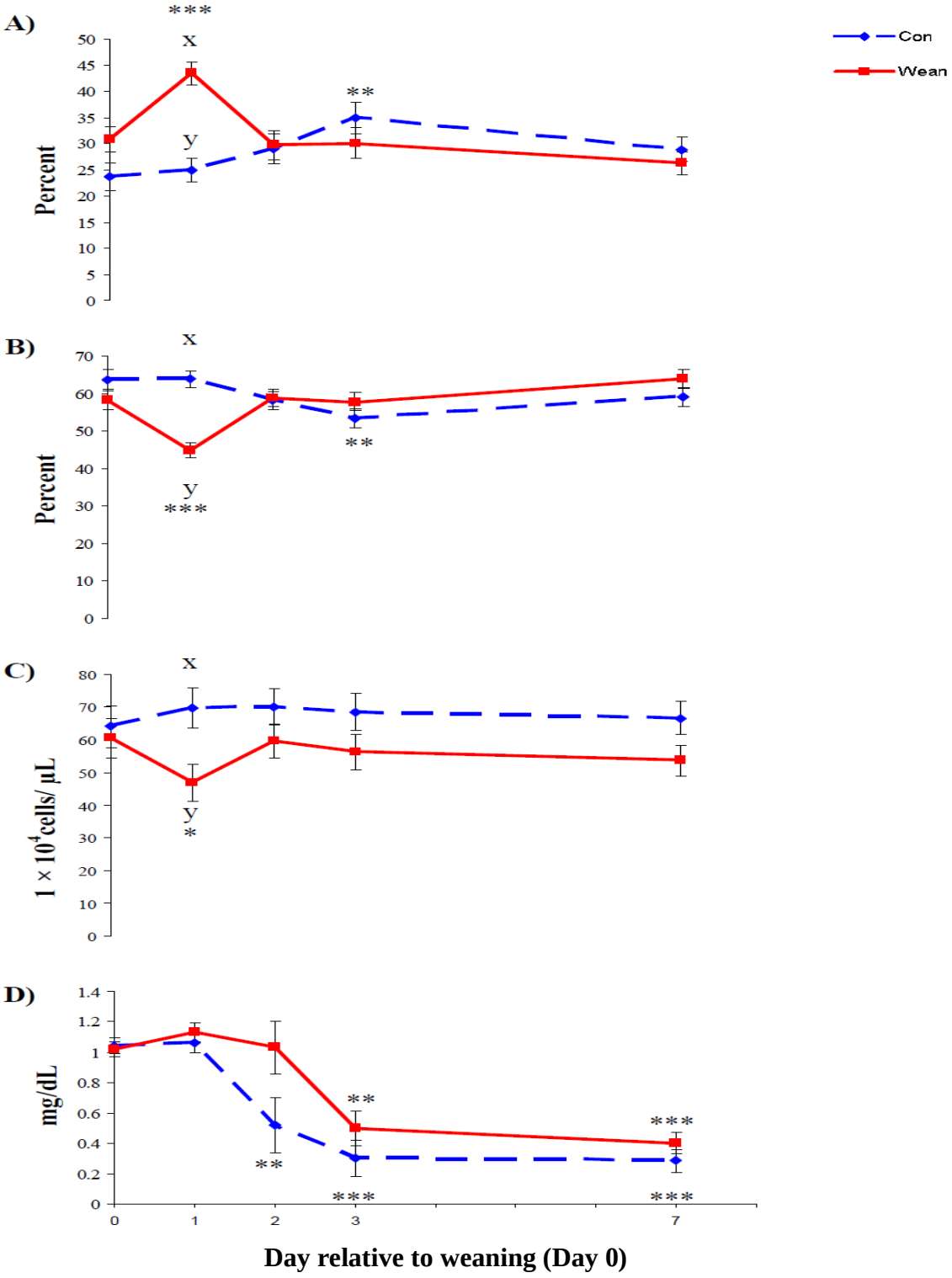

Figure 5.1. Effect of weaning at housing on haematological variables in control and weaned calves. Neutrophil percentage (**A**) rapidly increased on day 1 following weaning, indicating a profound stress response that was reciprocally matched by lymphocyte percentage (**B**). Platelet number (**C**) also decreased in response to weaning while haptoglobin (**D**) decreased in both weaned and control calves. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ Lsmeans differ relative to baseline (Day 0). ^{x,y}Lsmeans differ between location by $P < 0.05$.

5.3. Preliminary analysis of RNA-seq data

Forty-eight libraries representing 12 animals in two different treatments (control (n = 6) and weaned (n = 6)) on d 0, 1, 2 and 7 were prepared from total leukocyte mRNA. Collectively, this resulted in 1,089,256,422 sequenced reads with an average of 22,692,824 reads per lane (Table 5.4). Approximately 28% of reads, or 6,486,330, uniquely aligned to a single location on the reference genome and were retained for analysis, comparable to other recent RNA-seq studies (Sultan *et al*., 2008; Klostermeier *et al*., 2011). On average, 4,479,044 reads per lane failed to align, while a further 4,325,899 aligned to multiple genomic locations. A conservative approach was adapted towards identical reads aligning to the same genomic position whereby all but one of these reads was removed at each genomic location. An average of 7,402,570 reads per lane were removed due to their potential role in introducing PCR bias.

Table 5.4. Lane assignments and preliminary analysis of RNA-seq reads

Flowcell	Lane	Sample ID	Group	Raw reads	Failed to align	Aligned to multiple sites	Accepted alignments	Multiple Reads (PCR) removed	Retained Reads
100202	3	1755(4)	Con	23807742	4234985 (17.79%)	4364588 (18.33%)	15208169 (63.88%)	8139770	7068399 (29.69%)
100202	5	1755(2)	Con	24103370	3893758 (16.15%)	4792467 (19.88%)	15417145 (63.96%)	6989093	8428052 (34.97%)
100202	6	1755(3)	Con	22900561	3721237 (16.25%)	4298948 (18.77%)	14880376 (64.98%)	6124809	8755567 (38.23%)
100222	1	1755(6)	Con	25308344	4848638 (19.16%)	4515039 (17.84%)	15944667 (63.00%)	10689018	5255649 (20.77%)
100222	2	1758(2)	wean	24731241	4972679 (20.11%)	4434545 (17.93%)	15324017 (61.96%)	9606503	5717514 (23.12%)
100222	3	1758(3)	Wean	27200717	4667698 (17.16%)	5216682 (19.18%)	17316337 (63.66%)	9274689	8041648 (29.56%)
100222	4	1758(4)	Wean	27491775	4418265 (16.07%)	5246494 (19.08%)	17827016 (64.84%)	7142516	10684500 (38.86%)
100222	6	1758(6)	Wean	24913542	5853789 (23.50%)	4319498 (17.34%)	14740255 (59.17%)	5434274	9305981 (37.35%)
100222	7	1759(2)	Con	22956789	5761556 (25.10%)	4006293 (17.45%)	13188940 (57.45%)	6546383	6642557 (28.94%)
100222	8	1759(3)	Con	21469397	3958805 (18.44%)	4070117 (18.96%)	13440475 (62.60%)	6732008	6708467 (31.25%)
100225	1	1759(4)	Con	8309401	2409626 (29.00%)	1396116 (16.80%)	4503659 (54.20%)	1874430	2629229 (31.64%)
100225	2	1759(6)	Con	10953380	3589105 (32.77%)	1645410 (15.02%)	5718865 (52.21%)	2485393	3233472 (29.52%)
100225	3	1762(2)	Wean	17219415	3569406 (20.73%)	3263838 (18.95%)	10386171 (60.32%)	4666192	5719979 (33.22%)
100225	4	1762(3)	Wean	22511541	3744742 (16.63%)	4362419 (19.38%)	14404380 (63.99%)	7611763	6792617 (30.17%)
100225	6	1762(4)	Wean	21814888	3756135 (17.22%)	4117354 (18.87%)	13941399 (63.91%)	9381558	4559841 (20.90%)
100225	7	1762(6)	Wean	21543158	3703314 (17.19%)	4283210 (19.88%)	13556634 (62.93%)	7248825	6307809 (29.28%)
100225	8	1763(2)	Con	21734946	3699559 (17.02%)	4463100 (20.53%)	13572287 (62.44%)	7822737	5749550 (26.45%)
100309	1	1763(3)	Con	25761039	5210570 (20.23%)	5015198 (19.47%)	15535271 (60.31%)	10601554	4933717 (19.15%)
100309	2	1763(4)	Con	28973074	5462311 (18.85%)	6067176 (20.94%)	17443587 (60.21%)	10284606	7158981 (24.71%)
100309	3	1763(6)	Con	29314377	5567705 (18.99%)	6088391 (20.77%)	17658281 (60.24%)	10681849	6976432 (23.79%)
100309	4	1769(2)	Wean	26630192	4520797 (16.98%)	5119511 (19.22%)	16989884 (63.80%)	8763279	8226605 (30.89%)
100309	6	1769(3)	Wean	24587605	5183204 (21.08%)	4772581 (19.41%)	14631820 (59.51%)	7857133	6774687 (27.55%)
100309	7	1769(4)	Wean	24104306	5103971 (21.17%)	4597262 (19.07%)	14403073 (59.75%)	7550714	6852359 (28.43%)
100309	8	1769(6)	Wean	26321704	4798460 (18.23%)	5206822 (19.78%)	16316422 (61.99%)	8581174	7735248 (29.39%)
100316	1	1770(2)	Wean	24813826	4710674 (18.98%)	5224214 (21.05%)	14878938 (59.96%)	8872678	6006260 (24.21%)

100316	2	1770(3)	Wean	27345930	4670117 (17.08%)	5807108 (21.24%)	16868705 (61.69%)	9103730	7764975 (28.39%)
100316	3	1770(4)	Wean	27371688	5102727 (18.64%)	5806024 (21.21%)	16462937 (60.15%)	9508313	6954624 (25.41%)
100316	4	1770(6)	Wean	21700024	5126967 (23.63%)	4031807 (18.58%)	12541250 (57.79%)	6012884	6528366 (30.10%)
100316	6	1789(2)	Con	21848794	5425338 (24.83%)	4010789 (18.36%)	12412667 (56.81%)	6728162	5684505 (26.02%)
100316	7	1789(3)	Con	25587368	4816759 (18.82%)	5651481 (22.09%)	15119128 (59.09%)	8729209	6389919 (24.98%)
100316	8	1789(4)	Con	24325238	4560273 (18.75%)	4763625 (19.58%)	15001340 (61.67%)	8783726	6217614 (25.56%)
100409	1	1789(6)	Con	18569808	3851904 (20.74%)	3815774 (20.55%)	10902130 (58.71%)	6054730	4847400 (26.10%)
100409	2	1802(2)	Con	19768291	3889379 (19.67%)	3658744 (18.51%)	12220168 (61.82%)	6527900	5692268 (28.79%)
100409	3	1802(3)	Con	18668849	3996147 (21.41%)	3357507 (17.98%)	11315195 (60.61%)	5736113	5579082 (29.88%)
100409	4	1802(4)	Con	20172805	3780879 (18.74%)	3622253 (17.96%)	12769673 (63.30%)	6602469	6167204 (30.57%)
100409	6	1802(6)	Con	20364927	3743401 (18.38%)	3607275 (17.71%)	13014251 (63.91%)	7815146	5199105 (25.53%)
100409	7	1773(2)	Wean	22077884	4213821 (19.09%)	4308400 (19.51%)	13555663 (61.40%)	7126812	6428851 (29.12%)
100409	8	1773(3)	Wean	22878153	3747957 (16.38%)	4457866 (19.49%)	14672330 (64.13%)	5792572	8879758 (38.81%)
100421	1	1773(4)	Wean	16256030	3560915 (21.91%)	3011442 (18.53%)	9683673 (59.57%)	4454140	5229533 (32.17%)
100421	2	1773(6)	Wean	21827470	4003868 (18.34%)	4336738 (19.87%)	13486864 (61.79%)	6479381	7007483 (32.10%)
100421	3	1803(2)	Con	23087721	4549405 (19.70%)	4278948 (18.53%)	14259368 (61.76%)	7838335	6421033 (27.81%)
100421	4	1803(3)	Con	20985054	4247202 (20.24%)	3946304 (18.81%)	12791548 (60.96%)	6643163	6148385 (29.29%)
100421	6	1803(4)	Con	21954247	4514804 (20.56%)	4053299 (18.46%)	13386144 (60.97%)	7203517	6182627 (28.16%)
100421	7	1803(6)	Con	22488443	4575594 (20.35%)	4270961 (18.99%)	13641888 (60.66%)	7176634	6465254 (28.75%)
100421	8	1809(2)	Wean	21152860	4480619 (21.18%)	4099933 (19.38%)	12572308 (59.44%)	6797783	5774525 (27.29%)
100728	1	1809(3)	Wean	23718729	5774172 (24.34%)	3918368 (16.52%)	14026189 (59.14%)	7575608	6450581 (27.19%)
100728	3	1809(4)	Wean	25733471	5750247 (22.35%)	4444580 (17.27%)	15538644 (60.38%)	8011020	7527624 (29.25%)
100728	8	1809(6)	Wean	21896308	5250611 (23.98%)	3496640 (15.97%)	13149057 (60.05%)	7659087	5489970 (25.10%)
			Average	22692842	4479044	4325899	13887900	7402570	6485330

5.4. Differentially expressed genes and pathways between weaned and control calves on day 1, 2 and 7

5.4.1. Differentially expressed genes between weaned and control calves

There were 276 genes (human orthologs were identified for 215) differentially expressed between treatments on day 1 with 174 up-regulated in weaned animals and 102 down-regulated (Figure 5.2). This increased to 411 genes (human orthologs were identified for 322) differentially expressed between weaned and control calves on day 2 with 303 up-regulated and 108 down-regulated in weaned calves. By day 7, there were 1,218 genes (human orthologs were identified for 1,116) differentially expressed between the treatments with 1,136 genes up-regulated and 182 down-regulated in weaned calves. For a full list of differentially expressed genes, see Appendix 5. A list of DEG in common between time points can be found in Appendix 8.

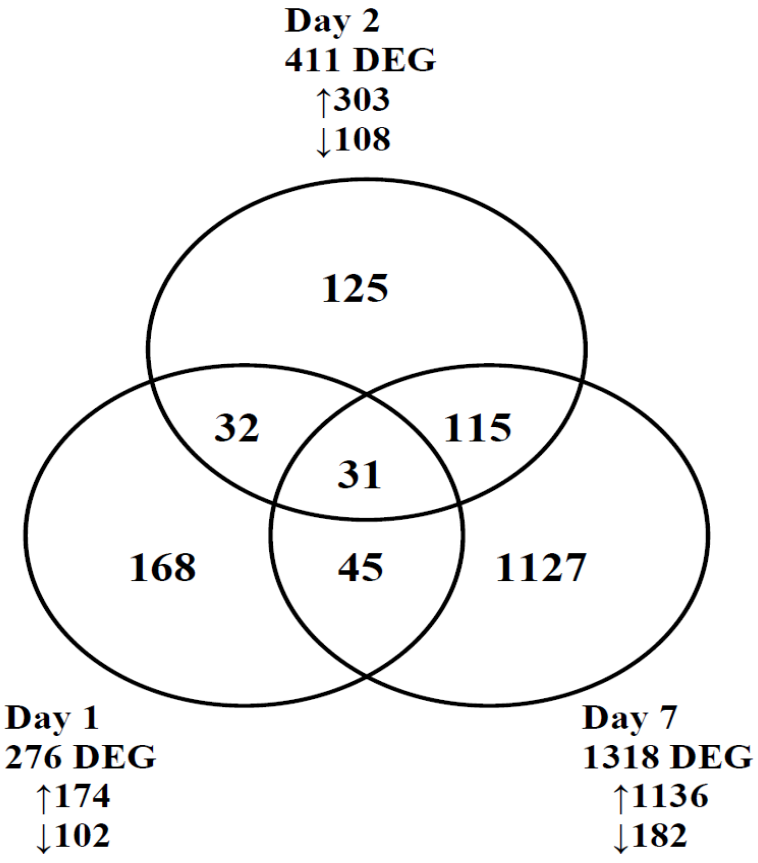

Figure 5.2. Venn diagram analysis of the number of differentially expressed genes between weaned and control calves.

5.4.2. Pathway and gene ontology (GO) analysis of differentially expressed genes to characterise the transcriptomic differences between weaned and control calves

In order to identify key differences between the weaned and control treatments, InnateDB pathway analysis was utilised and identified four main pathway categories, cytokine signalling, transmembrane transport, haemostasis and G-protein-coupled receptor (GPRC) signalling as statistically over-represented among differentially expressed genes in the weaned animals in comparison with controls (Table 5.5). Where a pathway was not significantly over-represented, no genes are listed. Appendix 9 contains a list of significantly over-represented pathways between weaned and control calves.

5.4.2.1. Cytokine signalling

On d 1, the cytokine signalling pathway was transcriptionally activated between treatments with nine genes in this pathway up-regulated in weaned calves. However, this pathway was not statistically over-represented on d 2 and d 7, despite the differential expression of a number of cytokines at these other time points.

5.4.2.2. Transmembrane transport

Similar to the cytokine signalling pathway, the transmembrane transport pathway was transcriptionally activated on d 1 with a number of differences observed between weaned and control calves. There was an up-regulation of six genes in weaned calves, all of which are members of the solute-carrier gene superfamily. Of the nine differentially expressed transmembrane transport pathway genes, eight were up-regulated on d 2. By d 7, this pathway was no longer significantly over-represented.

Table 5.5. Significantly differentially expressed pathways between weaned and control calves.

	Day post weaning		
Pathway	**Day 1**	**Day 2**	**Day 7**
Cytokine signalling	CCL24, CXCL5, FLT3, IFNK, CXCL8, KDR, CXCL7, TNFRSF11A, XCL2	**NS**	**NS**
Transmembrane transport	**AQP1**, SLC10A6, SLC12A3, SLC14A1, SLC26A3, SLC4A5, SLC5A11, **SLC6A5**	**SLC12A3**, SLC24A5, SLC26A3, SLC29A4, SLC29A10, SLC4A1, SLC5A7, SLC6A15, SLC6A2	**NS**
Haemostasis	MRVI1, PDE5A	**COL1A1**, **COL1A2**	**COL1A1**, **COL1A2**, α2β1
GPRC signalling	**NS**	**ADRB1**, **ADRB3**, HTR1B, **GLP1R**, **PDE4C**	GNAI1, RGS1, RGS13, RGS17, **RGS20**

RED indicates genes up-regulated in weaned calves versus control calves; **GREEN** indicates genes down-regulated in weaned calves versus control calves.
Genes are listed if two criteria are met: 1) they are significantly differentially expressed (fold change ≥ 2 and false discovery rate (FDR) < 0.05); 2) the pathway is significantly differentially expressed as identified by GOseq and InnateDB (FDR < 0.1).

5.4.2.3. Haemostasis

Two genes, phosphodiesterase 5A, cGMP-specific (PDE5A) and Inositol 1,4,5-triphosphate receptor-associated cGMP kinase substrate (MRVI1), were altered in the haemostatic pathway on d 1 with both up-regulated in weaned animals. This changed on d 2 with two genes, collagen type 1 alpha 1 (COL1A1) and collagen type 1 alpha 2 (COL1A2) down-regulated in weaned calves. These two genes were down-regulated on d 7, along with the up-regulation of a third gene, the surface integrin α2β1.

5.4.2.4. GPCR signalling

While the GPCR signalling pathway was not significantly over-represented on d 1, five genes were identified as playing a role in this pathway on d 2. Expression of both the beta-1 and -3 adrenoreceptors, responsible for activation of adenylate cyclase activity, was suppressed in weaned calves, while the 5-hydroxytryptamine (serotonin) receptor

1B (HTR1B) was up-regulated. However, on d 7, a different set of genes known as regulators of G-protein signalling were differentially expressed between treatments with five of the six genes up-regulated in weaned calves.

5.4.2.5. Gene ontology analysis of differentially expressed genes to characterise the transcriptomic differences between weaned and control calves

GOseq analysis did not identify any significant GO terms between weaned and control calves on d 1, although over 50 genes fell into the GO terms "chemokine activity", "integrin binding", "neutrophil activation", and "extracellular region" (Figure 5.3).

Thirteen enriched GO terms were returned for the differentially expressed genes on d 2, principally relating to the extracellular region and its components. Of the eight differentially expressed genes annotated to "extracellular matrix structural constituent", six were down-regulated in weaned animals, including the collagen genes collagen type I, III, IV and V. The GO term "extracellular region" encompassed the largest group of DEG on d 2 with fifty-one differentially expressed genes between weaned and control calves. This GO term was evenly split with twenty-five up-regulated and twenty-six down-regulated in weaned animals. Up-regulated genes included the platelet-derived growth factor C (PDGFC) and the lymphocyte apoptotic inhibitor, CD5L, among a range of other genes involved in extracellular matrix structure and function, particularly in the secretion of hormones, cytokines and other ligands. On d 7, 22 over-enriched GO terms, predominantly involved in mitotic cell division, were identified.

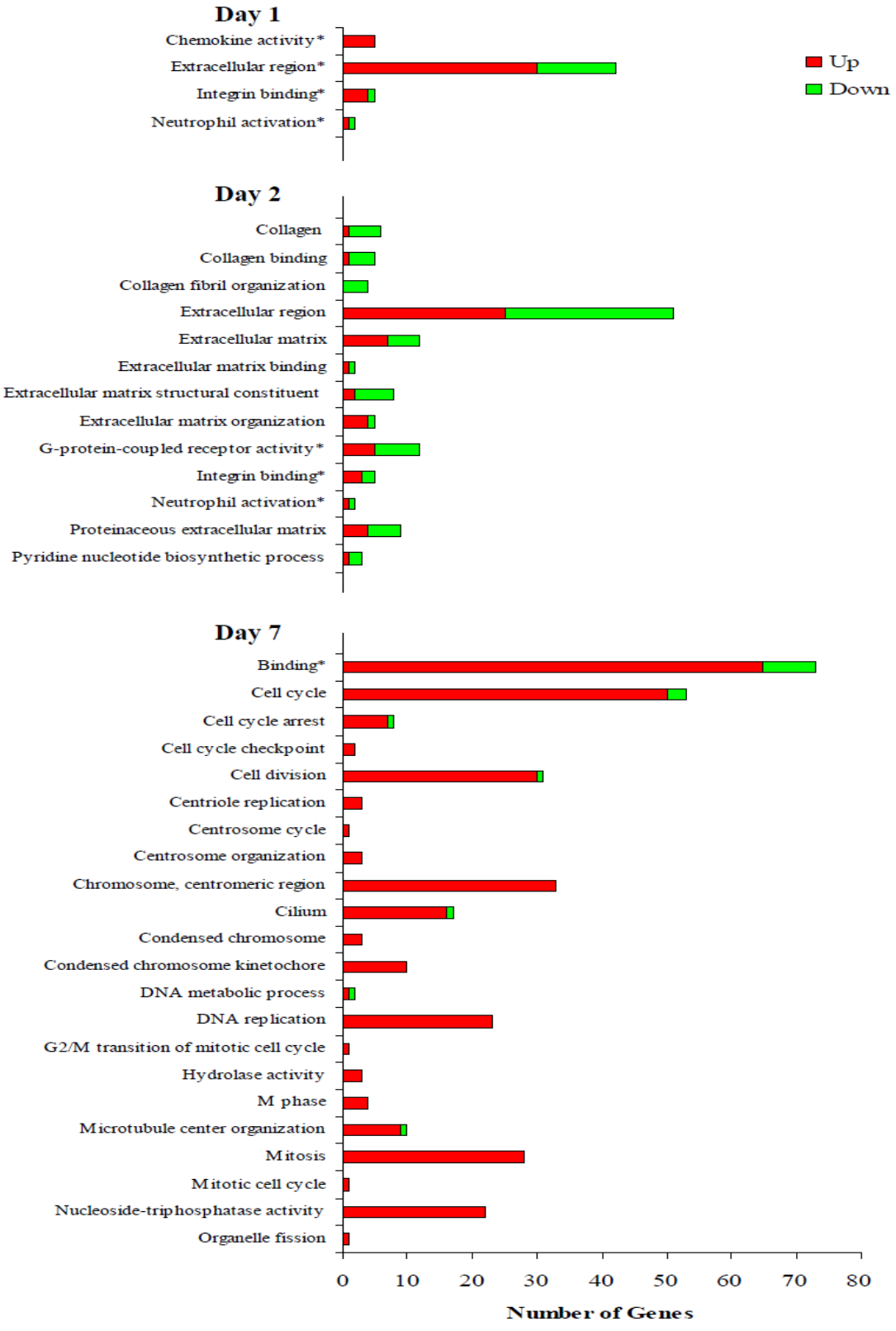

Figure 5.3. Significant GO terms returned by GOseq analysis of genes differentially expressed between weaned and control calves. On day 2, DEG were primarily assigned to GO terms involved in extracellular matrix structure and function, particularly in the secretion of hormones, cytokines and other ligands. However, by day 7, almost all DEG identified belonged to GO terms involved in mitotic cell division.

5.5. Differentially expressed genes and pathways to assess the effect of weaning stress on the leukocyte transcriptome

5.5.1. Genes differentially expressed in response to stress 1, 2 and 7 days following weaning

On day 1, 353 genes (human orthologs were identified for 274) were differentially expressed versus the pre-weaning baseline on day 0 with 281 up-regulated and 72 down-regulated (Figure 5.4). A similar trend in gene expression followed on day 2 with 360 genes (human orthologs were identified for 277) differentially expressed (265 up-regulated and 95 down-regulated) and day 7 with 370 genes (human orthologs were identified for 323) differentially expressed from baseline (272 up-regulated and 98 down-regulated). For a full list of differentially expressed genes, see Appendix 6. A list of DEG in common between time points can be found in Appendix 8.

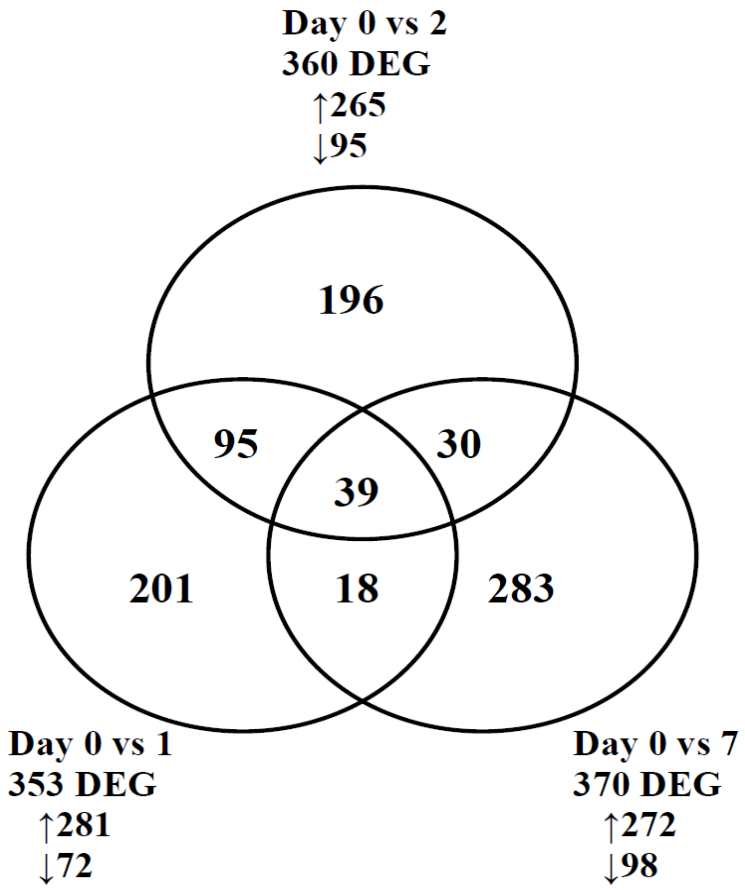

Figure 5.4. Venn diagram analysis of the number of differentially expressed genes on day 1, 2 and 7 following weaning.

5.5.2. Pathway and gene ontology (GO) analysis of differentially expressed genes to characterise the transcriptomic response of bovine leukocytes to stress 1, 2 and 7 days after weaning at housing

This analysis examined the stress response to weaning at housing by comparing gene expression on d 1, 2 and 7 with the pre-weaning baseline on d 0 (Table 5.6). Appendix 10 contains a list of significantly over-represented pathways for weaned calves on d 1, 2 and 7.

5.5.2.1. Cytokine signalling

On d 1, fourteen differentially expressed genes were annotated to the cytokine signalling pathway, with ten up-regulated following weaning including the neutrophil and lymphocyte chemoattractants CXCL5, CXCL7, CXCL8, CCL2, CCL24 and XCL2. This pathway remained significantly activated on d 2 post-weaning with twelve of the sixteen differentially expressed genes up-regulated. While cytokines, chemokines and their receptors accounted for all the differentially expressed genes in this pathway on d 1, by d 2, there were fewer chemokines induced, although a number of inflammatory mediators, including IL-1, were up-regulated. All four of the genes assigned to the cytokine signalling pathway on d 7 (IFN-γ, JAK2, LRPPRC, LIF) were up-regulated and represent a shift in the cytokine signalling network from chemotaxis towards mediated inflammatory response.

5.5.2.2. Transmembrane transport

A number of differentially expressed genes were involved in transmembrane transport, with seven of the eight increasing in response to weaning on d 1. These included two each of the glucose carrier family SLC5 and the potassium dependent sodium/calcium exchanger SLC24 family, in addition to the neurotransmitter transporter, SLC6A15. On

d 2, transmembrane transport remained similarly expressed to d 1, with members of the SLC5, SLC6 and SLC24 families being the most prominent of the 10 up-regulated genes in this pathway. By d 7, the transmembrane transport pathway was no longer significantly over-represented, although a number of genes involved in transport were still differentially expressed.

Table 5.6. Significantly differentially expressed pathways in weaned calves

	Day post weaning		
Pathway	**Day 1**	**Day 2**	**Day 7**
Cytokine signalling	**BMP6**, CCL2, CCL24, CXCL5, IL23R, IL5RA, CXCL8, LIFR, CXCL7, XCL2, AICDA, **COL1A1**, **COL1A2**, **IRS1**	**CCL19**, CXCL10, IL15RA, IL1A, IL23R, IL5RA, CXCL8, **MPL**, SKP1, PLCB4, TIAM2, AICDA, **COL1A1**, **COL1A2**, CCRL1	IFNG, JAK2, LRPPRC, LIF
Transmembrane transport	SLC24A1, SLC24A5, **SLC22A1**, SLC22A7, SLC5A11, SLC5A7, SLC6A15, NUP54	**CFTR**, **RHCG**, SLC1A1, SLC24A1, SLCA5, SLC2A13, SLC4A1, SLC5A7, SLC6A15, SLC6A2, SLC7A11, ABCA1, **ABCC11**	**NS**
Haemostasis	**COL1A1**, **COL1A2**, α2β1, CPB2, PLAT, SERPINB2, ANGPT2, PDE3A, PLA2G4A, TRPC3	**COL1A1**, **COL1A2**, α2β1, **COL3A1**, EDN1, PLA2G4A, TRPC6, **MPL**, P2RY12, TRPC3, IL1A, IL5RA, MME	**COL1A1**, **COL1A2**, **COL3A1**, α2β1, **ADCY8**, **EDNRB**, JAK2
GPRC signalling	**ADM**, CALCRL	**ADM**, **ADRB3**, **CCL19**, CCRL1, CXCL10, EDN1, **FZD4**, **FZD7**, OXTR, P2RY12, P2RY14, TSHB, **VIPR1**, ADCY10, **EREG**, FGF2, FZD3, IL1A, CXCL8, NRG2, RGS1, RGS11, TSHB, PLCB4, TRPC3, TRPC6, CAMP, PLCB4, RYR3, **CFTR**, CHRNA2, GHSR, **NMUR2**	**ADCY8**, CD80, FZD4, FZD7, IFNG, **LIF**, **RAPGEF4**, RGS1, RGS2, **TAC3**, TSHB, **ADM**, ADRB2, CCL4, **EDNRB**, HTR2B, **UTS2**

RED indicates genes up-regulated versus Day 0; **GREEN** indicates genes down-regulated versus Day 0.
Genes are listed if two criteria are met: 1) they are significantly differentially expressed (fold change ≥ 2 and false discovery rate (FDR) < 0.05); 2) the pathway is significantly differentially expressed as identified by GOseq and InnateDB (FDR < 0.1).

5.5.2.3. Haemostasis

Of the ten genes differentially expressed in the haemostatic pathway on d 1, eight were up-regulated from baseline expression levels, principally involved in inflammation and the adhesion of platelets while type I collagen (COL1A1 and COL1A2) was down-regulated. The down-regulation of these two genes was also found on d 2 and 7. However, the majority of the 13 genes involved in haemostasis on d 2 were up-regulated, being mostly involved in the adhesion of platelets to exposed collagen and the subsequent process of coagulation and immune cell activation. A shift in the haemostatic pathway occurred on d 7 with five of the seven differentially expressed genes in this pathway down-regulated from the pre-weaning baseline, although the $\alpha 2\beta 1$ integrin remained up-regulated.

5.5.2.4. GPCR signalling

Only two genes in the GPCR pathway, the multifunctional vasodilator adrenomedullin (ADM) and the ADM receptor, calcitonin receptor-like receptor (CALCRL), were altered on d 1 with a down-regulation of ADM accompanied by an up-regulation of CALCRL. However, on d 2 twenty-four of the thirty-three differentially expressed genes in the GPCR signalling pathway were up-regulated. These included significant numbers of both G-protein-coupled receptors and their diverse ligands, in addition to RGS genes. Despite a reduction of signalling in the GPCR pathway on d 7, seventeen genes were still altered with eleven having increased expression levels including cytokines, neuropeptides, GPCR and two members of the RGS family.

5.5.2.5. Gene ontology analysis of differentially expressed genes to characterise the transcriptomic response of bovine leukocytes to weaning stress at housing 1, 2 and 7 days after weaning

GOseq returned a single significant GO term on d 1 (Figure 5.5). A total of fifty-two genes were annotated to the GO term "Extracellular region", the majority of which (n = 36) were up-regulated following weaning. This term covers a broad group of genes primarily involved in cell signalling and includes a number of cytokines. Eighty-seven significant GO terms were returned as over-enriched on d 2. The two largest GO terms from d 2, "plasma membrane" and "integral to membrane" reveal that a vast majority of the annotated genes were involved in cell signalling, adhesion and ion transport. "Cell communication" and a number of GO terms associated with transmembrane transport similarly indicate the role of cell signalling. This had greatly reduced by d 7, where only six GO terms were associated with differentially expressed genes on d 7. The presence of the "extracellular region", "extracellular space", "extracellular matrix" and "proteinaceous extracellular matrix" indicate a role of a number of genes in cell signalling and interactions. "Extracellular region" covered 35 genes with 60 % of these up-regulated following weaning.

Figure 5.5. Significant GO terms returned by GOseq analysis of genes differentially expressed in response to weaning stress. A large number of GO terms were identified in response to weaning, predominantly involved in the extracellular region and plasma membrane. This indicates that a number of genes were involved in cell signalling and cytokine secretion. The profile on day 7 followed the earlier signature response to weaning although the magnitude was reduced.

5.6. Differentially expressed genes and pathways to assess the effect of housing stress on the leukocyte transcriptome

5.6.1. Genes differentially expressed in response to stress 1, 2 and 7 days following housing

On day 1, there were 110 genes (human orthologs were identified for 86) differentially expressed versus the pre-housing baseline on day 0 with 68 up-regulated and 42 down-regulated (Figure 5.6). This increased to 217 differentially expressed genes (human orthologs were identified for 167) on day 2 with 83 up-regulated and 134 down-regulated. However, a massive induction of genes occurred by day 7 with 1,240 differentially expressed (human orthologs were identified for 1,090) following housing (177 up-regulated, 1,063 down-regulated). For a full list of differentially expressed genes, see Appendix 7. A list of DEG in common between time points can be found in Appendix 8.

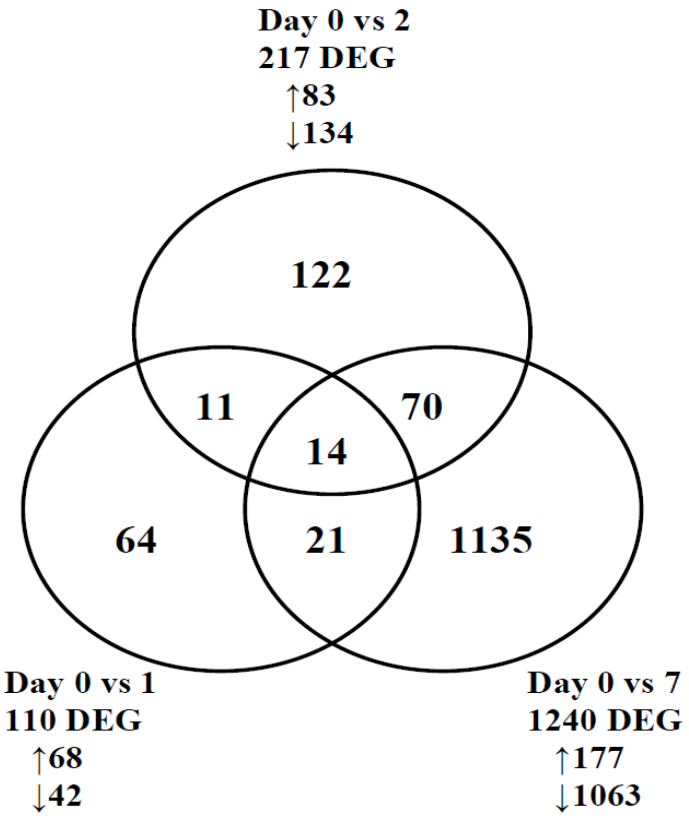

Figure 5.6. Venn diagram analysis of the number of differentially expressed genes on day 1, 2 and 7 following housing.

5.6.2. Pathway analysis of differentially expressed genes to characterise the transcriptomic response of bovine leukocytes to stress 1, 2 and 7 days following housing

This analysis examined the stress response to housing by comparing gene expression on d 1, 2 and 7 with the pre-housing baseline on d 0 (Table 5.7). Appendix 11 contains a list of significantly over-represented pathways for housed animals on d 1, 2 and 7.

5.6.2.1. Cytokine signalling

IL-13 and the platelet derived growth factor receptor (PDGFRB) were up-regulated following housing on d 1, while IL-1 and tumour necrosis factor receptor (TNFRSF11A) were down-regulated in this pathway. On d 2, CCBP2 and CCL22, involved in leukocyte trafficking, were up-regulated, while CCRL1 was down-regulated. The cytokine signalling pathway was not significantly over-represented on day 7.

5.6.2.2. Transmembrane transport

The transmembrane transport family was only significantly affected by housing on d 2 with three members of the ATP-binding cassette family differentially expressed, including the up-regulation of one member of the subfamily A and two members of the subfamily down-regulated.

5.6.2.3. Haemostasis

Four of the seven genes in the haemostasis pathway were up-regulated following housing on d 1 with all of them directly involved in platelet adhesion. However, the inflammatory mediator, IL-1, was down-regulated at this time. This pathway was not significantly over-represented on d 2 or d 7.

Table 5.7. Significantly differentially expressed pathways in housed control calves.

	Day following weaning		
Pathway	**Day 1**	**Day 2**	**Day 7**
Cytokine signalling	IL13, **IL1A**, PDGFRB, **TNFRSF11A**	CCBP2, CCL22, **CCRL1**	**NS**
Transmembrane transport	**NS**	ABCA13, **ABCC2**, **ABCC8**	**NS**
Haemostasis	GP1BA, **IL1A**, **MRVI1**, PLAT, PROC, **GNG12**, PDGFRB	**NS**	**NS**
GPRC signalling	PLAT, PROC, **SERPINA1**, IL13, **IL1A**, **WNT5B**, WNT8B	**C3AR1**, CCBP3, CCL22, **CCRL1**, GLP1R, **GNRH1**	**NS**

RED indicates genes up-regulated in weaned calves versus control calves; **GREEN** indicates genes down-regulated in weaned calves versus control calves.
Genes are only listed if two criteria are met: 1) they are significantly differentially expressed (fold change ≥ 2 and false discovery rate (FDR) < 0.05); 2) the pathway is significantly differentially expressed as identified by GOseq and InnateDB (FDR < 0.1).

5.6.2.4. GPCR signalling

A number of genes from the GPCR signalling pathway were affected by housing with four up-regulated on d 1. These genes were involved in inflammation and the Wnt family. On d 2, three of the six genes, involved in chemotaxis, were up-regulated. This pathway was not significantly over-represented on d 7.

5.6.2.5. Gene ontology analysis of differentially expressed genes to characterise the transcriptomic response of bovine leukocytes to housing stresss 1, 2 and 7 days after weaning

GOseq analysis revealed six significantly over-represented GO terms on d 1 following housing (Figure 5.7). The largest group of terms, "Extracellular region", included 22 genes, eighteen of which were up-regulated in response to housing. This is similar to the response seen at weaning, although fewer genes can be assigned to this category in the control calves. Three genes could be annotated to "response to lipopolysaccharide", although two of these were down-regulated relative to baseline. There were no significant GO terms on d 2. However, twenty-seven GO terms were identified on d 7. These were involved in the division of cells with fifty-one genes annotated to the largest category, "cell cycle". Of these, all but two genes were down-regulated.

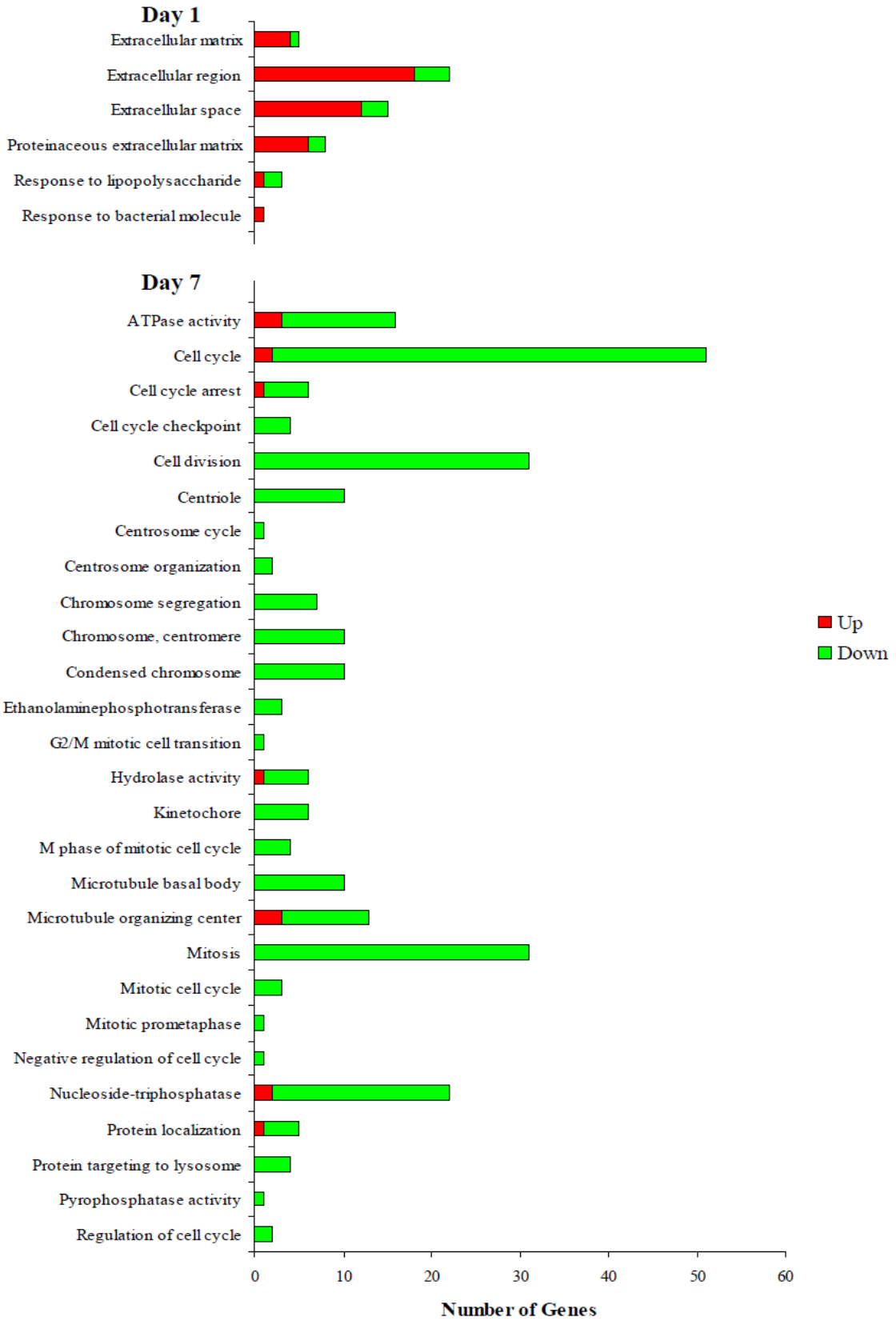

Figure 5.7. Significant GO terms returned by GOseq analysis of genes differentially expressed in response to housing stress. A similar response to weaned calves occurred in control calves on day 1 with an up-regulation of genes involved in the extracellular region. However, by day 7, there was a large down-regulation of genes in housed calves, predominantly involved in the cell cycle.

5.7. Analysis of differentially expressed exons and the promoter regions of differentially expressed genes to identify potential alternative splicing and transcription factors involved in the bovine stress response

5.7.1. Analysis of differentially expressed exons to identify potential alternative splicing events

Although power to detect alternative splicing is limited with single-end reads, a number of genes were identified which, when counting reads over the entire gene were not detected as differentially expressed, but which had at least one differentially expressed exon (Table 5.8). Genes with differentially expressed exons were mapped to their human orthologs and pathway analysis was carried out to identify whether any biological processes were statistically over-represented among these genes.

Ten genes were alternatively spliced between weaned and control calves on d 1. On d 2, three genes involved in metabolism were alternatively spliced (Appendix 12). The majority of alternative splicing was observed on d 7 with analysis revealing seventy-two over-represented pathways including mitotic cell cycle, metabolism of RNA, androgen receptor signalling, RNA transport and T cell receptor signalling.

A number of alternative splicing events occurred in weaned calves relative to pre-weaning with the most significant alterations occurring on d 1. Some of the most biologically and statistically significant pathways were toll receptor cascades, innate immune signalling, CD4 T cell receptor signalling and the phagosome. However, on d 2, transmembrane transport was the only over-represented term from the alternative splicing data.

While no alternative splicing events were identified relative to the pre-housing baseline in the control calves, nine genes were identified on d 2, predominantly involved in Wnt signalling. Pathway analysis of alternatively spliced genes on d 7 was similar to the profile of differentially expressed genes on d 7 with a number of pathways involved in cell cycle identified.

Table 5.8. Differentially expressed exons and estimates of alternate splicing

	Number of DEG	Number of DE exons	Number of genes with DE exons	Estimated number of Genes with alternate splicing*
Weaned versus Control				
Day 1	276	48	28	8
Day 2	411	97	56	19
Day 7	1318	2091	1217	689
Weaning response				
Day 0 vs 1	353	227	190	160
Day 0 vs 2	360	104	58	22
Day 0 vs 7	370	36	25	2
Housing response				
Day 0 vs 1	110	0	0	0
Day 0 vs 2	217	32	22	9
Day 0 vs 7	1240	3921	1734	1087

DEG = differentially expressed genes
DE = differentially expressed
*Non differentially expressed genes with differentially expressed exons

5.7.2. Identification of transcription factors involved in the bovine stress response based on common transcription factor binding sites of differentially expressed genes

Transcription factor analysis with oPOSSUM revealed a number of transcription factors to be responsible for differential gene expression between weaned and control calves (Table 5.9) with a similar pattern for weaned calves compared to the pre-weaning baseline (Table 5.10). The transcription factor analysis of differentially expressed genes from housed calves versus the pre-housing baseline revealed no over-represented transcription factor binding sites in the differentially expressed gene subsets on d 1, although significant TFBS were identified on d 2 and d 7 (Table 5.11).

Table 5.9. Significantly over-represented transcription factors based on transcription factor binding sites of up- and down-regulated genes between weaned and control calves

Treatment	Transcription factor	TF Class	No. submitted genes	No. included genes	Target gene hits	Target TFBS hits	Z-score	Fisher score
Weaned vs Control								
Day 1	**NFKB1**	REL	75	71	16	34	12.27	0.02442
Day 2	**SP1**	ZN-Finger, C2H2	94	87	64	286	10.27	0.008341
Day 7	Lhx3	Homeo	944	793	513	1868	36.1	0.00000000000906
	Foxd3	Homeo	944	793	536	2036	44.5	0.000000005882
	Prrx2	Homeo	944	793	673	6463	49.79	0.00000003235
	NKX3-1	Homeo	944	793	493	1607	31.43	0.00000009752
	SP1	ZN-Finger, C2H2	158	141	110	541	23.65	0.00001001
	SRY	HMG	944	793	624	3916	37.47	0.00005745
	MZF1 5-13	ZN-Finger, C2H2	158	141	113	572	15.92	0.0001504
	Pdx1	Homeo	944	793	670	6552	47.06	0.0002094
	Nkx2-5	Homeo	944	793	681	7005	47.62	0.0002818
	Foxa2	Forkhead	944	793	494	1733	35.57	0.0004601
	Sox5	HMG	944	793	629	4126	32.0	0.0007285
	REST	ZN-Finger, C2H2	158	141	6	6	11.4	0.009661

RED indicates analysis performed using up-regulated genes; **GREEN** indicates analysis performed using down-regulated genes

Table 5.10. Significantly over-represented transcription factors based on transcription factor binding sites of up- and down-regulated genes following weaning

Treatment	Transcription factor	TF Class	No. submitted genes	No. included genes	Target gene hits	Target TFBS hits	Z-score	Fisher score
Weaned								
Day 0 vs 1	SP1	ZN-Finger, C2H2	58	53	43	230	25.33	0.001226
	Sox5	HMG	215	170	141	1057	15.69	0.005115
	SRY	HMG	215	170	142	971	15.01	0.0005537
	Pdx1	Homeo	215	170	150	1556	14.9	0.001884
	Prrx2	Homeo	215	170	147	1500	14.77	0.001319
	NHLH1	bHLH	58	53	29	50	13.96	0.0001506
	Foxd3	Forkhead	215	170	117	474	13.7	0.001539
	Foxa2	Forkhead	215	170	118	415	12.17	0.0003255
	FOXI1	Forkhead	215	170	117	413	10.97	0.000663
	Lhx3	Homeo	215	170	118	429	10.06	0.000006085
Day 0 vs 2	Pdx1	Homeo	192	162	146	1511	14.61	0.0002197
	Prrx2	Homeo	192	162	143	1442	13.55	0.0001892
	Foxa2	Forkhead	192	162	115	403	11.93	0.00008997
	TEAD1	TEA	82	73	29	49	11.89	0.003366
	Lhx3	Homeo	192	162	117	427	11.53	0.0000002648
	Nkx2-5	Homeo	192	162	148	1576	11.09	0.0002961
	MEF2A	MADS	192	162	80	166	10.4	0.0002961
Day 0 vs 7	FOXI1	Forkhead	237	200	137	467	13.09	0.0003402
	Lhx3	Homeo	237	200	88	158	11.18	0.00001208

RED indicates analysis performed using up-regulated genes; **GREEN** indicates analysis performed using down-regulated genes

Table 5.11. Significantly over-represented transcription factors based on transcription factor binding sites of up- and down-regulated genes following housing

Treatment	Transcription factor	TF Class	No. submitted genes	No. included genes	Target gene hits	Target TFBS hits	Z-score	Fisher score
Weaned								
Day 0 vs 2	SP1	ZN-Finger, C2H2	73	67	51	214	10.01	0.005796
Day 0 vs 7	**NKX3-1**	Homeo	912	774	508	1688	33.86	0.0000000000007823
	Lhx3	Homeo	912	774	506	1962	39.0	0.00000000000108
	Foxd3	Forkhead	912	774	535	2055	40.72	0.00000000003793
	Prrx2	Homeo	912	774	659	6765	53.08	0.00000001522
	FOXI1	Forkhead	912	774	513	1723	27.93	0.00000002982
	Foxg1	Forkhead	912	774	360	735	17.0	0.0000003186
	Foxa2	Forkhead	912	774	504	1811	37.82	0.0000006219
	SP1	ZN-finger, C2H2	160	145	115	606	38.41	0.000001446
	HLF	bZip	912	774	329	543	14.13	0.000001766
	Pdx1	Homeo	912	774	553	6837	49.8	0.000007135
	MEF2A	MADS	912	774	337	635	14.14	0.0000095
	Sox5	HMG	912	774	626	4326	35.07	0.00001499
	SRY	HMG	912	774	611	4074	39.25	0.00003538
	USF1	bHLH-ZIP	160	145	103	313	14.65	0.00006722
	Mycn	bHLH-ZIP	160	145	105	336	13.43	0.00008339
	Nkx2-5	Homeo	912	774	688	7432	54.87	0.00009584
	Arnt	bHLH	160	145	105	349	13.42	0.0001749
	Myf	bHLH	160	145	93	232	10.76	0.0002006
	HNF4A	Nuclear Receptor	160	145	78	131	11.96	0.000236
	MAX	bHLH-ZIP	160	145	81	162	11.55	0.000236
	MZF1 5-13	ZN-finger, C2H2	160	145	115	641	30.55	0.0002943
	NHLH1	bHLH	160	145	62	110	12.74	0.0007532
	IRF1	TRP-Cluster	912	774	279	455	13.49	0.0008395
	SRF	MADS	160	145	16	19	13.14	0.001503

RED indicates analysis performed using up-regulated genes; **GREEN** indicates analysis performed using down-regulated genes

5.8. Chapter summary, discussion and conclusions

5.8.1. Chapter summary

Weaning of beef calves is a necessary husbandry practice and involves separating the calf from its mother, resulting in numerous stressful events including dietary change, social reorganisation and the cessation of the maternal-offspring bond and is often accompanied by housing. While much recent research has focused on the physiological response of the bovine immune system to stress in recent years, little is known about the molecular mechanisms modulating the immune response. Therefore, the objective of this study was to provide new insights into the molecular mechanisms underlying the physiological response to weaning at housing in beef calves using Illumina RNA-seq.

There was a treatment × sampling time interaction ($P < 0.001$) for total leukocyte number, neutrophil and lymphocyte percentage and N:L ratio following weaning where leukocyte, neutrophil, and the N:L ratio increased and lymphocyte percentage and platelet numbers decreased in relation to both baseline and control calves following weaning. Additionally, plasma cortisol concentration was higher ($P < 0.05$) in weaned calves following weaning while haptoglobin decreased in all animals in response to the stress of either housing or weaning. The leukocyte transcriptome was significantly altered for at least 7 days following either housing or weaning at housing. Analysis of differentially expressed genes revealed that four main pathways, cytokine signalling, transmembrane transport, haemostasis and G-protein-coupled receptor (GPRC) signalling were differentially regulated between control and weaned calves and underwent significant transcriptomic alterations in response to weaning stress on day 1, 2 and 7. Of particular note, chemokines, cytokines and integrins were consistently found to be up-regulated on each day following weaning. Evidence for alternative

splicing of genes was also detected, indicating a number of genes involved in the innate and adaptive immune response may be alternatively transcribed, including those responsible for toll receptor cascades and T cell receptor signalling.

This study represents the first application of RNA-Seq technology for genomic studies in bovine leukocytes in response to weaning stress. Weaning stress induces the activation of a number of cytokine, chemokine and integrin transcripts and may alter the immune system whereby the ability of a number of cells of the innate and adaptive immune system to locate and destroy pathogens is transcriptionally enhanced. Stress alters the homeostasis of the transcriptomic environment of leukocytes for at least 7 days following weaning, indicating long term effects of stress exposure in the bovine.

5.8.2. Chapter discussion

Weaning is a multifactorial stressor, often encompassing separation from the dam and peers, mixing with unfamiliar animals, housing, novel handling and a change in diet and environment. The results of the present study demonstrate that the leukocyte transcriptomic environment is profoundly altered by weaning stress, at least up to 7 days post-weaning and that a number of processes are transcriptionally activated to increase immune cell adhesion and migration, potentially serving to increase immune surveillance following exposure to weaning stress.

Neutrophilia resulting from a stressor, as seen in this study, has been frequently reported in both cattle (Hickey *et al*., 2003; Blanco *et al*., 2009; Lynch *et al*., 2010; O'Loughlin *et al*., 2011) and humans (Cole, 2008). This can partly be attributed to a surge of cortisol (Chang *et al*., 2004; Weber *et al*., 2006) which may result in a series of physiological alterations to neutrophil function including the release of a large number

of immature neutrophils from the bone marrow (Paape *et al*., 2003; Jones and Allison, 2007), an increase in neutrophil chemotaxis (Anderson *et al*., 1999) and a reduction in the neutrophil apoptosis rate (Chang *et al*., 2004; Madsen-Bouterse *et al*., 2006; Weber *et al*., 2006; Buckham Sporer *et al*., 2007). However, circulating cortisol concentrations peak early in the stress response and the infrequent sampling intervals employed in the current study likely missed the true peak (Sapolsky *et al*., 2000; Droste *et al*., 2008).

It has previously been reported that stress-induced cortisol surges inhibit CD62L (L-selectin) expression on bovine neutrophils, thus reducing endothelial adhesion and subsequent migration to sites of infection (Weber *et al*., 2004; Burton *et al*., 2005; Lynch *et al*., 2011). However, several other bovine transport (Buckham *et al*., 2007), castration (Pang *et al*., 2009) and weaning (O'Loughlin *et al*., 2011) studies have failed to identify this suppression transcriptionally. Furthermore, L-selectin expression may not be necessary to maintain leukocyte rolling as platelets have been reported to re-establish leukocyte trafficking in CD62L deficient mice (Diacovo *et al*., 1998). It is clear that a number of genes involved in integrin binding are up-regulated in weaned calves and that, while no difference in the expression of CD62L was identified, several other important integrins involved in cell adhesion and migration were up-regulated, in addition to a clear activation of platelets. The induction of a number of genes involved in transmembrane transport, including the glucose carrier family SLC5, the potassium dependent sodium/calcium exchanger SLC24, and the sodium and chloride dependent neurotransmitter transporter, SLC6A15, demonstrates an increased role for transmembrane transport of small molecules to leukocytes (He *et al*., 2009), aiding in functional activation.

Cytokines play an important role in the immune response, primarily functioning as inflammatory mediators of the innate immune system (Elenkov *et al*., 2005). The transcriptional induction of genes involved in chemotaxis provides a clear mechanism by which leukocyte function was altered. Chemokines are primarily involved in the recruitment of immune cells to inflammatory sites (Mantovani *et al*., 2006; Cardona *et al*., 2008), and their increased expression indicates that weaning stress results in transcriptional alterations that may enhance leukocyte recruitment and activation. Despite a preponderance of differentially expressed cytokine transcripts known to interact directly with neutrophils (Cascieri and Springer, 2000; Hirao *et al*., 2000; Craig *et al*., 2009), it is likely many of these cytokines also influenced a number of other cell populations including lymphocytes, monocytes and platelets (Henn *et al*., 1998; Badolato *et al*., 2006; Rostene *et al*., 2007; Oo and Adams, 2010). This is perhaps best demonstrated by the large induction of the GPCR signalling pathway with a number of regulators of G-protein signalling (RGS) genes remaining up-regulated throughout the course of this study, suggesting the rate of GPCR signalling was increased (Neubig and Siderovski, 2002). Transcriptional signatures suggest neutrophils, monocytes and lymphocytes may have had an enhanced capability to exit the vasculature and increase immune surveillance through an up-regulation of a number of genes responsible for chemotaxis and integrin binding, indicating enhanced leukocyte function and a modest inflammatory response to stress. This increased with the stressor as weaned calves had a longer, more potent inflammatory response than control calves.

Inflammation is tightly linked to haemostasis, an important innate immune defence mechanism primarily intended to arrest the bleeding process (Verhamme and Hoylaerts, 2009), although it has recently been shown that platelets have additional functionality and interact with cells of the innate immune system via secretion of cytokines,

chemokines and other inflammatory mediators (Von Hundelshausen and Weber, 2007; Gleissner *et al*., 2008; Smyth *et al*., 2009; Yeaman, 2010). In the current study, pathway analysis suggests that the process of haemostasis was activated as a result of weaning, and based on this activation, may have resulted in increased platelet aggregation, reducing the number of platelets freely available in circulation, as identified in weaned calves on day 1. A number of genes involved in collagen synthesis were differentially expressed with the overwhelming majority down-regulated in weaned animals, both in relation to control calves and the pre-weaning baseline. An early reaction to tissue damage is the release of collagen into blood vessels where it acts as a potent agonist of platelets and other leukocytes (Crowley *et al*., 2007). This is mediated by cytokine induced collagenase secretion along with the down-regulation of collagen mRNA by cortisol (Oikarinen *et al*., 1998; Cutroneo, 2003; Cutroneo and Sterling, 2004; Kahan *et al*., 2009), aiding in the rapid migration of leukocytes through the extracellular matrix (ECM) to sites of inflammation (Barreiro *et al*., 2007). An examination of genes involved in the extracellular region revealed the presence of a number of genes responsible for the activation and coagulation of platelets (Volger *et al*., 2007; Kumpers *et al*., 2008; Guiliano and Wheeler, 2009), in addition to platelet derived chemokines, such as CCL2 (Henn *et al*., 1998), CXCL5 (Gleissner *et al*., 2008), CXCL7 (Brandt *et al*., 2000) and platelet-derived growth factor C (PDGFC) (Wagsater *et al*., 2009) which may have facilitated the recruitment of neutrophils, monocytes, lymphocytes and other leukocytes to susceptible tissues, signifying that stress can transcriptionally activate an acute inflammatory response through interactions with platelets and the haemostatic system.

A large number of genes were differentially expressed on d 7 in weaned animals when compared with baseline expression values. While we have previously reported a

detectable signature of inflammatory candidate genes in bovine leukocytes 7 days following weaning (O'Loughlin *et al*., 2011), it was unexpected that the expression of such a large number of genes (>300) would remain altered for so long following weaning, although it has been previously reported that a number of physiological parameters, including plasma fibrinogen and haptoglobin concentrations, and total leukocyte number had not returned to baseline in weaned calves 35 days following weaning (Lynch *et al*., 2010a). Results from the current study indicate that weaning can have a profound impact on the leukocyte transcriptome for at least 7 days post-weaning and suggests that the stress of weaning can have far reaching consequences for the homeostasis of the transcriptome. The majority of these genes were up-regulated and principally involved in cell signalling and function with a number of cytokines involved in the Th1 response, suggesting that these effects may have been beneficial to the calves (Sorrells and Sapolsky, 2007). Conversely, an increase in the magnitude of the stressor can quickly result in deleterious effects (Dhabhar, 2008) while a prolonged inflammatory response can cause severe tissue damage (Eruslanov *et al*., 2005; Buckham Sporer *et al*., 2007).

The greatest induction of genes in the control calves also occurred on d 7, a rather unexpected result given that these animals were not additionally exposed to any other stressor. However, the profile for these calves was much different than that of the weaned animals with the cytokine signalling, transmembrane transport, haemostasis and GPCR signalling pathways all having returned to baseline by d 7. Rather, almost 86 % of the genes, which related to DNA replication and the cell cycle, were down-regulated on d 7 in relation to the pre-housing baseline. While it is clear that housing results in a molecular stress response on d 1 and 2, the results of d 7 require further examination as a similar effect was not found in the weaned treatment, despite these calves also being

housed in addition to weaning. However, further research is required to assess the long term effect of housing stress in weaned calves. In the first 2 days following housing, a similar stress induced gene expression profile is present in both housed and weaned calves, with a greater magnitude of induction in weaned calves exposed to the accumulative stress of housing, weaning and social reorganisation.

5.8.3. Chapter conclusions

A major strength of this study is that it represents the first application of RNA-Seq technology for genomic studies in bovine leukocytes in response to weaning stress. Weaning stress induced a significant number of transcriptional changes that are reflected in the physiological alterations identified in cellular distribution. The most important finding of this study was that simultaneously weaning and housing of calves produces a perturbation to the homeostasis of the leukocyte transcriptome which was still present 7 days following weaning. Weaning at housing induced activation of a number of cytokine, chemokine and integrin transcripts which may alter the immune system whereby the ability of a number of cells of the innate and adaptive immune systems to locate and destroy pathogens is enhanced. Weaning also appears to elicit an acute inflammatory response in calves. However, this may have potentially negative consequences for tissues that are at risk of damage due to inflammation. Additional research is required to align these two conclusions, although it is likely a case where an initial beneficial anti-pathogenic response gives way to damage due to inflammation over a number of days when the stressor is not eliminated. It is also clear that housing results in a less marked stress response than weaning, as identified by a reduced induction in gene expression following housing. Future studies should examine the processes that have been identified in this study as playing a critical role in immunocompetence of weaned calves and should principally address the ability of

calves to mount an immune response to viral and bacterial challenge following weaning or exposure to other stressors. This study has identified regulatory gene networks that are stress activated, and may be modulated by glucocorticoids in leukocytes, and provides a mechanistic framework to characterise the multifaceted nature of weaning stress adaptation in beef calves.

Chapter 6

General Discussion

6.1. Introduction

Stress is an important physiological response that is highly evolutionarily conserved, serving the purpose of preparing a rapid induction of processes necessary for the short-term survival of an animal. However, the current understanding of the complicated reaction to an environmental stressor, particularly in the context of livestock, is not entirely clear, particularly in relation to the effect a stressor may have on the immune status of an animal. Stress has long been implicated in the suppression of the immune system and an increase in disease susceptibility has often been reported during routine husbandry practices, including weaning (Hickey *et al*., 2003b; Arthington *et al*., 2005; Mitchell *et* al. 2008; Blanco *et al*., 2009; Carroll *et al*., 2009; Lynch *et al*., 2010a; 2010b; Kim *et al*. 2011), housing (Fisher *et al*., 1997b; 1997c; Hickey *et al*., 2003a; Gupta *et al*., 2007a), transportation (Earley and O'Riordan 2006a; Earley *et al*., 2006b; Gupta *et al*., 2007b; Buckham Sporer *et al*., 2007; 2008a; 2008b; Arthington *et al*., 2008; Earley and Murray 2010), and castration (Fisher *et al*., 1997a; Earley and Crowe, 2002; Ting *et al*., 2003a; 2003b; 2010; Pang *et al*. 2009a; 2009b; 2010; Marti *et al*., 2010). While these studies establish a definite perturbation to the immune system in response to stress, it is not clear what consequences they may have for the animal when exposed to infectious agents. Acute stressful events may increase the ability of the immune system to respond to infection (Saul *et al*., 2005; Viswanathan and Dhabhar, 2005; Dhabhar, 2008; 2009). It is only under conditions of chronic or extreme stress, more commonly referred to as distress, when immune suppression may occur.

Weaning is one of the most important husbandry stressors, typically combining multiple stressful events such as separation from the dam, social reorganisation, dietary change and housing. The accumulative nature of weaning means that a calf may

simultaneously undergo physical, psychological and social stress, triggering a powerful neuroendocrine response that can have a profound effect on the homeostasis of the immune system. Weaning has been reported to induce alterations in behaviour (Price *et al*., 2003; Haley *et al*., 2005; Enriquez *et al*. 2010; 2011; Lynch *et al*., 2011), hormones of the neuroendocrine system (Lefcourt and Elsasser, 1995; Hickey *et al*., 2003b), acute phase proteins (Arthington *et al*., 2005; Lynch *et al*., 2010b), and leukocyte cellular distribution patterns (Hickey *et al*., 2003b; Blanco *et al*., 2009; Lynch *et al*., 2010a; 2010b). It is unclear what degree of protection or susceptibility these alterations offer to the animal, although many have concluded that while it appeared the immune response may be suppressed, welfare was not negatively affected by weaning. However, if clinical signs of disease were identified due to immune suppression, animal welfare would have been severely compromised.

Therefore, a number of areas need to be examined in order to fully characterise the response to weaning stress which will help to improve both the health and welfare of beef calves at weaning. This thesis sought to characterise not only the physiological response to weaning stress, but also the molecular response in order to fully elucidate key mechanisms involved in the homeostatic perturbations, thus indicating health related connotations for weaned calves. Additionally, this thesis set out to identify novel molecular biomarkers that may serve as targets for therapeutics and also enable the identification of stressed calves that may be more susceptible to disease at weaning time. In order to achieve these goals, the following 3 objectives were carried out for this thesis:

1. Examine the effect of accumulative stressors (housing vs weaning at housing) on the physiological response to stress by measuring physiological variables

including leukocyte subsets, acute phase proteins, the neuroendocrine stress hormone, cortisol, and the potent neutrophil chemokine, CXCL8. This study provided the opportunity to identify if inflammatory mediators (CXCL8) are present following weaning and provided justification to carry out a molecular analysis of weaned calves (Chapter 3).

2. Examine the physiological and the molecular response to weaning stress using RT-qPCR. This study also sought to assess the gender effect of weaning, in addition to examining alternative weaning strategies with all calves housed 28 days prior to weaning and either weaned next to the dam, similar to a fenceline weaning system, or away from the dam (Chapter 4).

3. Characterise global transcriptomic alterations in gene expression of bovine leukocytes following either housing or weaning at housing in order to elucidate key mechanisms by which the bovine immune system responds to stress.

6.2. Discussion

While the physiological response to weaning stress has been adequately characterised, large gaps exist in the literature surrounding the adrenocortical and immunological response of beef calves to abrupt weaning. By predominantly using molecular techniques such as RT-qPCR and RNA-seq, and combining them with physiological measurements, this thesis sought to further shed light on the immunological response to stress and the implications these may have on health and animal welfare.

A common result between the three studies carried out in this thesis, neutrophilia was consistently found following weaning (Figure 6.1). In chapters 3 and 5, where calves were either housed (control) or weaned and housed (weaned), neutrophil number was found to be higher on day 1 relative to the pre-weaning baseline on day 0. In both cases, neutrophil number was also elevated in comparison to the control calves on day 1, indicating the effect of accumulative stressors on neutrophilia. It is likely that separating housing and weaning into two distinct events can reduce the accumulative effect of stressors (Lynch *et al*., 2010b). A surge in plasma cortisol concentration, even for a brief period of time, has been credited with increasing neutrophil number (Chang *et al*., 2004; Weber *et al*., 2006). This response is due to several physiological alterations to the neutrophil including the release of a large number of immature neutrophils from the bone marrow (Paape *et al*., 2003; Jones and Allison, 2007), an increase in neutrophil chemotaxis (Anderson *et al*., 1999) and a reduction in the rate of neutrophil apoptosis (Chang *et al*., 2004; Madsen-Bouterse *et al*., 2006; Weber *et al*., 2006; Buckham Sporer *et al*., 2007). While a different experimental model was employed in chapter 4, neutrophilia was still evident in all animals following weaning. Additionally, calves that were weaned away from the dam had a significantly higher neutrophil number following weaning than those that were weaned next to the dam. Not only can neutrophil number be used to differentiate weaned calves from housed calves, but also between calves weaned next to the dam and those weaned away from the dam.

A decrease in lymphocyte number has frequently been reported to accompany neutrophilia (Hickey *et al*., 2003b; Blanco *et al*. 2009; Lynch *et al*., 2010a). However, lymphocytopenia does not appear to be as sensitive as monitoring neutrophil number and was only detected in chapters 4 and 5. A decrease in lymphocyte number is

generally regarded as an indication of increased trafficking of lymphocytes from general circulation into tissues and organs that may be at risk of infection (Dhabhar, 2009). Perhaps more suitable than monitoring either neutrophil or lymphocyte number individually, the N:L ratio is a sensitive method of detecting fluctuations of the two cell populations (Zahorec *et al*., 2001) and was found to increase following weaning in all three studies undertaken for this thesis. Therefore, it appears that either neutrophil number of the N:L ratio should be routinely monitored in any future bovine stress studies and potentially incorporated into future prospective biomarker panels for bovine stress.

The measurement of platelets is important because of the role these cells play in inflammation (Von Hundelshausen and Weber, 2007). The results from this thesis suggest that platelet number is far too variable to be reliably used as a biomarker of stress in cattle. While chapter 3 found no alterations in platelet number, chapter 4 identified an increase in response to weaning while chapter 5 found a decrease in response to weaning. The decrease in platelet number found in chapter 5 was hypothesised to be as a result of increased clot formation following weaning, although it is not clear if this is the case. Future transciptomic studies, focusing specifically on platelets, are required to identify if this may be the case.

In response to weaning, haptoglobin was found to both increase (chapter 3) and decrease (chapter 5) in this thesis. Haptoglobin is typically known to increase in response to infection, inflammation and trauma, but is role in the stress response is complicated with conflicting reports that it increases (Arthington *et al*., 2005; Lomborg *et al*., 2008; Aich *et al*., 2009; Lynch *et al*., 2010b) or decreases (Arthington *et al*. 2003; Buckham Sporer *et al*., 2008b) in response to stress. The decrease identified in chapter

5 may be due to elevated baseline concentrations, in excess of 1.0 mg/mL, which were higher than the peak reported following weaning in chapter 3. Plasma SAA concentration was also found to increase in response to either weaning or housing in chapter 3, a finding similar to other studies (Arthington *et al*. 2003; Lomborg *et al*., 2008), but the high variability at baseline means the results may not be particularly meaningful. While APP may be of particular benefit in models where animals are challenged with an infection, it does not seem necessary to continue monitoring the APR in future stress studies as both the findings of this thesis, along the bulk of the literature, suggest that the great variability in the APR means APP are unreliable biomarkers of stress and offer little towards the characterisation of the post-stress environment. It is also not clear to what extend glucocorticoids play altering APP levels. Previously, it was shown that cortisol can induce SAA and haptoglobin synthesis in cytokine stimulated bovine hepatocyte cultures (Higuchi *et al*. 1994; Alsemgeest *et al*. 1996), but the same results were not found *in vivo* following cortisol infusion in cattle (Fisher *et al*. 1997; Ting *et al*., 2004).

In chapter 3, plasma CXCL8 was found to increase in response to both housing and weaning at housing, demonstrating a none-specific response to stress. That is to say, the stress of housing was sufficient to increase CXCL8 concentrations, and the additional stressor of weaning did not increase the magnitude of CXCL8. It is likely that this potent neutrophil chemokine was secreted by macrophages and endothelial cells (Waugh and Wilson, 2008; Gleissner *et al*., 2008) and played a role in neutrophil activation and the increased margination of neutrophils out of the vasculature (Hirao *et al*., 2000; Elenkov *et al*., 2005; Keller *et al*., 2005). Following weaning, neutrophil number increased, mainly due to the release of a large number of immature neutrophils from bone marrow (Paape *et al*., 2003; Jones and Allison, 2007) and a reduction in

neutrophil apoptosis (Chang *et al.*, 2004; Madsen-Bouterse *et al.*, 2006; Weber *et al.*, 2006; Buckham Sporer *et al.*, 2007). CXCL8 likely increased the chemotactic rate, reducing circulating neutrophil number over the course of several days. However, no data was collected for this thesis regarding the fate of these neutrophils upon exiting the vasculature and it is unclear as to whether these neutrophils survived to cause tissue damage or if they were removed by phagocytosing macrophages (Liu *et al.*, 1999).

The results of chapter 3 demonstrated that weaning stress can elicit a cytokine mediated inflammatory response. This led to the investigation, in chapter 4, of the gene expression of a number of candidate inflammatory markers, in addition to several other markers of immune competence. Primarily, chapter 4 built upon the conclusions of chapter 3 and established that, at least transcriptionally, there was a significant inflammatory reaction to weaning. The pro-inflammatory cytokines IL-1, CXCL8, IFN-γ and TNFα were all found to increase in expression following weaning, while the pathogen recognition receptor, TLR4, and the glucocorticoid (cortisol) receptor, GRα, were also found to be up-regulated. In addition to the induction of CXCL8, the surface marker CD62L was found to increase in expression, further suggesting the capacity of neutrophils to migrate out of the vasculature was increased following exposure to stress (Smalley and Ley, 2005). Additionally, the measurement of gene expression proved valuable in the detection of novel biomarkers of stress, predominantly because most had increased more than two-fold by sampling at d 1 and remained elevated at sampling on d 3 and d 7. Traditional measurements, such as neutrophil number and stress hormones, increase rapidly following the onset of a stress response but are often not detectable by 48-72 hrs, despite a continuation of a stressor. These molecular markers, which are sensitive enough to increase early and remain elevated for at least 7 d following weaning, are ideally suited to being included in an index with physiological

measurements (neutrophil number, etc) that can be used to asses stress and animal welfare in future studies.

However, the main objective of chapter 4 was to identify if weaning by itself was stressful on calves. Additionally, this was taken as an opportunity to assess an alternative weaning strategy, whereby fourteen calves were separated into a treatment where they were weaned next to the dam rather than the traditional practice of removing contact with the dam. This method effectively allowed the assessment of the strength of the maternal-offspring bond and served to look at the effect of psychological stress in calves. This was achieved by separating calves into one of two treatments, those weaned next to the dam and those weaned sufficiently distant from their dams that communication was prevented. Physiologically, a stronger stress response was detected in calves weaned away from the dam with higher neutrophil number and lower lymphocyte number versus those weaned next to the dam. However, no molecular response to treatment was identified, indicating that while a weaning stress response results in a strong induction in gene expression, the magnitude cannot differentiate between varying stress levels. An interesting result that did come from this study had to do with gender. Female calves had higher mRNA expression of CXCL8, IFN-γ and TNFα than male calves over the course of this study, suggesting that gene expression may aid in detecting the differential response to stress between male and female animals. Overall, the inflammatory response observed following weaning in these calves offered protection from disease, but the fact that the expression of so many inflammatory mediators remained elevated at d 7 indicates there may have been negative consequences for the health and welfare of the calves as inflammation can be severely damaging in the long-term (Eruslanov *et al*., 2005).

As chapter 4 firmly established the efficacy of studying the response of the leukocyte transcriptome to weaning stress, chapter 5 was used to address the global transcriptomic landscape in order to elucidate the key molecular pathways involved in the stress response and extrapolate the repercussions of these alterations for the immune status of the weaned beef calf. Physiologically, this study conformed to the classic stress response outlined throughout this thesis, predominantly involving neutrophilia with concurrent lymphopenia. A number of transcriptional changes were identified that were reflected in the physiological alterations identified in cellular distribution, predominantly involving the induction of a number of inflammatory cytokine and chemokine transcripts. This study further supported the conclusions of chapters 3 and 4, and the results indicate that, at least transcriptionally, weaning stress induces a mild activation of inflammatory mediators that alter the immune system to increase leukocyte margination and their ability to locate and destroy pathogens. While the early induction of an inflammatory response may contribute to pathogenic defence, the long-term nature of this response following weaning means that inflammatory induced damage of tissues may leave calves more susceptible to infection after a number of days (Eruslanov *et al*., 2005; Dhabhar, 2008)

However, perhaps the most striking result from the three studies is the long-term perturbation to immune homeostasis. The traditional physiological measurements of stress in cattle peak early and return to normal baseline levels within the first few days (Blanco *et al*., 2009; Enriquez *et al*., 2010; 2011; Lynch *et al*., 2010a). In chapter 3, plasma CXCL8 concentrations remained significantly elevated in both treatments (weaned and housed) at sampling 14 d after weaning. Chapter 4 reported that the gene expression of a number of cytokines and other molecular biomarkers were at least 2-fold elevated in relation to baseline at sampling 7 d following weaning while chapter 5

identified a significant induction (> 300) of genes remained at 7 d following weaning at housing. Therefore, stress results in a perturbation of immune function, at both a physiological (plasma CXCL8) and a molecular level for at least a week after weaning, a unique finding that has been repeated in different models throughout this thesis.

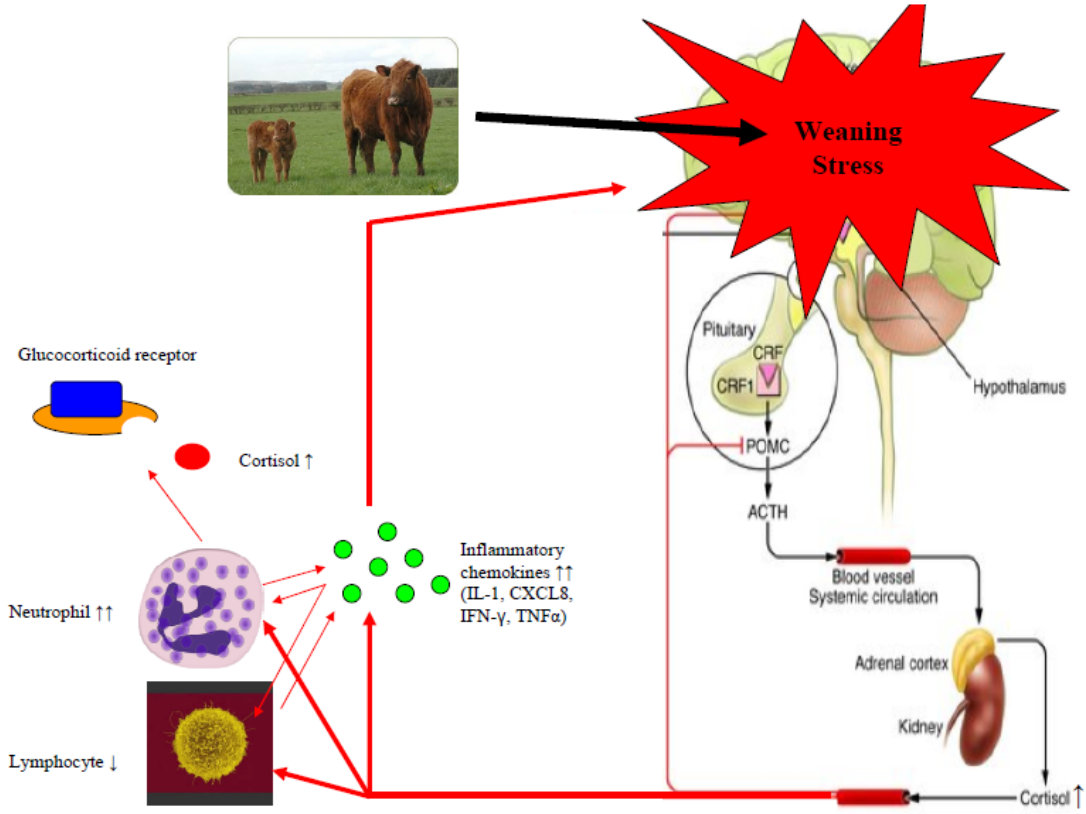

Figure 6.1. Model encompassing the interaction between the HPA axis, neutrophils, lymphoyctes, and inflammatory mediators following weaning stress.

6.3. Main conclusions

Based on the assessment of a number of physiological and molecular measurements of weaning stress in chapters 3, 4 and 5, the main conclusions drawn from this thesis are stated below:

1. Abrupt weaning is a stressful event for calves and results in alterations to the immune system that can be measured at both the physiological (Chapters 3, 4

and 5) and molecular (Chapter 4 and 5) levels, including increasing neutrophil and decreasing lymphocyte number.

2. Housing is a stressful event for calves and results in alterations to the immune system that can be measured at both the physiological (Chapters 3 and 5) and molecular (Chapter 5) levels, including increasing neutrophil number. However, housing appears to be a less stressful practice than weaning.

3. Weaning at housing, and housing, result in a rapid increase in plasma CXCL8 that remains elevated up to 14 d post-weaning (Chapter 3).

4. Weaning calves next to the dam results in a less marked physiological response than weaning calves away from the dam, although there was no difference in any of the molecular markers between the two treatments (Chapter 4).

5. There are a number of differences between male and female calves in gene expression of CXCL8, IFN-γ and TNFα where female calves have higher baseline expression levels and greater increases following exposure to stress (Chapter 4).

6. Weaning stress causes an increase in the gene expression of a number of inflammatory cytokines that remain elevated for at least 1 week. Additionally, mRNA transcripts of TLR4, GRα, Fas and CD62L also increase in response to weaning (Chapter 4).

7. Weaning at housing results in a greater transcriptomic induction of differentially expressed genes than housing alone (Chapter 5). While the magnitude was greater in weaned calves, both treatments experienced an increase in cytokine, chemokine and integrin transcripts, indicating an acute inflammatory response. Most notably, the cytokine signalling, haemostasis, transmembrane transport and GPCR signalling pathways are induced by weaning.

8. The immune system is primed by weaning stress to increase the ability of the innate and adaptive immune systems to locate and destroy foreign pathogens. However, in the long-term, this response can potentially cause severe tissue damage and lead to the development of pathology in the young calf (Chapter 5).

9. Weaning results in perturbations to the homeostasis of the immune system that are physiologically detectable up to 14 day following weaning (CXCL8, Chapter 3) and is detectable up to 7 days following weaning at the molecular level (Chapters 4 and 5).

10. By reducing the effect of accumulative stressors (weaning (near the dam vs away from the dam) vs housing vs weaning at housing), the stress response can be mitigated. Under ideal conditions, the stress response initially bolsters the immune response to pathogens and does not negatively impact on the welfare of cattle. However, where disease is present on farm, calves challenged in the first few weeks following weaning may be more susceptible to disease as a result of tissue damage induced by the inflammatory stress response.

6.4. Thesis implications and future work

The Animal Welfare, Recording and Breeding Scheme (AWRBS) for Suckler Herds (The Suckler Welfare Scheme) was launched by the Department of Agriculture in January 2008. The scheme provides the farmer €40 per animal that complies with the following conditions:

-All recording (including calf registration) is to be carried out through Animal Events.
-Record the following Pre Weaning Events (sample prewean form)

- •Date of Disbudding (Dehorning).
- •Date of Castration.
- •Date of Meal Feeding Introduction.

-Record the following Post Weaning Events

- •Date of Weaning.
- •Docility Score and Calf Quality Score (1-5 Scale).
- •Weight.

Regarding weaning, the AWRBS for Suckler Herds require graduated weaning where no more than half the calves in a herd are weaned at the one time. Additionally, if calves are weaned indoors, they must be weaned next to the dam. The results of this thesis suggest that this weaning strategy is beneficial for the calf and serves to reduce the level of stress placed on the calf at weaning. Additionally, deferring housing at weaning time, or housing 28 days prior to weaning, removes the accumulation of stressors and serves as a suitable method of reducing weaning stress.

The implications of the molecular results of this thesis require further research in order to fully elucidate if the observed inflammatory mechanisms serve to enhance the response to pathogens or if they result in deleterious effects due to tissue damage. Morbidity and mortality increase around the time of weaning (Galyean *et al*., 1999; Pinchak *et al*. 2004; Mitchell *et al*., 2008; Babcock *et al*., 2010). The increased morbidity found at weaning may be as a result of the comingling of unfamiliar cohorts which exposes animals to pathogens not previously encountered, particularly in housing. Whether this may be the case remains unclear and requires further examination of the weaning stress response.

A study should be undertaken to assess the effect of stress on the immune response to pathogenic challenge following weaning. A number of treatments are required for this study, including 1) abruptly weaned calves, 2) abruptly weaned calves challenged with a viral/bacterial pathogen or challenged with an initial viral infection followed by a secondary bacterial pathogen, 3) calves left with the dam, 4) calves left with the dam challenged with a viral/bacterial pathogen or challenged with an initial viral infection followed by a secondary bacterial pathogen. This study would ideally involve molecular and proteomic analysis of a number of leukocyte subsets including neutrophils, lymphocytes and monocytes. Additionally, bronchoalveolar lavage fluid should be collected to determine the response following leukocyte infiltration to the infected lung. This study would allow an accurate assessment of the immune response to a viral and bacterial challenge, and a comparison between the response and survival rate of stressed and non-stressed infected calves will allow accurate conclusions to be drawn on the implications of stress for the immune system (Mitchell *et al*., 2008). An immune challenge should occur at several points following weaning including at weaning, and then 1, 3, 7 and 14 days following weaning. The results of this thesis

suggest that certainly on d 1, and perhaps on d 3, the weaned calves may respond better to disease than the control calves. However, by d 7 to 14, the prolonged inflammatory response in weaned calves may result in soft tissue damage, leaving these calves more susceptible to disease than control calves. Additionally, the effect of stress on vaccination should be examined, as the timing of vaccination may play a role in its efficacy. This is particularly relevant in light of the conclusions of this thesis which demonstrate the inflammatory nature of the stress response.

This thesis has demonstrated that through the progression of research into stress related illness, the mechanisms of disease susceptibility can be sufficiently characterised to elucidate potential therapeutic targets for vaccination and treatment of disease. Additionally, through the identification of specific triggers responsible for immune alterations, this work may be suitable for informing future legislative decisions on animal welfare.

Chapter 7

Bibliography

ABRAHAM, G., GOTTSCHALK, J. & UNGEMACH, F. R. (2004) Possible Role of Dexamethasone in Sensitizing the Beta-2-Adrenergic Receptor System in vivo in Calves during Concomitant Treatment with Clenbuterol. *Pharmacology,* 72**,** 196-204.

ADAMS, J. (2008) Transcriptome: connecting the genome to gene function. *Nature Education,* 1.

ADCOCK, I. M., NASUHARA, Y., STEVENS, D. A. & BARNES, P. J. (1999) Ligand-induced differentiation of glucocorticoid receptor (GR) trans-repression and transactivation: preferential targetting of NF-κB and lack of I-κB involvement. *British Journal of Pharmacology,* 127**,** 1003-1011.

AGARWAL, S. K. & MARSHALL, G. D. (1998) Glucocorticoid-Induced Type 1/Type 2 Cytokine Alterations in Humans: A Model for Stress-Related Immune Dysfunction. *Journal of Interferon & Cytokine Research,* 18**,** 1059-1068.

AICH, P., BABIUK, L. A., POTTER, A. A. & GRIEBEL, P. (2009) Biomarkers for Prediction of Bovine Respiratory Disease Outcome. *OMICS: A Journal of Integrative Biology,* 13**,** 199-209.

AICH, P., JALAL, S., CZUBA, C., SCHATTE, G., HERZOG, K., OLSON, D. J. H., ROSS, A. R. S., POTTER, A. A., BABIUK, L. A. & GRIEBEL, P. (2007) Comparative Approaches to the Investigation of Responses to Stress and Viral Infection in Cattle. *OMICS: A Journal of Integrative Biology,* 11**,** 413-434.

AKGUL, C., MOULDING, D. A. & EDWARDS, S. W. (2001) Molecular control of neutrophil apoptosis. *FEBS Letters,* 487**,** 318-322.

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P. (2002) Molecular Biology of the Cell, 4th edition. *Garland Science, New York.*

ALCOCK, J. & BRAINARD, A. H. (2008) Hemostatic containment - An evolutionary hypothesis of injury by innate immune cells. *Medical Hypotheses,* 71**,** 960-968.

ALSEMGEEST, S., LAMBOOY, I., WIERENGA, H., DIELEMAN, S., MEERKERK, B., VAN EDEREN, A. & TA, N. (1995) Influence of physical stress on the plasma concentration of serum amyloid-A (SAA) and haptoglobin (Hp) in calves. *The Veterinary Quarterly,* 17**,** 9-12.

AMADORI, M., ARCHETTI, I. L., FRASNELLI, M., BAGNI, M., OLZI, E., CARONNA, G. & LANTERI, M. (1997) An Immunological Approach to the Evaluation of Welfare in Holstein Frisian cattle. *Journal of Veterinary Medicine, Series B,* 44**,** 321-327.

AMARAL, L. A. N., DÍAZ-GUILERA, A., MOREIRA, A. A., GOLDBERGER, A. L. & LIPSITZ, L. A. (2004) Emergence of complex dynamics in a simple model of signaling networks. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 15551-15555.

ANDERSON, B. H., WATSON, D. L. & COLDITZ, I. G. (1999) The Effect of Dexamethasone on Some Immunological Parameters in Cattle. *Veterinary Research Communications,* 23**,** 399-413.

ANDERSON, N. L., POLANSKI, M., PIEPER, R., GATLIN, T., TIRUMALAI, R. S., CONRADS, T. P., VEENSTRA, T. D., ADKINS, J. N., POUNDS, J. G., FAGAN, R. & LOBLEY, A. (2004) The Human Plasma Proteome. *Molecular & Cellular Proteomics,* 3**,** 311-326.

ANGEN, B., MUTTERS, R., CAUGANT, D. A., OLSEN, J. E. & BISGAARD, M. (1999) Taxonomic relationships of the *Pasteurella haemolytica* complex as evaluated by DNA-DNA hybridizations and 16s rRNA sequencing with proposal of *Mannheimia haemolytica gen. nov., comb. nov., Mannheimia granulomatis comb. nov., Mannheimia glucosida spa nov., Mannheimia ruminalis spa nov. and Mannheimia varigena sp. nova. International Journal of Systematic Bacteriology,* 49**,** 67-86.

APPLEBY, M. (1999) What Should We Do About Animal Welfare? . Oxford: Blackwell Science.

APPLEBY, M. C. & SANDOE, P. T. (2002) Philosophical Debate on the Nature of Well-being: Implications for Animal Welfare. *Animal Welfare,* 11**,** 283-294.

ARONSON, J. K. (2005) Biomarkers and surrogate endpoints. *British Journal of Clinical Pharmacology,* 59**,** 491-494.

ARTHINGTON, J. D., EICHER, S. D., KUNKLE, W. E. & MARTIN, F. G. (2003) Effect of transportation and commingling on the acute-phase protein response, growth, and feed intake of newly weaned beef calves. *J. Anim Sci.,* 81**,** 1120-1125.

ARTHINGTON, J. D., QIU, X., COOKE, R. F., VENDRAMINI, J. M. B., ARAUJO, D. B., CHASE, C. C., JR. & COLEMAN, S. W. (2008b) Effects of preshipping management on measures of stress and performance of beef steers during feedlot receiving. *J. Anim Sci.,* 86**,** 2016-2023.

ARTHINGTON, J. D., SPEARS, J. W. & MILLER, D. C. (2005) The effect of early weaning on feedlot performance and measures of stress in beef calves. *J. Anim Sci.,* 83**,** 933-939.

AUER, P. L. & DOERGE, R. W. (2010) Statistical Design and Analysis of RNA Sequencing Data. *Genetics,* 185**,** 405-416.

AUERNHAMMER, C. J. & MELMED, S. (2001) The central role of SOCS-3 in integrating the neuro-immunoendocrine interface. *The Journal of Clinical Investigation,* 108**,** 1735-1740.

AUPHAN, N., DIDONATO, J. A., ROSETTE, C., HELMBERG, A. & KARIN, M. (1995) Immunosuppression by Glucocorticoids: Inhibition of NF-κB Activity Through Induction of IκB Synthesis. *Science,* 270**,** 286-289.

BABCOCK, A. H., RENTER, D. G., WHITE, B. J., DUBNICKA, S. R. & SCOTT, H. M. (2010) Temporal distributions of respiratory disease events within cohorts of feedlot

cattle and associations with cattle health and performance indices. *Preventive Veterinary Medicine,* 97**,** 198-219.

BADOLATO, R., SOZZANI, S., LUKACS, N. W. & SCHALLER, M. (2006) Lymphocyte trafficking and chemokine receptors during pulmonary disease. IN PARNHAM, M. J. (Ed.) *Lymphocyte Trafficking in Health and Disease.* Birkhäuser Basel.

BAILEY, M. T., KINSEY, S. G., PADGETT, D. A., SHERIDAN, J. F. & LEBLEBICIOGLU, B. (2009) *Social stress enhances IL-1beta and TNF-alpha production by Porphyromonas gingivalis lipopolysaccharide-stimulated CD11b+ cells.*

BAMBERGER, C. M., SCHULTE, H. M. & CHROUSOS, G. P. (1996) Molecular Determinants of Glucocorticoid Receptor Function and Tissue Sensitivity to Glucocorticoids. *Endocr Rev,* 17**,** 245-261.

BARNARD, C. (2007) Ethical regulation and animal science: why animal behaviour is special. *Animal Behaviour,* 74**,** 5-13.

BARNETT, V. & LEWIS, T. (1994) Outliers in Statistical Data, third ed.,. *Willey, Chichester, UK.*

BARREIRO, O., DE LA FUENTE, H., MITTELBRUNN, M. & SÁNCHEZ-MADRID, F. (2007) Functional insights on the polarized redistribution of leukocyte integrins and their ligands during leukocyte migration and immune interactions. *Immunological Reviews,* 218**,** 147-164.

BARTHOLOME, B., SPIES, C. M., GABER, T., SCHUCHMANN, S., BERKI, T., KUNKEL, D., BIENERT, M., RADBRUCH, A., BURMESTER, G.-R., LAUSTER, R., SCHEFFOLD, A. & BUTTGEREIT, F. (2004) Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. *FASEB J.*, 18**,** 70-80.

BAUER, M. E. (2005) Stress, glucocorticoids and ageing of the immune system. *Stress,* 8**,** 69-83.

BENJAMINI, Y. & HOCHBERG, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological),* 57**,** 289-300.

BERGQUIST, J., TARKOWSKI, A., EKMAN, R. & EWING, A. (1994) Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 12912-12916.

BETHIN, K. E., VOGT, S. K. & MUGLIA, L. J. (2000) Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation. *Proceedings of the National Academy of Sciences,* 97**,** 9317-9322.

BIERHAUS, A., HUMPERT, P. M. & NAWROTH, P. P. (2004) NF-kB as a molecular link between psychosocial stress and organ dysfunction. *Pediatric Nephrology,* 19**,** 1189-1191.

BIERHAUS, A., WOLF, J., ANDRASSY, M., ROHLEDER, N., HUMPERT, P. M., PETROV, D., FERSTL, R., VON EYNATTEN, M., WENDT, T., RUDOFSKY, G., JOSWIG, M., MORCOS, M., SCHWANINGER, M., MCEWEN, B., KIRSCHBAUM, C. & NAWROTH, P. P. (2003) A mechanism converting psychosocial stress into mononuclear cell activation. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 1920-1925.

BLACK, P. H. (2002) Stress and the inflammatory response: A review of neurogenic inflammation. *Brain, Behavior, and Immunity,* 16**,** 622-653.

BLANCO, M., CASASÚS, I. & PALACIO, J. (2009) Effect of age at weaning on the physiological stress response and temperament of two beef cattle breeds. *animal,* 3**,** 108-117.

BLEKHMAN, R., MARIONI, J. C., ZUMBO, P., STEPHENS, M. & GILAD, Y. (2010) Sex-specific and lineage-specific alternative splicing in primates. *Genome Research,* 20**,** 180-189.

BOAVENTURA, V. S., SANTOS, C. S., CARDOSO, C. R., DE ANDRADE, J., DOS SANTOS, W. L. C., CLARÊNCIO, J., SILVA, J. S., BORGES, V. M., BARRAL-NETTO, M., BRODSKYN, C. I. & BARRAL, A. (2010) Human mucosal

leishmaniasis: Neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. *European Journal of Immunology,* 40**,** 2830-2836.

BOEHM, U., KLAMP, T., GROOT, M. & HOWARD, J. C. (1997) Cellular Responses to Interferon-γ. *Annual Review of Immunology,* 15**,** 749-795.

BOISSY, A., ARNOULD, C., CHAILLOU, E., DÉSIRÉ, L., DUVAUX-PONTER, C., GREIVELDINGER, L., LETERRIER, C., RICHARD, S., ROUSSEL, S., SAINT-DIZIER, H., MEUNIER-SALAÜN, M. C., VALANCE, D. & VEISSIER, I. (2007) Emotions and cognition: a new approach to animal welfare. *Animal Welfare,* 16**,** 37-43.

BOLAND, H. T., SCAGLIA, G., SWECKER, W. S. & BURKE, N. C. (2008) Effects of Alternate Weaning Methods on Behavior, Blood Metabolites, and Performance of Beef Calves. *The Professional Animal Scientist,* 24**,** 539-551.

BOYSEN, P. & STORSET, A. K. (2009) Bovine natural killer cell. *Veterinary Immunology and Immunopathology,* 130, 163-177.

BRANDT, E., PETERSEN, F., LUDWIG, A., EHLERT, J. E., BOCK, L. & FLAD, H. D. (2000) The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. *Journal of Leukocyte Biology,* 67**,** 471-478.

BRAUN, C. M., HUANG, S.-K., BASHIAN, G. G., KAGEY-SOBOTKA, A., LICHTENSTEIN, L. M. & ESSAYAN, D. M. (1997) Corticosteroid modulation of

human, antigen-specific Th1 and Th2 responses. *Journal of Allergy and Clinical Immunology,* 100**,** 400-407.

BROOM, D. (2011) A History of Animal Welfare Science. *Acta Biotheoretica***,** 1-17.

BROOM, D. M. (1991) Animal welfare: concepts and measurement. *J. Anim Sci.,* 69**,** 4167-4175.

BROOM, D. M. (2006) Behaviour and welfare in relation to pathology. *Applied Animal Behaviour Science,* 97**,** 73-83.

BROTMAN, D. J., GOLDEN, S. H. & WITTSTEIN, I. S. (2007) The cardiovascular toll of stress. *The Lancet,* 370**,** 1089-1100.

BRUN-HANSEN, H. C., KAMPEN, A. H. & LUND, A. (2006) Hematologic values in calves during the first 6 months of life. *Veterinary Clinical Pathology,* 35**,** 182-187.

BUCKHAM SPORER, K. R., BURTON, J. L., EARLEY, B. & CROWE, M. A. (2007) Transportation stress in young bulls alters expression of neutrophil genes important for the regulation of apoptosis, tissue remodeling, margination, and anti-bacterial function. *Veterinary Immunology and Immunopathology,* 118**,** 19-29.

BUCKHAM SPORER, K. R., WEBER, P. S. D., BURTON, J. L., EARLEY, B. & CROWE, M. A. (2008b) Transportation of young beef bulls alters circulating physiological parameters that may be effective biomarkers of stress. *J. Anim Sci.,* 86**,** 1325-1334.

BUCKHAM SPORER, K. R., XIAO, L., TEMPELMAN, R. J., BURTON, J. L., EARLEY, B. & CROWE, M. A. (2008a) Transportation stress alters the circulating steroid environment and neutrophil gene expression in beef bulls. *Veterinary Immunology and Immunopathology,* 121**,** 300-320.

BUDZYNSKA, M. & WEARY, D. M. (2008) Weaning distress in dairy calves: Effects of alternative weaning procedures. *Applied Animal Behaviour Science,* 112**,** 33-39.

BUJAK, J. S. & ROOT, R. K. (1974) The Role of Peroxidase in the Bactericidal Activity of Human Blood Eosinophils. *Blood,* 43**,** 727-736.

BUMILLER, A., GÖTZ, F., ROHDE, W. & DÖRNER, G. (1999) Effects of repeated injections of interleukin 1[beta] or lipopolysaccharide on the HPA axis in the newborn rat. *Cytokine,* 11**,** 225-230.

BURDICK, N. C., AGADO, B. J., RANDEL, R. D., NEUENDORFF, D. A., CARROLL, J. A., VANN, R. C., CHITKO-MCKOWN, C. G., LAWHON, S. D. & WELSH, J., T. H. (2010) Endogenous cortisol: Acute modulation of cytokine gene expression in bovine PBMCs. *Journal of Animal Science Supplement,* 88**,** E-Suppl 3, Abstract 61.

BURKE, N. C., SCAGLIA, G., BOLAND, H. T. & SWECKER JR, W. S. (2009) Influence of two-stage weaning with subsequent transport on body weight, plasma lipid peroxidation, plasma selenium, and on leukocyte glutathione peroxidase and glutathione

reductase activity in beef calves. *Veterinary Immunology and Immunopathology,* 127**,** 365-370.

BURRAGE, P. S., MIX, K. S. & BRINCKERHOFF, C. E. (2006) Matrix metalloproteinases: role in arthritis. *Frontiers in Bioscience,* 11**,** 529-543.

BURTON, J. L., MADSEN, S. A., CHANG, L.-C., WEBER, P. S. D., BUCKHAM, K. R., VAN DORP, R., HICKEY, M.-C. & EARLEY, B. (2005) Gene expression signatures in neutrophils exposed to glucocorticoids: A new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. *Veterinary Immunology and Immunopathology,* 105**,** 197-219.

BUSTIN, S., BEAULIEU, J.-F., HUGGETT, J., JAGGI, R., KIBENGE, F., OLSVIK, P., PENNING, L. & TOEGEL, S. (2010) MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology,* 11**,** 74.

BUSTIN, S. A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology,* 29**,** 23-39.

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem,* 55**,** 611-622.

BUTTGEREIT, F. & SCHEFFOLD, A. (2002) Rapid glucocorticoid effects on immune cells. *Steroids,* 67**,** 529-534.

CARDONA, A. E., LI, M., LIU, L., SAVARIN, C. & RANSOHOFF, R. M. (2008) Chemokines in and out of the central nervous system: much more than chemotaxis and inflammation. *Journal of Leukocyte Biology,* 84**,** 587-594.

CARROLL, J. A., ARTHINGTON, J. D. & CHASE, C. C., JR. (2009) Early weaning alters the acute-phase reaction to an endotoxin challenge in beef calves. *J. Anim Sci.,* 87**,** 4167-4172.

CARROLL, J. A. & FORSBERG, N. E. (2007) Influence of Stress and Nutrition on Cattle Immunity. *Veterinary Clinics of North America: Food Animal Practice,* 23**,** 105-149.

CARSTENS, E. & MOBERG, G. P. (2000) Recognizing pain and distress in laboratory animals. *Institute for laboratory animal research journal,* 41.

CASCIERI, M. A. & SPRINGER, M. S. (2000) The chemokine/chemokine-receptor family: potential and progress for therapeutic intervention. *Current Opinion in Chemical Biology,* 4**,** 420-427.

CECILIANI, F., POCACQUA, V., MIRANDA-RIBERA, A., BRONZO, V., LECCHI, C. & SARTORELLI, P. (2007) Alpha(1)-acid glycoprotein modulates apoptosis in bovine monocytes. *Veterinary Immunology and Immunopathology,* 116, 145-152.

CHANG, L.-C., MADSEN, S. A., TOELBOELL, T., WEBER, P. S. D. & BURTON, J. L. (2004) Effects of glucocorticoids on Fas gene expression in bovine blood neutrophils. *J Endocrinol,* 183**,** 569-583.

CHANG, P., DUNHAM, J., NUZHDIN, S. & ARBEITMAN, M. (2011) Somatic sex-specific transcriptome differences in Drosophila revealed by whole transcriptome sequencing. *BMC Genomics,* 12**,** 364.

CHAPLIN, D. D. (2010) Overview of the immune response. *Journal of Allergy and Clinical Immunology,* 125**,** S3-S23.

CHARMANDARI, E., KINO, T. & CHROUSOS, G. P. (2004) Glucocorticoids and Their Actions: An Introduction. *Annals of the New York Academy of Sciences,* 1024**,** 1-8.

CHEN, L. & ZHENG, S. (2009) Studying alternative splicing regulatory networks through partial correlation analysis. *Genome Biology,* 10.

CHESNOKOVA, V. & MELMED, S. (2002) Minireview: Neuro-Immuno-Endocrine Modulation of the Hypothalamic-Pituitary-Adrenal (HPA) Axis by gp130 Signaling Molecules. *Endocrinology,* 143**,** 1571-1574.

CHIN, A. C., LEE, W. D., MURRIN, K. A., MORCK, D. W., MERRILL, J. K., DICK, P. & BURET, A. G. (2000) Tilmicosin Induces Apoptosis in Bovine Peripheral Neutrophils in the Presence or in the Absence of Pasteurella haemolytica and Promotes

Neutrophil Phagocytosis by Macrophages. *Antimicrob. Agents Chemother.,* 44**,** 2465-2470.

CHROUSOS, G. P. (1998) Stressors, Stress, and Neuroendocrine Integration of the Adaptive Response: The 1997 Hans Selye Memorial Lecture. *Annals of the New York Academy of Sciences,* 851**,** 311-335.

CHROUSOS, G. P. (2009) Stress and disorders of the stress system. *Nat Rev Endocrinol,* 5**,** 374-381.

CHROUSOS, G. P., CHARMANDARI, E. & KINO, T. (2004) Glucocorticoid Action Networks--An Introduction to Systems Biology. *J Clin Endocrinol Metab,* 89**,** 563-564.

CHURCH, J. S. & HUDSON, R. J. (1999) Comparison of the stress of abrupt and interval weaning of farmed wapiti calves (Cervus elaphus). *Small Ruminant Research,* 32**,** 119-124.

CLARKE, B. L., GEBHARDT, B. M. & BLALOCK, J. E. (1993) Mitogen-stimulated lymphocytes release biologically active corticotropin. *Endocrinology,* 132**,** 983-988.

CLOONAN, N. & GRIMMOND, S. M. (2008) Transcriptome content and dynamics at single-nucleotide resolution. *Genome Biology,* 9.

COLE, S. W. (2008) Social regulation of leukocyte homeostasis: The role of glucocorticoid sensitivity. *Brain, Behavior, and Immunity,* 22**,** 1049-1055.

CONNOR, T. J., BREWER, C., KELLY, J. P. & HARKIN, A. (2005) Acute stress suppresses pro-inflammatory cytokines TNF-α and IL-1β independent of a catecholamine-driven increase in IL-10 production. *Journal of neuroimmunology,* 159**,** 119-128.

COQUERET, O. (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends in Cell Biology,* 13**,** 65-70.

COUNCIL, F. A. W. (2009) Farm Animal Welfare in Britain: Past, Present and Future.

COX, G. (1995) Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *The Journal of Immunology,* 154**,** 4719-4725.

CRAIG, A., MAI, J., CAI, S. & JEYASEELAN, S. (2009) Neutrophil Recruitment to the Lungs during Bacterial Pneumonia. *Infect. Immun.,* 77**,** 568-575.

CRONEY, C. C. & NEWBERRY, R. C. (2007) Group size and cognitive processes. *Applied Animal Behaviour Science,* 103**,** 215-228.

CROWLEY, D. J., KANAKARIS, N. K. & GIANNOUDIS, P. V. (2007) Debridement and wound closure of open fractures: The impact of the time factor on infection rates. *Injury,* 38**,** 879-889.

CROXTALL, J., CHOUDHURY, Q. & FLOWER, R. (2000) Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a

receptor-dependent, transcription-independent mechanism. *British Journal of Pharmacology,* 21**,** 57-94.

CUNNINGHAM, J., EMMETT T. , WADA, E., CARTER, D. B., TRACEY, D. E., BATTEY, J. F. & DE SOUZAL, E. B. (1992) In situ Histochemical Localization of Type I Interleukin-1 Receptor Messenger RNA in the Central Nervous System, Pituitary, and Adrenal Gland of the Mouse. *The Journal of Neuroscience,* 12**,** 1101-1114.

CURTIN, N. M., BOYLE, N. T., MILLS, K. H. G. & CONNOR, T. J. (2009) Psychological stress suppresses innate IFN-γ production via glucocorticoid receptor activation: Reversal by the anxiolytic chlordiazepoxide. *Brain, Behavior, and Immunity,* 23**,** 535-547.

CUTRONEO, K. R. (2003) How is Type I procollagen synthesis regulated at the gene level during tissue fibrosis. *Journal of Cellular Biochemistry,* 90**,** 1-5.

CUTRONEO, K. R. & STERLING, K. M. (2004) How do glucocorticoids compare to oligo decoys as inhibitors of collagen synthesis and potential toxicity of these therapeutics? *Journal of Cellular Biochemistry,* 92**,** 6-15.

DAWKINS, M. S. (1990) From an animal's point of view: Motivation, fitness, and animal welfare. *Behavioral and Brain Sciences.*

DE BOSSCHER, K. & HAEGEMAN, G. (2009) Minireview: Latest Perspectives on Antiinflammatory Actions of Glucocorticoids. *Mol Endocrinol,* 23**,** 281-291.

DE BOSSCHER, K., VANDEN BERGHE, W. & HAEGEMAN, G. (2003) The Interplay between the Glucocorticoid Receptor and Nuclear Factor-{kappa}B or Activator Protein-1: Molecular Mechanisms for Gene Repression. *Endocr Rev,* 24**,** 488-522.

DE BOSSCHER, K., VANDEN BERGHE, W., VERMEULEN, L., PLAISANCE, S. P., BOONE, E. & HAEGEMAN, G. (2000) Glucocorticoids repress NF-κB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 3919-3924.

DE KLOET, E. R., VREUGDENHIL, E., OITZL, M. S. & JOELS, M. (1998) Brain Corticosteroid Receptor Balance in Health and Disease. *Endocr Rev,* 19**,** 269-301.

DEEPAK, S., KOTTAPALLI, K., RAKWAL, R., OROS, G., RANGAPPA, K., IWAHASHI, H., MASUO, Y. & AGRAWAL, G. (2007) Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. *Current Genomics,* 8**,** 234-251.

DEINZER, R., GRANRATH, N., STUHL, H., TWORK, L., IDEL, H., WASCHUL, B. & HERFORTH, A. (2004) Acute stress effects on local Il-1[beta] responses to pathogens in a human in vivo model. *Brain, Behavior, and Immunity,* 18**,** 458-467.

DELVAEYE, M. & CONWAY, E. M. (2009) Coagulation and innate immune responses: can we view them separately? *Blood,* 114**,** 2367-2374.

DEROO, B. J. & ARCHER, T. K. (2001) Glucocorticoid receptor-mediated chromatin remodeling in vivo. *Oncogene,* 20**,** 3039-3046.

DESBOROUGH, J. P. (2000) The stress response to trauma and surgery. *British Journal of Anaesthesia,* 85**,** 109-117.

DÉSIRÉ, L., BOISSY, A. & VEISSIER, I. Emotions in farm animals:: a new approach to animal welfare in applied ethology. *Behavioural Processes,* 60**,** 165-180.

DHABHAR, F. S. (2002a) A Hassle a Day May Keep the Doctor Away: Stress and the Augmentation of Immune Function. *Integr. Comp. Biol.,* 42**,** 556-564.

DHABHAR, F. S. (2002b) Stress-induced augmentation of immune function--The role of stress hormones, leukocyte trafficking, and cytokines. *Brain, Behavior, and Immunity,* 16**,** 785-798.

DHABHAR, F. S. (2008) Enhancing versus Suppressive Effects of Stress on Immune Function: Implications for Immunoprotection versus Immunopathology. *Allergy, Asthma & Clinical Immunology,* 4**,** 2-11.

DHABHAR, F. S. (2009) A hassle a day may keep the pathogens away: The fight-or-flight stress response and the augmentation of immune function. *Integr. Comp. Biol.,* 49**,** 215-236.

DHABHAR, F. S. & MCEWEN, B. S. (1997) Acute Stress Enhances while Chronic Stress Suppresses Cell-Mediated Immunityin Vivo:A Potential Role for Leukocyte Trafficking. *Brain, Behavior, and Immunity,* 11**,** 286-306.

DHABHAR, F. S., MILLER, A. H., MCEWEN, B. S. & SPENCER, R. L. (1995) Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. *J Immunol,* 154**,** 5511-5527.

DIACOVO, T. G., CATALINA, M. D., SIEGELMAN, M. H. & ANDRIAN, U. H. V. (1998) Circulating Activated Platelets Reconstitute Lymphocyte Homing and Immunity in L-selectin–Deficient Mice. *The Journal of Experimental Medicine,* 187**,** 197-204.

DIAMANDIS, E. P. & VAN DER MERWE, D.-E. (2005) Plasma Protein Profiling by Mass Spectrometry for Cancer Diagnosis: Opportunities and Limitations. *Clinical Cancer Research,* 11**,** 963-965.

DIXIT, V. D., MARAHRENS, M. & PARVIZI, N. (2001) Transport stress modulates adrenocorticotropin secretion from peripheral bovine lymphocytes. *J. Anim Sci.,* 79**,** 729-734.

DRENNAN, M. J. & MCGEE, M. (2009) Performance of spring-calving beef suckler cows and their progeny to slaughter on intensive and extensive grassland management systems. *Livestock Science,* 120**,** 1-12.

DROSTE, S. K., DE GROOTE, L., ATKINSON, H. C., LIGHTMAN, S. L., REUL, J. M. H. M. & LINTHORST, A. C. E. (2008) Corticosterone Levels in the Brain Show a

Distinct Ultradian Rhythm but a Delayed Response to Forced Swim Stress. *Endocrinology,* 149**,** 3244-3253.

DUGAN, A., GREGERSON, K., GARDNER, J., NEELY, A., BUCKLEY, D., NOEL, G., OGLE, C. & HORSEMAN, N. (2007) Chronic Psychogenic Stress (predator exposure) Improved Survival and Altered the Cytokine/Chemokine Profile in Two Sepsis Models *The Journal of Immunology,* 178**,** 44.1-44.3.

DUNN, A. J. (2000) Cytokine Activation of the HPA Axis. *Annals of the New York Academy of Sciences,* 917**,** 608-617.

DUNN, A. J., ADRIANA DEL REY, G. P. C. & HUGO, O. B. (2007) The HPA Axis and the Immune System: A Perspective. *NeuroImmune Biology*. Elsevier.

EARLEY, B. & CROWE, M. A. (2002) Effects of ketoprofen alone or in combination with local anesthesia during the castration of bull calves on plasma cortisol, immunological, and inflammatory responses. *J. Anim Sci.,* 80**,** 1044-1052.

EARLEY, B., FISHER, A. D. & O'RIORDAN, E. G. (2006a) Effects of pre-transport fasting on physiological, haematological and immunological responses to a 12-h journey by road. *Irish Journal of Agricultural and Food Research,* 45**,** 39-50.

EARLEY, B., FISHER, A. D. & O'RIORDAN, E. G. (2006b) Effects of pre-transport fasting on the physiological responses of young cattle to 8-hour road transport. *Irish Journal of Agricultural and Food Research,* 45**,** 51-60.

EARLEY, B. & MURRAY, M. (2010) The effect of road and sea transport on inflammatory, adrenocortical, metabolic and behavioural responses of weanling heifers. *BMC Veterinary Research,* 6**,** 36.

EARLEY, B. & O'RIORDAN, E. G. (2006) Effects on transporting bulls at different space allowances on physiological, haematological and immunological responses to a 12 h journey by road. *Irish Journal of Agricultural and Food Research,* 45**,** 39-50.

ECKERSALL, P., DUTHIE, S., SAFI, S., MOFFATT, D., HORADAGODA, N., DOYLE, S., PARTON, R., BENNETT, D. & FITZPATRICK, J. (1999) An automated biochemical assay for haptoglobin: Prevention of interference from albumin. *Comparative Haematology International,* 9**,** 117-124.

EICHER, S. D., MCMUNN, K. A., HAMMON, H. M. & DONKIN, S. S. (2004) Toll-like receptors 2 and 4, and acute phase cytokine gene expression in dexamethasone and growth hormone treated dairy calves. *Veterinary Immunology and Immunopathology,* 98**,** 115-125.

ELENKOV, I., PAPANICOLAOU, D., WILDER, R. & CHROUSOS, G. (1996) Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. *Proceedings of the Association of American Physicians,* 108**,** 374-381.

ELENKOV, I. J. (2004) Glucocorticoids and the Th1/Th2 Balance. *Annals of the New York Academy of Sciences,* 1024, 138-146.

ELENKOV, I. J. & CHROUSOS, G. P. (1999) Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease. *Trends in Endocrinology and Metabolism,* 10**,** 359-368.

ELENKOV, I. J. & CHROUSOS, G. P. (2002) Stress Hormones, Proinflammatory and Antiinflammatory Cytokines, and Autoimmunity. *Annals of the New York Academy of Sciences,* 966**,** 290-303.

ELENKOV, I. J., IEZZONI, D. G., DALY, A., HARRIS, A. G. & CHROUSOS, G. P. (2005) Cytokine Dysregulation, Inflammation and Well-Being. *Neuroimmunomodulation,* 12**,** 255-269.

ELSASSER, T. H., KAHL, S., STEELE, N. C. & RUMSEY, T. S. (1997) Nutritional Modulation of Somatotropic Axis-cytokine Relationships in Cattle: A Brief Review. *Comparative Biochemistry and Physiology Part A: Physiology,* 116**,** 209-221.

ELSIK, C. G., TELLAM, R. L., WORLEY, K. C. & CONSORTIUM, T. B. G. S. A. A. (2009) The Genome Sequence of Taurine Cattle: A Window to Ruminant Biology and Evolution. *Science,* 324**,** 522-528.

ENGLER, H., DAWILS, L., HOVES, S., KURTH, S., STEVENSON, J. R., SCHAUENSTEIN, K. & STEFANSKI, V. (2004) Effects of social stress on blood leukocyte distribution: the role of [alpha]- and [beta]-adrenergic mechanisms. *Journal of neuroimmunology,* 156**,** 153-162.

ENRIQUEZ, D., HOTZEL, M. & UNGERFELD, R. (2011) Minimising the stress of weaning of beef calves: a review. *Acta Veterinaria Scandinavica,* 53**,** 28.

ENRÍQUEZ, D. H., UNGERFELD, R., QUINTANS, G., GUIDONI, A. L. & HÖTZEL, M. J. (2010) The effects of alternative weaning methods on behaviour in beef calves. *Livestock Science,* 128**,** 20-27.

ENSMINGER, M. & PERRY, R. (1997) Beef Cattle Science, Seventh Edition, Part B Danville, IL: Interstate Publishers, Inc. p.727.

ERUSLANOV, E. B., LYADOVA, I. V., KONDRATIEVA, T. K., MAJOROV, K. B., SCHEGLOV, I. V., ORLOVA, M. O. & APT, A. S. (2005) Neutrophil Responses to Mycobacterium tuberculosis Infection in Genetically Susceptible and Resistant Mice. *Infect. Immun.,* 73**,** 1744-1753.

EVANKO, D. S., THIYAGARAJAN, M. M., SIDEROVSKI, D. P. & WEDEGAERTNER, P. B. (2001) Gβγ Isoforms Selectively Rescue Plasma Membrane Localization and Palmitoylation of Mutant Gαs and Gαq. *Journal of Biological Chemistry,* 276**,** 23945-23953.

FEDURCO, M., ROMIEU, A., WILLIAMS, S., LAWRENCE, I. & TURCATTI, G. (2006) BTA, a novel reagent for DNA attachment on glass and efficient generation of solid-phase amplified DNA colonies. *Nucleic Acids Research,* 34**,** e22.

FERNANDEZ-BOTRAN, R. (1991) Soluble cytokine receptors: their role in immunoregulation. *The FASEB Journal,* 5**,** 2567-2574.

FISHER, A. D., CROWE, M. A., O'KIELY, P. & ENRIGHT, W. J. (1997a) Growth, behaviour, adrenal and immune responses of finishing beef heifers housed on slatted floors at 1.5, 2.0, 2.5 or 3.0 m2 space allowance. *Livestock Production Science,* 51**,** 245-254.

FISHER, A. D., CROWE, M. A., O'NUALLAIN, E. M., MONAGHAN, M. L., PRENDIVILLE, D. J., O'KIELY, P. & ENRIGHT, W. J. (1997a) Effects of suppressing cortisol following castration of bull calves on adrenocorticotropic hormone, in vitro interferon-gamma production, leukocytes, acute-phase proteins, growth, and feed intake. *J. Anim Sci.,* 75**,** 1899-1908.

FISHER, A. D., CROWE, M. A., PRENDIVILLE, D. J. & ENRIGHT, W. J. (1997b) Indoor space allowance: effects on growth, behaviour, adrenal and immune responses of finishing beef heifers. *Animal Science,* 64**,** 53-62.

FLESHNER, M., BRENNAN, F. X., NGUYEN, K., WATKINS, L. R. & MAIER, S. F. (1996) RU-486 blocks differentially suppressive effect of stress on in vivo anti-KLH immunoglobulin response. *Am J Physiol Regul Integr Comp Physiol,* 271**,** R1344-1352.

FLO, T. H., SMITH, K. D., SATO, S., RODRIGUEZ, D. J., HOLMES, M. A., STRONG, R. K., AKIRA, S. & ADEREM, A. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature,* 432**,** 917-921.

FRANCHIMONT, D., GALON, J., GADINA, M., VISCONTI, R., ZHOU, Y. J., ARINGER, M., FRUCHT, D. M., CHROUSOS, G. P. & O'SHEA, J. J. (2000)

Inhibition of Th1 Immune Response by Glucocorticoids: Dexamethasone Selectively Inhibits IL-12-Induced Stat4 Phosphorylation in T Lymphocytes. *J Immunol,* 164**,** 1768-1774.

FRASER, D. (2008) Understanding animal welfare: the science in its cultural context. *Wiley Blackwell, Chichester*.

FRASER, D. & WEARY, D. (2003) Quality of life for farm animals: linking science, ethics and animal welfare. *The Well-being of Farm Animals: Challenges and Solutions. Edited by Benson GJ, Rollin BE.* , Blackwell Publishing, Oxford**,** 39-60.

FRITH, M. C., PHEASANT, M. & MATTICK, J. S. (2005) Genomics: The amazing complexity of the human transcriptome. *Eur J Hum Genet,* 13**,** 894-897.

FULLER, C. W., MIDDENDORF, L. R., BENNER, S. A., CHURCH, G. M., HARRIS, T., HUANG, X., JOVANOVICH, S. B., NELSON, J. R., SCHLOSS, J. A., SCHWARTZ, D. C. & VEZENOV, D. V. (2009) The challenges of sequencing by synthesis. *Nat Biotech,* 27**,** 1013-1023.

FULTON, R. W. (2009) Bovine respiratory disease research (1983-2009). *Animal Health Research Reviews,* 10**,** 131-139.

GAJEWSKI, T. F. & FITCH, F. W. (1988) Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN- gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J Immunol,* 140**,** 4245-4252.

GALLIGAN, C. L. & COOMBER, B. L. (2000) Effects of human IL-8 isoforms on bovine neutrophil function in vitro. *Veterinary Immunology and Immunopathology,* 74**,** 71-85.

GALYEAN, M. L., PERINO, L. J. & DUFF, G. C. (1999) Interaction of cattle health/immunity and nutrition. *Journal of Animal Science,* 77**,** 1120-1134.

GAN, L. & LI, L. (2006) Regulations and roles of the interleukin-1 receptor associated kinases (IRAKs) in innate and adaptive immunity. *Immunologic Research,* 35**,** 295-302.

GARDNER, D. G. (2001) Mechanisms of hormone action. *Basic and Clinical Endocrinology***,** 59-79.

GAYRARD, V., ALVINERIE, M. & TOUTAIN, P. L. (1996) Interspecies variations of corticosteroid-binding globulin parameters. *Domestic Animal Endocrinology,* 13**,** 35-45.

GERSZTEN, R. E., ACCURSO, F., BERNARD, G. R., CAPRIOLI, R. M., KLEE, E. W., KLEE, G. G., KULLO, I., LAGUNA, T. A., ROTH, F. P., SABATINE, M., SRINIVAS, P., WANG, T. J., WARE, L. B. & FOR THE, N. C. P. P. (2008) Challenges in translating plasma proteomics from bench to bedside: update from the NHLBI Clinical Proteomics Programs. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 295**,** L16-L22.

GLASER, R. & KIECOLT-GLASER, J. K. (2005) Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol,* 5**,** 243-251.

GLASS, E. J., BAXTER, R., LEACH, R. J. & JANN, O. C. (2011) Genes controlling vaccine responses and disease resistance to respiratory viral pathogens in cattle. *Veterinary Immunology and Immunopathology,* In Press, Corrected Proof.

GLEISSNER, C. A., VON HUNDELSHAUSEN, P. & LEY, K. (2008) Platelet Chemokines in Vascular Disease. *Arterioscler Thromb Vasc Biol,* 28**,** 1920-1927.

GOLDFIEN, A. (2001) Adrenal Medulla. *Basic and Clinical Endocrinology***,** 399 - 421.

GONYOU, H. & STOOKEY, J. (1987) Maternal and neonatal behavior. *The Veterinary clinics of North America. Food animal practice,* 3**,** 231-249.

GOUJON, E., PARNET, P., LAYE, S., COMBE, C., KELLEY, K. W. & DANTZER, R. (1995) Stress Downregulates Lipopolysaccharide-Induced Expression of Proinflammatory Cytokines in the Spleen, Pituitary, and Brain of Mice. *Brain, Behavior, and Immunity,* 9**,** 292-303.

GRAVELEY, B. (2010) The developmental transcriptome of Drosophila melanogaster. *Genome Biology,* 11**,** I11.

GRIFFIN, D., CHENGAPPA, M. M., KUSZAK, J. & MCVEY, D. S. (2010) Bacterial Pathogens of the Bovine Respiratory Disease Complex. *Veterinary Clinics of North America: Food Animal Practice,* 26**,** 381-394.

GRIFFIN, J. F. T. (1989) Stress and immunity: A unifying concept. *Veterinary Immunology and Immunopathology,* 20**,** 263-312.

GRIMBLE, R. F. (1994) 2. Impact of nutrients on cytokine biology in infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 88**,** 615-619.

GUNN, G. J. & STOTT, A. W. (1998) Economic losses associated with calf pneumonia. *Cattle Practice,* 6**,** 259.

GUPTA, B. B. P. & LALCHHANDAMA, K. (2002) Molecular mechanisms of glucocorticoid action. *Current Science Association,* 83**,** 1103-1111.

GUPTA, S., EARLEY, B. & CROWE, M. A. (2007a) Pituitary, adrenal, immune and performance responses of mature Holstein × Friesian bulls housed on slatted floors at various space allowances. *The Veterinary Journal,* 173**,** 594-604.

GUPTA, S., EARLEY, B. & CROWE, M. A. (2007b) Effect of 12-hour road transportation on physiological, immunological and haematological parameters in bulls housed at different space allowances. *The Veterinary Journal,* 173**,** 605-616.

GUYON, A., MASSA, F., ROVÈRE, C. & NAHON, J.-L. (2008) How cytokines can influence the brain: A role for chemokines? *Journal of neuroimmunology,* 198**,** 46-55.

HACHIYA, O., TAKEDA, Y., MIYATA, H., WATANABE, H., YAMASHITA, T. & SENDO, F. (1995) Inhibition by Bacterial Lipopolysaccharide of Spontaneous and

TNF-α-Induced Human Neutrophil Apoptosis In Vitro. *Microbiology and Immunology,* 39**,** 715-723.

HADDAD, J., J., NAYEF, E. S. & BARED, S.-G. (2002d) Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *Journal of neuroimmunology,* 133**,** 1-19.

HADDAD, J. J. (2002c) Cytokines and related receptor-mediated signaling pathways. *Biochemical and Biophysical Research Communications,* 297**,** 700-713.

HADDAD, J. J. & LAND, S. (2002a) Redox/ROS regulation of lipopolysaccharide-induced mitogen-activated protein kinase (MAPK) activation and MAPK-mediated TNF-α biosynthesis. *British Journal of Pharmacology,* 135**,** 520-536.

HADDAD, J. J. & LAND, S. C. (2002b) Amiloride Blockades Lipopolysaccharide-Induced Proinflammatory Cytokine Biosynthesis in an Ikappa B-alpha /NF-kappa B-Dependent Mechanism . Evidence for the Amplification of an Antiinflammatory Pathway in the Alveolar Epithelium. *Am. J. Respir. Cell Mol. Biol.,* 26**,** 114-126.

HALEY, D. B., BAILEY, D. W. & STOOKEY, J. M. (2005) The effects of weaning beef calves in two stages on their behavior and growth rate. *Journal of Animal Science,* 83**,** 2205-2214.

HARHAY, G., SMITH, T., ALEXANDER, L., HAUDENSCHILD, C., KEELE, J., MATUKUMALLI, L., SCHROEDER, S., VAN TASSELL, C., GRESHAM, C., BRIDGES, S., BURGESS, S. & SONSTEGARD, T. (2010) An atlas of bovine gene

expression reveals novel distinctive tissue characteristics and evidence for improving genome annotation. *Genome Biology,* 11**,** R102.

HASLETT, C. (1999) Granulocyte Apoptosis and Its Role in the Resolution and Control of Lung Inflammation. *Am. J. Respir. Crit. Care Med.,* 160**,** S5-11.

HE, L., VASILIOU, K. & NEBERT, D. W. (2009) Analysis and update of the human solute carrier (SLC) gene superfamily. *Human Genomics,* 3**,** 195-206.

HEANEY, M. L. & GOLDE, D. W. (1996) Soluble cytokine receptors. *Blood,* 87**,** 847-857.

HENN, V., SLUPSKY, J. R., GRAFE, M., ANAGNOSTOPOULOS, I., FORSTER, R., MULLER-BERGHAUS, G. & KROCZEK, R. A. (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature,* 391**,** 591-594.

HERMAN, J. P., OSTRANDER, M. M., MUELLER, N. K. & FIGUEIREDO, H. (2005) Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 29**,** 1201-1213.

HERZOG, K. (2007) The effects of weaning stress on the serum protein profile of calves: A proteomic analysis. *Ph.D Thesis,* University of Saskatchewan, Saskatchewan, Canada.

HICKEY, M. C., DRENNAN, M. & EARLEY, B. (2003b) The effect of abrupt weaning of suckler calves on the plasma concentrations of cortisol, catecholamines, leukocytes, acute-phase proteins and in vitro interferon-gamma production. *J. Anim Sci.,* 81**,** 2847-2855.

HICKEY, M. C., EARLEY, B. & FISHER, A. D. (2003a) The Effect of Floor Type and Space Allowance on Welfare Indicators of Finishing Steers. *Irish Journal of Agricultural and Food Research,* 42**,** 89-100.

HIGUCHI, R., DOLLINGER, G., WALSH, P. S. & GRIFFITH, R. (1992) Simultaneous Amplification and Detection of Specific DNA Sequences. *Nat Biotech,* 10**,** 413-417.

HINGORANI, R., BI, B., DAO, T., BAE, Y., MATSUZAWA, A. & CRISPE, I. N. (2000) CD95/Fas Signaling in T Lymphocytes Induces the Cell Cycle Control Protein p21cip-1/WAF-1, Which Promotes Apoptosis. *J Immunol,* 164**,** 4032-4036.

HIRAO, Y., KANDA, T., ASO, Y., MITSUHASHI, M. & KOBAYASHI, I. (2000) Interleukin-8—An Early Marker for Bacterial Infection. *Lab Medicine,* 31**,** 39-44.

HO SUI, S. J., MORTIMER, J. R., ARENILLAS, D. J., BRUMM, J., WALSH, C. J., KENNEDY, B. P. & WASSERMAN, W. W. (2005) oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. *Nucleic acids research,* 33**,** 3154-3164.

HOEBEN, D., BURVENICH, C. & MASSART-LEËN, A.-M. (1998) Glucocorticosteroids and in vitro effects on chemiluminescence of isolated bovine blood granulocytes. *European Journal of Pharmacology,* 354**,** 197-203.

HOFFMAN-GOETZ, L. & PEDERSEN, B. K. (1994) Exercise and the immune system: a model of the stress response? *Immunology Today,* 15**,** 382-387.

HOLLOWAY, A. F., RAO, S. & SHANNON, M. F. (2002) Regulation of cytokine gene transcription in the immune system. *Molecular Immunology,* 38**,** 567-580.

HU, X., LI, W.-P., MENG, C. & IVASHKIV, L. B. (2003) Inhibition of IFN-{gamma} Signaling by Glucocorticoids. *J Immunol,* 170**,** 4833-4839.

HUANG, W. & KHATIB, H. (2010) Comparison of transcriptomic landscapes of bovine embryos using RNA-Seq. *BMC Genomics,* 11**,** 711.

HUBBARD, C. & SCOTT, K. (2011) Do farmers and scientists differ in their understanding and assessment of farm animal welfare? *Animal Welfare,* 20**,** 79-87.

IKEGAMI, H., SONO, Y., NAGAI, K., YOSHIHIRO, T., INOUE, E., KOBAYASHI, N., MATSUHASHI, T., OHTANI, T., NAKAGAWA, M., MORIMOTO, K. & MATSUMOTO, K. (2008) Discovery of A Protein Biomarker Candidate Related to Carcass Weight In Japanese Black Beef Cattle (Wagyu). *Journal of Proteomics and Bioinformatics,* S2.

IRWIN, M., PATTERSON, T., SMITH, T., L. , CALDWELL, C., BROWN, S., A., GILLIN, J. C. & GRANT, I. (1990) Reduction of immune function in life stress and depression. *Biological psychiatry,* 27**,** 22-30.

IVANOV, I. & GRITSENKO, V. (2009) Comparative Activities of Cattle and Swine Platelet Microbicidal Proteins. *Probiotics and Antimicrobial Proteins,* 1**,** 148-151.

JANEWAY. C. A., TRAVERS, M., WALPORT, M. & SCLOMCHICK, M. J. (2005) Immunobiology, the immune system in health and disease. *6th edition Garland Science Publication, New York.*

JENSEN, J. L. (2002) Behavioural disturbances, stress and welfare. *In: Jensen P (ed) The ethology of domestic animals.,* CABI, Wallingford**,** 79-98.

JOHNSON, J. D., O'CONNOR, K. A., DEAK, T., STARK, M., WATKINS, L. R. & MAIER, S. F. (2002) Prior Stressor Exposure Sensitizes LPS-Induced Cytokine Production. *Brain, Behavior, and Immunity,* 16**,** 461-476.

JONES, M. L. & ALLISON, R. W. (2007) Evaluation of the Ruminant Complete Blood Cell Count. *Veterinary Clinics of North America: Food Animal Practice,* 23**,** 377-402.

JU, J., KIM, D. H., BI, L., MENG, Q., BAI, X., LI, Z., LI, X., MARMA, M. S., SHI, S., WU, J., EDWARDS, J. R., ROMU, A. & TURRO, N. J. (2006) Four-color DNA sequencing by synthesis using cleavable fluorescent nucleotide reversible terminators. *Proceedings of the National Academy of Sciences,* 103**,** 19635-19640.

KAHAN, V., ANDERSEN, M. L., TOMIMORI, J. & TUFIK, S. (2009) Stress, immunity and skin collagen integrity: Evidence from animal models and clinical conditions. *Brain, Behavior, and Immunity,* 23**,** 1089-1095.

KANSAS, G. S. (1996) Selectins and their ligands: current concepts and controversies. *Blood,* 88**,** 3259-3287.

KEHRLI, M. E., BURTON, J. L., NONNECKE, B. J., LEE, E. K. & ROLAND, D. S. (1999) Effects of stress on leukocyte trafficking and immune responses: Implications for vaccination. *Advances in Veterinary Medicine.* Academic Press.

KELLER, M., SPANOU, Z., SCHAERLI, P., BRITSCHGI, M., YAWALKAR, N., SEITZ, M., VILLIGER, P. M. & PICHLER, W. J. (2005) T Cell-Regulated Neutrophilic Inflammation in Autoinflammatory Diseases. *The Journal of Immunology,* 175**,** 7678-7686.

KELLEY, K. W. (1980) Stress and immune function: a bibliographic review. *Annales de recherches veterinaires. Annals of veterinary research,* 11**,** 445-78.

KIM, M.-H., YANG, J.-Y., UPADHAYA, S. D., LEE, H.-J., YUN, C.-H. & HA, J. K. (2011) The stress of weaning influences serum levels of acute-phase proteins, iron-binding proteins, inflammatory cytokines, cortisol, and leukocyte subsets in Holstein calves. *Journal of Veterinary Science,* 12**,** 151-157.

KLOSTERMEIER, U., BARANN, M., WITTIG, M., HASLER, R., FRANKE, A., GAVRILOVA, O., KRECK, B., SINA, C., SCHILHABEL, M., SCHREIBER, S. &

ROSENSTIEL, P. (2011) A tissue-specific landscape of sense/antisense transcription in the mouse intestine. *BMC Genomics,* 12**,** 305.

KNIGHTS, M. & SMITH, G. W. (2007) Decreased ACTH secretion during prolonged transportation stress is associated with reduced pituitary responsiveness to tropic hormone stimulation in cattle. *Domestic Animal Endocrinology,* 33**,** 442-450.

KONO, K., TAKAHASHI, A., IIZUKA, H., FUJII, H., SEKIKAWA, T. & MATSUMOTO, Y. (2001) Effect of oesophagectomy on monocyte-induced apoptosis of peripheral blood T lymphocytes. *British Journal of Surgery,* 88**,** 1110-1116.

KOOMEN, J. M., LI, D., XIAO, L.-C., LIU, T. C., COOMBES, K. R., ABBRUZZESE, J. & KOBAYASHI, R. (2005) Direct Tandem Mass Spectrometry Reveals Limitations in Protein Profiling Experiments for Plasma Biomarker Discovery. *Journal of Proteome Research,* 4**,** 972-981.

KORT, W. J. (1994) The effect of chronic stress on the immune system. *Advanced Neuroimmunology,* 4**,** 1-11.

KROEZE, W. K., SHEFFLER, D. J. & ROTH, B. L. (2003) G-protein-coupled receptors at a glance. *Journal of Cell Science,* 116**,** 4867-4869.

KUBISTA, M., ANDRADE, J. M., BENGTSSON, M., FOROOTAN, A., JONÁK, J., LIND, K., SINDELKA, R., SJÖBACK, R., SJÖGREEN, B., STRÖMBOM, L., STÅHLBERG, A. & ZORIC, N. The real-time polymerase chain reaction. *Molecular Aspects of Medicine,* 27**,** 95-125.

KUMPERS, P., LUKASZ, A., DAVID, S., HORN, R., HAFER, C., FAULHABER-WALTER, R., FLISER, D., HALLER, H. & KIELSTEIN, J. (2008) Excess circulating angiopoietin-2 is a strong predictor of mortality in critically ill medical patients. *Critical Care,* 12**,** R147.

KURASH, J. K., SHEN, C.-N. & TOSH, D. (2004) Induction and regulation of acute phase proteins in transdifferentiated hepatocytes. *Experimental Cell Research,* 292**,** 342-358.

LA BAER, J. & YAMAMOTO, K. R. (1994) Analysis of the DNA-binding affinity, Sequence Specificity and Context Dependence of the Glucocorticoid Receptor Zinc Finger Region. *Journal of Molecular Biology,* 239**,** 664-688.

LABAER, J. (2005) So, You Want to Look for Biomarkers (Introduction to the Special Biomarkers Issue)†. *Journal of Proteome Research,* 4**,** 1053-1059.

LABRANCHE, T. P., EHRICH, M. F. & EYRE, P. (2010) Characterization of bovine neutrophil β2-adrenergic receptor function. *Journal of Veterinary Pharmacology and Therapeutics,* 33**,** 323-331.

LANGMEAD, B., TRAPNELL, C., POP, M. & SALZBERG, S. (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology,* 10**,** R25.

LAWRENCE, A. B. (2008) Applied animal behaviour science: Past, present and future prospects. 115**,** 1-24.

LAY JR, D. C., FRIEND, T. H., RANDEL, R. D., BOWERS, C. L., GRISSOM, K. K., NEUENDORFF, D. A. & JENKINS, O. C. (1998) Effects of restricted nursing on physiological and behavioral reactions of Brahman calves to subsequent restraint and weaning. *Applied Animal Behaviour Science,* 56**,** 109-119.

LE NEINDRE, P. (1989) Influence of cattle rearing conditions and breed on social relationships of mother and young. *Applied Animal Behaviour Science,* 23**,** 117-127.

LEDBETTER, T. K., PAAPE, M. J. & DOUGLASS, L. W. (2001) Cytotoxic effects of peroxynitrite, polymorphonuclear neutrophils, free-radical scavengers, inhibitors of myeloperoxidase, and inhibitors of nitric oxide synthase on bovine mammary secretory epithelial cells. *American Journal of Veterinary Research,* 62**,** 286-293.

LEE, P. C. (1996) The meanings of weaning: Growth, lactation, and life history. *Evolutionary Anthropology: Issues, News, and Reviews,* 5**,** 87-98.

LEE, P. C., MAJLUF, P. & GORDON, I. J. (1991) Growth, weaning and maternal investment from a comparative perspective. *Journal of Zoology,* 225**,** 99-114.

LEFCOURT, A. M., BITMAN, J., KAHL, S. & WOOD, D. L. (1993) Circadian and Ultradian Rhythms of Peripheral Cortisol Concentrations in Lactating Dairy Cows. *Journal of Dairy Science,* 76**,** 2607-2612.

LEFCOURT, A. M. & ELSASSER, T. H. (1995) Adrenal responses of Angus x Hereford cattle to the stress of weaning. *Journal of Animal Science,* 73**,** 2669-2676.

LEY, K., LAUDANNA, C., CYBULSKY, M. I. & NOURSHARGH, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol,* 7**,** 678-689.

LINDEMANN, S., TOLLEY, N. D., DIXON, D. A., MCINTYRE, T. M., PRESCOTT, S. M., ZIMMERMAN, G. A. & WEYRICH, A. S. (2001) Activated platelets mediate inflammatory signaling by regulated interleukin 1β synthesis. *The Journal of Cell Biology,* 154**,** 485-490.

LIPPOLIS, J. D. & REINHARDT, T. A. (2005) Proteomic survey of bovine neutrophils. *Veterinary Immunology and Immunopathology,* 103**,** 53-65.

LIU, Y., COUSIN, J. M., HUGHES, J., VAN DAMME, J., SECKL, J. R., HASLETT, C., DRANSFIELD, I., SAVILL, J. & ROSSI, A. G. (1999) Glucocorticoids Promote Nonphlogistic Phagocytosis of Apoptotic Leukocytes. *The Journal of Immunology,* 162**,** 3639-3646.

LIVAK, K. J. & SCHMITTGEN, T. D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. *Methods,* 25**,** 402-408.

LOBERG, J. M., HERNANDEZ, C. E., THIERFELDER, T., JENSEN, M. B., BERG, C. & LIDFORS, L. (2008) Weaning and separation in two steps—A way to decrease

stress in dairy calves suckled by foster cows. *Applied Animal Behaviour Science,* 111**,** 222-234.

LOMBORG, S., NIELSEN, L., HEEGAARD, P. & JACOBSEN, S. (2008) Acute phase proteins in cattle after exposure to complex stress. *Veterinary Research Communications,* 32**,** 575-582.

LUISI, B. F., XU, W. X., OTWINOWSKI, Z., FREEDMAN, L. P., YAMAMOTO, K. R. & SIGLER, P. B. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature,* 352**,** 497-505.

LUPOLI, B., JOHANSSON, B., UVN, AUML, S-MOBERG, K. & SVENNERSTEN-SJAUNJA, K. (2001) Effect of suckling on the release of oxytocin, prolactin, cortisol, gastrin, cholecystokinin, somatostatin and insulin in dairy cows and their calves. *Journal of Dairy Research,* 68**,** 175-187.

LYNCH, E., EARLEY, B., MCGEE, M. & DOYLE, S. (2010c) Characterisation of physiological and immunological responses in beef cows to abrupt weaning and subsequent housing. *BMC Veterinary Research,* 6**,** 37.

LYNCH, E. M., EARLEY, B., MCGEE, M. & DOYLE, S. (2010a) Effect of abrupt weaning at housing on leukocyte distribution, functional activity of neutrophils, and acute phase protein response of beef calves. *BMC Veterinary Research,* 6**,** 39.

LYNCH, E. M., EARLEY, B., MCGEE, M. & DOYLE, S. (2010b) Effect of post-weaning management practices on physiological and immunological responses of weaned beef calves. *Irish Journal of Agricultural and Food Research (In review)*.

LYNCH, E. M., EARLEY, B., MCGEE, M. & DOYLE, S. (2011) 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. *BMC Veterinary Research, In Review.*

LYNN, D., WINSOR, G., CHAN, C., RICHARD, N., LAIRD, M., BARSKY, A., GARDY, J., ROCHE, F., CHAN, T., SHAH, N., LO, R., NASEER, M., QUE, J., YAU, M., ACAB, M., TULPAN, D., WHITESIDE, M., CHIKATAMARLA, A., MAH, B., MUNZNER, T., HOKAMP, K., HANCOCK, R. & BRINKMAN, F. (2008) InnateDB: facilitating systems-level analyses of the mammalian innate immune response. *Mol Syst Biol,* 4.

MACKENZIE, A. M., DRENNAN, M., ROWAN, T. G., DIXON, J. B. & CARTER, S. D. (1997) Effect of transportation and weaning on humoral immune responses of calves. *Research in Veterinary Science,* 63**,** 227-230.

MACPHEE, I. A. M., TURNER, D. R. & OLIVEIRA, D. B. G. (2000) The role of endogenous steroid hormones in the generation of T helper 2‐mediated autoimmunity in mercuric chloride‐treated Brown–Norway rats. *Immunology,* 99**,** 141-146.

MADDEN, K. S. (2003) Catecholamines, sympathetic innervation, and immunity. *Brain, Behavior, and Immunity,* 17 Suppl 1**,** S5-10.

MADSEN-BOUTERSE, S. A., ROSA, G. J. M. & BURTON, J. L. (2006) Glucocorticoid Modulation of Bcl-2 Family Members A1 and Bak during Delayed Spontaneous Apoptosis of Bovine Blood Neutrophils. *Endocrinology,* 147**,** 3826-3834.

MAIER, S. F. & WATKINS, L. R. (1998) Cytokines for psychologists: Implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychological Review,* 105**,** 83 - 107.

MAMANOVA, L., ANDREWS, R. M., JAMES, K. D., SHERIDAN, E. M., ELLIS, P. D., LANGFORD, C. F., OST, T. W. B., COLLINS, J. E. & TURNER, D. J. (2010) FRT-seq: amplification-free, strand-specific transcriptome sequencing. *Nat Meth,* 7**,** 130-132.

MANTOVANI, A., BONECCHI, R. & LOCATI, M. (2006) Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol,* 6**,** 907-918.

MARIONI, J. C., MASON, C. E., MANE, S. M., STEPHENS, M. & GILAD, Y. (2008) RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. *Genome Research,* 18**,** 1509-1517.

MARTI, S., VELARDE, A., DE LA TORRE, J. L., BACH, A., ARIS, A., SERRANO, A., MANTECA, X. & DEVANT, M. (2010) Effects of ring castration with local

anesthesia and analgesia in Holstein calves at 3 months of age on welfare indicators. *J. Anim Sci.,* 88**,** 2789-2796.

MASTORAKOS, G., WEBER, J. S., MAGIAKOU, M. A., GUNN, H. & CHROUSOS, G. P. (1994) Hypothalamic-pituitary-adrenal axis activation and stimulation of systemic vasopressin secretion by recombinant interleukin-6 in humans: potential implications for the syndrome of inappropriate vasopressin secretion. *J Clin Endocrinol Metab,* 79**,** 934-939.

MCCUDDEN, C., HAINS, M., KIMPLE, R., SIDEROVSKI, D. & WILLARD, F. (2005) G-protein signaling: back to the future. *Cellular and Molecular Life Sciences,* 62**,** 551-577.

MCEWEN, B. S. (2008) Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *European Journal of Pharmacology,* 583**,** 174-185.

MCGEE, M., DRENNAN, M. & CAFFREY, P. (2005) Effect of suckler cow genotype on milk yield and pre-weaning calf performance. *Irish Journal of Agricultural and Food Research.*

MCLOUGHLIN, R. M., HURST, S. M., NOWELL, M. A., HARRIS, D. A., HORIUCHI, S., MORGAN, L. W., WILKINSON, T. S., YAMAMOTO, N., TOPLEY, N. & JONES, S. A. (2004) Differential Regulation of Neutrophil-Activating Chemokines by IL-6 and Its Soluble Receptor Isoforms. *The Journal of Immunology,* 172**,** 5676-5683.

MCMAHON, M., GERICH, J. & RIZZA, R. (1988) Effects of glucocorticoids on carbohydrate metabolism. *Diabetes/Metabolism Reviews,* 4**,** 17-30.

MEDZHITOV, R. & JANEWAY, C. A. (1997) Innate Immunity: The Virtues of a Nonclonal System of Recognition. *Cell,* 91**,** 295-298.

MELTZER, J. C., MACNEIL, B. J., SANDERS, V., PYLYPAS, S., JANSEN, A. H., GREENBERG, A. H. & NANCE, D. M. (2004) Stress-induced suppression of in vivo splenic cytokine production in the rat by neural and hormonal mechanisms. *Brain, Behavior, and Immunity,* 18**,** 262-273.

MENCH, J. A. (2008) Farm animal welfare in the U.S.A.: Farming practices, research, education, regulation, and assurance programs. *Applied Animal Behaviour Science,* 113**,** 298-312.

MENGE, C. & DEAN-NYSTROM, E. A. (2008) Dexamethasone Depletes [gamma][delta]T Cells and Alters the Activation State and Responsiveness of Bovine Peripheral Blood Lymphocyte Subpopulations. *Journal of Dairy Science,* 91**,** 2284-2298.

METZKER, M. L. (2005) Emerging technologies in DNA sequencing. *Genome Research,* 15**,** 1767-1776.

METZKER, M. L. (2010) Sequencing technologies - the next generation. *Nat Rev Genet,* 11**,** 31-46.

MILLER, J. N. & MILLER, J. C. (2000) Statistics and Chemometrics for Analytical Chemistry, fourth ed. *Pearson, Harlow.*

MIRANDA-RIBERA, A., LECCHI, C., BRONZO, V., SCACCABAROZZI, L., SARTORELLI, P., FRANCIOSI, F. & CECILIANI, F. (2010) Down-regulatory effect of alpha$_1$-acid glycoprotein on bovine neutrophil degranulation. *Comparative Immunology, Microbiology and Infectious Diseases,* 33, 291-306.

MITCHELL, G. B., CLARK, M. E., SIWICKY, M. & CASWELL, J. L. (2008) Stress alters the cellular and proteomic compartments of bovine bronchoalveolar lavage fluid. *Veterinary Immunology and Immunopathology,* 125**,** 111-125.

MOBERG, G. P. & MENCH, J. A. (2000) The biology of animal stress: Basic principles and applications for animal welfare. *Ed. CABI Publishing, Wallingford, Oxon, UK.*

MORINOBU, A. & KUMAGAI, S. (1998) Cytokine measurement at a single-cell level to analyze human Th1 and Th2 cells. *Rinsho Byori,* 46**,** 908-914.

MORMÈDE, P., ANDANSON, S., AUPÉRIN, B., BEERDA, B., GUÉMENÉ, D., MALMKVIST, J., MANTECA, X., MANTEUFFEL, G., PRUNET, P., VAN REENEN, C. G., RICHARD, S. & VEISSIER, I. (2007) Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Physiology & Behavior,* 92**,** 317-339.

MORTAZAVI, A., WILLIAMS, B. A., MCCUE, K., SCHAEFFER, L. & WOLD, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Meth,* 5**,** 621-628.

MOYNIHAN, J. A., KARP, J. D., COHEN, N. & ADER, R. (2000) *Immune deviation following stress odor exposure: role of endogenous opioids.*

MULHOLLAND, P. J., SELF, R. L., HENSLEY, A. K., LITTLE, H. J., LITTLETON, J. M. & PRENDERGAST, M. A. (2006) A 24 h corticosterone exposure exacerbates excitotoxic insult in rat hippocampal slice cultures independently of glucocorticoid receptor activation or protein synthesis. *Brain Research,* 1082**,** 165-172.

MUNCK, A. & NÁRAY-FEJES-TÓTH, A. (1992) The ups and downs of glucocorticoid physiology Permissive and suppressive effects revisited. *Molecular and Cellular Endocrinology,* 90**,** C1-C4.

MURATA, H. (2007) Stress and acute phase protein response: An inconspicuous but essential linkage. *The Veterinary Journal,* 173**,** 473-474.

MURATA, H., SHIMADA, N. & YOSHIOKA, M. (2004) Current research on acute phase proteins in veterinary diagnosis: an overview. *The Veterinary Journal,* 168**,** 28-40.

NAGATA, S. & GOLSTEIN, P. (1995) The Fas death factor. *Science,* 267**,** 1449-1456.

NATHAN, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol,* 6**,** 173-182.

NELSON, E. E. & PANKSEPP, J. (1998) Brain Substrates of Infant-Mother Attachment: Contributions of Opioids, Oxytocin, and Norepinephrine. *Neuroscience & Biobehavioral Reviews,* 22**,** 437-452.

NEUBIG, R. R. & SIDEROVSKI, D. P. (2002) Regulators of G-Protein signalling as new central nervous system drug targets. *Nat Rev Drug Discov,* 1**,** 187-197.

NEUFELD-COHEN, A., TSOORY, M. M., EVANS, A. K., GETSELTER, D., GIL, S., LOWRY, C. A., VALE, W. W. & CHEN, A. (2010) A triple urocortin knockout mouse model reveals an essential role for urocortins in stress recovery. *Proceedings of the National Academy of Sciences.*

NEUVIANS, T. P., SCHAMS, D., BERISHA, B. & PFAFFL, M. W. (2004) Involvement of Pro-Inflammatory Cytokines, Mediators of Inflammation, and Basic Fibroblast Growth Factor in Prostaglandin F2{alpha}-Induced Luteolysis in Bovine Corpus Luteum. *Biol Reprod,* 70**,** 473-480.

NEWBERRY, R. C. & SWANSON, J. C. (2008) Implications of breaking mother-young social bonds. *Applied Animal Behaviour Science,* 110**,** 3-23.

NG, G., GEORGE, S. R., ZASTAWNY, R. L., CARON, M., BOUVIER, M., DENNIS, M. & O'DOWD, B. F. (1993) Human serotonin1B receptor expression in Sf9 cells:

Phosphorylation, palmitoylation, and adenylyl cyclase inhibition. *Biochemistry,* 32**,** 11727-11733.

NIKUNEN, S., HÄRTEL, H., ORRO, T., NEUVONEN, E., TANSKANEN, R., KIVELÄ, S. L., SANKARI, S., AHO, P., PYÖRÄLÄ, S., SALONIEMI, H. & SOVERI, T. (2007) Association of bovine respiratory disease with clinical status and acute phase proteins in calves. *Comparative Immunology, Microbiology and Infectious Diseases,* 30**,** 143-151.

(NRC), N. R. C. (1992) Recognition and Alleviation of Pain and Distress in Laboratory Animals. Washington, DC: National Academy Press.

NUYTTENS, B. P., THIJS, T., DECKMYN, H. & BROOS, K. (2011) Platelet adhesion to collagen. *Thrombosis Research,* 127, Supplement 2**,** S26-S29.

O'CONNOR, K. A., JOHNSON, J. D., HANSEN, M. K., WIESELER, F. J. L., MAKSIMOVA, E., WATKINS, L. R. & MAIER, S. F. (2003) Peripheral and central proinflammatory cytokine response to a severe acute stressor. *Brain Research,* 991**,** 123-132.

O'LOUGHLIN, A., MCGEE, M., WATERS, S., DOYLE, S. & EARLEY, B. (2011) Examination of the bovine leukocyte environment using immunogenetic biomarkers to assess immunocompetence following exposure to weaning stress. *BMC Veterinary Research,* 7**,** 45.

ODORE, R., BADINO, P., RE, G., BARBERO, R., CUNIBERTI, B., D'ANGELO, A., GIRARDI, C., FRACCARO, E. & TARANTOLA, M. (2011) Effects of housing and short-term transportation on hormone and lymphocyte receptor concentrations in beef cattle. *Research in Veterinary Science,* 90**,** 341-345.

ODORE, R., D'ANGELO, A., BADINO, P., BELLINO, C., PAGLIASSO, S. & RE, G. (2004) Road transportation affects blood hormone levels and lymphocyte glucocorticoid and [beta]-adrenergic receptor concentrations in calves. *The Veterinary Journal,* 168**,** 297-303.

OIKARINEN, HAAPASAARI, SUTINEN & TASANEN (1998) The molecular basis of glucocorticoid-induced skin atrophy: topical glucocorticoid apparently decreases both collagen synthesis and the corresponding collagen mRNA level in human skin in vivo. *British Journal of Dermatology,* 139**,** 1106-1110.

OO, Y. H. & ADAMS, D. H. (2010) The role of chemokines in the recruitment of lymphocytes to the liver. *Journal of Autoimmunity,* 34**,** 45-54.

OPPENHEIM, J. (2001) Cytokines: Past, Present, and Future. *International Journal of Hematology,* 74**,** 3-8.

OSHLACK, A., ROBINSON, M. & YOUNG, M. (2010) From RNA-seq reads to differential expression results. *Genome Biology,* 11**,** 220.

OSHLACK, A. & WAKEFIELD, M. (2009) Transcript length bias in RNA-seq data confounds systems biology. *Biology Direct,* 4**,** 14.

OWENS, D., MCGEE, M., BOLAND, T. & O'KIELY, P. (2009) Rumen fermentation, microbial protein synthesis, and nutrient flow to the omasum in cattle offered corn silage, grass silage, or whole-crop wheat. *J. Anim Sci.,* 87**,** 658-668.

PAAPE, M. J., BANNERMAN, D., D. , ZHAO, X. & LEE, J.-W. (2003) The bovine neutrophil: Structure and function in blood and milk. *Vet. Res.,* 34**,** 597-627.

PAAPE, M. J., WERGIN, W. P., GUIDRY, A. J. & PEARSON, R. E. (1979) Leukocytes-Second Line of Defense Against Invading Mastitis Pathogens. *Journal of Dairy Science,* 62**,** 135-153.

PADGETT, D. A. & GLASER, R. (2003) How stress influences the immune response. *Trends in Immunology,* 24**,** 444-448.

PANG, W., EARLEY, B., SWEENEY, T., GATH, V. & CROWE, M. (2009a) Temporal patterns of inflammatory gene expression in local tissues after banding or burdizzo castration in cattle. *BMC Veterinary Research,* 5**,** 36.

PANG, W. Y., EARLEY, B., MURRAY, M., SWEENEY, T., GATH, V. & CROWE, M. A. (2010) Banding or Burdizzo castration and carprofen administration on peripheral leukocyte inflammatory cytokine transcripts. *Research in Veterinary Science,* In Press, Corrected Proof.

PANG, W. Y., EARLEY, B., SWEENEY, T. & CROWE, M. A. (2006) Effect of carprofen administration during banding or burdizzo castration of bulls on plasma cortisol, in vitro interferon-γ production, acute-phase proteins, feed intake, and growth. *Journal of Animal Science,* 84**,** 351-359.

PANG, W. Y., EARLEY, B., SWEENEY, T., PIRANI, S., GATH, V. & CROWE, M. A. (2009b) Effects of banding or burdizzo castration of bulls on neutrophil phagocytosis and respiratory burst, CD62-L expression, and serum interleukin-8 concentration. *J. Anim Sci.,* 87**,** 3187-3195.

PARIANTE, C. M., PEARCE, B. D., PISELL, T. L., SANCHEZ, C. I., PO, C., SU, C. & MILLER, A. H. (1999) The Proinflammatory Cytokine, Interleukin-1{alpha}, Reduces Glucocorticoid Receptor Translocation and Function. *Endocrinology,* 140**,** 4359-4366.

PARK, Y., SCHOENE, N. & HARRIS, W. (2002) Mean platelet volume as an indicator of platelet activation: methodological issues. *Platelets,* 13**,** 301-306.

PASCUAL-LE TALLEC, L. & LOMBES, M. (2005) The Mineralocorticoid Receptor: A Journey Exploring Its Diversity and Specificity of Action. *Mol Endocrinol,* 19**,** 2211-2221.

PELLETIER, H., SAWAYA, M. R., KUMAR, A., WILSON, S. H. & KRAUT, J. (1994) Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. *Science,* 264**,** 1891-1903.

PETERSEN, F., FLAD, H. D. & BRANDT, E. (1994) Neutrophil-activating peptides NAP-2 and IL-8 bind to the same sites on neutrophils but interact in different ways. Discrepancies in binding affinities, receptor densities, and biologic effects. *The Journal of Immunology,* 152**,** 2467-2478.

PHILLIPS, W. A., JUNIEWICZ, P. E., ZAVY, M. T. & VON TUNGELN, D. L. (1989) The effect of the stress of weaning and transport on white blood cell patterns and fibrinogen concentration of beef calves on different genotypes. *Canadian Journal of Animal Science,* 69**,** 333-340.

PIÉ, S., LALLÈS, J. P., BLAZY, F., LAFFITTE, J., SÈVE, B. & OSWALD, I. P. (2004) Weaning Is Associated with an Upregulation of Expression of Inflammatory Cytokines in the Intestine of Piglets. *The Journal of Nutrition,* 134**,** 641-647.

PINCHAK, W. E., TOLLESON, D. R., MCCLOY, M., HUNT, L. J., GILL, R. J., ANSLEY, R. J. & BEVERS, S. J. (2004) Morbidity effects on productivity and profitability of stocker cattle grazing in the Southern Plains. *Journal of Animal Science,* 82**,** 2773-2779.

PLAGEMANN A, STAUDT A, GOTZ F, MALZ U, ROHDE W, RAKE A & G, D. (1998) Long-term Effects of Early Postnatally Administered Interleukin-1-beta on the Hypothalamic-Pituitary-Adrenal (HPA) Axis in Rats. *Endocrine Regulations,* 32**,** 77-85.

POLLOCK, J. M., ROWAN, T. G., DIXON, J. B., CARTER, S. D. & KELLY, D. F. (1991) Estimation of immunity in the developing calf: Cellular and humoral responses

to keyhole limpet haemocyanin. *Veterinary Immunology and Immunopathology,* 29**,** 105-113.

PRASSE, K., LATIMER, K. & MAHAFFEY, E. (2003) Duncan and Prasse's Veterinary Laboratory Medicine: Clinical Pathology, 4th Ed. *Wiley-Blackwell*.

PREISLER, M. T., WEBER, P. S. D., TEMPELMAN, R. J., ERSKINE, R. J., HUNT, H. & BURTON, J. L. (2000) Glucocorticoid Receptor Expression Profiles in Mononuclear Leukocytes of Periparturient Holstein Cows. *Journal of Dairy Science,* 83**,** 38-47.

PRICE, E. O., HARRIS, J. E., BORGWARDT, R. E., SWEEN, M. L. & CONNOR, J. M. (2003) Fenceline contact of beef calves with their dams at weaning reduces the negative effects of separation on behavior and growth rate. *J. Anim Sci.,* 81**,** 116-121.

PRUETT, S. B. (2003) Stress and the immune system. *Pathophysiology,* 9**,** 133-153.

PRUETT, S. B. & FAN, R. (2001) Quantitative modeling of suppression of IgG1, IgG2a, IL-2 and IL-4 responses to antigen in mice treated with exogenous corticosterone or restraint stress. *Journal of Toxicology and Environmental Health, Part A: Current Issues,* 62**,** 175 - 189.

PUGH, N., SIMPSON, A. M. C., SMETHURST, P. A., DE GROOT, P. G., RAYNAL, N. & FARNDALE, R. W. (2010) Synergism between platelet collagen receptors defined using receptor-specific collagen-mimetic peptide substrata in flowing blood. *Blood,* 115**,** 5069-5079.

QIU, X., ARTHINGTON, J. D., RILEY, D. G., CHASE, C. C., PHILLIPS, W. A., COLEMAN, S. W. & OLSON, T. A. (2007) Genetic effects on acute phase protein response to the stresses of weaning and transportation in beef calves. *Journal of Animal Science,* 85**,** 2367-2374.

RAINARD, P., RIOLLET, C., POUTREL, B. & PAAPE, M. J. (2000) Phagocytosis and killing of Staphylococcus aureus by bovine neutrophils after priming by tumor necrosis factor-α and the des-arginine derivative of C5a. *American Journal of Veterinary Research,* 61**,** 951-959.

RASBY, R. (2007) Early Weaning Beef Calves. *Veterinary Clinics of North America: Food Animal Practice,* 23**,** 29-40.

RAUSCH, P. G. & MOORE, T. G. (1975) Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. *Blood,* 46**,** 913-919.

REED, J. C. (2000) Mechanisms of Apoptosis. *The American Journal of Pathology,* 157**,** 1415-1430.

REICHARDT, H. M., TUCKERMANN, J. P., GÖTTLICHER, M., VUJIC, M., WEIH, F., ANGEL, P., HERRLICH, P. & SCHÜTZ, G. (2001) Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *EMBO J.*, 20**,** 7168-7173.

REICHE, E. M. V., NUNES, S. O. V. & MORIMOTO, H. K. (2004) Stress, depression, the immune system, and cancer. *The Lancet Oncology,* 5**,** 617-625.

REINHARDT, V. & REINHARDT, A. (1981) Natural sucking performance and age of weaning in zebu cattle (Bos indicus). *The Journal of Agricultural Science,* 96**,** 309-312.

RIFAI, N. & GERSZTEN, R. E. (2006) Biomarker Discovery and Validation. *Clin Chem,* 52**,** 1635-1637.

RINALDI, M., LI, R. W. & CAPUCO, A. V. (2010) Mastitis associated transcriptomic disruptions in cattle. *Veterinary Immunology and Immunopathology,* 138**,** 267-279.

RIONDATO, F., D'ANGELO, A., MINISCALCO, B., BELLINO, C. & GUGLIELMINO, R. (2008) Effects of road transportation on lymphocyte subsets in calves. *The Veterinary Journal,* 175**,** 364-368.

RIVIER, C. (1994) Stimulatory effect of interleukin-1β on the hypothalamic-pituitary-adrenal axis of the rat: influence of age, gender and circulating sex steroids. *J Endocrinol,* 140**,** 365-372.

ROBERTS, A., TRAPNELL, C., DONAGHEY, J., RINN, J. & PACHTER, L. (2011) Improving RNA-Seq expression estimates by correcting for fragment bias. *Genome Biology,* 12**,** R22.

ROBINSON, M. & OSHLACK, A. (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology,* 11**,** R25.

ROBINSON, M. D., MCCARTHY, D. J. & SMYTH, G. K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics,* 26**,** 139-140.

ROSENBAUM, D. M., RASMUSSEN, S. G. F. & KOBILKA, B. K. (2009) The structure and function of G-protein-coupled receptors. *Nature,* 459**,** 356-363.

ROSTÈNE, W., GUYON, A., KULAR, L., GODEFROY, D., BARBIERI, F., BAJETTO, A., BANISADR, G., CALLEWAERE, C., CONDUCTIER, G., ROVÈRE, C., MÉLIK-PARSADANIANTZ, S. & FLORIO, T. (2011) Chemokines and chemokine receptors: New actors in neuroendocrine regulations. *Frontiers in Neuroendocrinology,* 32**,** 10-24.

ROSTENE, W., KITABGI, P. & PARSADANIANTZ, S. M. (2007) Chemokines: a new class of neuromodulator? *Nat Rev Neurosci,* 8**,** 895-903.

ROZEN, S. & SKALETSKY, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*.

SAAMA, P. M., JACOB, J. B., KEHRLI, M. E., FREEMAN, A. E., KELM, S. C., KUCK, A. L., TEMPELMAN, R. J. & BURTON, J. L. (2004) Genetic Variation in Bovine Mononuclear Leukocyte Responses to Dexamethasone. *Journal of Dairy Science,* 87**,** 3928-3937.

SACCÒ, M., VALENTI, G., CORVI MORA, P., WU, F. & RAY, D. (2002) DHEA, a selective glucocorticoid receptor antagonist: its role in immune system regulation and metabolism. *Journal of Endocrinological Investigation,* 25**,** 81-82.

SAFIEH-GARABEDIAN, B., POOLE, S., HADDAD, J. J., MASSAAD, C. A., JABBUR, S. J. & SAADÉ, N. E. (2002) The role of the sympathetic efferents in endotoxin-induced localized inflammatory hyperalgesia and cytokine upregulation. *Neuropharmacology,* 42**,** 864-872.

SAIKI, R. K., SCHARF, S., FALOONA, F., MULLIS, K. B., HORN, G. T., ERLICH, H. A. & ARNHEIM, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science,* 230**,** 1350-1354.

SAITOH, 0., KUBO, Y., ODOGIRI, M., ICHIKAWA, M., YAMAGATA, K. & SEKINE, T. (1999) RGS7 and RGS8 differentially accelerate G protein mediated modulation of K+ currents. *Journal of Biological Chemistry,* 274, 9899-9904.

SALICRÚ, A. N., SAMS, C. F. & MARSHALL, G. D. (2007) Cooperative effects of corticosteroids and catecholamines upon immune deviation of the type-1/type-2 cytokine balance in favor of type-2 expression in human peripheral blood mononuclear cells. *Brain, Behavior, and Immunity,* 21**,** 913-920.

SAMAHA, F. F. & KAHN, M. L. (2006) Novel Platelet and Vascular Roles for Immunoreceptor Signaling. *Arterioscler Thromb Vasc Biol,* 26**,** 2588-2593.

SAPOLSKY, R. M. (2004) Organismal stress and telomeric aging: An unexpected connection. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 17323-17324.

SAPOLSKY, R. M., ROMERO, L. M. & MUNCK, A. U. (2000) How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions. *Endocr Rev,* 21**,** 55-89.

SARTIN, J. L., ELSASSER, T. H., GUNTER, D. R. & MCMAHON, C. D. (1998) Endocrine modulation of physiological responses to catabolic disease. *Domestic Animal Endocrinology,* 15**,** 423-429.

SAUL, A. N., OBERYSZYN, T. M., DAUGHERTY, C., KUSEWITT, D., JONES, S., JEWELL, S., MALARKEY, W. B., LEHMAN, A., LEMESHOW, S. & DHABHAR, F. S. (2005) Chronic Stress and Susceptibility to Skin Cancer. *J. Natl. Cancer Inst.,* 97**,** 1760-1767.

SANDERS, V. (1997) Immune system interactions with other organ systems: Neuroimmunology. *Comprehensive Toxicology: Toxicology of the Immune System, Elsevier Press,* 5**,** 175-186.

SANDERS, V. M. & STRAUB, R. H. (2002) Norepinephrine, the β-Adrenergic Receptor, and Immunity. *Brain, Behavior, and Immunity,* 16**,** 290-332.

SCHEINMAN, R. I., COGSWELL, P. C., LOFQUIST, A. K. & BALDWIN, A. S., JR. (1995) Role of Transcriptional Activation of IkappaBalpha in Mediation of Immunosuppression by Glucocorticoids. *Science,* 270**,** 283-286.

SCHRODER, K., HERTZOG, P. J., RAVASI, T. & HUME, D. A. (2004) Interferon-{gamma}: an overview of signals, mechanisms and functions. *J Leukoc Biol,* 75**,** 163-189.

SEKI, E., DE MINICIS, S., OSTERREICHER, C. H., KLUWE, J., OSAWA, Y., BRENNER, D. A. & SCHWABE, R. F. (2007) TLR4 enhances TGF-β signaling and hepatic fibrosis. *Nat Med,* 13**,** 1324-1332.

SERPELL, J. A. (2003) Anthropomorphism and anthropomorphic selection: beyond the 'cute response'. *Society and Animals,* 11**,** 83.

SETH, M., LAMONT, E. A., JANAGAMA, H. K., WIDDEL, A., VULCHANOVA, L., STABEL, J. R., WATERS, W. R., PALMER, M. V. & SREEVATSAN, S. (2009) Biomarker Discovery in Subclinical Mycobacterial Infections of Cattle. *PLoS ONE,* 4**,** e5478.

SEYLE, H. (1946) The general adaptive syndrome and diseases of adaptation. *Journal of Clinical Endocrinology,* 6**,** 117-230.

SEYLE, H. (1973) The evolution of the stress concept. *American Scientist,* 61**,** 692-699.

SEYLE, H. (1975) Stress without distress. New York: New American Library.

SHAHRARA, S., PARK, C. C., TEMKIN, V., JARVIS, J. W., VOLIN, M. V. & POPE, R. M. (2006) RANTES Modulates TLR4-Induced Cytokine Secretion in Human Peripheral Blood Monocytes. *J Immunol,* 177**,** 5077-5087.

SHANKER, A. (2010) Adaptive control of innate immunity. *Immunology Letters,* 131**,** 107-112.

SHENDURE, J. A., PORRECA, G. J. & CHURCH, G. M. (2001) Overview of DNA Sequencing Strategies. *Current Protocols in Molecular Biology.* John Wiley & Sons, Inc.

SHEPHARD, R. J. & SHEK, P. N. (1999) Effects of Exercise and Training on Natural Killer Cell Counts and Cytolytic Activity: A Meta-Analysis. *Sports Medicine,* 28**,** 177-195.

SIEGFORD, J. M., BUSKIRK, D. D. & SHARRA, M. K. (2007) Behaviour of beef calves weaned by traditional, fenceline and two-step methods. *Journal of Animal Science,* 90, (Suppl 1)**,** 365.

SIMMS, D., CIZDZIEL, P. E. & CHOMCZYNSKI, P. (1993) TRIzol: A new reagent for optimal single-step isolation of RNA. *Focus,* 15**,** 532-535.

SIMON, S., LAMOUREUX, L., PLEWS, M., STOBART, M., LEMAISTRE, J., ZIEGLER, U., GRAHAM, C., CZUB, S., GROSCHUP, M. & KNOX, J. D. (2008) The

identification of disease-induced biomarkers in the urine of BSE infected cattle. *Proteome Science,* 6**,** 23.

SINGER, A., ADORO, S. & PARK, J.-H. (2008) Lineage fate and intense debate: myths, models and mechanisms of CD4/CD8 lineage choice. *Nature Reviews Immunology,* 8**,** 788-801.

SLADEK, Z. & RYSANEK, D. (2001) Neutrophil apoptosis during the resolution of bovine mammary gland injury. *Research in Veterinary Science,* 70**,** 41-46.

SLOMINSKI, A. (2007) A nervous breakdown in the skin: stress and the epidermal barrier. *The Journal of Clinical Investigation,* 117**,** 3166-3169.

SMALLEY, D. M. & LEY, K. (2005) L-selectin: mechanisms and physiological significance of ectodomain cleavage. *Journal of Cellular and Molecular Medicine,* 9**,** 255-266.

SMITH, E. M. & BLALOCK, J. E. (1981) Human lymphocyte production of corticotropin and endorphin-like substances: association with leukocyte interferon. *Proceedings of the National Academy of Sciences,* 78**,** 7530-7534.

SMYTH, S. S., MCEVER, R. P., WEYRICH, A. S., MORRELL, C. N., HOFFMAN, M. R., AREPALLY, G. M., FRENCH, P. A., DAUERMAN, H. L., BECKER, R. C. & FOR THE PLATELET COLLOQUIUM, P. (2009) Platelet functions beyond hemostasis. *Journal of Thrombosis and Haemostasis,* 7**,** 1759-1766.

SORRELLS, S. F., CASO, J. R., MUNHOZ, C. D. & SAPOLSKY, R. M. (2009) The Stressed CNS: When Glucocorticoids Aggravate Inflammation. *Neuron,* 64**,** 33-39.

SORRELLS, S. F. & SAPOLSKY, R. M. (2007) An inflammatory review of glucocorticiod actions in the CNS. *Brain, Behavior, and Immunity*, 21, 259-272.

STAHN, C. & BUTTGEREIT, F. (2008) Genomic and nongenomic effects of glucocorticoids. *Nat Clin Pract Rheum,* 4**,** 525-533.

STEFANO, G. B. & SMITH, E. M. (1996) Adrenocorticotropin -- A central trigger in immune responsiveness: Tonal inhibition of immune activation. *Medical Hypotheses,* 46**,** 471-478.

STEHULOVÁ, I., LIDFORS, L. & SPINKA, M. (2008) Response of dairy cows and calves to early separation: Effect of calf age and visual and auditory contact after separation. *Applied Animal Behaviour Science,* 110**,** 144-165.

STERLING, P. & EYER, J. (1988) Allostasis, a new paradigm to explain arousal pathology. *In: Fisher, S., Reason, J. (Eds.), Handbook of Life Stress, Cognition and Health*, John Wiley & Sons, New York**,** 629-649.

STERNBERG, E. M. (1997) Neural-immune Interactions in Health and Disease. *The Journal of Clinical Investigation,* 100**,** 2641-2647.

STERNBERG, E. M. (2006) Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol,* 6**,** 318-328.

STEVENS-FELTEN SY, B. D. (1997) Noradrenergic and peptidergic innervation of lymphoid organs. *Chemical Immunology,* 69**,** 99-131.

STEVENSON, J. R., WESTERMANN, J., LIEBMANN, P. M., HÖRTNER, M., RINNER, I., FELSNER, P., WÖLFLER, A. & SCHAUENSTEIN, K. (2001) Prolonged alpha-adrenergic stimulation causes changes in leukocyte distribution and lymphocyte apoptosis in the rat. *Journal of neuroimmunology,* 120**,** 50-57.

STORDEUR, P., ZHOU, L., BYL, B., BROHET, F., BURNY, W., DE GROOTE, D., VAN DER POLL, T. & GOLDMAN, M. (2003) Immune monitoring in whole blood using real-time PCR. *Journal of Immunological Methods,* 276**,** 69-77.

STUART, E. T. & DAVID, H. B. (2010) Innate immunity. *The Journal of allergy and clinical immunology,* 125**,** S24-S32.

SUBRAMANIAM, N., CAIRNS, W. & OKRET, S. (1997) Studies on the mechanism of glucocorticoid-mediated repression from a negative glucocorticoid response element from the bovine prolactin gene. *DNA Cell Biology,* 16**,** 153-163.

SULTAN, M., SCHULZ, M. H., RICHARD, H., MAGEN, A., KLINGENHOFF, A., SCHERF, M., SEIFERT, M., BORODINA, T., SOLDATOV, A., PARKHOMCHUK, D., SCHMIDT, D., O'KEEFFE, S., HAAS, S., VINGRON, M., LEHRACH, H. & YASPO, M.-L. (2008) A Global View of Gene Activity and Alternative Splicing by Deep Sequencing of the Human Transcriptome. *Science,* 321**,** 956-960.

SWAIN, S. D., JUTILA, K. L. & QUINN, M. T. (2000) Cell-surface lactoferrin as a marker for degranulation of specific granules in bovine neutrophils. *American Journal of Veterinary Research,* 61**,** 29-37.

TADICH, N., GALLO, C., BUSTAMANTE, H., SCHWERTER, M. & VAN SCHAIK, G. (2005) Effects of transport and lairage time on some blood constituents of Friesian-cross steers in Chile. *Livestock Production Science,* 93**,** 223-233.

TEMPELMAN, R. J., SAAMA, P. M., FREEMAN, A. E., KELM, S. C., KUCK, A. L., KEHRLI JR, M. E. & BURTON, J. L. (2002) Genetic Variation in Bovine Neutrophil Sensitivity to Glucocorticoid Challenge. *Acta Agriculturae Scandinavica, A,* 52**,** 189-202.

THADIKKARAN, L., SIEGENTHALER, M. A., CRETTAZ, D., QUELOZ, P.-A., SCHNEIDER, P. & TISSOT, J.-D. (2005) Recent advances in blood-related proteomics. *PROTEOMICS,* 5**,** 3019-3034.

THE_GENE_ONTOLOGY_CONSORTIUM (2000) Gene ontology: tool for the unification of biology. *Nature Genetics,* 25**,** 25-29.

THOMAS, T. J., WEARY, D. M. & APPLEBY, M. C. (2001) Newborn and 5-week-old calves vocalize in response to milk deprivation. *Applied Animal Behaviour Science,* 74**,** 165-173.

TING, S. T. L., EARLEY, B. & CROWE, M. A. (2003b) Effect of repeated ketoprofen administration during surgical castration of bulls on cortisol, immunological function, feed intake, growth, and behavior. *J. Anim Sci.,* 81**,** 1253-1264.

TING, S. T. L., EARLEY, B., HUGHES, J. M. L. & CROWE, M. A. (2003a) Effect of ketoprofen, lidocaine local anesthesia, and combined xylazine and lidocaine caudal epidural anesthesia during castration of beef cattle on stress responses, immunity, growth, and behavior. *J. Anim Sci.,* 81**,** 1281-1293.

TING, S. T. L., EARLEY, B., & CROWE, M. A. (2004) Effect of cortisol infusion patterns and castration on metabolic and immunological indices of stress response in cattle. *Domestic Animal Endocrinology*., 26, 329-349.

TING, S. T. L., EARLEY, B., VEISSIER, I., GUPTA, S. & CROWE, M. A. (2010) Effects of Burdizzo castration on CO2 laser induced thermal nociception of Holstein-Friesian calves of different ages. *Applied Animal Behaviour Science*, 126**,** 12-18.

TRAPNELL, C., WILLIAMS, B. A., PERTEA, G., MORTAZAVI, A., KWAN, G., VAN BAREN, M. J., SALZBERG, S. L., WOLD, B. J. & PACHTER, L. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotech,* 28**,** 511-515.

TURNBULL, A. V. & RIVIER, C. (1995) Regulation of the HPA Axis by Cytokines. *Brain, Behavior, and Immunity,* 9**,** 253-275.

TURNBULL, A. V. & RIVIER, C. L. (1999) Regulation of the Hypothalamic-Pituitary-Adrenal Axis by Cytokines: Actions and Mechanisms of Action. *Physiol. Rev.,* 79**,** 1-71.

TURRO, E., SU, S.-Y., GONCALVES, A., COIN, L., RICHARDSON, S. & LEWIN, A. (2011) Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. *Genome Biology,* 12**,** R13.

ULRICH-LAI, Y. M. & HERMAN, J. P. (2009) Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci,* 10**,** 397-409.

VALLEJO, M. (1994) Transcriptional control of gene expression by cAMP-response element binding proteins. *Journal of Neuroimmunology,* 6**,** 587-596.

VALLIERES, L. & RIVEST, S. (1999) Interleukin-6 Is a Needed Proinflammatory Cytokine in the Prolonged Neural Activity and Transcriptional Activation of Corticotropin-Releasing Factor during Endotoxemia. *Endocrinology,* 140**,** 3890-3903.

VAN KAMPEN, C. & MALLARD, B. A. (1997) Effects of peripartum stress and health on circulating bovine lymphocyte subsets. *Veterinary Immunology and Immunopathology,* 59**,** 79-91.

VAN OOSTVELDT, K., PAAPE, M. J., DOSOGNE, H. & BURVENICH, C. (2002) Effect of apoptosis on phagocytosis, respiratory burst and CD18 adhesion receptor expression of bovine neutrophils. *Domestic Animal Endocrinology,* 22**,** 37-50.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* 3**,** research0034.1 - research0034.11.

VEISSIER, I., BUTTERWORTH, A., BOCK, B. & ROE, E. (2008) European approaches to ensure good animal welfare. *Applied Animal Behaviour Science,* 113**,** 279-297.

VEISSIER, I. & LE NEINDRE, P. (1989) Weaning in calves: Its effects on social organization. *Applied Animal Behaviour Science,* 24**,** 43-54.

VEISSIER, I., LE NEINDRE, P. & GAREL, J. P. (1990) Decrease in cow-calf attachment after weaning. *Behavioural Processes,* 21**,** 95-105.

VERHAMME, P. & HOYLAERTS, M. (2009) Hemostasis and inflammation: two of a kind? *Thrombosis Journal,* 7**,** 15.

VISWANATHAN, K. & DHABHAR, F. S. (2005) Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 5808-5813.

VOGEL, C., DE SOUSA ABREU, R., KO, D., LE, S.-Y., SHAPIRO, B. A., BURNS, S. C., SANDHU, D., BOUTZ, D. R., MARCOTTE, E. M. & PENALVA, L. O. (2010) Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol Syst Biol,* 6.

VOLGER, O. L., FLEDDERUS, J. O., KISTERS, N., FONTIJN, R. D., MOERLAND, P. D., KUIPER, J., VAN BERKEL, T. J., BIJNENS, A.-P. J. J., DAEMEN, M. J. A. P., PANNEKOEK, H. & HORREVOETS, A. J. G. (2007) Distinctive Expression of Chemokines and Transforming Growth Factor-{beta} Signaling in Human Arterial Endothelium during Atherosclerosis. *Am J Pathol,* 171**,** 326-337.

VON BORELL, E. H. (2001) The biology of stress and its application to livestock housing and transportation assessment. *Journal of Animal Science,* 79**,** E260-E267.

VON HUNDELSHAUSEN, P. & WEBER, C. (2007) Platelets as Immune Cells: Bridging Inflammation and Cardiovascular Disease. *Circ Res,* 100**,** 27-40.

VON HUNDELSHAUSEN, P., KOENEN, R. R. & WEBER, C. (2009) Platelet-Mediated Enhancement of Leukocyte Adhesion. *Microcirculation,* 16**,** 84-96.

WAGNER, J. G. & ROTH, R. A. (2000) Neutrophil Migration Mechanisms, with an Emphasis on the Pulmonary Vasculature. *Pharmacological Reviews*, 52**,** 349-374.

WÅGSÄTER, D., ZHU, C., BJÖRCK, H. M. & ERIKSSON, P. (2009) Effects of PDGF-C and PDGF-D on monocyte migration and MMP-2 and MMP-9 expression. *Atherosclerosis*, 202**,** 415-423.

WALL, M. A., POSNER, B. A. & SPRANG, S. R. (1998) Structural basis of activity and subunit recognition in G protein heterotrimers. *Structure (London, England : 1993),* 6**,** 1169-83.

WANG, Z., GERSTEIN, M. & SNYDER, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**,** 57-63.

WATKINS, L. R. & MAIER, S. F. (1999) Implications of immune-to-brain communication for sickness and pain. *Proceedings of the National Academy of Sciences,* 96**,** 7710-7713.

WAUGH, D. J. J. & WILSON, C. (2008) The Interleukin-8 Pathway in Cancer. *Clinical Cancer Research,* 14**,** 6735-6741.

WEARY, D. M., JASPER, J. & HÖTZEL, M. J. (2008) Understanding weaning distress. *Applied Animal Behaviour Science,* 110**,** 24-41.

WEAVER, R. F. (2008) Molecular Biology, 4th Edition. *McGraw-Hill Higher Education, New York.*

WEBER, P. S. D., MADSEN-BOUTERSE, S. A., ROSA, G. J. M., SIPKOVSKY, S., REN, X., ALMEIDA, P. E., KRUSKA, R., HALGREN, R. G., BARRICK, J. L. & BURTON, J. L. (2006) Analysis of the bovine neutrophil transcriptome during glucocorticoid treatment. *Physiol. Genomics,* 28**,** 97-112.

WEBER, P. S. D., TOELBOELL, T., CHANG, L.-C., TIRRELL, J. D., SAAMA, P. M., SMITH, G. W. & BURTON, J. L. (2004) Mechanisms of glucocorticoid-induced down-regulation of neutrophil L-selectin in cattle: evidence for effects at the gene-expression level and primarily on blood neutrophils. *J Leukoc Biol,* 75**,** 815-827.

WEBSTER, A. J. F. (2001) Farm Animal Welfare: the Five Freedoms and the Free Market. *The Veterinary Journal,* 161**,** 229-237.

WEBSTER, J. (1995) Animal Welfare *A Cool Eye Towards Eden,* Oxford: Blackwell Science.

WEBSTER, J. C., OAKLEY, R. H., JEWELL, C. M. & CIDLOWSKI, J. A. (2001) Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative β isoform: A mechanism for the generation of glucocorticoid resistance. *Proceedings of the National Academy of Sciences,* 98**,** 6865-6870.

WEBSTER MARKETON, J. I. & GLASER, R. (2008) Stress hormones and immune function. *Cellular Immunology,* 252**,** 16-26.

WENINGER, S. C., DUNN, A. J., MUGLIA, L. J., DIKKES, P., MICZEK, K. A., SWIERGIEL, A. H., BERRIDGE, C. W. & MAJZOUB, J. A. (1999) Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 8283-8288.

WERR, J., JOHANSSON, J., ERIKSSON, E. E., HEDQVIST, P., RUOSLAHTI, E. & LINDBOM, L. (2000) Integrin α2β1 (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood,* 95**,** 1804-1809.

WETTERBOM, A., AMEUR, A., FEUK, L., GYLLENSTEN, U. & CAVELIER, L. (2010) Identification of novel exons and transcribed regions by chimpanzee transcriptome sequencing. *Genome Biology,* 11**,** R78.

WEYRICH, A. S. & ZIMMERMAN, G. A. (2004) Platelets: signaling cells in the immune continuum. *Trends in Immunology,* 25**,** 489-495.

WIEGERS, G. J. & REUL, J. M. H. M. (1998) Induction of cytokine receptors by glucocorticoids: functional and pathological significance. *Trends in Pharmacological Sciences,* 19**,** 317-321.

WIGMORE, S. J., KENNETH, C. H., FEARON, C. H., MAINGAY, J. P., LAI, B.S., & ROSS, J. A. (1997) Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes. *Endocrinology and Metabolism*, 273: E720-E726.

WU, W., YAMAURA, T., MURAKAMI, K., MURATA, J., MATSUMOTO, K., WATANABE, H. & SAIKI, I. (2000) Social isolation stress enhanced liver metastasis of murine colon 26-L5 carcinoma cells by suppressing immune responses in mice. *Life Sciences,* 66**,** 1827-1838.

YAGI, Y., SHIONO, H., CHIKAYAMA, Y., OHNUMA, A., NAKAMURA, I. & YAYOU, K. (2004) Transport stress increases somatic cell counts in milk, and enhances

the migration capacity of peripheral blood neutrophils of dairy cows. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science,* 66**,** 381-7.

YANG, E. V. & GLASER, R. (2000) Stress-induced immunomodulation: impact on immune defenses against infectious disease. *Biomedecine & Pharmacotherapy,* 54**,** 245-250.

YANG, S. S., TU, Z., CHEUNG, F., XU, W., LAMB, J., JUNG, H.-J., VANCE, C. & GRONWALD, J. (2011) Using RNA-Seq for gene identification, polymorphism detection and transcript profiling in two alfalfa genotypes with divergent cell wall composition in stems. *BMC Genomics,* 12**,** 199.

YEAMAN, M. (2010) Platelets in defense against bacterial pathogens. *Cellular and Molecular Life Sciences,* 67**,** 525-544.

YI, Z., YING, Z., JUNYING, M., GREGORY, H., CHARLES, S., XIULI, S., TINGTING, C. & DELING, Y. (2008) Chronic restraint stress promotes immune suppression through Toll-like receptor 4-mediated phosphoinositide 3-kinase signaling. *Journal of neuroimmunology,* 204**,** 13-19.

YIN, D., TUTHILL, D., MUFSON, R. A. & SHI, Y. (2000) Chronic Restraint Stress Promotes Lymphocyte Apoptosis by Modulating Cd95 Expression. *The Journal of Experimental Medicine,* 191**,** 1423-1428.

YIN, D., ZHANG, Y., STUART, C., MIAO, J., ZHANG, Y., LI, C., ZENG, X., HANLEY, G., MOORMAN, J., YAO, Z. & WOODRUFF, M. (2006) Chronic restraint

stress modulates expression of genes in murine spleen. *Journal of neuroimmunology,* 177**,** 11-17.

YOSHIKAWA, M., TANI, F., ASHUCAGA, T., YOSHIMURA, T. & CHIBA, H. (1986) Purification and Characterization of an Opioid Antagonist from a Peptic Digest of Bovine κ-Casein *Agriculatural and Biological Chemistry,* 50**,** 2951-2954.

YOUNG, M., WAKEFIELD, M., SMYTH, G. & OSHLACK, A. (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology,* 11**,** R14.

ZAHOREC, R. (2001) Ratio of neutrophil to lymphocyte counts--rapid and simple parameter of systemic inflammation and stress in critically ill. *Bratislavské lekárske listy,* 102**,** 5-14.

ZAVY, M., JUNIEWICZ, P., PHILLIPS, W. & VONTUNGELN, D. (1992) Effect of initial restraint, weaning, and transport stress on baseline and ACTH-stimulated cortisol responses in beef calves of different genotypes. *American Journal of Veterinary Research,* 53**,** 551-557.

ZHANG, Y., WOODRUFF, M., ZHANG, Y., MIAO, J., HANLEY, G., STUART, C., ZENG, X., PRABHAKAR, S., MOORMAN, J., ZHAO, B. & YIN, D. (2008a) Toll-like receptor 4 mediates chronic restraint stress-induced immune suppression. *Journal of neuroimmunology,* 194**,** 115-122.

ZHANG, Y., ZHANG, Y., MIAO, J., HANLEY, G., STUART, C., SUN, X., CHEN, T. & YIN, D. (2008b) Chronic restraint stress promotes immune suppression through Toll-

like receptor 4-mediated phosphoinositide 3-kinase signaling. *Journal of neuroimmunology,* 204**,** 13-19.

ZHOU, D., SHANKS, N., RIECHMAN, S. E., LIANG, R., KUSNECOV, A. W. & RABIN, B. S. (1996) Interleukin 6 Modulates lnterleukin-1– and Stress-Induced Activation of the Hypothalamic-Pituitary-Adrenal Axis in Male Rats. *Neuroendocrinology,* 63**,** 227-236.

ZINDER, O. & DAR, D. E. (1999) Neuroactive steroids: their mechanism of action and their function in the stress response. *Acta Physiologica Scandinavica,* 167**,** 181-188.

Chapter 8

Appendix

Appendix 1. Effect of post-weaning management on total leukocyte, neutrophil and lymphocyte number, neutrophil:lymphocyte (N:L) ratio, eosinophil and monocyte number in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± SE.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	11	T	S	T×S
Total Leukocytes	FB	9.3 ± 0.64	9.6^{x} ± 0.81	9.2 ± 0.71	9.3 ± 0.72	10.2 ± 0.51	7.8 ± 0.69	NS	NS	NS
($\times10^3$cells/μL)	FA	9.9 ± 0.64	10.7^{xy} ± 0.81	10.2 ± 0.71	10.2 ± 0.72	10.8 ± 0.51	9.8 ± 0.69			
	MB	9.4 ± 0.65	9.7^{xy} ± 0.83	9.6 ± 0.72	9.7 ± 0.74	10.8^{a} ± 0.52	9.1 ± 0.71			
	MA	10.4 ± 0.68	12.1^{by} ± 0.87	10.4 ± 0.75	9.7 ± 0.77	10.2 ± 0.54	8.4^{a} ± 0.74			
Neutrophils	FB	1.9^{x} ± 0.55	2.5^{x} ± 0.73	2.6^{b} ± 0.54	2.8^{a} ± 0.53	3.4^{c} ± 0.41	1.8 ± 0.41	NS	*******	0.07
($\times10^3$cells/μL)	FA	2.1^{x} ± 0.55	3.2^{cx} ± .072	2.6 ± 0.54	2.9 ± 0.53	3.0^{b} ± 0.40	2.4 ± 0.41			
	MB	1.9^{x} ± 0.56	2.7^{bx} ± 0.72	2.4^{a} ± 0.54	2.5 ± 0.53	3.7^{c} ± 0.40	2.3 ± 0.41			
	MA	3.5^{y} ± 0.55	5.6^{cy} ± 0.72	3.9 ± 0.54	3.2 ± 0.53	3.7 ± 0.41	2.1^{a} ± 0.41			
Lymphocytes	FB	7.2^{xy} ± 0.29	7.2 ± 0.24	6.7^{ax} ± 0.18	6.6^{xy} ± 0.27	6.8^{x} ± 0.27	6.1^{bx} ± 0.30	******	******	NS
($\times10^3$cells/μL)	FA	7.7^{x} ± 0.29	7.7 ± 0.24	7.4^{y} ± 0.17	7.2^{x} ± 0.26	7.6^{y} ± 0.27	7.2^{y} ± 0.30			
	MB	7.2^{xy} ± 0.29	7.0 ± 0.24	6.7^{x} ± 0.18	7.3^{x} ± 0.26	6.8^{x} ± 0.27	6.7^{xy} ± 0.30			
	MA	6.7^{y} ± 0.31	6.8 ± 0.25	6.3^{x} ± 0.18	6.3^{y} ± 0.28	6.3^{x} ± 0.28	6.0^{x} ± 0.31			
N:L Ratio	FB	0.27^{x} ± 0.07	0.36^{ax} ± 0.10	0.42^{b} ± 0.08	0.46^{a} ± 0.07	0.52^{b} ± 0.06	0.31 ± 0.05	NS	***	*
	FA	0.28^{x} ± 0.07	0.43^{bx} ± 0.10	0.35 ± 0.07	0.39 ± 0.07	0.42 ± 0.06	0.34 ± 0.05			
	MB	0.27^{xy} ± 0.07	0.40^{bx} ± 0.10	0.39^{b} ± 0.08	0.38 ± 0.07	0.58^{c} ± 0.06	0.35 ± 0.05			
	MA	0.49^{y} ± 0.07	0.76^{cy} ± 0.10	0.55 ± 0.07	0.46 ± 0.07	0.56 ± 0.06	0.32 ± 0.05			
Monocytes	FB	0.67 ± 0.07	0.51 ± 0.05	0.58 ± 0.06	0.50 ± 0.06	0.53 ± 0.06	0.46 ± 0.07	NS	NS	NS
($\times10^3$cells/μL)	FA	0.61 ± 0.07	0.55 ± 0.05	0.59 ± 0.06	0.64 ± 0.06	0.60 ± 0.06	0.60 ± 0.07			
	MB	0.55 ± 0.07	0.50 ± 0.05	0.61 ± 0.06	0.53 ± 0.06	0.54 ± 0.06	0.60 ± 0.07			
	MA	0.58 ± 0.07	0.58 ± 0.05	0.69 ± 0.06	0.64 ± 0.06	0.66 ± 0.06	0.63 ± 0.07			
Eosinophils	FB	0.13 ± 0.03	0.12^{x} ± 0.02	0.13^{xy} ± 0.02	0.12^{xy} ± 0.04	0.12 ± 0.03	0.07^{a} ± 0.03	NS	NS	NS
($\times10^3$cells/μL)	FA	0.11 ± 0.03	0.13^{x} ± 0.02	0.14^{x} ± 0.02	0.23^{ax} ± 0.04	0.13 ± 0.03	0.09 ± 0.03			
	MB	0.17 ± 0.03	0.09^{bxy} ± 0.02	0.12^{xy} ± 0.02	0.13^{xy} ± 0.04	0.14 ± 0.03	0.10 ± 0.03			
	MA	0.12 ± 0.03	0.08^{y} ± 0.02	0.09^{y} ± 0.02	0.12^{y} ± 0.04	0.14 ± 0.03	0.15 ± 0.03			

Females weaned beside the dam (FB; n = 7); Females weaned away from dam (FA; n = 7); Males weaned beside the dam (MB; n = 7); Males weaned away from dam (MA; n = 7). T = treatment, S = sampling time, T×S = treatment × sampling time interactions, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

Appendix 2. Effect of weaning induced stress on red blood cell (RBC) number, haemoglobin (HGB) concentration, haematocrit (HCT) %, mean cell haemoglobin concentration (MCHC) and platelet number in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning						*P*-Values		
Variable		0	1	2	3	7	11	T	S	T×S
RBC ($\times 10^6$ cells/µL)	FB	10.1 ± 0.47	10.3 ± 0.49	9.9 ± 0.44	10.2 ± 0.42	10.0^{x} ± 0.41	6.4^{c} ± 0.62	NS	***	NS
	FA	10.2 ± 0.48	10.3 ± 0.49	9.7^{b} ± 0.45	10.4 ± 0.42	10.2^{xy} ± 0.41	6.6^{c} ± 0.63			
	MB	10.8 ± 0.49	10.9 ± 0.50	10.6 ± 0.46	10.9 ± 0.43	10.7^{xy} ± 0.42	7.3^{c} ± 0.64			
	MA	11.3 ± 0.48	11.0 ± 0.49	10.8^{b} ± 0.44	11.2 ±0.42	11.2^{y} ± 0.41	5.9^{c} ± 0.62			
HGB (g/dL)	FB	12.2 ± 0.22	12.5 ± 0.17	12.4 ± 0.19	12.4 ± 0.15	12.0 ± 0.22	12.1 ± 0.24	NS	NS	NS
	FA	12.2 ± 0.22	12.4 ± 0.17	12.5 ± 0.19	12.7^{a} ± 0.15	12.2 ± 0.22	12.1 ± 0.24			
	MB	12.8 ± 0.22	12.7 ± 0.17	12.4 ± 0.19	12.8 ± 0.15	12.3 ± 0.22	12.4 ± 0.24			
	MA	12.5 ± 0.22	12.3 ± 0.17	12.1 ± 0.19	12.4 ± 0.15	12.4 ± 0.22	12.1 ± 0.24			
HCT (%)	FB	33.1 ± 1.3	33.5 ± 1.2	32.2 ± 1.0	33.8 ± 0.9	32.6 ± 1.0	21.4^{c} ± 1.8	NS	**	NS
	FA	32.6 ± 1.3	32.9 ± 1.2	31.0^{a} ± 1.0	33.9 ± 0.9	32.4 ± 1.0	21.5^{c} ± 1.8			
	MB	34.3 ± 1.3	34.1 ± 1.2	32.9^{a} ± 1.1	33.9 ± 1.0	33.3 ± 1.0	23.2^{c} ± 1.9			
	MA	34.8 ± 1.3	33.3 ± 1.2	33.5 ± 1.1	34.1 ± 1.1	34.2 ± 1.02	18.0^{c} ± 1.9			
MCHC (g/dL)	FB	36.5 ± 0.45	37.0 ± 0.45	38.0^{ax} ± 0.58	36.7 ± 0.69	36.7^{x} ± 0.27	59.7^{c} ± 4.45	NS	*	**
	FA	37.2 ± 0.45	37.5 ± 0.45	40.3^{cy} ± 0.58	37.0 ± 0.69	37.4^{y} ± 0.27	57.0^{c} ± 4.47			
	MB	37.4 ± 0.46	37.2 ± 0.45	37.5^{x} ± 0.59	37.5 ± 0.70	37.0^{xy} ± 0.27	58.0^{c} ± 4.52			
	MA	36.7 ± 0.46	37.8^{a} ± 0.45	37.1^{x} ± 0.58	36.8 ± 0.69	36.7^{xy} ± 0.27	68.1^{c} ± 4.49			
Platelet ($\times 10^3$ cells/µL)	FB	772.9 ± 84.4	877.0^{xy} ± 68.7	855.1^{x} ± 61.7	807.3^{x} ± 60.7	690.9 ± 52.9	516.1^{a} ± 49.6	*	***	**
	FA	774.6 ± 84.7	846.4^{x} ± 68.9	882.0^{x} ± 61.9	696.0^{x} ± 60.9	691.5 ± 53.1	483.2^{b} ± 49.6			
	MB	838.7 ± 84.3	1066.4^{cy}± 68.6	1070.4^{cy}± 61.6	1031.7^{by} ±60.6	758.8 ± 52.8	543.1^{b} ± 49.4			
	MA	877.2 ± 84.1	1020.4^{ay}± 68.4	1005.5^{ay}± 61.5	996.0^{ay} ± 60.5	816.8 ± 52.8	440.5^{c} ± 49.3			

Females weaned beside the dam (FB; n = 7); Females weaned away from dam (FA; n = 7); Males weaned beside the dam (MB; n = 7); Males weaned away from dam (MA; n = 7). T = treatment, S = sampling time, T×S = treatment × sampling time interactions, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

Appendix 3. Effect of weaning induced stress on the relative gene expression of IL-1ß, IL-2, IL-4, CXCL8, IFN-γ, TNFα and lymphotoxin in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning				*P*-Values		
Variable		0	1	3	7	T	S	T×S
IL-1β	FB	11.8 ± 2.5	15.5 ± 3.9	18.7 ± 3.8	19.6^{b} ± 2.7	NS	***	NS
	FA	9.0 ± 2.5	13.4 ± 3.9	19.5^{a} ± 3.8	21.7^{c} ± 2.7			
	MB	6.6 ± 2.5	23.0^{c} ± 3.9	17.0^{a} ± 3.8	20.5^{c} ± 2.7			
	MA	7.6 ± 2.5	22.2^{c} ± 3.9	18.9^{b} ± 3.8	25.8^{c} ± 2.7			
IL-2	FB	9.9 ± 2.6	15.5^{b} ± 4.8	8.0 ± 3.8	11.8 ± 2.8	NS	NS	NS
	FA	9.6 ± 2.6	13.3 ± 4.8	8.7 ± 3.8	9.7 ± 2.7			
	MB	9.4 ± 2.6	14.6 ± 4.8	15.8 ± 3.8	9.2 ± 2.7			
	MA	9.3 ± 2.6	7.1 ± 4.8	7.8 ± 3.8	6.0 ± 2.7			
IL-4	FB	15.1 ± 3.2	15.6^{xy}± 5.4	8.3 ± 2.5	12.8 ± 3.1	NS	NS	NS
	FA	7.8 ± 3.2	13.0^{xy}± 5.4	10.5 ± 2.5	12.3 ± 2.9			
	MB	10.7 ± 3.2	24.9^{ax}± 5.4	13.5 ± 2.5	8.1 ± 2.9			
	MA	12.4 ± 3.2	7.7^{y} ± 5.4	9.5 ± 2.5	7.9 ± 2.9			
CXCL8	FB	5.1^{xy} ± 3.0	16.8^{a} ± 4.3	7.0 ± 2.2	12.4 ± 4.9	NS	*	NS
	FA	9.7^{x} ± 3.0	15.1 ± 4.3	12.5 ± 2.2	13.7 ± 4.9			
	MB	3.2^{y} ± 3.0	11.5 ± 4.3	9.9^{a} ± 2.2	5.5 ± 4.9			
	MA	4.4^{y} ± 3.0	8.0 ± 4.3	7.5 ± 2.2	6.9 ± 4.9			
IFN-γ	FB	21.6^{x} ± 4.8	66.8^{cx}± 13.7	43.7 ± 14.7	23.8± 10.7	NS	***	**
	FA	17.3^{xy}± 4.8	54.4^{bxy}±13.7	24.9 ± 14.7	40.1^{b}±10.1			
	MB	6.6^{y} ± 4.8	15.2^{y} ± 14.0	34.3^{c} ±14.7	21.6^{a}±10.1			
	MA	11.5^{xy}± 4.8	31.9^{ay}± 13.7	27.2 ± 15.1	17.6± 10.1			
TNF-α	FB	2.5 ± 0.4	4.8^{bx} ± 0.7	2.9 ± 0.5	4.4^{b} ± 0.7	NS	***	NS
	FA	2.4 ± 0.4	4.4^{bxy} ± 0.7	3.6^{a} ± 0.5	4.5^{c} ± 0.7			
	MB	2.6 ± 0.4	3.3^{xy} ± 0.7	3.9^{b} ± 0.5	3.2 ± 0.7			
	MA	2.3 ± 0.4	2.7^{y} ± 0.7	3.1 ± 0.5	3.0 ± 0.7			
Lymphotoxin	FB	106.2 ± 19.9	133.0 ± 27.9	96.8 ± 17.2	116.9^{x}±13.7	NS	NS	NS
	FA	99.9 ± 17.5	89.9 ± 27.9	87.9 ± 16.8	93.8^{xy}± 13.7			
	MB	90.3 ± 17.9	102.7 ± 27.9	80.7 ± 16.8	64.0^{ay}± 13.7			
	MA	79.8 ± 17.5	76.7 ± 27.9	59.9 ± 16.8	71.3^{y} ± 13.7			

Females weaned beside the dam (FB; n = 7); Females weaned away from dam (FA; n = 7); Males weaned beside the dam (MB; n = 7); Males weaned away from dam (MA; n = 7). T = treatment, S = sampling time, T × S = treatment × sampling time interactions, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

Appendix 4. Effect of weaning induced stress on the relative gene expression of nuclear factor kappa B (NFκB), p21, CD62l, haptoglobin and bactericidal/permeability increasing protein (BPI) in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning				*P*-Values		
Variable		0	1	3	7	T	S	T×S
NFκB1	FB	2.4 ± 0.5	3.0 ± 0.5	2.6 ± 0.5	2.9^{xy} ± 0.5	NS	*	NS
	FA	2.4 ± 0.5	3.4 ± 0.5	2.7 ± 0.5	4.1^{bx} ± 0.5			
	MB	2.5 ± 0.5	3.1 ± 0.5	3.0 ± 0.5	2.5^{y} ± 0.5			
	MA	2.7 ± 0.5	3.1 ± 0.5	3.1 ± 0.5	2.8^{xy} ± 0.5			
NFκB2	FB	2.4 ± 0.5	2.8 ± 0.5	3.1 ± 0.4	3.0 ± 0.4	NS	NS	NS
	FA	2.3 ± 0.5	3.2 ± 0.5	2.6 ± 0.4	2.8 ± 0.4			
	MB	3.5 ± 0.5	2.9 ± 0.5	2.7 ± 0.4	2.9 ± 0.4			
	MA	3.1 ± 0.5	3.0 ± 0.5	2.3 ± 0.4	2.4 ± 0.4			
p21	FB	6.3 ± 1.7	4.1 ± 0.9	4.0 ± 1.4	5.7 ± 1.4	NS	NS	NS
	FA	4.6 ± 1.7	6.5 ± 0.9	6.9 ± 1.4	5.7 ± 1.4			
	MB	6.2 ± 1.7	3.8 ± 0.9	6.8 ± 1.4	6.2 ± 1.4			
	MA	7.9 ± 1.7	5.4 ± 0.9	5.2 ± 1.4	4.9 ± 1.4			
CD62L	FB	29.3 ± 8.5	32.8 ± 9.5	35.8 ± 7.8	42.2^{a} ± 8.9	NS	***	NS
	FA	23.5 ± 8.5	31.9 ± 9.5	42.2^{b} ± 7.8	46.3^{b} ± 8.9			
	MB	22.0 ± 8.5	37.6 ± 9.5	41.4^{b} ± 7.8	34.2 ± 8.9			
	MA	29.3 ± 8.5	36.8 ± 9.5	30.6 ± 7.8	37.6 ± 8.9			
Haptoglobin	FB	12.8 ± 5.8	11.3 ± 9.0	13.0 ± 7.1	13.2 ± 5.1	NS	NS	NS
	FA	6.5 ± 5.8	30.2^{b} ± 9.0	25.6^{b} ± 7.1	20.1^{b} ± 5.1			
	MB	18.1 ± 6.1	9.9 ± 9.0	16.4 ± 7.1	13.2 ± 5.1			
	MA	12.1 ± 5.8	13.9 ± 9.0	15.6 ± 7.1	14.3 ± 5.1			
BPI	FB	20.7 ± 4.7	15.1 ± 4.7	16.0 ± 3.8	16.2 ± 3.5	NS	***	NS
	FA	15.3 ± 4.7	16.9 ± 4.7	13.4 ± 3.8	11.2 ± 3.5			
	MB	17.5 ± 4.7	22.1 ± 4.7	16.8 ± 3.8	7.3^{b} ± 3.5			
	MA	19.6 ± 4.7	12.8 ± 4.7	12.8 ± 3.8	9.5^{b} ± 3.5			

Females weaned beside the dam (FB; n = 7); Females weaned away from dam (FA; n = 7); Males weaned beside the dam (MB; n = 7); Males weaned away from dam (MA; n = 7). T = treatment, S = sampling time, T × S = treatment × sampling time interactions, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

Appendix 5. Effect of weaning induced stress on the relative gene expression of toll-like receptor (TLR) 4, glucocorticoid receptor (GR)α and Fas in weaned beef calves. The values are expressed as least squares means (Lsmeans) ± s.e.

		Days Post Weaning				*P*-Values		
Variable		0	1	3	7	T	S	T×S
TLR4	FB	5.6 ± 1.9	6.2 ± 2.2	7.6 ± 2.3	11.8^{a} ± 3.8	NS	***	NS
	FA	4.0 ± 1.9	6.9 ± 2.2	9.1^{b} ± 2.3	9.0 ± 3.8			
	MB	4.2 ± 1.9	9.1^{a} ± 2.2	8.8^{b} ± 2.3	11.1^{b} ± 3.8			
	MA	6.8 ± 1.9	11.0^{a} ± 2.2	10.1 ± 2.3	14.2^{b} ± 3.8			
GRα	FB	8.6 ± 1.3	27.5^{cx} ± 4.8	13.3 ± 6.1	22.9^{a} ± 5.9	NS	***	NS
	FA	6.7 ± 1.3	20.6^{bxy} ±4.8	16.3^{a} ± 6.1	16.2^{a} ± 5.9			
	MB	4.8 ± 1.3	14.9^{ay} ± 4.8	25.3^{c} ± 6.1	14.1^{a} ± 5.9			
	MA	5.6 ± 1.3	16.1^{axy} ±4.8	13.1 ± 6.1	13.1 ± 5.9			
Fas	FB	11.7 ± 2.9	31.6^{a} ± 10.4	20.1 ± 9.1	40.7^{a} ± 14.9	NS	***	NS
	FA	10.2 ± 2.9	33.6^{b} ± 10.4	22.8 ± 9.1	25.2 ± 14.9			
	MB	7.4 ± 2.9	31.2^{a} ± 10.4	29.0^{c} ± 9.1	33.4^{a} ± 14.9			
	MA	9.4 ± 2.9	38.1^{b} ± 10.4	33.8^{b} ± 9.1	41.6^{b} ± 14.9			

Females weaned beside the dam (FB; n = 7); Females weaned away from dam (FA; n = 7); Males weaned beside the dam (MB; n = 7); Males weaned away from dam (MA; n = 7). T = treatment, S = sampling time, T × S = treatment × sampling time interactions, 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 $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

[x,y]Between rows, Lsmeans differ between treatments by $P < 0.05$.

Further supplementary data relating to Chapter 5 can be found in the attachment entitled "Supplementary data for RNA-seq data."