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Interactions between the neurophysiological and immune systems as objective measures of animal welfare

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Abstract

Objective measures are essential to provide a scientific basis for assessing the welfare of sheep and cattle in Australian production systems. The aim of the research described herein was to develop methodologies to examine the link between neurophysiological changes associated with stress responses and the function of peripheral blood leukocytes and to apply these methodologies to investigate responses to chronic stressors in order to provide information on pre-pathological states indicative of an animal's failure to adapt and cope.

The results represent the utilisation of leukocytes as target tissues to measure cellular stress responses in sheep and cattle. A panel of leukocyte markers has been identified that will be suitable for application to a range of studies to underpin animal welfare research. Selected measures have been applied to three experimental models of stress in sheep and cattle. These experimental models included animal welfare outcomes of livestock road transport practices (sheep), the development of a fear model of chronic stress in cattle, and the amelioration of heat stress in feedlot cattle by dietary means.

Executive Summary

The welfare status of sheep and cattle in the production environments and management systems used in Australia is attracting increased community attention as attitudes towards animals change. Objective measures are needed to provide a scientific basis for assessing the welfare of sheep and cattle in Australian production systems.

The aim of the current studies was to identify a panel of leukocyte markers, immune responses and biochemical changes that can be employed in the monitoring of neurophysiological responses to stress in cattle and sheep and that will provide scientifically-defensible objective measures of animal welfare.

The objectives of the current project were through postdoctoral research to:

- Acquire a detailed knowledge of interactions between stress response outputs of the nervous system and immune function in the disciplines of psychoneuroimmunology and stress physiology
- Develop laboratory methods to investigate interactions between the nervous system and immune function
- Apply the methods above to measure nervous system interactions with immune function in experimental models of stress under investigation through Meat and Livestock Australia's Animal Welfare Objective Measures Research Program
- Present scientific results in seminars, in scientific papers in international refereed journals and at conferences
- Undergo training to develop skills for the efficient performance of scientific research and for project development

This project has developed and provided technical validation of a suite of new assays for measuring interactions between the neurophysiological and immune systems of cattle and sheep and has commenced the process of identifying the impact of husbandry stressors on the biological pathways that the assays monitor. The assays are available now for use by animal welfare scientists, and if further investment is made in their application by the Australian Meat and Livestock Industry, they have the potential within five years to be a substantial component of a framework for objective assessment of animal welfare and a valuable tool for description of the welfare outcomes of husbandry practices.

The project has also built capacity through the development of skills and methodologies and their transfer to other welfare researchers though scientific publications and collaborations in linked projects.

Contents

1 Background

Since the field of animal welfare science was established approximately 40 years ago (Mench, 1998) many advances in animal welfare research have been made, however substantial knowledge gaps remain and as such there is a need to enhance the scale and focus of research in this field. The welfare status of sheep and cattle in the production environments and management systems used in Australia is attracting increased community attention as attitudes towards animals change. There is an increasing recognition in the community that animals possess an intrinsic value, that they are important sources of food, fibre and other products, that animals play a key role in the history and development of our nation and that animal

industries have an ongoing economic importance to Australia. In addition, there are increasing requirements for accreditation of the animal welfare credentials of products from farm animals to maintain access to international markets. Objective measures are needed to provide a scientific basis for assessing the welfare of sheep and cattle in Australian production systems.

It is well recognised that stress can compromise the capacity of the immune system to protect against disease. In cattle this link between stress and immune function underlies the increased risk of bovine respiratory disease (shipping fever) at feedlot entry. Previous work in the CRC for Cattle and Beef Quality developed a panel of tests to measure immune competence in cattle and examined the impact of a range of husbandry practices on these measures of immune competence. A number of cell surface markers on leukocytes were sensitive indicators of stress responses in cattle. The opportunity now exists to extend these early findings through focussed studies on the link between neurophysiological changes associated with stress responses and the function of peripheral blood leukocytes. The rationale for this approach is two fold. Firstly, neurophysiological changes are an important component of the generalised stress response and are likely to contribute to changes in behaviour and the emotional state of animals. However, measuring neurophysiological changes is difficult. Peripheral blood leukocytes express receptors for many hormones and neurotransmitters and traffic through tissue sites such as peripheral lymph nodes where they are exposed to neurotransmitters such as neuropeptide Y before returning to the peripheral circulation. Within the peripheral circulation they provide an easily accessible population of cells that can be readily assayed. Peripheral blood leukocytes might therefore provide a window on neurophysiological changes. The second rationale moves on from using peripheral blood leukocytes as a proxy for neurophysiological changes associated with stress to examine the biological significance of the links between the nervous system and immune function.

Immune measures assess the competence of an animal's immune system. Immunocompetence, which is clearly important in resistance against disease, may be compromised by welfare challenges. Immune measures include the relative populations of white blood cells, laboratory-based assays of white blood cell function, and measurement of biochemical analytes produced by or relevant to the immune system and disease (e.g. interleukins, interferons, acute phase proteins). Measurement of immune competence has two potential applications. Firstly, acute changes in immune function following exposure to a stressor provide an additional measure that integrates a number of the homeostatic drivers of the impact of the stressor on the animal. Secondly, basal immune function when an animal is stabilized or adapted within its production environment is likely to be associated with disease risk. Thus immune measures can provide a warning of decreased disease resistance and the pre-pathological consequences of welfare challenges for an animal (Fell *et al.,* 1999); however, to this point they remain difficult to measure outside the laboratory, and there has been insufficient effort to identify widely-applicable and easily measurable markers of immunocompetence. These factors highlight the importance of developing scientifically robust models to examine neurophysiological and immune system interactions in response to stress.

The concept of "bidirectional communication" between immune and neuroendocrine systems has its molecular basis in the commonality in the pathways of action of immunoregulators, neurotransmitters, hormones and the expression of their respective receptors. The immune system and hypothalamic-pituitary-adrenal (HPA) axis are intricately connected. It is well established that chronic and acute stress, both impact the functioning of the HPA axis. For example, it is well established that neuropeptide Y exerts potent immunologic effects both *in vitro* and *in vivo.* These include differentiation of T helper cells, monocyte mediator release, NK cell activation and immune cell redistribution (reviewed in Bedoui *et al.,* 2003). A bimodal role for the Y1 receptor of neuropeptide Y on immune cells has been suggested – strong negative regulator of T cells and a key activator of APC function (Whenway, 2005). It is also well established that glucocorticoids are a family of steroids necessary for the regulation of energy metabolism and the immune and inflammatory responses. These compounds exert their effect through their interaction with the glucocoticoid receptor (GR) and that complex's subsequent association with DNA. All normal mammalian tissues examined to date have been shown to contain glucocorticoid receptor

The heat shock or stress proteins, which are among the most highly conserved proteins in nature, are found in all organisms from bacteria to humans and are induced in response to a mild, generally non-lethal stress. These proteins were originally observed in cells exposed to a mild heat shock and hence the name heat shock proteins (hsps). Later experiments reported that these proteins were also induced on exposure of cells to environmental stressors other than heat, such as inflammation, microbial infection (viral and

bacterial), psychological stress, oxidative stress and cytotoxins. The more general term ‘stress protein’ has thus been applied to this set of proteins, however it is important to note that stress proteins are also expressed constitutively in the unstressed cell making them ideal candidates for examination at both basal levels and following an acute stress event.

An important physiological function for the hsps is their role in the assembly and transport of newly synthesised proteins within cells, as well as in the removal of denatured proteins. The hsps are therefore important in preventing damage and in cellular repair processes after injury. There is well documented evidence that increased production of hsps protect cells against subsequent lethal stress induced by e.g. oxidative stress, cytotoxins, heat stress and cellular damage after ischaemia or sepsis-induced injury. There is now increasing evidence that the hsps play key roles as prominent antigens in the humoral and cellular immune responses mediated by antibodies and T cells, respectively (Zugel & Kaufmann, 1999; Basu & Srivastava, 2000).

The roles of hsps in immune response can be characterised by three main categories. The first involves the ability of hsps of the cytosol and the endoplasmic reticulum to non-covalently bind immunogenic peptides such as MHC class I epitopes and their elongated precursors. Secondly, hsp-peptide complexes are able to be cross-presented to CD8 T lymphocytes via the MHC class I pathway. It has been proposed that hsp-chaperoned antigenic peptides are endocytosed by antigen presenting cells (APC) through the common heat shock protein receptor CD91. Thirdly, hsps have the ability to promote dendritic cell maturation and the release of pro-inflammatory cytokines. These characteristics of hsps make them prime candidates to study the links between the stress response and the immune response.

The aim therefore of the current studies was to identify a panel of leukocyte markers, immune responses and biochemical changes that can be employed in the monitoring of neurophysiological responses to stress in cattle and sheep and that will provide scientifically-defensible objective measures of animal welfare.

2 Project Objectives

Through postdoctoral research to:

- Acquire a detailed knowledge of interactions between stress response outputs of the nervous system and immune function in the disciplines of psychoneuroimmunology and stress physiology
- Develop laboratory methods to investigate interactions between the nervous system and immune function
- Apply the methods above to measure nervous system interactions with immune function in experimental models of stress under investigation through Meat and Livestock Australia’s Animal Welfare Objective Measures Research Program
- Present scientific results in seminars, in scientific papers in international refereed journals and at conferences
- Undergo training to develop skills for the efficient performance of scientific research and for project development

3 Methodology

3.1 Methodological Development

During the course of this project methodologies have been developed to examine the interactions between the neurophysiological and immune systems of livestock. Specific protocols for each methodology are presented in Appendix I of this report.

3.1.1 Flow cytometry

3.1.1.1 Measurement of hsp70 expression in ovine and bovine CD14+ monocytes

Stress proteins or heat shock proteins (hsp) as they are otherwise known, are a group of highly conserved proteins that represent between 2% and 15% of total cellular protein and are expressed by every living organism (Morimoto *et al.,* 1994). As the name implies they are expressed when cells are exposed to stressful stimuli such as: free radical attack, UV light, ozone or fever, however they are also expressed constitutively in the unstressed cell (Welch, 1992). These proteins allow cells to adapt to gradual changes in their environment. The main functions of hsps are to regulate apoptosis and to act as intracellular molecular chaperones that facilitate protein folding, biogenesis and assembly (Morimoto *et al.,* 1994). The stress proteins belong to a multigene family and range in size from 8 to 150 kDa. Hsps are classified according to their molecular weight, for example the 70 kDa protein is named hsp 70, and each hsp species has a unique mechanism of action (Craig *et al.,* 1993, Whitley *et al.,* 1999).

It has been reported that flow cytometry is a rapid, easy and quantitative method of determining intracellular heat shock protein (hsp) 70 expression, in individual cells from a heterogeneous population such as peripheral blood mononuclear cells (PBMC) (Bachelet *et al.,* 1998). Flow cytometric methods have also demonstrated that hsp70 is constitutively expressed in human leukocytes but that the level of expression varies considerably between different cell types (Oeheler *et al.,* 2001). This method holds several advantages over the more traditional methods of determining levels of heat shock protein expression, such as western immunoblot and 1D-SDS-PAGE. These advantages include the specific information provided by this technique as to which cells are expressing the protein, and the significantly smaller volume of blood required to conduct the flow cytometric analysis.

The literature clearly indicates that the optimal temperature of a mild, non-lethal heat shock of human leukocytes to examine the induction of stress proteins is 42.5 °C. There is, however, no clear indication in the literature as to whether this temperature is also applicable to bovine and ovine blood samples. The current studies aim to determine the optimal experimental conditions for the measurement of leukocyte hsp70 in cattle and sheep leukocytes.

3.1.1.2 Determination of leukocyte phenotype following cryopreservation of whole blood samples

Immunophenotyping of leukocyte subsets in peripheral whole blood is a valuable tool used to evaluate immune system changes. Pinto *et al.,* (2005) reported that fixing and freezing of human whole blood and isolated peripheral blood mononuclear cells (PBMCs) before or after cell surface staining was a reliable method for later determination of cell surface subset percentages by flow cytometry. There are currently no reports of this methodology being applied to blood samples from sheep or cattle. The vast majority of analyses of cell surface and intracellular molecules by flow cytometry require fresh blood samples therefore development of methodologies that preserve the viability of samples without altering the cellular phenotype would be an invaluable tool for the current project and any future research in this area. The issue of lack of access to fresh blood samples is a common problem as many livestock research projects are conducted in remote sites without access to a flow cytometer. The current studies seek to examine whether fixing whole blood samples, then freezing at -80°C prior to staining for determination of leukocyte cell surface markers (CD4, CD8, WC1 [gamma-delta T cell receptor] and BB2 [B cell marker] and an intracellular molecule (glucocorticoid receptor) is a method that has the potential to be applied to livestock trials.

3.1.1.3 Use of a cryopreservation system for immunophenotyping livestock blood samples

Access to fresh blood samples is a common problem encountered by researchers when samples are collected from remote or on-farm sites that do not have access to a flow cytometer or personnel trained in

the required staining techniques. The methodology developed in milestone 1 (Method 2: Determination of leukocyte phenotype following cryopreservation of whole blood samples) displayed potential for application to cattle blood samples, however was deemed unsuitable for use in sheep samples.

The development of a cryopreservation system for human lymphocyte phenotyping was driven by the need for a method to immunophenotype blood samples from HIV-positive patients in remote and resource-poor settings without access to suitably equipped laboratory facilities. It has recently been reported that human whole blood samples collected in Cyto-Chex® blood collection tubes are stabilised for marker phenotype for at least 7 days following blood collection (Warrino *et al.,* 2005).

The current studies seek to determine whether sheep and cattle peripheral blood collected in CytoChex® blood collection tubes can be used for determination of percentages of leukocyte cell surface markers (CD4, CD8, WC1 [gamma-delta T cell receptor] and BB2 [B cell marker] and the intracellular molecule (glucocorticoid receptor [GR]).

3.1.1.4 Measurement of glucocorticoid receptor in leukocyte subpopulations

At the cellular level, the actions of glucocorticoids, such as cortisol, are mediated by their interaction with the intracellular glucocorticoid receptor. The glucocorticoid receptor (GR) is a member of the ligand-dependant nuclear receptor family whose role is to integrate host immune responses with physiological homeostatic balance that includes the repression of transcriptional responses to inflammatory signals. Specifically, it has been demonstrated that GR can interfere with the activities of a number of signal-dependant transcription factors including NF-κB and activator protein 1 (AP-1) family members (reviewed in Ogawa *et al.,* 2005).

The aim of the current study was to determine whether glucocorticoid receptor expression could be measured in cattle and sheep leukocyte sub-populations using fluorescein-conjugated dexamethsone (a corticosteroid analog and GR ligand).

3.1.1.5 Modification of staining techniques for flow cytometry to suit a 96-well plate format

Flow cytometric methods are frequently used in the laboratory for the determination of expression levels of immunological markers. The methods used for such staining are well developed, however when large numbers of samples are received it becomes difficult for one person to complete such staining protocols in a timely manner. A number of researchers have adapted staining protocols to suit a 96-well plate format to allow the processing of a larger number of samples at the same time. Methods developed in the course of the current project have therefore been adapted to suit such a format.

3.1.2 Western immunoblot

Western immunoblotting is a semi-quantitative method of detecting a protein of interest in a given sample of tissue homogenate or extract. Gel electrophoresis is used to separate proteins which are then transferred to a membrane which, in turn, is probed with an antibody specific for the target protein. Protein levels in samples can be quantitated using densitometric analysis. The method is also useful for determining whether an antibody is specifically binding the target protein.

3.1.3 Immunohistochemistry

Immunohistochemistry is a method used to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.

3.1.4 ELISA

Although stress proteins were traditionally considered to be intracellular molecules, the discovery of hsp60 and hsp70 in the peripheral circulation of ‘normal’ individuals has led to the suggestion that hsps may, in

fact, also be intercellular signalling molecules involved in the regulation or prevention/protection against pathophysiological processes involving immunorecognition or cross-recognition of hsps (Pockley *et al.,* 1998). Whether the release of hsp70 into the circulation is due to cell damage or via active secretion remains to be determined. It has been suggested that an increase in serum hsps may be a biomarker for disease as increases in circulating hsp have been detected in patients with infections (Giraldo *et al.,* 1999; Njemini *et al.,* 2003), cardiovascular disease (Pockley *et al.,* 2000; Xu *et al.,* 2000) renal vascular disease (Wright *et al.,* 2000), pre-eclampsia (Jirecek *et al.,* 2002) and diabetic ketoacidosis (Oglesbee *et al.,* 1995). A study by Pittet *et al.* (2002) detected hsp70 in the serum of severely traumatised patients within 30 minutes of sustaining an injury.

3.1.5 qRT-PCR

Real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a method that provides accurate and reproducible quantitation of gene copies (Heid *et al.,* 1996). The current studies aim to validate the use of a set of primers for the detection of hsp70 by real time qRT-PCR in future studies.

3.1.6 Oxidative stress

Free radicals are highly reactive molecules containing one or more unpaired electrons, that originate from nitrogen (reactive nitrogen species RNS) or oxygen (reactive oxygen species ROS). ROS are derived primarily from oxygen as a part of normal metabolism and biological processes including muscle contraction, hormone secretion and immune defence processes (Halliwell & Gutteridge, 1999). ROS are also generated by a myriad of other mechanisms including: cigarette smoke, environmental pollutants, UV light, ionizing radiation and virus induced activation of phagocytes. Examples of ROS include hydrogen peroxide (H_2O_2), the hydroxyl radical (OH*), superoxide (O_2*), hyperchlorous acid HOCl), the peroxyl radicals (ROO*) and singlet oxygen ($^1\Delta O_2$). Oxidative stress can be defined as the disturbance in the balance between production of ROS (free radicals) and an organism's antioxidant defenses (Sies, 1991). These antioxidant defence systems can be enzymatic or non-enzymatic. The enzymatic system couples metal ions such as Cu, Mn, Se and Zn, with the active site of metal-ion-dependant enzymes including superoxide dismutase, catalase and glutathione peroxidase. Antioxidants that are nonenzymatic include vitamin C, vitamin E and β-carotene.

If the oxidant/antioxidant balance is tipped in favour of oxidative stress, the result can be lipid peroxidation, protein oxidation and DNA damage (Halliwell & Gutteridge, 1999). Three possible mechanisms of imposing oxidative stress are: a decrease in antioxidants, an increase in the production of ROS, or an increase in the availability of transition metal ions so that a greater proportion of metabolically produced radicals such as O_2^{*-} and H_2O_2 are converted to damaging OH^* radicals (Gutteridge & Halliwell, 1994).

It has been suggested that the immune system may be particularly susceptible to oxidative stress, hence oxidative stress has been implicated in the pathophysiology of a number of disease states such as arthritis (Maurice *et al.,* 1997), arteriosclerosis (Halliwell & Gutteridge, 1999), cancer and neurodegenerative disorders such as Alzheimer's and Parkinson's Disease (Halliwell & Gutteridge, 1999).

3.1.6.1 Lipid Peroxidation

Lipid peroxidation is a radical-initiated chain reaction that is initiated by the abstraction of a hydrogen atom from an unsaturated fatty acid and is self-propagating in cell membranes. Once a hydrogen atom is removed from a second lipid, a new ROS is produced facilitating the formation of numerous products. The greater the number of double bonds in a fatty acid side chain, the easier the removal of a hydrogen atom, hence monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) are more resistant to free radical attack than are polyunsaturated fatty acids (PUFA) (Halliwell & Chirico, 1993). PUFAs are major constituents of cellular membranes. Once a cell membrane has undergone lipid peroxidation, rigidity results, which in turn can give rise to a loss of selective permeability and, at worst, a loss of integrity (Davies, 2000). A measure of oxidative stress is the detection, in plasma, of lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAD). The aldehydes are water-soluble products and as such are able to diffuse from membranes into other subcellular compartments (Davies, 2000). It has been suggested that lipid peroxidation products can also act as cross-linking reagents that

may play a role in protein aggregation, inhibition of enzyme function and the formation of mutation-inducing DNA adducts (Davies, 2000).

3.1.6.2 Protein Oxidation

Metal-catalyzed oxidation of proteins may result in introduction of carbonyl groups into amino acid residues of proteins, which are detectable by reaction of these groups with carbonyl-specific reagents (Levine *et al.,* 1990). Metal catalyzed protein oxidation may also result in cross-linking of proteins or protein fragmentation. The mechanism by which metal-catalyzed oxidation of proteins occurs involves a metal cation capable of redox cycling binding to a divalent cation binding site on the protein. Reaction with O_2 or H_2O_2 generates a ROS capable of oxidizing amino acid residues at or near the cation-binding site (Stadtman, 1989). Other methods of detection and quantitation of protein modification include loss of catalytic activity, loss of histidine residues, changes in surface hydrophobicity, and changes in the ultraviolet spectrum of the protein (Levine *et al.,* 1990). Subsequent to ubiquitination, oxidatively modified proteins are selectively degraded, by multicatalytic proteinase complexes called proteasomes, to amino acids, which are reused for *de novo* protein synthesis (Davies, 1987). Oxidized amino acids are either eliminated or used as carbon sources for ATP synthesis (Davies, 2000). Periods of prolonged oxidative stress may be problematic, however, if the proteolytic system is unable to maintain adequate levels of digestion, as oxidized proteins may cross-link or form extensive hydrophobic bonds, which are detrimental to normal cell functions (Davies, 2000).

3.2 Methodological Application to Experimental Models of stress

Selected methodologies from Section 3.1 were applied to three experimental models of stress in livestock.

3.2.1 Animal welfare outcomes of livestock road transport practices

3.2.1.1 Background

The research reported here represents a subset of immunological data collected from the fourth in a series of investigations examining the animal welfare outcomes of land transport practices for cattle and sheep (AHW.055) where animal welfare outcomes were determined during and post-transport for different curfew and transport duration combinations. This project was designed to develop scientifically defensible quantification of the animal welfare outcomes of specific Australian livestock transport practices.

Curfew is the generic term used in livestock industries for the practice of enforced food or food and water deprivation prior to transport, sale or slaughter. During transport, it is inevitable that there will be short to moderate periods of restricted access to food and water. Whilst food and water deprivation will normally occur during transport, the period of deprivation can be substantially extended if animals undergo a significant curfew period prior to transport. Curfews are applied because of the demands and selling conditions of transport operators, livestock buyers and abattoir management, respectively. Curfews are typically 6 – 12 h in duration and the primary reason for their use is to reduce the gastrointestinal volume (empty out) prior to transport, thus reducing the total amount of excreta in trucks and the level of faecal soiling on animals which can have impacts for food safety.

3.2.1.2 Aim

The aim of this experiment was to quantify the effect of pre-transport food and water deprivation (curfew) on the immunological response to transport in a small cohort of Merino lambs and to validate the use of the glucocorticoid receptor assay developed in B.AWW.0184 in experimental models of stress.
A total of one hundred and eighty Merino lambs were used in the experiment. The lambs were approximately 6-7 months of age and comprised both ewes and wethers. Of the total, there were 120 focal animals (all ewes) where detailed measurements were recorded. For the current experiments, a small sub-set of animals was selected from two separate transport journeys of identical length, one included a curfew period and the other did not n = 6 animals in each transport journey.

The experiment was approved by the CSIRO Livestock Industries FD McMaster Animal Ethics Committee (Approval No. 07/05).

3.2.1.3 Experimental treatments

For the current studies a small subset of the cohort was randomly selected from two truck journeys of identical length (24 h) (TJ4 – animals were sampled pre-transport, on arrival and 24 h after arrival; TJ6 animals underwent a 24 h food and water curfew prior to transport, followed by the same sampling regime as for TJ4).

3.2.1.4 Blood sampling

Each animal was blood sampled via jugular venipuncture on 6 occasions; prior to and on completion of the curfew period and 0, 24, 48 and 72 h post-transport. The sample taken on completion of the curfew treatment was also defined as the pre-transport sample. Two blood samples were taken at each time point (6 ml EDTA and 10 ml serum vacutainers).

An aliquot of whole blood was analysed for the haematology variables haemoglobin (HGB), red cell counts (RCC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), white blood cells count (WBC) and differential cell counts of neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), using a Cell-Dyn Haematology Analyser 3500R (Abbott Diagnostics, CA, USA).

Detection of intracellular glucocorticoid receptor (GR) was conducted using the appropriate extracellular marker antibodies in conjunction with intracellular fluorochrome-labelled antibodies. These assays were performed on blood samples collected prior to and on completion of the curfew period and 0 and 24 h post-transport.

Serum cortisol was determined using a Spectria Cortisol RIA (Orion Dianostica, Espoo, FIN).

3.1.2.5 Statistical analysis

Prior to analysis data was transformed using either log (base e) or square root arcsine transformations to improve data normality The significance of treatment differences was determined using ASREML (UNIX version 1.99c). The effect of treatment on haematological values, serum cortisol and leukocyte glucocorticoid receptor expression were determined using a repeated measures model with time x treatment fitted as fixed effects, pre-treatment levels fitted as covariates and animal fitted as a random effect. Non-significant fixed effects were removed from the model prior to final analysis. P values < 0.05 were considered significant.

3.2.2 Development of a fear model of chronic stress in cattle

3.2.2.1 Background

The research reported here represents a subset of immunological data collected from the “Development of a fear model of chronic stress in cattle”. This protocol was part of the Cooperative Research Centre (CRC) for Beef Genetic Technologies, under program 3 of the CRC, ‘Adaptation and Animal Welfare’. Program 3 aims to develop and determine robust, scientifically defensible measures and critical thresholds that define the welfare status of cattle when exposed to conditions that elicit a sustained stress response; it is anticipated that these thresholds (or lower levels) will be available for use by animal welfare policy makers, regulatory agencies and the beef industry by 2011. Stress models being used are chronic fear and nutrient deprivation. This protocol is for the chronic fear model.

The experiment in this protocol (and others to be conducted at other locations) will also serve to quantify gene expression changes in peripheral tissues in response to chronic fear and nutrient deprivation. The decision to undertake these analyses will be predicated on the evidence that HPA (hypothalamic-pituitary-adrenal) dysfunction was achieved.

3.2.2.2 Aim

To develop a chronic fear stressor model in cattle and to quantify the behavioural and physiological responses of the animals. The latter will include leukocyte gene expression and cellular markers of stress to identify relevant biochemical pathways, both partially understood (eg. HPA axis).

The experiment used a total of 36 Murray Grey calves (steers) that were about 10 months of age with similar experiential and genetic backgrounds. There were 3 treatments (control, procedures imposed 3 times weekly and procedures imposed 5 times per week – see below). The experiment involved 3 time replicates of 12 experimental animals per replicate. Animals were housed in 12 individual pens 4 x 5 m, with 4 animals of each treatment represented in each replicate.

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3.2.2.3 Experimental treatments

Electrical stimuli

Numerous studies indicate that electric shocks elicit acute behavioural and physiological stress responses. Laboratory studies using a variety of mammals have shown the effectiveness of electric shock to study traumatic avoidance learning and handling studies with farm animals have shown that electric stimulation activates the HPA axis.

Appearance of a novel object

The appearance of a novel object stimulus will involve novel objects (rubber snake, physical prod, soft object {ball/plastic container} falling from roof). These will be released from behind a solid section at the front of the pens or outside of the shed so that animals cannot view the human and the objects will suddenly appear close to the animal. The objects will be used in a pseudo-randomised manner so that over the period of one week each animal receives a similar number of exposures for each object.

Flashing lights

There is little information in the literature on the effects of flashing lights on the acute behavioural and physiological stress responses. However anecdotal observations by the authors indicate at least acute behavioural responses to flashing lights. Unfamiliar flashing lights are likely to be perceived by animals as novel and high intensity stimuli, situations in which Gray (1987) reports will elicit fear.

Unpredictability

Predictability and control of the environment are important for animals. The response to aversive stimulation is always likely to be more effective (ie assist coping) if there is some accurate anticipation of what is likely to happen and if there is some control (eg. avoidance responses are possible). In contrast, unpredictability of aversive stimulation challenges coping.

Animals were randomly allocated to 1 of 3 treatments which were imposed over 4 weeks:

1. Control – animals wore an electronic shock collar but received no electrical stimulation or other stimuli.
2. Exposure to fear-provoking stimuli (3 times per week) – animals were exposed to 5 fear-provoking stimuli over a period of 20 min on each of the three days/week. The stimuli included the appearance of a novel object, flashing lights and electric shock and only one stimulus was used on each day of exposure; the treatment to be imposed was selected in a random manner. For the electric shock, the animals wore an electronic shock collar used to train dogs (pulse of 65 V at 45 mA for duration of 2 s). Intensity and characteristics of the lights were varied to increase variability and thus unpredictability. The treatments occurred in the home pen.
3. Exposure to fear-provoking stimuli (5 times per week) – The same treatment as above except that the fear-provoking stimuli occurred 5 days per week over the 4 week period.

3.2.2.4 Blood sampling

Blood samples were collected from each animal at four time points (pre-treatment, mid-way through treatment cycle, post-treatment and recovery). Samples were collected by jugular venipuncture into 1 x Cytochex blood collection tube and 6 x EDTA tubes. Cytochex blood collection tubes were kept refrigerated and transported on ice to CSIRO Armidale within 24 h of collection. EDTA tubes were centrifuged at 2500 rpm for 10 min. Plasma was collected and frozen at -20°C. Analyses completed on blood samples included leukocyte phenotyping, expression of intra-cellular glucocorticoid receptor (GR) in leukocyte subpopulations and haematology.

The blood samples were used to determine whether the exposure of animals to fear stress provokes an immune response and whether the markers examined may be useful in identifying which animals are more prone to suffer from chronic fear stress.

An aliquot of whole blood was analysed for the haematology variables haemoglobin (HGB), red cell counts (RCC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), white blood cells count (WBC) and differential cell counts of neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), using a Cell-Dyn Haematology Analyser 3500R (Abbott Diagnostics, CA, USA).

Leukocyte phenotyping using flurochrome-labelled extracellular marker antibodies was conducted using methods as previously reported (B.AWW.0184 Milestone 1). Detection of intracellular glucocorticoid receptor (GR) was conducted using the appropriate extracellular marker antibodies in conjunction with intracellular fluorochrome-labelled antibodies as previously reported (B.AWW.0184 Milestone 3 interim report).

In addition RNA was isolated from blood samples for microarray analysis of a selected set of stress induced leukocyte changes (to be completed on the basis of demonstrated immunological changes from the complete data set).

3.2.2.5 Statistical analysis

Prior to analysis data was transformed using either log (base e) or square root arcsine transformations to improve data normality The significance of treatment differences was determined using ASREML (UNIX version 1.99c). The effect of treatment on haematological values, extracellular markers and leukocyte glucocorticoid receptor expression were determined using a repeated measures model with time x treatment fitted as fixed effects, pre-treatment levels fitted as covariates and animal fitted as a random effect. Non-significant fixed effects were removed from the model prior to final analysis. P values < 0.05 were considered significant.

3.2.3 Amelioration of heat stress in feedlot cattle by dietary means

3.2.3.1 Background

Mortality and stress caused by the onset of heat waves is a major concern to feedlot operators, because of the financial impact and adverse publicity. Major efforts have been made to ameliorate the impact of such events, in the form of provision of shade and water sprays in feedlot, but additionally it has been suggested that appropriate management of feeding time and dietary composition may reduce the effects of environmental heat stress by avoiding coincidental peaks of heat from the environment and metabolic sources; the latter provides about 75% of total heat load on the feedlot animal. Body temperature in mammals follows a circadian rhythm, typically of magnitude between 0.5 and 1.2^{o}C but may be 3^{o}C in cattle subject to heat stress, at which stage rate of cellular metabolism increases significantly and adds to the heat load. Conditions in the proposed experiment will be set to avoid severe heat stress.

It has been suggested that the temperature–humidity index (THI), a parameter widely used to describe heat load on humans, is a good indicator of stressful thermal climatic conditions (McDowell et al. 1976). The THI is derived from a combination of wet and dry bulb air temperatures for a particular day and expressed as:

$$THI=0.72(W°C+D°C)+40.6$$

(Where W°C=wet bulb and D°C=dry bulb. Temperature–humidity index values of 70 or less are considered comfortable, 75–78 stressful, and values greater than 78 cause extreme distress and animals are unable to maintain thermoregulatory mechanisms or normal body temperature).

In a review on the effects of heat stress on extensively farmed ruminants, Silanikove (2000) stated that the impacts of increased heat stress in cattle include reduced grazing time (partly as a result of animals seeking shade), reduced feed intake, increased body temperature, increased respiration rate, and weight loss. In dairy cows, heat stress reduces milk yield, reduces milk fat and protein content, and decreases

reproduction rates (Jones & Hennesey, 2000). High-producing dairy cows are the most susceptible to increases in the THI. Heat stress days with THI > 80 lead to a substantial effect on reproduction of dairy cows, particularly for Holstein-Friesian. When assessing the impact of climate change on THI, it is important to assess, not just the change in the mean, but also the change in the number of extreme days (Howden et al, 1999).

The response of beef cattle to THI is similar to the response of dairy cattle, although Bos indicus cattle seem to be about 10% more tolerant than Bos taurus. All cattle require significantly more water when under stress. Significant stress is experienced at a THI of 80, and a recovery period is important in minimising production losses (Davison et al. 1996).

The aim of this project was to provide 'proof of concept' that the total heat load (environmental and metabolic) in feedlot cattle exposed to a high environmental heat load can be diminished by appropriate ration formulation. The industry problem being addressed is the economic loss and adverse publicity caused by feedlot mortalities during heat waves. A promising result would lead to feedlot trials to validate the result in practical situation, and dispersion of appropriate advice to the industry through MLA and other channels. The outcome sought is another tool to reduce heat stress in feedlot animals and accordingly improve animal welfare and the social acceptability of Australia's livestock products in the marketplace.

3.2.3.2 Animals

The experiment used 20 *B. taurus* approximately 6-8 months of age (weighing 345.1 ± 6.5 kg). Duration of the main experiment was 22 weeks, of which the first 10 weeks comprised adaptation to diets and training. For the heat stress trial, animals were divided into five groups of 4, one group being a "spare" group

In order to fine-tune conditions in the climate rooms, the "spare" cattle were subjected to the temperature/humidity conditions used in these rooms which produced the degree of stress desired for this study.

3.2.3.3 Diets

Feedlot type diets, containing approximately 80-85% concentrate and at least 15% roughage were fed at 2.7% of live-weight per day. The diets were similar, except that one contained wheat, the other sorghum. The ration was fed at approximately 0800 and 1500 daily.

3.2.3.4 Experimental treatments

Housing was in an animal house, either in individual pens in the main complex or in a climate room. During the application of heat load in the climate chambers, exposure was limited to 3 days if a panting score of 3.0 for 2 consecutive hours for at least 3 animals was achieved by the 3rd day, otherwise a 4-day heat load was applied.

3.2.3.5 Analysis of blood samples performed at CSIRO Armidale

Blood samples were collected by jugular venipuncture before treatment and on exit from the climate room. Samples were collected into 1 x Cytochex blood collection tube and 1 x EDTA tube. Cytochex blood collection tubes were kept refrigerated and transported on ice to CSIRO Armidale within 24 h of collection. EDTA tubes were centrifuged at 2500 rpm for 10 min. Plasma was collected and frozen at -20°C. Analyses completed on blood samples included leukocyte phenotyping, expression of intra-cellular glucocorticoid receptor (GR) and heat shock protein (hsp) 70 in leukocyte subpopulations and haematological parameters.

The blood samples were used to determine whether the exposure of animals to heat stress provokes an immune response and whether the markers examined may be useful in identifying which animals are more prone to suffer from heat stress.

An aliquot of whole blood was analysed for the haematology variables haemoglobin (HGB), red cell counts (RCC), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), white blood cells count (WBC) and differential cell counts of neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), using a Cell-Dyn Haematology Analyser 3500R (Abbott Diagnostics, CA, USA).

Leukocyte phenotyping using flurochrome-labelled extracellular marker antibodies was conducted using methods as previously reported (B.AWW.0184 Milestone 1). Detection of intracellular glucocrticoid receptor (GR) and hsp70 was conducted using the appropriate extracellular marker antibodies in conjunction with intracellular fluorochrome-labelled antibodies as previously reported (B.AWW.0184 Milestone 1 and Milestone 3 interim report).

3.2.3.6 Statistical analysis

Data was transformed using either log (base e) or square root arcsine transformations to improve data normality. Data was analysed with multivariate repeated measures analysis using Systat 9 (SPSS Inc.). Predictive factors were determined using a backwards, stepwise, univariate regression analysis. P values < 0.05 were considered significant.

4 Results and Discussion

4.1 Results and Discussion – Methodological development

4.1.1 Flow cytometry

4.1.1.1 Measurement of hsp70 expression in ovine and bovine CD14+ monocytes

4.1.1.1.1 Temperature dependant hsp70 expression

All animals displayed low basal hsp70 expression in CD14+ monocytes and upregulated expression patterns following heat shock, consistent with the published literature. A series of experiments were conducted to establish the appropriate heat shock temperature for optimal induction of hsp70 in ovine and bovine leukocytes. Figures 1 and 2 are representative results for the percentage of ovine and bovine CD14+ leukocytes expressing hsp70 over a range of temperatures. These results indicated that the optimum temperature for heat shock induction of hsp70 in leukocytes is 43.5°C for both sheep and cattle.

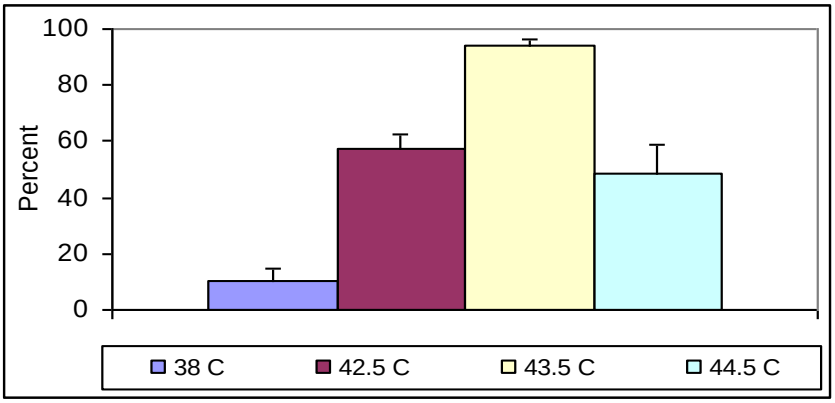


Figure 1: Temperature dependant ovine CD14+ leukocyte hsp70 expression.
Values are means ± sem (n=4)

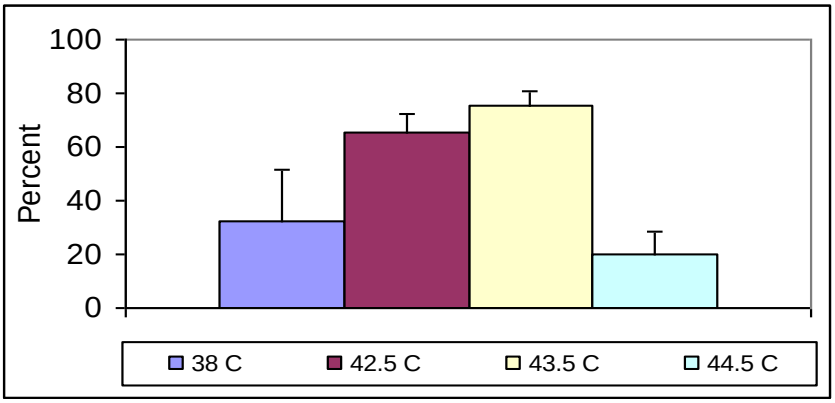


Figure 2: Temperature dependant bovine CD14+ leukocyte hsp70 expression.
Values are means ± sem (n = 4)

Additional experiments were conducted to determine optimal experimental conditions such as appropriate anticoagulant, storage of samples and the use of commercial reagents.

4.1.1.1.2 Anticoagulant comparison

Use of the anticoagulant, EDTA allows both haematologic and flow cytometric analysis to be performed on the same sample. The anticoagulant routinely used for hsp70 analysis by flow cytometry is lithium heparin. A comparison of staining patterns for hsp70 in leukocytes collected in vcacutainers containing EDTA and lithium heparin anticoagulants was performed. The results demonstrated that EDTA could be routinely used for this assay as there was no significant difference between results obtained for samples collected in EDTA or lithium heparin as shown in Figure 3.

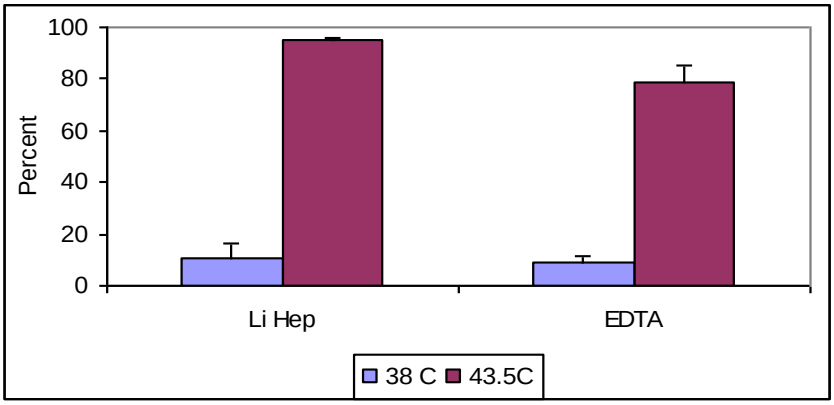


Figure 3: Anticoagulant comparison for ovine blood
Values are means ± sem (n=6). P > 0.05 for 38°C and 43.5°C

4.1.1.1.3 24h storage of blood

Researchers do not always have access to fresh blood samples. A series of experiments was conducted to determine whether leukocyte hsp70 analysis could be conducted on stored blood samples. Blood samples were kept at room temperature in the dark for 24 h prior to staining for leukocyte hsp70 analysis. The results demonstrated that when compared to staining patterns of fresh samples, those stored for 24 h showed a reduction in inducibility of hsp70 following heat shock and greater variation in individual responses was evident as denoted by greater standard errors. The marked changes that occur in the pattern of leukocyte hsp70 expression following a 24 h delay in staining indicate that fresh blood samples are required for optimal hsp70 expression profiles.

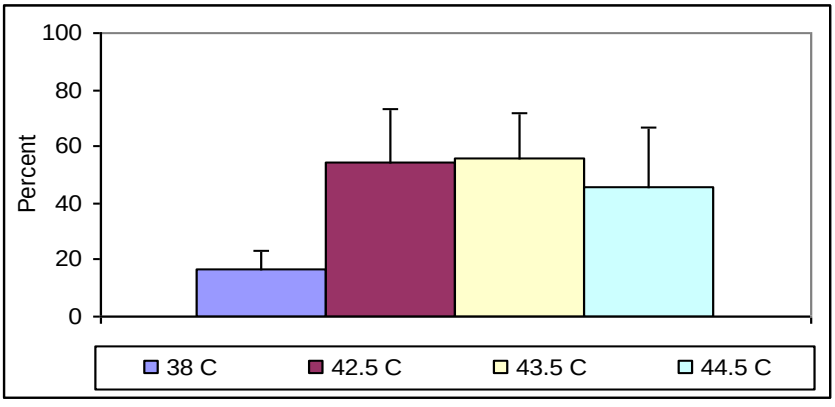


Figure 4: Temperature dependant ovine CD14+
leukocyte hsp70 expression after storage of blood for 24h
at room temperature. Values are means ± sem (n=4)

4.1.1.1.4 Protocol comparison

Variability in the methodology of intracellular staining protocols for flow cytometry may influence experimental results. Two protocols for detection of leukocyte hsp70 expression by flow cytometry were compared. The first protocol utilized standard reagents, whilst the second protocol took advantage of commercially available lysis and permeablizing solutions. The results as illustrated in Figure 5 demonstrated that the use of commercially available reagents and the use of alternative methods not requiring such reagents yielded similar results for leukocyte hsp70 expression.

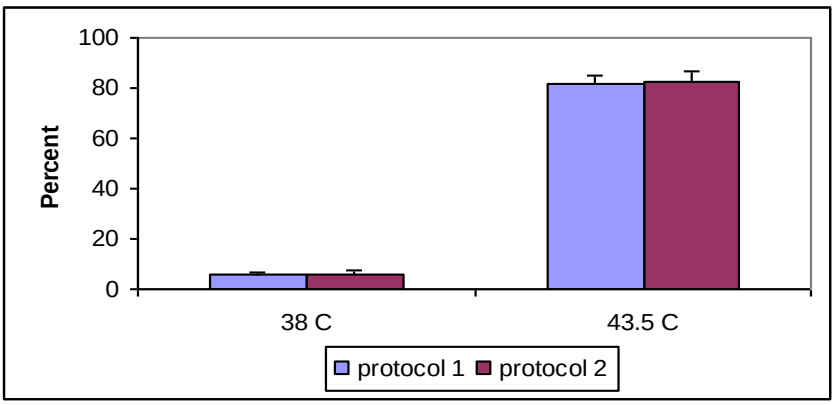


Figure 5: Comparison of staining protocols for ovine whole blood.
Values are means ± sem (n=6). P > 0.05 for 38°C and 43.5°C.

Discussion

The application of a mild, transient, non-lethal heat shock to cells has been widely reported to induce the expression of a number of heat shock proteins (hsp). The current methodology has been adapted from a method developed during my PhD for use in human leukocytes. The results of these recent studies have demonstrated that evaluation of leukocyte hep70 expression by flow cytometry is a robust, reproducible method for use with cattle and sheep blood.

The current studies have determined that the optimum temperature for heat shock of leukocytes from sheep and cattle is 43.5°C. This temperature provided the greatest stress response without a loss of cell viability. The application of a mild, transient, non-lethal, *in vitro* heat shock is a technique that allows the researcher to examine the cellular stress response which is a highly conserved defence mechanism. As such, leukocyte hsp70 expression may be a useful indicator of an organism's adaptation to environmental/physiological stress. It has been demonstrated that hsp70 expression is induced *in vivo* in a range of cell types in response to a variety of stressors including increases in body temperature (>1.5°C), ether anesthesia, surgery, restraint stress, water immersion stress, ischemia, trauma, hemodynamic overload and exercise (Reviewed in Rokutan et al., 1998). A simple translation of *in vitro* to *in vivo* temperatures for induction of heat shock proteins is generally not considered to be meaningful.

It has also been determined that the best results are obtained from fresh samples. The pattern of hsp70 expression following heat shock were not consistent with the results obtained from the fresh blood samples, suggesting that storage of blood prior to staining for hsp70 will not provide an accurate Indication of the stress responsiveness of these cells. The current studies also revealed that EDTA is a suitable anticoagulant for use in blood collections for flow cytometric analysis of hsp70 in leukocytes.The benefit of being able to stain cells for flow cytometric analysis of hsp70 expression from blood collected in tubes containing EDTA is that it avoids the necessity of collecting more than one blood sample per animal.

This method is currently undergoing further development to determine the patterns of expression of hsp70 in a range of leukocyte sub-types. The method will then be applied to test the cross-reactivity of other hsp antibodies such as hsp27 and heme-oxygenase (HO-1).

4.1.1.2 Determination of leukocyte phenotype following cryopreservation of whole blood samples

Results

Fresh blood samples were stained with antibodies to the extracellular and intracellular markers as listed above. Additional blood samples were fixed with paraformaldehyde then frozen at -80°C for 7 days. These samples were thawed and stained in the same manner as the fresh samples. The results, as illustrated in Figures 6 demonstrated that for ovine samples there were significant differences in the staining profiles for all markers when fresh samples were compared with cryopreserved samples. Figure 7 illustrated that for bovine samples there was a significant difference for the expression of glucocorticoid receptor in fresh and cryopreserved samples, however, there were no differences for any of the extracellular markers examined.

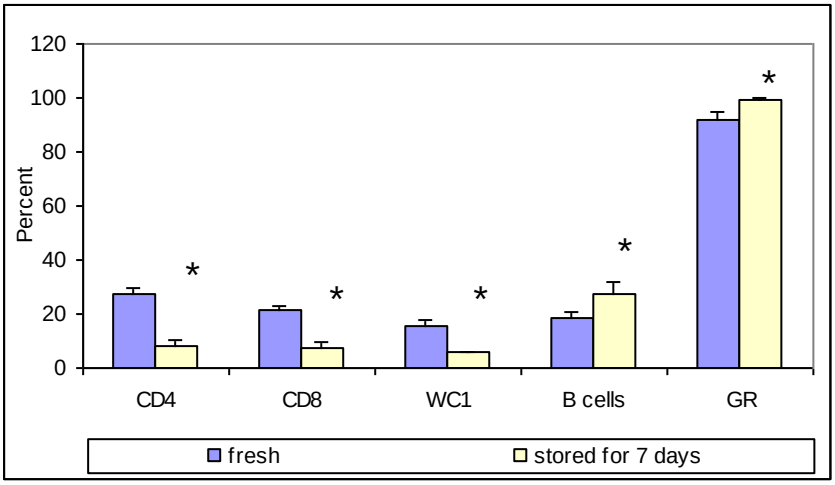


Figure 6: Comparison of flow cytometric markers in fresh and frozen ovine blood samples. WC1 = γδ T cell receptor, GR = glucocorticoid receptor. Values are means ± sem (n=4).* P < 0.05 for all markers

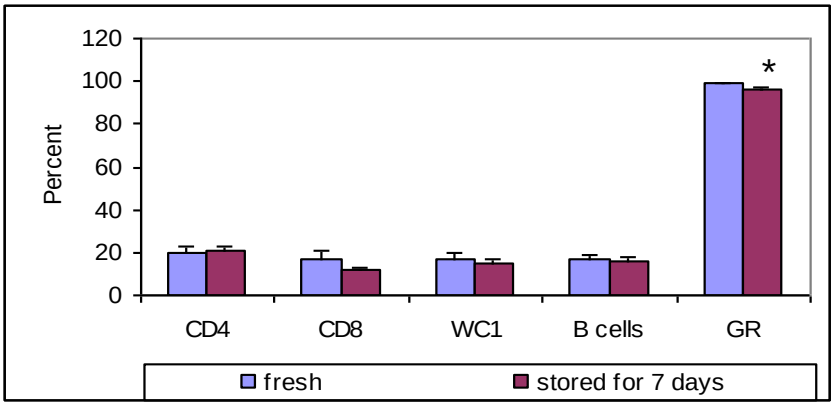


Figure 7: Comparison of flow cytometric markers in fresh and frozen bovine blood samples. WC1 = γδ T cell receptor, GR = glucocorticoid receptor. Values are means ± sem (n=4). * P < 0.05 ; P > 0.05 for all other markers

Discussion

Lymphocyte phenotyping is an invaluable tool used to monitor immune responses. The results of the current studies have demonstrated that fixing and freezing whole blood for later staining with antibodies to extracellular markers is a suitable method for phenotyping bovine leukocytes. This method does not appear to be suitable for intracellular staining of the glucocorticoid receptor. The method is also unsuitable for use with sheep blood samples. This technique will have great benefits for the current project and for future research in this area as access to fresh blood samples is a common problem encountered by researchers when samples are collected from remote or on-farm sites that do not have access to a flow cytometer or personnel trained in the required staining techniques. This methodology will allow the classification of leukocyte markers in samples that were previously unable to be characterised in this manner. This methodology will therefore be able to be employed in a greater range of experiments involving immune responses in cattle.

4.1.1.3 Use of a cryopreservation system for immunophenotyping livestock blood samples

4.1.1.3.1 Results for percentage of cells expressing each marker

Results comparing the percentage of cells expressing each receptor from EDTA blood samples and Cytochex blood samples are illustrated in figures 8 (sheep) and 9 (cattle)

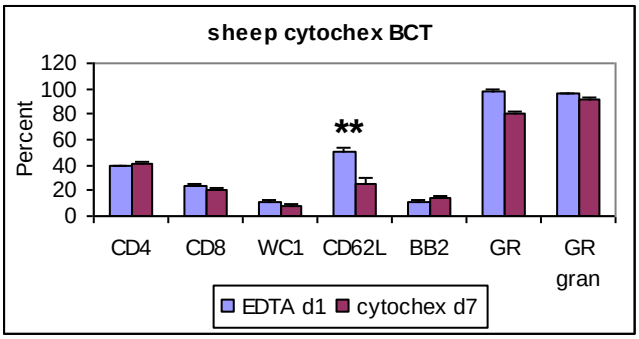


Figure 8: Values are means ± sem.
****** CD62L p=0.007, GR p=0.009

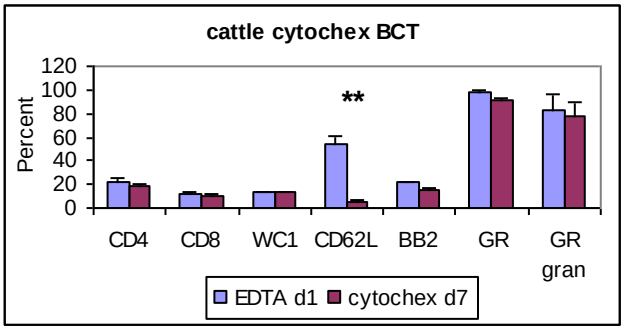


Figure 9: Values are means ± sem.
******CD62L p=0.009

4.1.1.3.2 Haematology results

Results comparing haematological values from EDTA blood samples and Cytochex blood samples are illustrated in Table 1.

Table 1: Haematological parameters

Parameter	Sheep			Cattle		
	EDTA	CytoChex	P value	EDTA	CytoChex	P value
WBC 10*6/mL	4.407 ± 0.323	5.008 ± 0.506	0.099	9.136 ± 1.427	9.512 ± 0.845	0.718
NEU 10*6/mL	2.386 ± 0.298	2.556 ± 0.378	0.242	3.35 ± 0.25	3.408 ± 0.301	0.482
LYM 10*6/mL	1.601 ± 0.127	2.125 ± 0.11	**0.002**	4.65 ± 1.091	4.352 ± 0.849	0.772
MONO 10*6/mL	0.324 ± 0.062	0.168 ± 0.055	0.087	1.101 ± 0.094	1.029 ± 0.132	0.723
EOS 10*6/mL	0.039 ± 0.016	0.04 ± 0.012	0.912	0.001 ± 0.001	0.058 ± 0.048	0.346
BAS 10*6/mL	0.057 ± 0.022	0.119 ± 0.098	0.632	0.034 ± 0.003	0.665 ± 0.075	**0.014**
RBC 10*9/mL	10.82 ± 0.456	11.12 ± 0.741	0.412	10.12 ± 0.636	10.41 ± 0.585	**0.031**
HGB g/dL	13.48 ± 0.681	13.27 ± 0.679	0.697	11.59 ± 0.427	11.84± 0.584	0.292
HCT %	13.48 ± 0.681	43.74 ± 3.114	0.082	31.6 ± 1.112	34.45 ± 0.69	**0.023**
MCV fL	36.63 ± 0.362	39.32 ± 0.542	**0.005**	31.34 ± 0.959	33.25 ± 1.33	**0.036**
MCH pg	12.44 ± 0.158	12 ± 0.627	0.545	11.49 ± 0.335	11.38 ± 0.086	0.707
MCHC g/dL	33.97 ± 0.117	30.5 ± 1.458	0.151	36.66 ± 0.067	34.32 ± 1.073	0.148
PLT 10*6/mL	709.8 ± 78.93	647.8 ± 83.62	**0.018**	866.1 ± 40.34	901.1 ± 70.19	0.383

Discussion

Lymphocyte phenotyping is an invaluable tool used to monitor immune responses. The results of the current studies have demonstrated that sheep and cattle blood samples collected in Cytochex blood collection tubes are suitable for immunophenotyping for up to 7 days following collection. The current studies revealed that there was no difference in percentages of cells expressing the extracellular markers CD4, CD8, WC1 and BB2 between samples collected in EDTA and stained fresh or samples collected in Cytochex BCT and stained after 7 days of storage. The current studies also revealed that Cytochex samples are unsuitable for determination of the cell activation marker CD62L (also known as L-selectin) suggesting that such cryopreserved samples are not suitable to use in functional assays.

The results of the intracellular staining suggest that it is possible that these samples may also be suitable for determination of glucocorticoid receptor expression; however a larger study will be required before a definitive statement on intracellular staining of cryopreserved samples can be made.

Similarly, for haematological analysis of blood samples, these preliminary results indicate that it may be possible to use Cytohex samples for haematology, however, due to observed differences in cell scatter plots on the haematology auto-analyser, a study involving a larger number of samples will be required before a definitive statement can be made regarding the suitability of these samples for haematology.

The use of Cytochex BCT for immunophenotyping will have great benefits for the current project and for future research in this area as access to fresh blood samples is a common problem encountered by researchers when samples are collected from remote or on-farm sites that do not have access to a flow cytometer or personnel trained in the required staining techniques. This methodology will allow the classification of leukocyte markers in samples that were previously unable to be characterised in this manner. This methodology will therefore be able to be employed in a greater range of experiments involving immune responses in sheep and cattle.

4.1.1.4 Measurement of glucocorticoid receptor in leukocyte subpopulations

Results

Percentages of CD4, CD8, WC1 and BB2 lymphocytes and CD14 monocytes expressing the glucocorticoid receptor are illustrated in Figures 10 (cattle) and 11 (sheep).

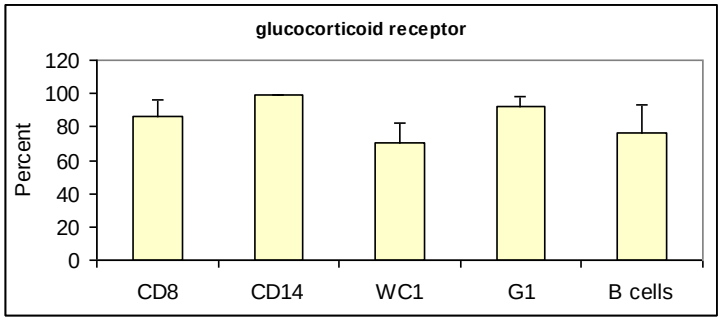


Figure 10: Cattle leukocyte GR expression profiles. Values are means ± sem.

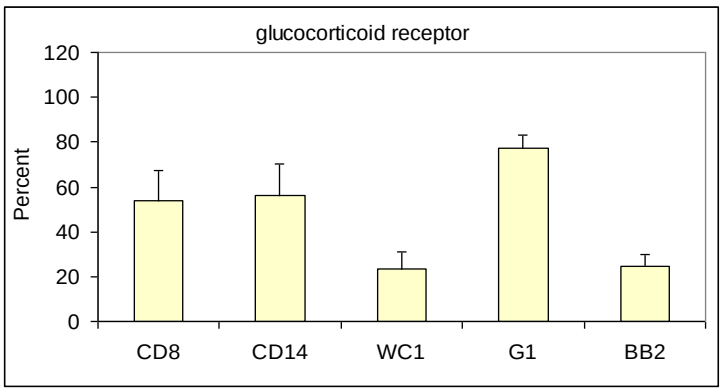


Figure 11: Sheep leukocyte GR expression profiles. Values are means ± sem

Discussion

The results of the current studies revealed that leukocyte glucocorticoid receptor was differentially expressed in leukocyte subtypes in both cattle and sheep. Furthermore it was noted that there were species differences in these expression profiles.

The importance of glucocorticoid receptor expression to the current project is two-fold. Firstly, it has been reported that lymphocyte GR expression and cortisol levels are inversely associated in various human psychiatric conditions (Yehuda *et al.,* 1993). Furthermore, it has been reported that increased glucocorticoid levels result in a down-regulation of GR expression and a restriction of GR signalling (Wallace & Sidlowski, 2001). One potential application of this assay therefore could be to monitor the negative feedback of GR expression in immune cells during chronic hyperactivity of the HPA axis in livestock species. Secondly, there is increasing evidence to suggest a link between the stress-induced cortisol response and the cellular heat shock response. *In vivo* studies of chronic glucocorticoid treatment decreased the heat shock-induced hsp70 expression in fish liver (Basu *et al.,* 2001) and rat brain (Barr & Dokas, 1999) and the heat shock-induced hsp90 mRNA accumulation in trout hepatocytes (Sathiyaa *et al.,* 2001). Whilst the mechanism for this attenuation has not yet been elucidated, it has been suggested that GR-mediated inhibition of HSF-1 may play a role in the observed attenuated heat-shock response (Wadekar *et al.,* 2001). As the development of a method for measuring hsps in leukocytes from livestock species was reported in milestone 1 of the current project, the two methodologies could be utilised to investigate possible links between hsp and GR expression and therefore could increase our understanding of the interplay between cellular stress responses and activity of the HPA axis.

4.1.2 Detection of hsp70 by Western immunoblot

Results

Expression of hsp70 in sheep and cattle leukocytes was measured by Western immunoblot (Figure 12). Densitometric analysis of this expression relative to that of β-actin (Figure 13) will be a useful measure for the comparison of treatment effects on individual animals. The results of this study have demonstrated that the antibody used (anti-hsp70 clone C92)is, in fact, specifically binding the hsp70 protein. As this clone is also used in flow cytometric analysis of hsp70 it adds further evidence to suggest that the antibody cross-reacts with sheep and cattle leukocytes.

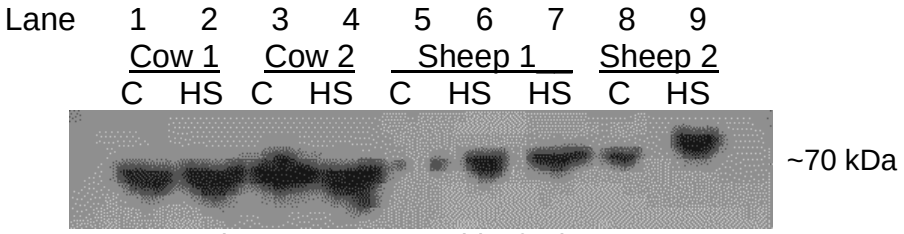


Figure 12: Western blot for hsp70

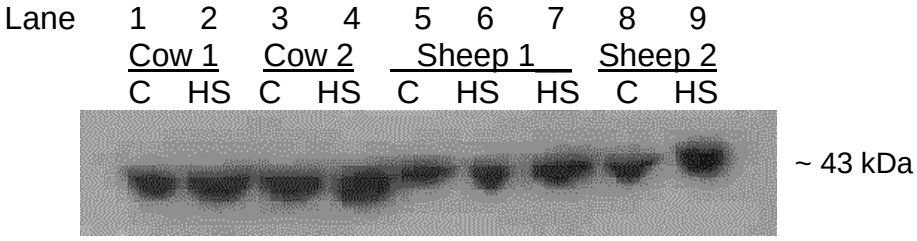


Figure 13: Western blot for β-actin

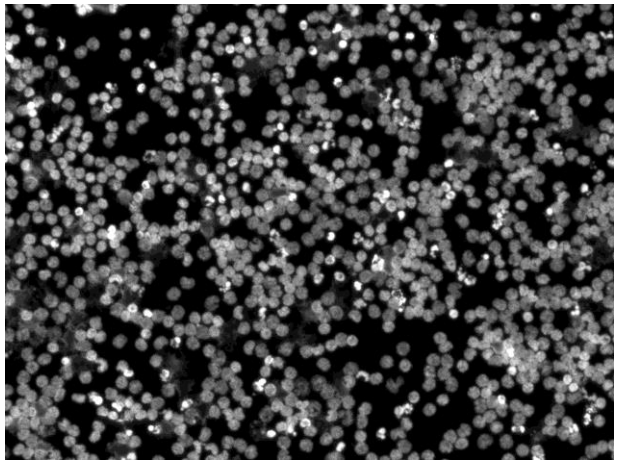
4.1.3 Immunohistochemistry

Results

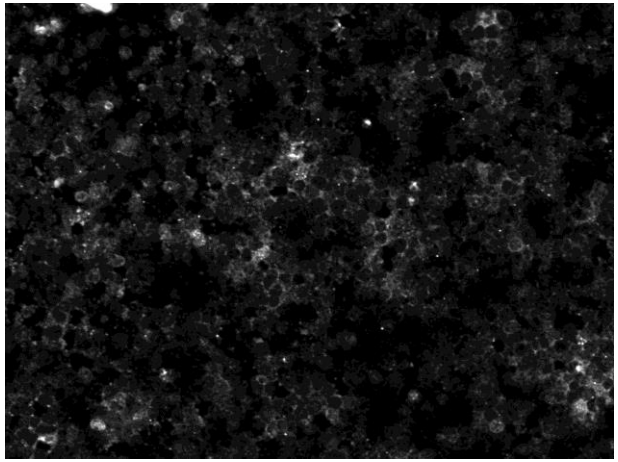
As illustrated in the images that follow the presence of hsp70 was detected in a range of ovine and bovine tissues (blood – at basal temperature and following heat shock, mammary tissue, jejunum and testis. Glucocorticoid receptor was also detected in these tissues.

Results
Results of hsp70 staining in PBMC smears

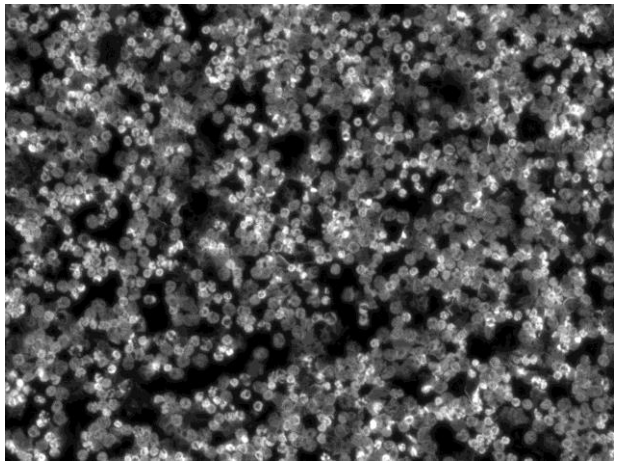
Cattle blood smears stained with hsp70-FITC and DAPI



DAPI



Hsp70-FITC control



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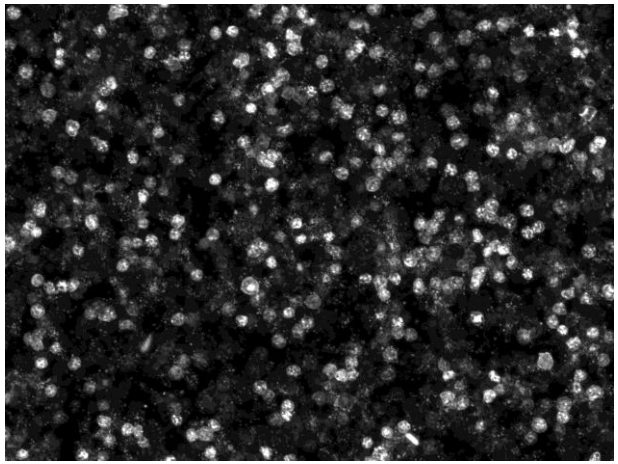
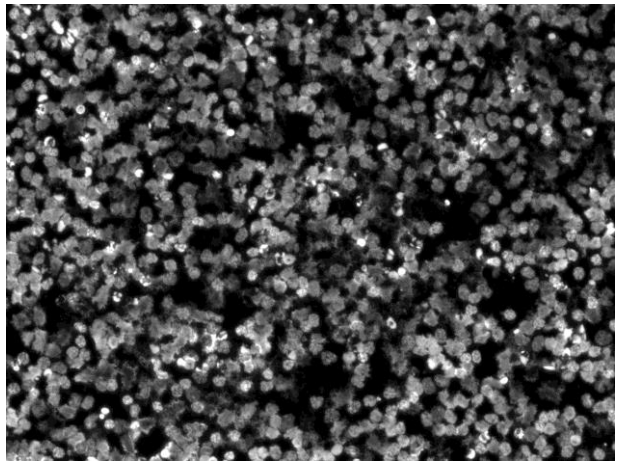
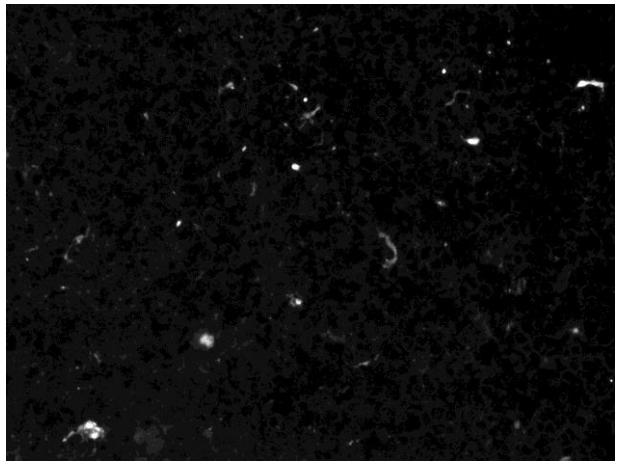


Hsp70-FITC heat shock

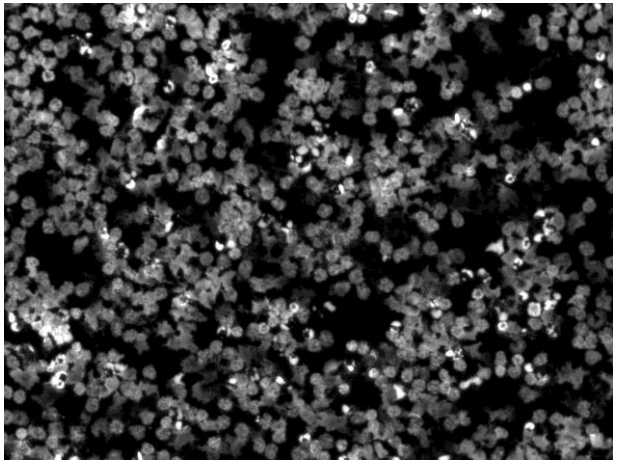


DAPI

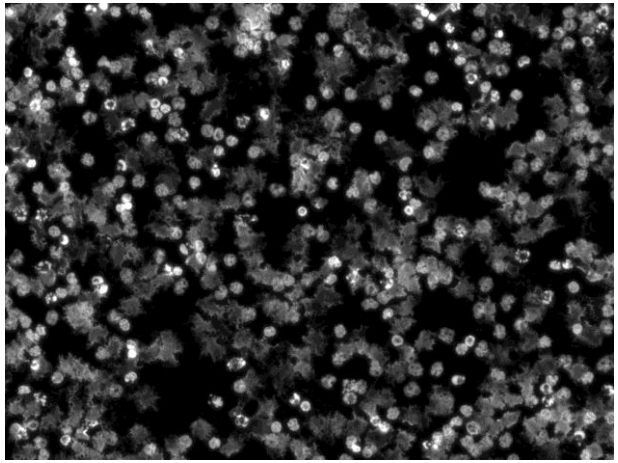


IgG1-FITC isotype control

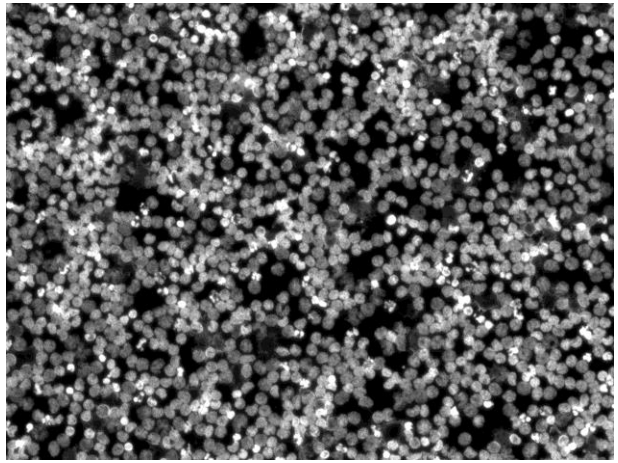
Sheep blood smears stained with hsp70-FITC and DAPI



DAPI



DAPI



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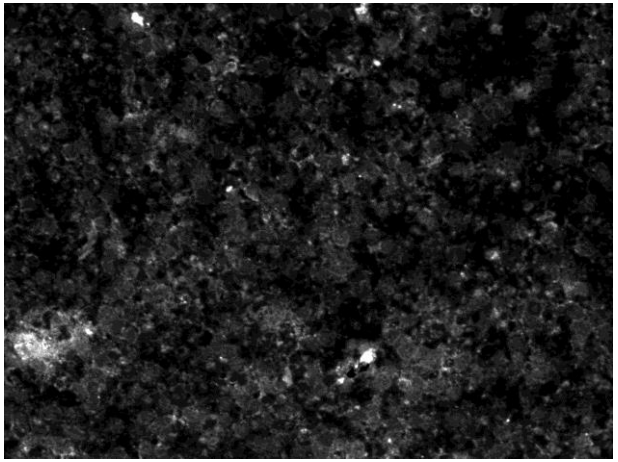
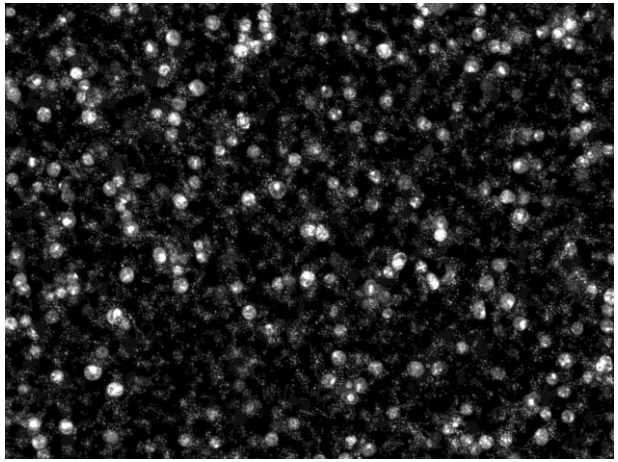
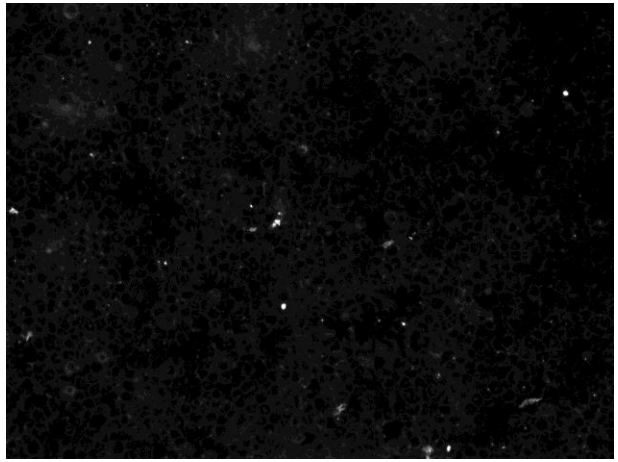


Hsp70-FITC control



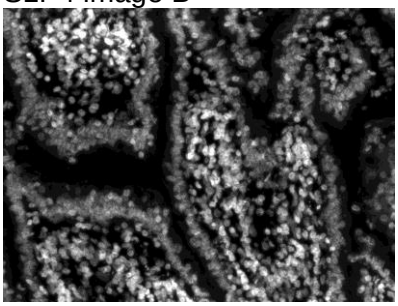
Hsp70-FITC heat shock



IgG1-FITC isotype control

Results of staining in ovine gut tissue

S1P4 image B



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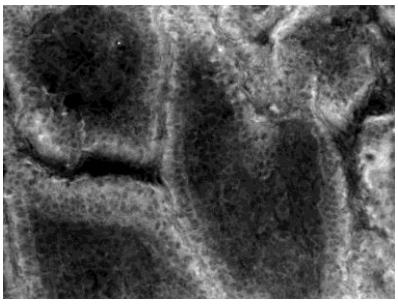
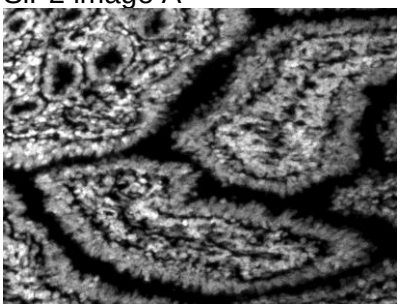


Dex-FITC

SiP2 image A



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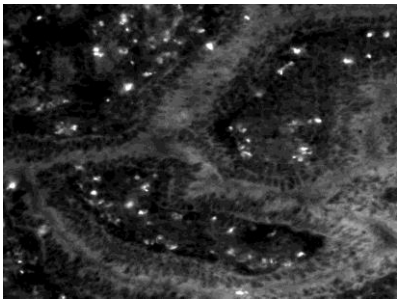
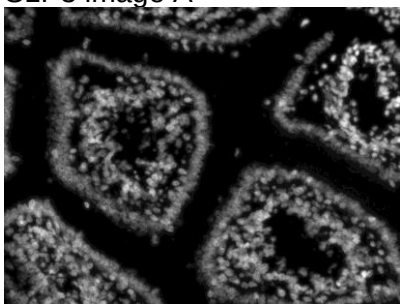
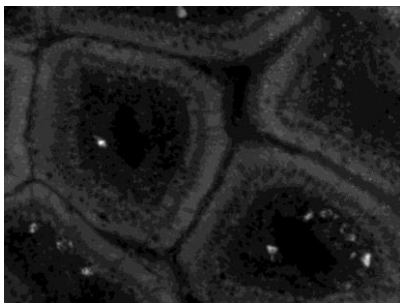


Dex-FITC

S1P3 image A

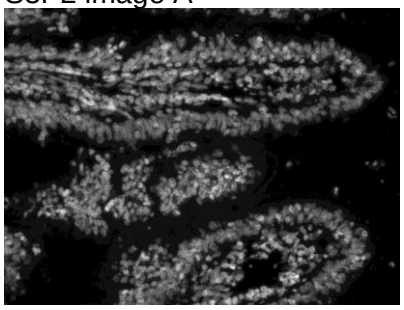


DAPI



Hsp70-FITC

S3P2 image A



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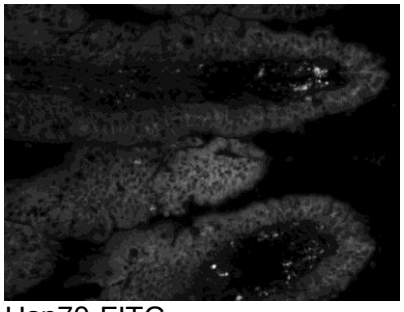
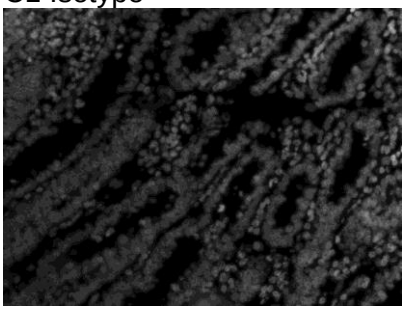


Hsp70-FITC

C1 Isotype



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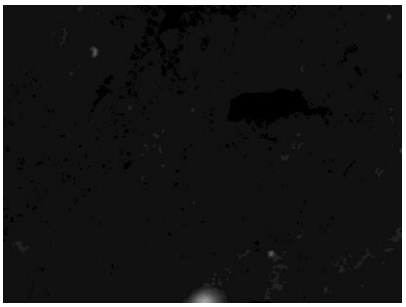
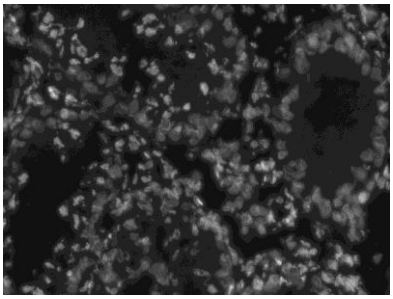


Isotype control IgG1-FITC

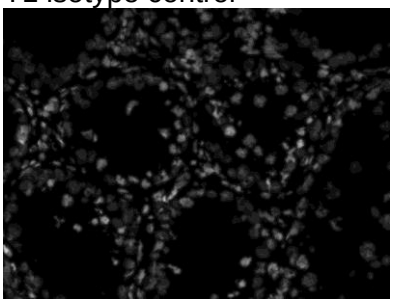
Results of staining in bovine testis

T3 dex

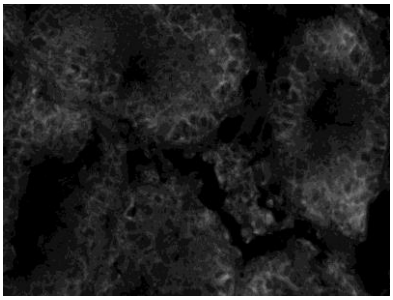


DAPI

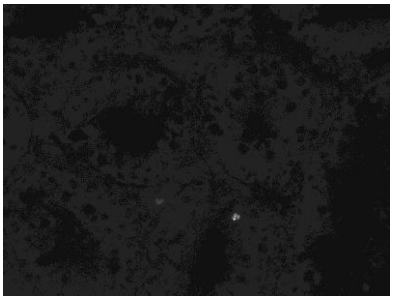
T1 isotype control



DAPI

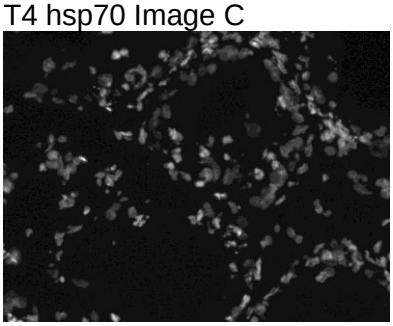


Dexamethasone-FITC

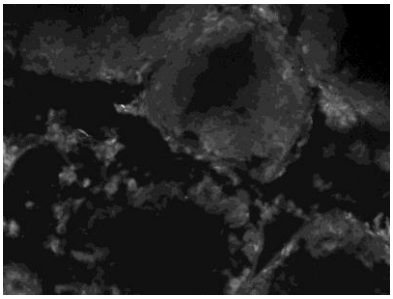


Isotype control IgG1-FITC

T4 hsp70 Image C



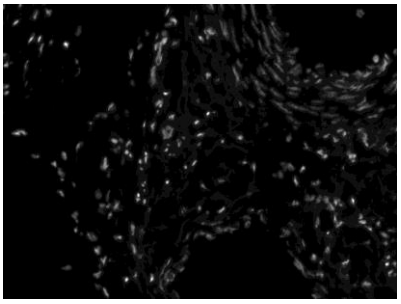
DAPI



Hsp70-FITC

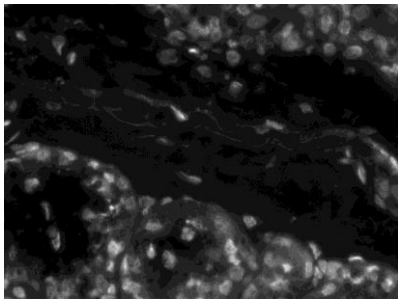
Results of staining in ovine mammary issue

RHS



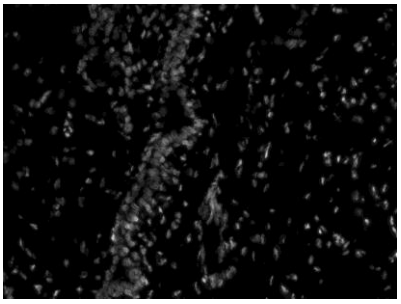
DAPI

LHS

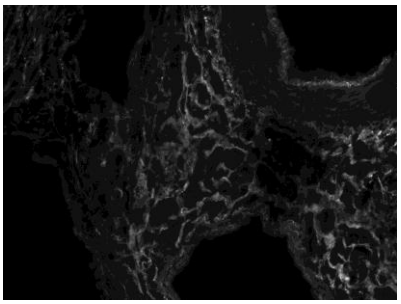


DAPI

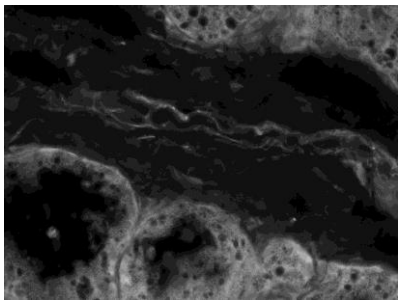
RHS



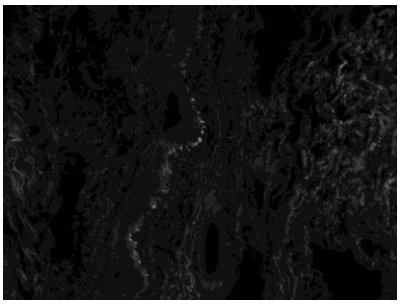
DAPI



Hsp70-fITC

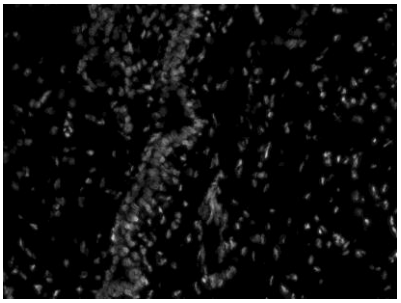


Dexamethasone-FITC



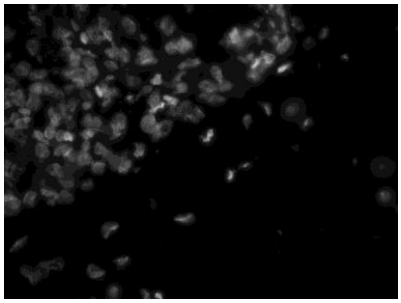
Sotype control

LHS

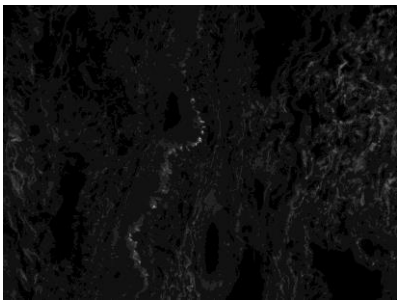


DAPI

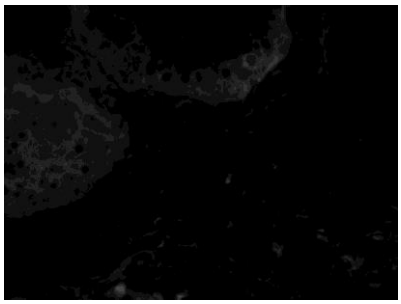
LHS



DAPI



Hsp70-FITC



Isotype control

4.1.4 ELISA

Results

The Surveyor™ IC human/mouse/rat total hsp70 immunoassay kit has not previously been tested for use in livestock species. The results of the assay as illustrated in Figure 15 indicate that the assay is suitable for the detection of hsp70 in bovine plasma. It was also determined by serial dilution of the plasma samples that the optimum working dilution for samples is 1:16 (Figure 15).

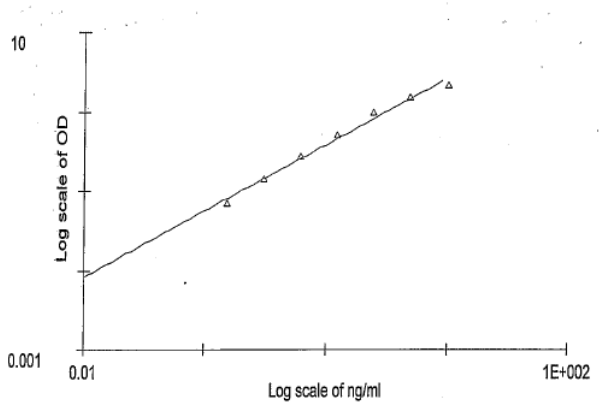


Figure 14: Typical standard curve for hsp70 (ng/ml)

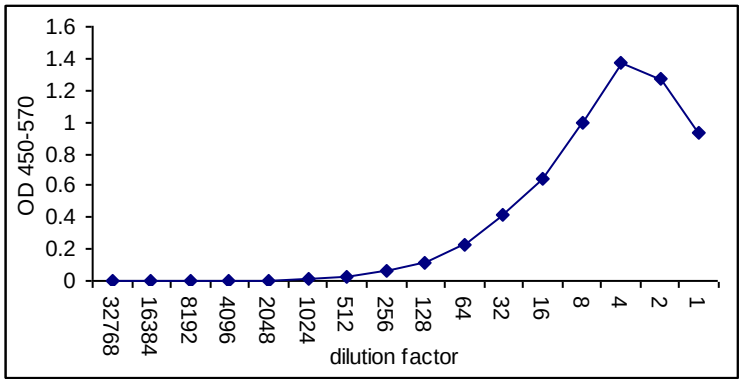


Figure 15: Calibration curve for detection of hsp70 in cattle plasma

4.1.5 qRT-PCR

In summary the current studies have checked primers, sequences and melt curves suggesting that a single specific amplicon exists for each product tested in bovine tissue. Future studies will be required to test the efficiencies prior to performing real time analysis.

The specificity of all primer sets used in this study were assessed by melt-curve analysis (Figure 1), agarose gel electrophoresis (Figure 17) and sequencing of amplification products. Each primer set was used to generate amplification products from pooled RNA samples in a qRT-PCR assay run under standard conditions. Melt curves were analysed for the presence of diverse peaks with different T_ms or plateaus (indicating the presence of non-specific products) and peaks with T_ms much lower than that of the specific product (indicating the presence of PD).

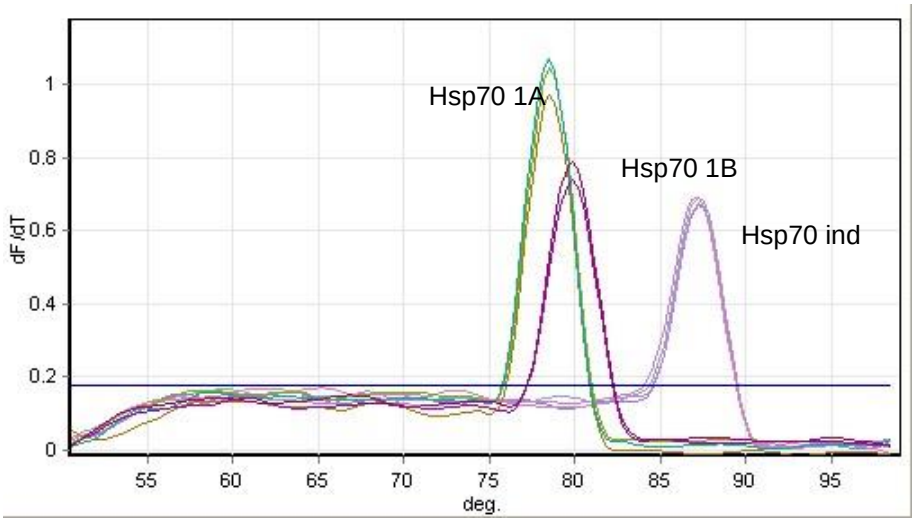


Figure 16: Melt curve analysis displaying peaks representing each specific product.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12

Figure 17: Gel electrophoresis of bovine amplification products. Lanes 1&12 contain a 100bp ladder (intense band = 500bp) (Promega). Lanes 2, 3, 4 & 5 are empty, lanes 6 & 7 hsp70 ind, lanes 8 & 9 hsp70 1A, lanes 10 & 11 hsp70 1B.

4.1.6 Oxidative stress

4.1.6.1 Lipid peroxidation

Results

All animals showed an increase in the concentration of thiobarbituric acid reactive substances (TBARS) after a 3 hr incubation of plasma with the free radical generator AAPH (Figures 18 & 19).

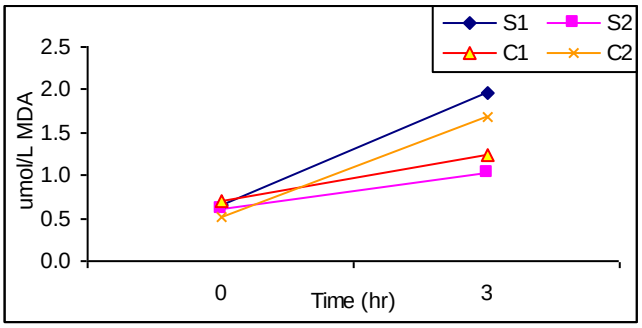


Figure 18: Sheep and cattle lipid peroxidation values

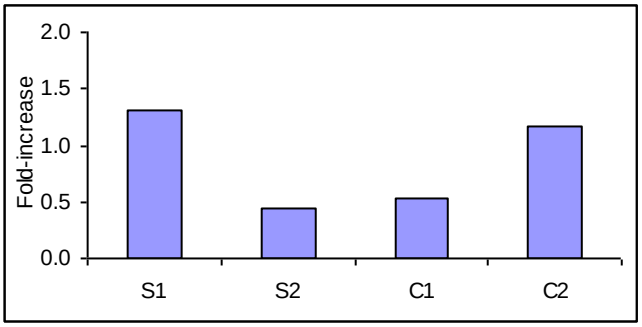


Figure 19: Fold-increase in TBARS (3 hr/0 hr) after oxidative stress

4.1.6.2 Protein oxidation

Results

The presence of protein oxidation products (protein carbonyls) was observed in ovine and bovine plasma samples.

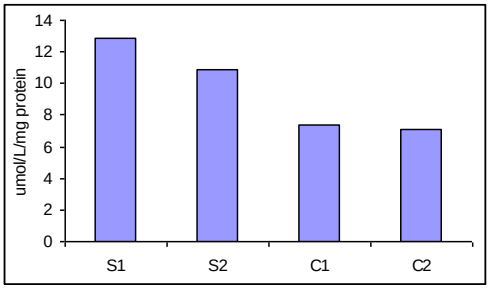


Figure 20: Sheep and cattle protein carbonyl values

4.2 Results and Discussion – Methodological application

4.2.1 Animal welfare outcomes of livestock road transport practices

4.2.1.1 Glucocorticoid receptor

Significant differences were observed in GR expression profiles in several leukocyte subsets as illustrated in Figures 21a, 21b, 22a and 22b.

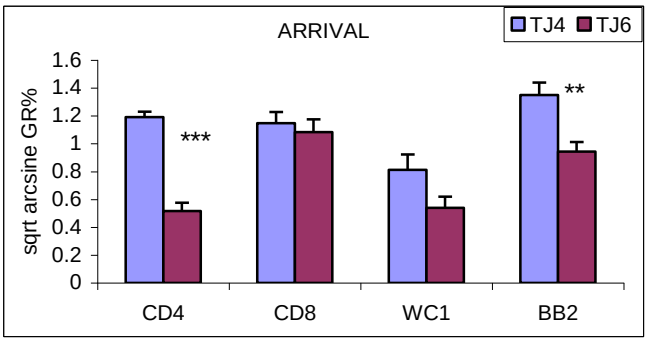


Figure 21a: Percentage of cells expressing GR on arrival CD4GR p<0.001, BB2GR p<0.01. Values are mean transformed values ± sem, n = 6 for each treatment group

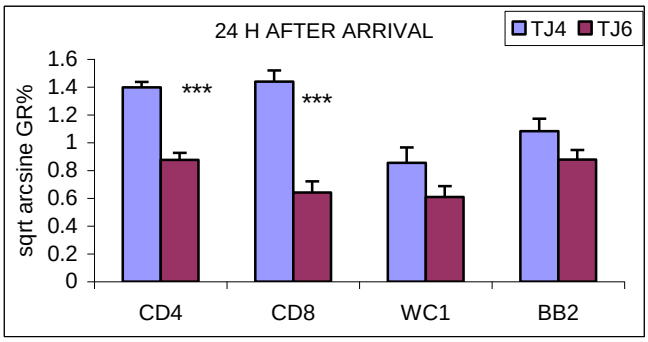


Figure 21b: Percentage of cells expressing GR 24h after arrival CD4GR p<0.001, CD8GR p<0.001. Values are mean transformed values ± sem, n = 6 for each treatment group

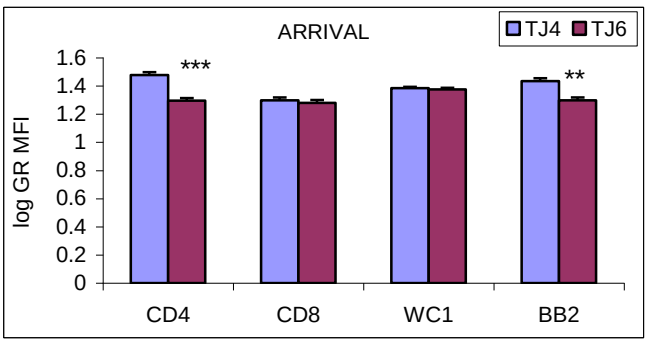


Figure 22a: MFI of GR expression on arrival CD4GR p<0.001, BB2GR p<0.01. Values are mean transformed values ± sem, n = 6 for each treatment group

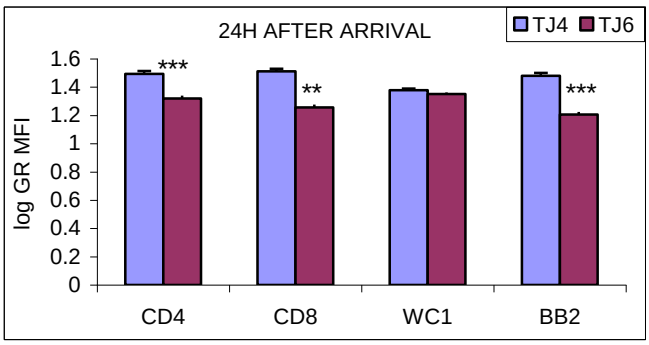
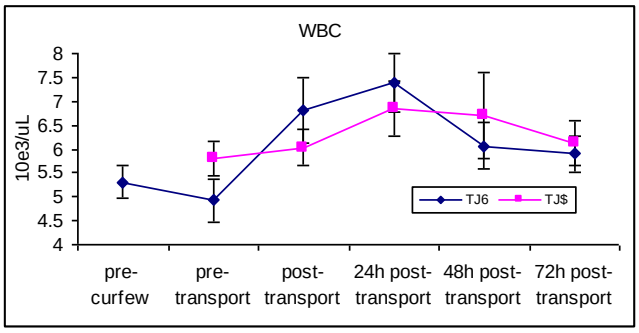
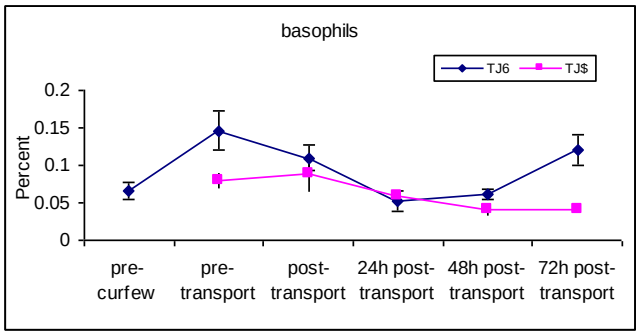


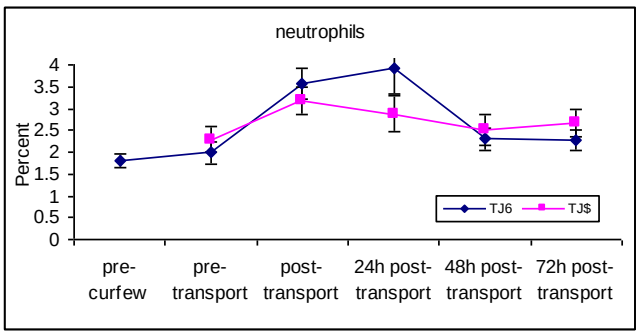
Figure 22b: MFI of GR expression 24h after arrival CD4GR p<0.001, CD8GR p<0.01, BB2GR p<0.001. Values are mean transformed values ± sem, n = 6 for each treatment group

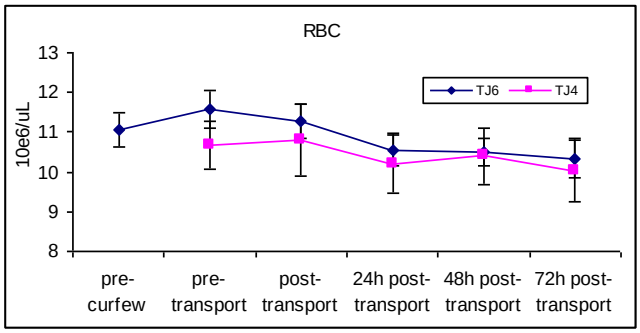
4.2.1.2 Haematology

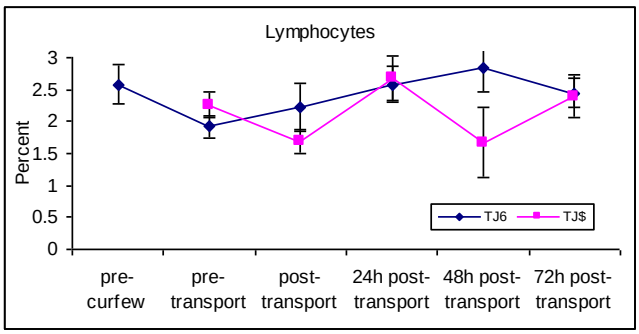
The pre-curfew means for the blood chemistry and haematology variables (Table 2) generally conformed to the accepted normal clinical ranges for sheep (as listed in Kaneko et al 1997). All values returned to normal within 72 h of the transport journey (Table 2).

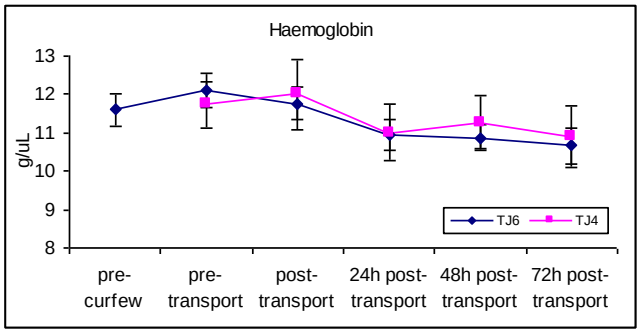


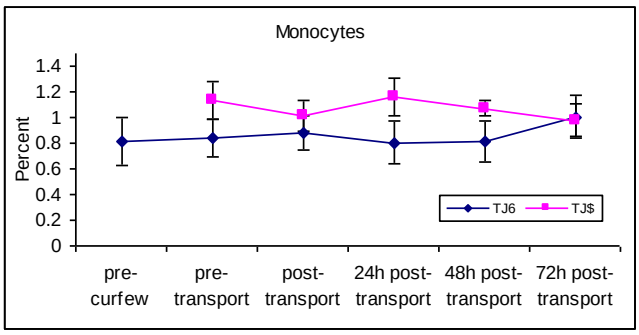


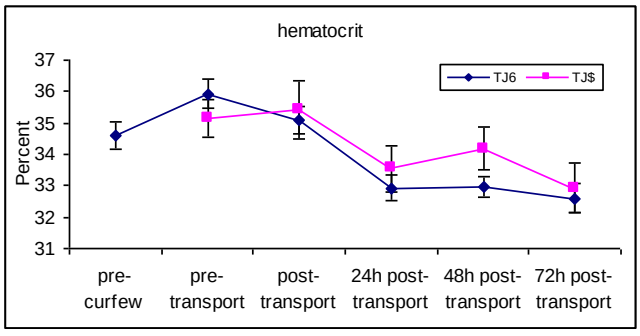


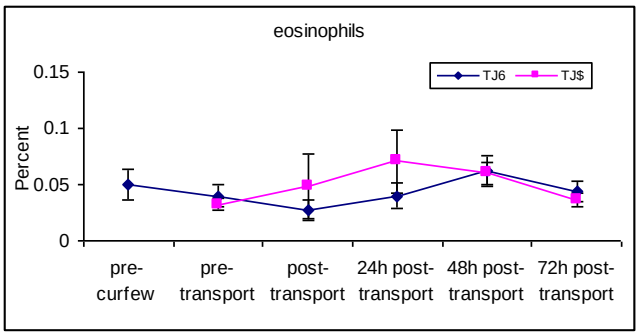












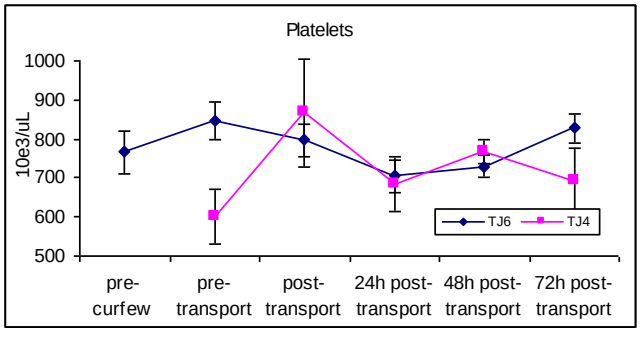


Table 2: Raw haematology data

	Ref range	Units	TJ4					TJ6					
			Pre-transport	Post-transport	24 h	48 h	72 h	Pre-curfew	Pre-transport	Post-transport	24 h	48 h	72 h
HGB	9 – 14	g/μL	11.7 ± 0.3	12 ± 0.3	11 ± 0.3	11.3 ± 0.2	10.9 ± 0.3	11.6 ± 0.1	12.1 ± 0.1	11.8 ± 0.1	10.9 ± 0.2	10.9 ± 0.1	10.7 ± 0.1
RCC	6 – 16	10^6/μL	10.7 ± 0.2	10.8 ± 0.2	10.2 ± 0.3	10.4 ± 0.4	10 ± 0.3	11.1 ± 0.1	11.6 ± 0.2	11.3 ± 0.2	10.6 ± 0.2	10.5 ± 0.2	10.3 ± 0.2
PLT	250-750	10^3/μL	601 ± 72	867 ± 137	683 ± 70	767 ± 32	694 ± 83	766 ± 55	848 ± 48	798 ± 41	705 ± 42	729 ± 28	827 ± 37
HCT	24 – 50	%	35.1 ± 0.6	35.4 ± 0.9	33.5 ± 0.7	34.2 ± 0.7	32.9 ± 0.8	34.6 ± 0.4	35.9 ± 0.5	35.1 ± 0.4	32.9 ± 0.4	33 ± 0.3	32.6 ± 0.5
MCV	23 – 48	fl	33 ± 0.7	32.8 ± 0.7	33 ± 0.7	33.2 ± 1.2	32.9 ± 0.7	31.3 ± 0.7	31.1 ± 0.7	31.2 ± 0.7	31.3 ± 0.7	31.5 ± 0.7	31.7 ± 0.7
MCH		Pg	11 ± 0.2	11.1 ± 0.2	10.8 ± 0.2	10.9 ± 0.3	10.9 ± 0.2	10.5 ± 0.2	10.5 ± 0.2	10.5 ± 0.2	10.4 ± 0.2	10.4 ± 0.1	10.3 ± 0.2
MCHC	31 – 38	g/dL	33.4 ± 0.6	34 ± 0.5	32.8 ± 0.3	33 ± 0.2	33.1 ± 0.3	33.6 ± 0.3	33.7 ± 0.2	33.5 ± 0.3	33.2 ± 0.3	33 ± 0.4	32.7 ± 0.3
WBC	4 – 12	10^3/μL	5.81 ± 0.4	6.03 ± 0.4	6.84 ± 0.6	6.69 ± 0.9	6.13 ± 0.5	5.31 ± 0.4	4.93 ± 0.5	6.8 ± 0.7	7.39 ± 0.6	6.07 ± 0.5	5.9 ± 0.4
NEU	0.7 – 6	%	2.29 ± 0.3	3.18 ± 0.3	2.87 ± 0.4	2.52 ± 0.4	2.67 ± 0.3	1.8 ± 0.2	1.98 ± 0.3	3.56 ± 0.4	3.91 ± 0.6	2.3 ± 0.3	2.28 ± 0.2
LYM	2 – 9	%	2.26 ± 0.2	1.7 ± 0.2	2.68 ± 0.4	3 ± 0.6	2.39 ± 0.3	2.58 ± 0.3	1.92 ± 0.2	2.22 ± 0.4	2.58 ± 0.3	2.83 ± 0.4	2.45 ± 0.2
MON	0 – .75	%	1.14 ± 0.2	1.01 ± 0.1	1.17 ± 0.2	1.0 ± 0.1	0.98 ± 0.1	0.82 ± 0.2	0.84 ± 0.2	0.88 ± 0.1	0.81 ± 0.2	0.82 ± 0.2	1.01 ± 0.2
EOS	0 – 1	%	0.03 ± 0.01	0.05 ± 0.03	0.07 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
BAS	0 – 0.4	%	0.08 ± 0.01	0.09 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.15 ± 0.01	0.11 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.12 ± 0.02

Values are means ± sem, n = 6 for each treatment group.

4.2.1.3 Serum cortisol

There were no differences in serum cortisol values (Figure 23).

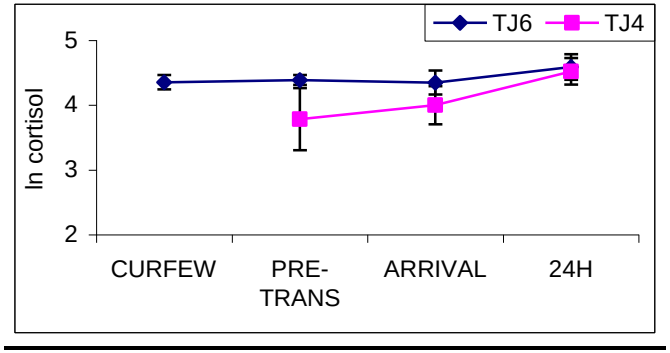


Figure 23: Serum cortisol. Values are mean transformed values ± sem, n = 6 for each treatment group.

Discussion

Glucocorticoids exert a wide range of physiological effects such as glucose homeostasis; protein, lipid, and carbohydrate metabolism; development; neurobiology; and programmed cell death. Glucocorticoids are produced by the adrenal cortex and are under the control of the HPA axis. Glucocorticoids exert potent immunosuppressive and antiinflammatory actions in a cell type–specific manner largely through the interruption of cytokine-mediated pathways. These antiinflammatory actions are also complemented by the ability of glucocorticoids to induce apoptosis in many cells including thymocytes, blood monocytes, and peripheral T cells (Necela & Cidlowski, 2004).

The glucocorticoid receptor (GR) is a member of the ligand-dependant nuclear receptor family whose role is to integrate host immune responses with physiological homeostatic balance that includes the repression of transcriptional responses to inflammatory signals. Specifically, it has been demonstrated that GR can interfere with the activities of a number of signal-dependant transcription factors including NF-κB and activator protein 1 (AP-1) family members, (reviewed in Ogawa *et al.,* 2005).

The results of the current study indicate that subjecting healthy, grass-fed lambs to pre-transport periods of food and water deprivation (24 h compared with 0 h controls) prior to 24 h transportation resulted in a decrease in both the percentage of CD4+ T lymphocytes and BB2+ B lymphocytes expressing the glucocorticoid receptor, combined with a reduction in the staining intensity (MFI) of GR in these cells. These changes remained apparent 24 h after arrival. Furthermore there appeared to be a delayed response in CD8+ T lymphocytes with a reduction in both the percentage of these cells expressing GR as well as a reduced MFI 24 h after arrival. As further measurements were not taken after this time point we are unable to determine whether these effects were transient and when they returned to baseline levels. If any further work was to be conducted to understand the likely impacts of the change in leukocyte GR expression a much larger cohort and an extended sampling schedule would be recommended. It should be noted, however, that other immunological parameters either remained unchanged (cortisol) or alterations were observed up to 48h post-transport, (eg. White cell counts) therefore it is conceivable that alterations in leukocyte GR expression would be similarly transient suggesting that there are likely to be no implications of this result for industry practice.

Odore et al (2004) reported a significant decrease in lymphocyte glucocorticoid receptor and β-adrenergic receptor following transport in 6 month old calves on arrival. This down-regulation of GR and β-AR was associated with significant increases in serum cortisol and catecholamine levels. These observations returned to normal 24 h after transportation. These patterns account for endocrine modifications induced by stress conditions and may reflect a possible impairment of the immune system functions. However, the stress induced condition may be considered transient, since circulating hormone levels and lymphocyte AR and GR concentrations returned to physiological values within 24 h, thus suggesting a rapid restoration of the endocrine profile and, probably of the immune system functions. The homologous GR down-regulation occurs in many glucocorticoid-sensitive tissues in human and rodents. Studies have indicated that the rate of GR gene transcription is decreased by glucocorticoid-activated GRs, leading to 30–80% reduction in GR mRNA, which is responsible for the reduction in receptor concentrations. Moreover, the homologous down-regulation of steroid receptors is dose- and time-dependent, reversible upon hormone withdrawal and occurs at both transcriptional and post-transcriptional levels (Reviewed in Odore et al., 2004)

GR were down-regulated in bovine mononuclear leukocytes in association with increased adrenal secretion of cortisol at calving. These results indicated that glucocorticoid receptor down-regulation is also associated with altered phenotype or function (or both) of lymphocytes and monocytes. This possibility should be substantiated because it could explain increased disease susceptibility in periparturient dairy cows (Preisler et al., 2000). A reduction in GR in peripheral tissues of patients suffering depression has also been reported (Gomley et al., 1985). More recently, reduced expression of GR in mice was shown to cause depression-like behaviours and HPA-axis dysfunction following exposure to stress (Ridder et al., 2005).

The response of leukocyte GR expression levels is likely to be transient and coupled with information in the literature and overall results of this project (as reported in AWW.055 milestone 9 report) healthy, grass-fed lambs to 24 h food and water deprivation compared with 0 h before road transport of 24 h did

not adversely affect animal welfare. Furthermore the results of the current study have validated the use of the glucocorticoid receptor assay developed in B.AWW.0184 in experimental models of stress.

4.2.2 Development of a fear model of chronic stress in cattle

4.2.2.1 Haematology

There were no significant differences in any of the haematological parameters measured.

Table 3: Mean haematological parameters of control animals

Parameter	Units	Reference Range *	control			
			Baseline	Pre-treatment	Post-treatment	recovery
HGB	g/μL	*9.0-15.3*	14 ± 0.3	14.6 ± 0.3	13.2 ± 0.4	13.7 ± 0.3
RCC	10^6/μL	*5.2-13.1*	9.8 ± 0.21	10.3 ± 0.32	8.81 ± 0.32	8.98 ± 0.23
PLT	10^3/μL	*193-582*	671 ± 85	417 ± 49	381 ± 45	514 ± 35
HCT	%	*22.0-47.0*	41.7 ± 0.85	43.9 ± 1.59	38.3 ± 1.58	41.5 ± 1.07
MCV	Fl	*30.2-70.2*	42.7 ± 0.97	43.4 ± 0.73	43.6 ± 1.34	46.3 ± 1.02
MCH	Pg	*10.0-23.0*	14.3 ± 0.42	14.2 ± 0.33	15.1 ± 0.38	15.3 ± 0.35
MCHC	g/dL	*28.0-38.1*	33.6 ± 0.66	33.5 ± 0.8	34.9 ± 0.78	33.1 ± 0.52
WCC	10^3/μL	*5.0-19.8*	4.02 ± 0.43	4.24 ± 0.61	4.78 ± 0.47	3.97 ± 0.57
NEU	10^3/μL	*1.1-15.8*	0.18 ± 0.16	0.02 ± 0	0.78 ± 0.28	0.4 ± 0.26
LYM	10^3/μL	*2.3-10.1*	2.83 ± 0.36	3.27 ± 0.29	3.28 ± 0.24	2.95 ± 0.32
MON	10^3/μL	*0.09-1.26*	0.89 ± 0.34	0.89 ± 0.37	0.58 ± 0.26	0.56 ± 0.24
EOS	10^3/μL	*0.06-1.85*	0.07 ± 0.06	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01
BAS	10^3/μL	*0.04-0.08*	0.03 ± 0.01	0.03 ± 0.01	0.08 ± 0.02	0.04 ± 0.01

Table 4 Mean haematological parameters of animals exposed to treatments 3 times/week

Parameter	Units	Reference Range *	3 treatments/week			
			Baseline	Pre-treatment	Post-treatment	Recovery
Hgb	g/μL	*9.0-15.3*	13.9 ± 0.4	14.2 ± 0.4	13 ± 0.31	13.4 ± 0.48
RCC	10^6/μL	*5.2-13.1*	9.43 ± 0.23	10.2 ± 0.25	8.93 ± 0.24	8.93 ± 0.35
Plt	10^3/μL	*193-582*	591 ± 88	384 ± 41	319 ± 57	446 ± 45
HCT	%	*22.0-47.0*	41 ± 1.29	42.8 ± 1.08	36.2 ± 0.68	39.2 ± 1.36
MCV	Fl	*30.2-70.2*	43.5 ± 1.12	42.1 ± 1.09	40.8 ± 1.12	44.1 ± 1.08
MCH	Pg	*10.0-23.0*	14.7 ± 0.32	14 ± 0.45	14.7 ± 0.41	15.2 ± 0.74
MCHC	g/dL	*28.0-38.1*	33.0 ± 0.42	33.3 ± 0.51	36 ± 0.2	34.5 ± 1.17
WCC	10^3/μL	*5.0-19.8*	4.47 ± 0.57	3.95 ± 0.35	4.48 ± 0.4	4.55 ± 0.73
Neu	10^3/μL	*1.1-15.8*	0.06 ± 0.02	0.03 ± 0.01	0.93 ± 0.46	0.84 ± 0.27
Lym	10^3/μL	*2.3-10.1*	2.94 ± 0.47	3.07 ± 0.21	2.5 ± 0.28	3.01 ± 0.55
Mon	10^3/μL	*0.09-1.26*	1.4 ± 0.26	0.79 ± 0.25	0.9 ± 0.35	0.57 ± 0.19
Eos	10^3/μL	*0.06-1.85*	0.04 ± 9.01	0.02 ± 0.01	0.05 ± 0.02	0.09 ± 0.04
Baso	10^3/μL	*0.04-0.08*	0.04 ± 0.01	0.03 ± 0.01	0.1 ± 0.02	0.03 ± 0.01

Table 5: Mean haematological parameters of animals exposed to treatments 5 times/week

Parameter	Units	Reference range *	5 treatments/week			
			Baseline	Pre-treatment	Post-treatment	Recovery
Hgb	g/μL	*9.0-15.3*	14.1 ± 0.33	14 ± 0.19	12.8 ± 0.36	13 ± 0.3
RCC	10^6/μL	*5.2-13.1*	9.49 ± 0.21	9.23 ± 0.45	8.63 ± 0.26	9.01 ± 0.25
Plt	10^3/μL	*193-582*	579 ± 52	460 ± 31	397 ± 45	560 ± 35
HCT	%	*22.0-47.0*	42.3 ± 0.75	40 ± 1.91	36.2 ± 1.03	39.8 ± 1.06
MCV	Fl	*30.2-70.2*	44.6 ± 0.46	43.5 ± 0.64	42.1 ± 0.91	44.2 ± 0.65
MCH	Pg	*10.0-23.0*	14.9 ± 0.35	15.8 ± 1.16	14.9 ± 0.36	14.5 ± 0.34

MCHC	g/dL	*28.0-38.1*	33.5 ± 0.46	36.2 ± 1.41	35.3 ± 0.5	32.9 ± 0.76
WCC	$10^3/\mu L$	*5.0-19.8*	3.59 ± 0.34	3.46 ± 0.43	4.97 ± 0.44	3.71 ± 0.5
Neu	$10^3/\mu L$	*1.1-15.8*	0.04 ± 0.01	0.05 ± 0.03	0.78 ± 0.29	0.38 ± 0.21
Lym	$10^3/\mu L$	*2.3-10.1*	2.78 ± 0.18	2.82 ± 0.18	3.5 ± 0.33	2.86 ± 0.43
Mon	$10^3/\mu L$	*0.09-1.26*	0.72 ± 0.39	0.53 ± 0.26	0.6 ± 0.3	0.33 ± 0.11
Eos	$10^3/\mu L$	*0.06-1.85*	0.02 ± 0	0.02 ± 0.01	0.03 ± 0.02	0.08 ± 0.05
Baso	$10^3/\mu L$	*0.04-0.08*	0.03 ± 0.01	0.03 ± 0.01	0.06 ± 0.02	0.06 ± 0.02

Values are mean raw values ± sem, n = 12 for each treatment group.

4.2.2.2 Lymphocyte subsets

There were no significant differences in percentages of circulating CD4+ T cells, CD8+ T cells, WC1+ T cells or BB2+ B cells.

Table 6: Mean percentages of circulating lymphocyte subpopulations

SAMPLE	TREATMENT	CD4+	CD8+	WC1+	BB2+
baseline	3 weekly	0.489 ± 0.035	0.352 ± 0.042	0.386 ± 0.026	0.482 ± 0.027
	5 weekly	0.512 ± 0.036	0.403 ± 0.024	0.339 ± 0.046	0.429 ± 0.022
	control	0.516 ± 0.016	0.391 ± 0.012	0.415 ± 0.010	0.519 ± 0.031
pre-treatment	3 weekly	0.602 ± 0.026	0.409 ± 0.030	0.343 ± 0.027	0.516 ± 0.032
	5 weekly	0.605 ± 0.009	0.414 ± 0.021	0.418 ± 0.034	0.457 ± 0.021
	control	0.535 ± 0.013	0.386 ± 0.010	0.384 ± 0.023	0.500 ± 0.045
post-treatment	3 weekly	0.555 ± 0.031	0.503 ± 0.042	0.386 ± 0.048	0.471 ± 0.039
	5 weekly	0.563 ± 0.066	0.432 ± 0.056	0.305 ± 0.074	0.488 ± 0.061
	control	0.520 ± 0.019	0.399 ± 0.036	0.362 ± 0.044	0.507 ± 0.026
recovery	3 weekly	0.592 ± 0.030	0.438 ± 0.053	0.348 ± 0.037	0.471 ± 0.027
	5 weekly	0.626 ± 0.014	0.369 ± 0.023	0.341 ± 0.021	0.566 ± 0.034
	control	0.580 ± 0.013	0.406 ± 0.019	0.391 ± 0.026	0.542 ± 0.026

Values are mean transformed values ± sem, n = 12 for each treatment group.

4.2.2.3 Glucocorticoid receptor expression

The only significant difference observed was in the percentages of circulating $WC1^+$ γδ T cells expressing the glucocorticoid receptor (Figure 24).

Table 7: Mean percentages of circulating lymphocyte subpopulations

SAMPLE	TREATMENT	CD4+	CD8+	WC1+	BB2+	CD14+
baseline	3 weekly	1.54 ± 0.01	1.56 ± 0.00	1.53 ± 0.02	1.54 ± 0.01	1.45 ± 0.04
	5 weekly	1.52 ± 0.01	1.55 ± 0.01	1.52 ± 0.02	1.54 ± 0.01	1.47 ± 0.02
	Control	1.53 ± 0.01	1.57 ± 0.00	1.54 ± 0.01	1.51 ± 0.01	1.51 ± 0.02
pre-treatment	3 weekly	1.42 ± 0.03	1.54 ± 0.01	1.39 ± 0.08	1.40 ± 0.03	1.42 ± 0.03
	5 weekly	1.41 ± 0.02	1.53 ± 0.01	1.40 ± 0.13	1.38 ± 0.04	1.33 ± 0.05
	Control	1.47 ± 0.02	1.54 ± 0.01	1.53 ± 0.08	1.45 ± 0.03	1.40 ± 0.05
post-treatment	3 weekly	1.51 ± 0.02	1.55 ± 0.01	1.52 ± 0.03	1.43 ± 0.04	1.36 ± 0.07
	5 weekly	1.54 ± 0.01	1.54 ± 0.01	1.54 ± 0.01	1.44 ± 0.03	1.38 ±0.05
	Control	1.54 ± 0.01	1.54 ± 0.01	1.56 ± 0.01	1.37 ± 0.04	1.37 ± 0.03
recovery	3 weekly	1.52 ± 0.02	1.50 ± 0.03	1.53 ± 0.02	1.46 ± 0.02	1.37 ± 0.06
	5 weekly	1.50 ± 0.01	1.48 ± 0.04	1.57 ± 0.00	1.44 ± 0.03	1.42 ± 0.05
	Control	1.51 ± 0.02	1.49 ± 0.02	1.54 ± 0.01	1.41 ± 0.03	1.25 ± 0.09

Values are mean transformed values ± sem, n = 12 in each treatment group

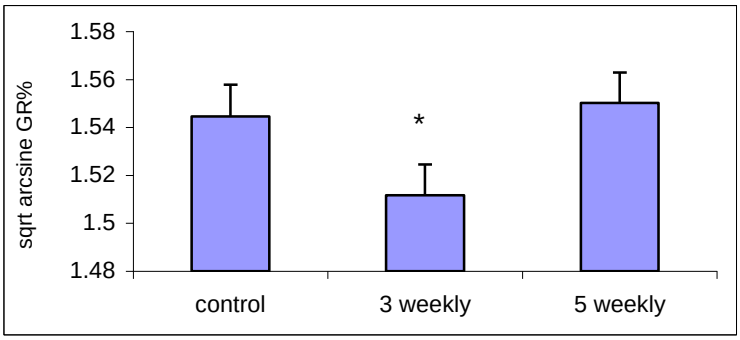


Figure 24: Glucocorticoid receptor expression in WC1+ T lymphocyte (* p<0.05). Values are mean transformed values ± sem, n = 12 for each treatment group.

There was a significant effect of treatment on growth rate (P=0.016). Growth rate during the 4 weeks of the treatment period was significantly less in the 5-times-weekly treatment than in the Control treatment (Table 8; mean growth rate for Control, 3-times-weekly and 5-times-weekly treatments expressed as estimated marginal means were 0.724, 0.553 and 0.343 kg/day, respectively). The difference between the Control and 5-times-weekly treatments was significant at P=0.006, whilst differences between the 3-times-weekly and 5-times-weekly treatments showed a trend (P=0.059) and there were no differences between Control and the 3-times-weekly treatments (P=0.110). Mean body weights for each replicate and treatment allocations at the start of the experiment did not differ (P>0.05; Table 9).

Table 8. Growth rate (estimated marginal means and Standard Errors (SE); kg/day) over the 4 weeks of treatment imposition

Parameter	Treatment			SE	P value
	Control	3-weekly	5-weekly		
Growth rate	0.724[b]	0.553	0.343[a]	0.064	0.016

[ab]different letters denote a significant difference (P<0.01)

Table 9. Body weight (kg) for each replicate (Rep) and treatment (Tmt) of cattle on arrival and at the start of week 1 of the treatment period (estimated marginal means and Standard Errors (SE))

Rep/Tmt	On arrival	SE	Week 1	SE
Replicate				
1	231.0	1.93	236.5	2.16
2	257.1	1.58	265.2	2.36
3	334.3	2.89	340.9	2.35
Treatment				
Control	271.1	13.68	282.2	13.00
3-weekly	272.3	12.94	283.8	14.78
5-weekly	278.9	13.45	276.7	12.79

Mean daytime total cortisol concentrations showed a significant effect of treatment (P=0.004). Mean cortisol concentrations for the 5-times-weekly treatment were significantly higher than both the 3-times-weekly (P=0.004) and Control (P=0.002) treatments. (Table11; mean daytime total cortisol concentrations for Control, 3-times-weekly and 5-times-weekly treatments were 3.4, 4.2 and 8.0 ng/mL, respectively).

While the maximum cortisol response (log_{10} transformed) to an intramuscular injection of ACTH was higher in the 5-times-weekly treatment compared to the Control and 3-times-weekly treatments, the difference was not significant (P>0.05). The estimated log_{10} mean values for the Control, 3-times-weekly and 5-times-weekly treatments were 1.88, 1.93 and 2.02 log_{10}ng/mL, respectively. The overall

standard error was 0.062. The untransformed mean values for the respective treatments were 78.2, 88.5 and 107.8 ng/mL.

The data for the maximum cortisol response to an intravenous infusion of CRH was $\log_{10}$ transformed prior to analysis. There was a significant effect of treatment on the transformed data using the Kruskal Wallis test (P=0.025) and a similar trend using the untransformed data (P=0.051) (Table 10). Follow-up tests for non-parametric analysis of variance are very conservative; however, using the untransformed data the maximum mean cortisol response to CRH was higher in the 5-times-weekly treatment than both the Control and 3-times-weekly treatments (P=0.020). The untransformed estimated mean values for the Control, 3-times-weekly and 5-times-weekly treatments were 43.2, 54.3 and 72.3 ng/mL, respectively.

Table 10. Mean maximum cortisol response to CRH ($\log_{10}$ ng/mL) in week 5 after treatment imposition, which occurred over a period of 4 weeks, had stopped. The untransformed data are shown in parentheses) (estimated marginal means and Standard Errors (SE))

Parameter	Treatment			SE	P value
	Control	3-weekly	5-weekly		
Cortisol response to CRH	1.64 (43.2)	1.73 (54.3)	1.85 (72.3)	0.040	0.025

There were no effects of treatment on haptoglobin concentrations in the week 5 samples (P>0.05; Table 11).

Table 11. Mean total daytime cortisol concentrations (ng/mL) and mean haptoglobin concentrations (from a single blood sample) in week 5 after treatment imposition, which occurred over a period of 4 weeks, had stopped (estimated marginal means and Standard Errors (SE))

Parameter	Treatment			SE	P value
	Control	3-weekly	5-weekly		
Cortisol	3.5[a]	4.2	8.0[b]	0.608	0.004
Haptoglobin[1]	0.17	0.09	0.20	0.09	0.693

[ab]different letters denote a significant difference (P<0.01); [1]corrected for haemoglobin interference in the assay.

There were no effects of treatment on differential white cell counts or the derived neutrophil:lymphocyte ratio (P>0.05; Table 12). Analyses of these data using measurements from week 1 as a covariate showed a significant effect of covariate for total white blood cell count (P<0.05) but not for the other haematology variables; there was still no treatment effect (P>0.05).

Table 12. Total and differential white cell counts and neutrophil:lymphocyte ratio (N:L) at week 1 (1) (before stimulus imposition) and week 5 (5) (after stimulus imposition had ended) (estimated marginal means and overall Standard Errors (SE))

Variable	Treatment						SE
	Control		3-times-weekly		5-times-weekly		
	1	5	1	5	1	5	
Total white cell count (10^6/mL)	8.6	8.9	7.2	8.5	7.4	8.0	0.55
Neutrophil[1] (%)	40.2	29.3	35.6	27.6	38.8	31.1	3.99
Lymphocyte[1] (%)	50.6	60.0	55.5	61.8	52.3	58.5	4.53
N:L	0.84	0.53	0.68	0.56	0.77	0.62	0.108

[1] expressed as a percentage of total white blood cell count

Discussion

It has been widely reported that both the amygdala and the serotonergic system play important roles in the regulation of fear and anxiety in a number of different species (reviewed in Bauman & Amaral, 2005). The activation of the sympathetic nervous system–adrenal medulla and the hypothalamic–pituitary–adrenal system are the main neuroendocrine responses associated with negative emotions (reviewed in Forkman et al, 2007).

In rats a repeated 2 hour confrontation and social defeat with submission on 7 consecutive days results in a reduced total WBC count, granulocytes and B-lymphocytes and a reduced percentage of lymphocytes, specifically CD4+ and CD8+T cells directly after the last defeat session. A reduced percentage of CD4+ and CD8+ T cells is still seen 7 days after the last defeat with submission. Interestingly, these long-term changes are not seen in rats who are defeated but do not show submissive behaviour (subdominants), indicating that individual coping styles are important mediators of long-term immunological changes following stress (Stefanski, 1998). It is therefore expected that the imposition of a chronic fear model of stress would result in some measurable immunological and neuroendocrine changes.

It is the opinion of the Principal Investigator that the data presented in this report are sufficient to conclude the treatment involving a combination of the novel object, flashing light and electric shocks imposed individually for a total of 5-times a week (5-times-weekly) resulted in a chronic stress response, compared to the control treatment. Evidence of a chronic stress response is based on increased daytime mean cortisol concentrations, increased cortisol response to CRH and reduced growth rate. There was also a higher cortisol response to ACTH, although the increase was not statistically significant.

The results presented here provide some evidence of a chronic stress response in the 5-times-weekly treatment. This is based on evidence of a sustained elevation in mean daytime cortisol concentrations, reduced growth rate and an increased maximum cortisol response to CRH (the hypothalamic hormone that regulates the pituitary-adrenal ACTH-cortisol axis). While there was also an increased maximum cortisol response to ACTH, the difference was not statistically significant.

However, given that only one of the parameters measured at CSIRO Armidale displayed a significant difference (WC1+ GR expression $p=0.04$) there is conflicting evidence as to whether the treatments imposed elicited a chronic fear response.

Curiously, these results suggest that GR expression is attenuated in the group exposed to the stressor 3 times per week with the control group and the 5 times per week group displaying similar results. It is possible that in the 5 treatments per week group homeostatic mechanisms maintained GR levels at baseline levels suggesting that the animals in the 5 treatments per week group underwent habituation to the treatments. If this were the case we would expect to see evidence of stress in other parameters in the 3 treatments per week group. Alternatively, it is possible that all animals including control animals experienced an equal level of stress as a result of increased handling during the experiment resulting in no differences in response to treatment.

There were no treatment effects on differential white cell counts or the derived neutrophil:lymphocyte ratio. This was surprising as in many species this ratio is a relatively sensitive indicator of stress and is particularly associated with increased cortisol concentrations. However, for sampling at both weeks 1 and 5 there was a minimum period of at least 40 h following surgery to implant the catheter and thus any acute response may have been missed.

It needs to be remembered that the stimuli were imposed for a period of 4 weeks and then stopped with the final blood sampling period occurring 5, 6 and 7 days later for the daytime cortisol concentrations and the responses to ACTH and CRH, respectively. Thus, treatment imposition had stopped for at least 4 days prior to sampling.

While the effects of the 3-times-weekly treatment were generally not statistically significant, for a number of the variables the response was an intermediate one between the Control and 5-times-weekly treatments. In the context of this experiment this suggests that the frequency of imposing the stimuli was an important factor, although it remains to be tested whether duration is equally important ie. a similar treatment effect may occur in the 3-times-weekly treatment if it was imposed for a longer period of time or for longer bouts.

Taken together, these data suggest that it is worthwhile to analyse the white blood cell RNA extracts collected 4 times throughout each replicate of the experiment to identify genetic markers of chronic stress based on a fear model and progress the CRC's stated outcome to utilise these markers to develop new measurable indices and welfare guidelines for cattle production.

4.2.3 Amelioration of heat stress in feedlot cattle by dietary means

4.2.3.1 Physiological measurements

When statistical analysis was performed on mean physiological measurements collected on day 3 of the heat stress treatment imposition, two variables (maximum temperature increase and the diurnal temperature change) displayed significant differences as a result of diet (Table 13).

Table 13: Physiological measurements

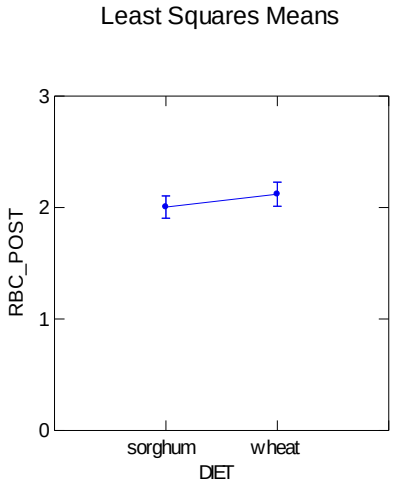
parameter	wheat	sorghum	P
Weight (kg)	357 ± 6.2	335 ± 9.8	0.064
RR 3h	109.6 ± 5.9	95.1 ± 7.0	0.62
RR max	120.3 ± 6.5	109 ± 7.2	0.88
PS 3 h	1.4 ± 0.2	1.2 ± 0.3	0.39
PS max	1.6 ± 0.3	1.3 ± 0.3	0.31
CT 3h (°C)	39.9 ± 0.3	39.2 ± 0.2	0.15
CT max (°C)	40.1 ± 0.3	38.8 ± 0.2	0.11
TC max Temp inc (°C)	1.7 ± 0.1	1.3 ± 0.1	**0.017**
Diurnal Temp change (°C)	1.0 ± 0.1	0.6 ± 0.1	**0.002**
Eat time 25% (h)	2.4 ± 1.1	1.9 ± 0.8	0.79
Eat time 50% (h)	7.7 ± 2.3	5.4 ± 2.1	0.49

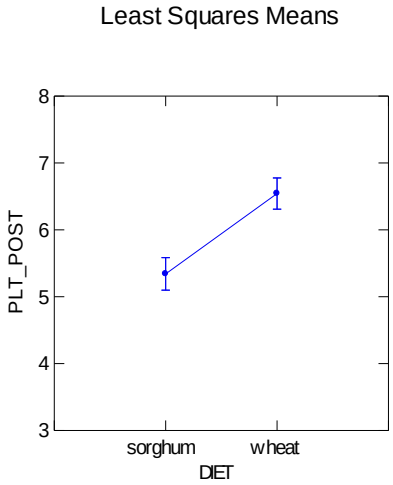
RR 3h: Mean respiration rate between 1200 and 1500 on day 3 of heat exposure
PS 3h: Mean stress score between 1200 and 1500 on day 3 of heat exposure
RR max: Max respiration rate between 1200 and 1500 on day 3 of heat exposure
PS max: Max stress score between 1200 and 1500 on day 3 of heat exposure
Core Temp 3 h; core temp between 1200 and 1500 on day 3 of heat exposure
Core Temp max: Max core temp rate between 1200 and 1500 on day 3 of heat
TC: Temp change from min value (^{o}C) and max increase on day 3
Diurnal TC: Accentuation of diurnal temperature range due to heat exposure.
Eat time, 25, 50%. Time (h) to eat 25% and 50% of the grain-based ration.

When measurements for the four time periods (1200, 1300, 1400 and 1500) over three days was analysed, ANOVA for RR, PS and CT result in significant differences as P= 0.00001, 0.0002 and <0.000001. When the analysis is repeated using only day 3 data (representing the extreme of the stress and the day before blood samples were collected) the ANOVA for RR, PS and CT result in values of P= 0.38, 0.043 and 0.004 respectively (Note: time of day was a non-significant factor in all analyses).

4.2.3.2 Haematology

There was no effect of diet when comparing pre- and post-treatment haematological values. When the influence of diet on post-treatment haematological values was examined, diet was significant for PLT (p= 0.026) with the trend being for higher PLT counts in animals fed wheat. PS at 3 h was a predictive factor for post-treatment PLT values (p = 0.016). Diet was also significant for RCC (p= 0.019) with the trend being for higher RCC counts in animals fed wheat. RR at 3 h was a predictive factor (p = 0.05) for post-treatment RCC values. Predictive factors for other haematological parameters were: HGB RR at 3 h (p = 0.04), HCT RR at 3 h (p = 0.06), MCHC PS at 3 h (p = 0.021).

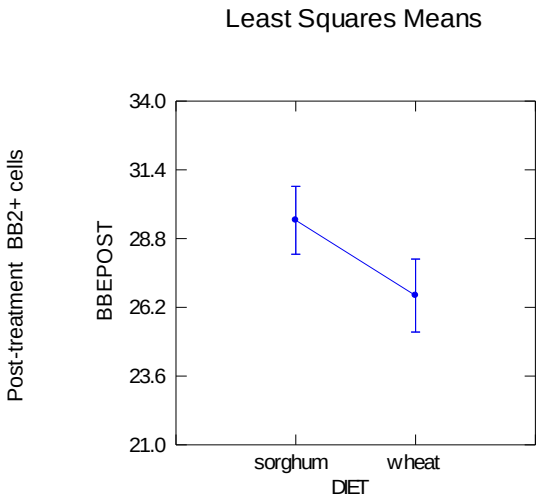




4.2.3.3 Percentages of major lymphocyte subsets

When comparing the effect of diet on the difference between pre- and post-treatment measures of lymphocyte subpopulations there were no significant differences in the percentages of lymphocytes expressing the CD4+ T cell marker, CD8+ T cell marker, WC1+ gamma delta T cell marker or BB2+ B cell marker. There was no difference in the CD4:CD8 ratio.

When the influence of diet on post-treatment lymphocyte subpopulations was examined, diet was significant for BB2+ (p = 0.038) with the trend being for a greater percentage of BB2+ cells in animals fed sorghum. For CD8+ T lymphocytes predictive factors were RR 3 h (p = 0.014) and CT 3 h (p = 0.039).

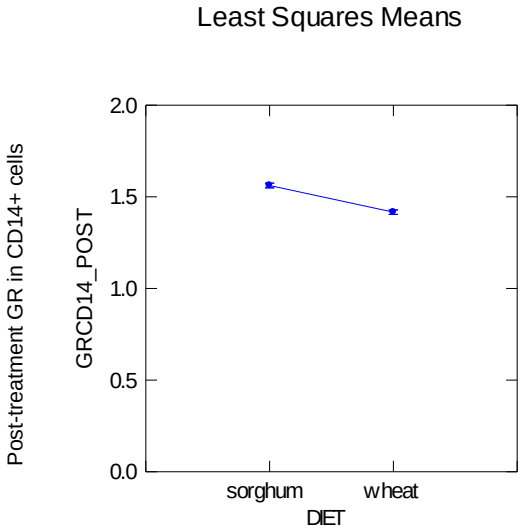


4.2.3.4 Glucocorticoid receptor

When comparing the effect of diet on the difference between pre- and post-treatment measures of leukocyte GR expression no significant differences were observed. There was, however, a trend towards significance for GR in CD14+ cells (p = 0.066).

When the influence of diet on post-treatment GR values was examined, diet was highly significant (p= 0.001) with the trend being for higher GR expression in animals fed sorghum. CT and RR at 3 h were also predictive factors for post-treatment CD14+ GR expression (p = 0.002 and p = 0.005 respectively).

Although there was no effect of diet on the difference between pre- and post-treatment GR in CD4+, CD8+, WC1+ and BB2+ cells, there were observed predictive factors for the post-treatment values. These were: CD4+ GR PS at 3 h (p = 0.01), CD8+ GR PS 3 h (p = 0.05) and weight (p = 0.001), WC1+ GR PS 3 h (p = 0.06).



4.2.3.5 Hsp70 expression in leukocyte sub-populations

When comparing the effect of diet on the difference between pre- and post-treatment measures of leukocyte hsp70 expression significant differences were observed in CD4+ cells (p = 0.041) and CD14+ cells (p = 0.012). There was no effect of diet in hsp70 expression in CD8+, WC1+ or BB2+ cells.

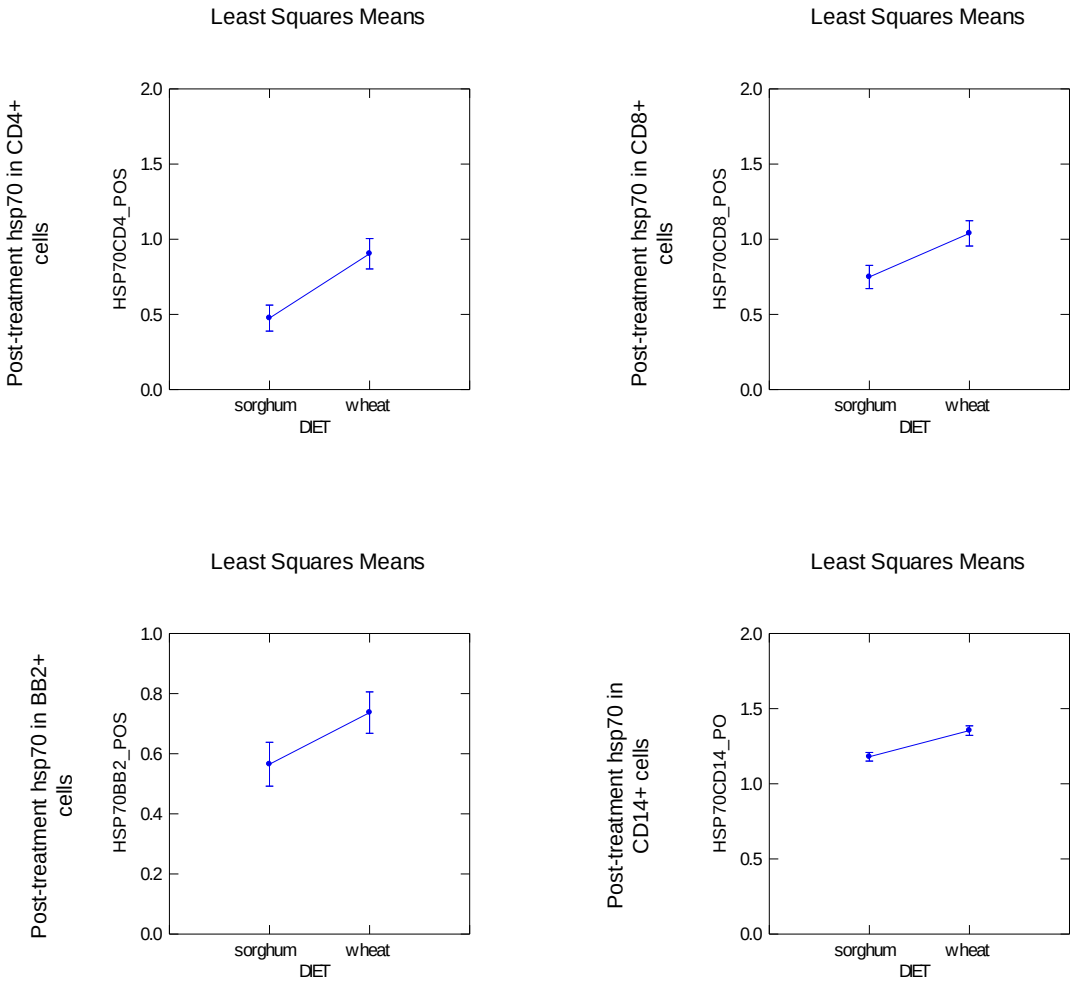
When the measurement of cellular stress and immune function are analysed significant differences are observed between pre- and post-treatment hsp70 values (expressed as percentage of cells expressing the protein) (Table 14).

Table 14: Effect of diet on the difference between pre- and post-treatment values

parameter	P
Th lymphocyte hsp70	0.041
monocyte hsp70	0.012

Th = T helper cells

When the influence of diet on post-treatment hsp70 values was examined, diet was significant for both CD4+ cells (p= 0.023) and CD14+ cells (p = 0.005) with the trend being for higher hsp70 expression in animals fed wheat. Weight was a predictive factor for post-treatment CD4+ hsp70 (p = 0.04). RR at 3 h and PS at 3 h were predictive for post-treatment CD14+ hsp70 expression (p = 0.002 and p = 0.043 respectively). Although there was no effect of diet on the difference between pre- and post-treatment hsp70 in CD8+ cells, diet was a predictive factor for the post-treatment values (p = 0.033) with the trend towards higher CD8+ hsp70 in animals fed wheat.



When the highly significant physiological measurement diurnal temperature change is fitted to the analysis as a covariate it' is strongly predictive for a number of post-treatment values (Table 15).

Table 15: Influence of diurnal temperature change on post-treatment values

parameter	P
Tγδ lymphocyte hsp70	0.049
Monocyte hsp70	0.030
Th lymphocyte GR	0.026
Tγδ lymphocyte GR	<0.001
B lymphocyte GR	0.02
MCH	0.003
platelets	0.027

Tγδ = gamma-delta T cells
GR = glucocorticoid receptor

Collectively the data in Tables 13 – 15 lend evidence to the concept that the heat stress response as a whole-body phenomenon with intracellular, tissue, and systemic components.

Discussion

The general homeostatic responses to thermal stress in mammals include raised respiration rates panting, drooling, reduced heart rates and profuse sweating decreased feed intake as well as reduced milk production (Reviewed in Silanikove, 2000). The physiological observations made during the current study indicate that there was an effect of diet on physiological parameters of stress with the trend being for a reduced capacity to cope with heat stress in animals fed wheat.

Diet was a significant factor in post-treatment platelet and red cell counts. Physiological stress scores such as panting score and respiration rate were predictive for a number of post-treatment red blood cell parameters including platelet count, red cell counts, haemoglobin, hematocrit and MCHC. A study in rats showed that after heat stress hematocrit, total plasma protein and lactate increased significantly while triglycerides decreased (Michel et al., 2007).

In the current study no changes in circulating lymphocyte subsets were detected. In a previous human study, patients suffering from heatstroke displayed a number of lymphocyte subset changes when compared to control and heat stressed patients (Hammami et al., 1998) suggesting that circulating lymphocyte numbers are not affected by heat stress but are altered as a result of heat stroke. Heat stress is defined as a physical response by the body to the total heat burden to which it is subjected by both external (air temperature, humidity, air movement, radiant heat from surroundings) and internal (metabolic heat load from physical or chemical activity) factors which could result in fatigue, heat exhaustion, heat cramps and heat stroke Heat stroke is a severe condition caused by impairment of the body's temperature-regulating abilities, resulting from prolonged exposure to excessive heat and characterized by cessation of sweating, severe headache, high fever, hot dry skin, and in serious cases collapse and coma.

There was a trend towards significance for GR expression in CD14+ cells when the effect of diet on the difference between pre- and post-treatment GR expression was examined. Diet was strongly predictive of higher GR in CD14+ cells of animals fed sorghum. Furthermore, core temperature and respiration rate were highly predictive for post-treatment CD14+ GR expression. In other leukocyte sub-populations (CD4+, CD8+ and WC1+) panting score was a predictive factor for post-treatment GR expression. These results support the suggestion of HPA axis involvement in heat stress response in cattle. In rats plasma corticosterone levels decreased and there was a trend towards increased ACTH suggesting that failure to cope with heat stress may involve HPA axis impairment (Michel *et al.,* 2007) Christison and Johnson (1972) reported that in cattle plasma cortisol increased within 20 min of exposure to acute heat stress, and reached a plateau within 2 h. It has also been reported that plasma cortisol rises markedly when cattle are acutely exposed to high environmental temperatures and decreases during the chronic phase (Habeeb et al., 1992).

The results of the current studies indicate that leukocyte heat shock protein expression was influenced by diet. Specifically, these effects were observed in CD4+ T helper cells and CD14+ monocytes. Furthermore, post-treatment hsp70 levels were influenced by diet in CD4+, CD8+, CD14+ and BB2+ cells, with the trend being for higher hsp70 levels in animals fed wheat. It has been suggested that an increase in cellular hsp70 is a defence mechanism for protection against cellular damage as a result of hyperthermia and that activation of hsp genes is positively correlated with cell injury score. These factors indicate that hsps act as a biological indicator for changes in stress levels giving support for the hypothesis that lower cellular hsp70 levels indicate higher heat tolerance (Lacetera et al., 2006).

There is increasing evidence to suggest a link between the stress-induced cortisol response and the cellular heat shock response. The Hsp70 and Hsp90 machineries are indispensable for GR folding, its hormone binding, transport to the nucleus, activation of transcription, nuclear retention and degradation. The modulation of GR activity by the Hsp70 and Hsp90 chaperone machines is a complex process, modulated by changes in the concentrations of their respective cochaperones, which probably respond to changes in the cellular environment. (Grad & Picard, 2007) *In vivo* studies of chronic glucocorticoid treatment decreased the heat shock-induced hsp70 expression in fish liver (Basu et al., 2001) and rat brain (Barr & Dokas, 1999) and the heat shock-induced hsp90 mRNA accumulation in trout hepatocytes (Sathiyaa *et al.,* 2001). Whilst the mechanism for this attenuation has not yet been elucidated, it has been suggested that GR-mediated inhibition of HSF-1 may be a possible mechanism for this attenuated heat-shock response (Wadekar *et al.,* 2001). It has been reported that increased glucocorticoid levels result in a down-regulation of GR expression and a restriction of GR signalling (Wallace & Sidlowski, 2001). Furthermore it has been reported that in fish hepatocytes elevated cortisol levels, typically seen in animals exposed to chronic stressors, affected hsp70 turnover in heat-shocked cells and the observed cortisol-attenuated hsp70 stress response may involve proteasome-mediated GR degradation (Boone & Vijayan, 2002). It has also been demonstrated that hsp70 expression in rat adrenal cortex is upregulated in response to restraint stress indicating a role for hsp70 in the physiologic stress response mediated by the HPA axis (Blake et al., 1991).

While the relative feeding value of wheat and sorghum are similar (Maner, 1987), sorghum has a lower metabolisable energy (ME) and crude protein content (CP) than other grains and is commonly used as a feed source in Queensland feedlots whereas wheat has a relatively high ME and CP between 7 and 15%

and is usually only fed at 50% of total grain due to risk of acidosis (Sneath & Wood, 2006). In dairy cows responses to increased concentration of dietary fat, and reduced protein or fibre concentration on improved milk yield in diets of heat-stressed cows are well documented and may be explained by a reduction in metabolic heat production under high environmental temperatures (West, 1999). In restricted-feeding studies, Mader et al (1999) housed feedlot steers under thermoneutral or hot environmental conditions. Steers were offered a 6% roughage finishing diet *ad libitum* (HE), offered the same diet restricted to 85 to 90% of *ad libitum* DMI levels (RE), or offered a 28% roughage diet *ad libitum* (HR). Steers fed the HR diet tended to have lower respiratory rates and significantly lower body temperatures under hot conditions than HE- and RE-fed steers, whereas RE-fed steers had significantly lower body temperature than HE-fed steers. The lower body temperature of the HR- and RE-fed steers would indicate that ME intake prior to exposure to excessive heat load influences the ability of cattle to cope with the challenge of hot environments and that lowering ME intake can lower body temperature (Reviewed in Mader, 2003).

Some researchers have suggested that the work spent in eating is a possible contributor for the higher heat increment of fibrous diets (Orskov and MacLeod, 1990). Replacing wheat hay with soy hulls to dairy cows can serve as thermoregulatory means by enabling the cows to consume their feed in more and shortened meals, with reduced feeding costs. However, the contribution of this tool to the overall energy budget of the heat stressed lactating cow seems to be small (Arieli et al., 2004). In dairy cattle it is well documented that reducing dietary fibre content can aid as a means for reducing heat increment in heat stressed cows, and part of that mitigation effect might be related to the reducing acetate/propionate ratio in the rumen (West, 1999). Making a change in the ruminal fermentation pattern, to improve the acetate/propionate balance, may therefore result in reduced metabolic heat production (MacRae and Lobley, 1982).

It should be noted however, that until details of the fibre content and feeding cost of the diets used in the current study are made available in the final analysis of results, a complete discussion regarding any effects of these factors on the current results is unable to be undertaken.

The results of this component of the study indicate that there are differences in the cellular stress response to heat stress in cattle fed different diets with the cattle fed a wheat diet displaying greater stress response than those fed sorghum.

5 Success in Achieving Objectives

The objectives of the current postdoctoral project (as detailed in Section 2) have been achieved with

- the acquisition of knowledge of interactions between the neurophysiological and immune systems in livestock;
- the development of a range of methodologies to examine the interaction between the two systems;
- the application of selected methodologies to three individual experimental models of stress in livestock;
- the presentation of results in a number of seminars, at an international conference and in a publication in an international, peer-reviewed scientific journal;
- and by development of skills for the efficient performance of scientific research and for project development.

6 Impact on Meat and Livestock Industry – now & in five years time

The promotion of world-class animal welfare standards is vital in the agricultural sector where healthy animals and sound welfare practices contribute significantly to improving production and meeting community expectations (AAWS, 2005). It has recently been reported that whilst somewhat varied, consumer expectations are universal in the desire to be sure that the meat, eggs and dairy products purchased at the supermarket are produced in a way that promotes healthy animals and allows the animals the ability to perform natural behaviours (Berg & Hammerstrom, 2006; Vanhonacker et al., 2007). Human-animal interaction is a fundamental part of Australian livestock production systems and it has been demonstrated that these interactions can limit livestock productivity and welfare (Hemsworth & Coleman, 1998). There is therefore, a significant need for a more comprehensive understanding of the biology of animal welfare and its measurement.

This project has developed and provided technical validation of a suite of new assays for measuring interactions between the neurophysiological and immune systems of cattle and sheep and has commenced the process of identifying the impact of husbandry stressors on the biological pathways that the assays monitor. The assays are available now for use by animal welfare scientists, and if further investment is made in their application by the Australian Meat and Livestock Industry, they have the potential within five years to be a substantial component of a framework for objective assessment of animal welfare and a valuable tool for description of the welfare outcomes of husbandry practices.

7 Conclusions and Recommendations

The results of this work represent the utilisation of leukocytes as target tissues to measure cellular stress responses in sheep and cattle. A panel of leukocyte markers has been identified that will be suitable for application to a range of studies to underpin animal welfare research. Selected measures have been applied to three experimental models of stress in sheep and cattle. These experimental models included animal welfare outcomes of livestock road transport practices (sheep), the development of a fear model of chronic stress in cattle, and the amelioration of heat stress in feedlot cattle by dietary means. The assays are available now for use by animal welfare scientists, and if further investment is made in their application by the Australian Meat and Livestock Industry, they have the potential within five years to be a substantial component of a framework for objective assessment of animal welfare and a valuable tool for description of the welfare outcomes of husbandry practices. The project has also built capacity through the development of skills and methodologies and their transfer to other welfare researchers though scientific publications and collaborations in linked projects.

The results described herein were achieved during a 2 year postdoctoral fellowship. Given the scope for further development and validation of the use of the panel of markers in assessing the impacts of livestock husbandry practices it is recommended that future postdoctoral fellowships of this nature be offered for a period of three years tin order o achieve the greatest possible outcomes for the Australian Meat and Livestock Industry.

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9 Appendices

9.1 Appendix 1

Protocol 1: Measurement of hsp70 expression in ovine and bovine CD14+ monocytes

Materials & Methods

Materials

4.5 ml EDTA vacutainer blood collection tubes, 4.5 ml Lithium heparin vacutainer blood collection tubes (BD Biosciences)
5 ml Falcon polystyrene tubes (BD Biosciences)
PBA – PBS + BSA + Azide (Final conc. 0.5% BSA 0.1% sodium azide)
0.6% saponin in PBS/BSA
Paraformaldehyde (1 % in PBS)
7 – 8% formalin in PBS
BD FACS Lysing solution (BD Biosciences)
BD Perm-2 (BD Biosciences)

Antibodies
Anti-CD14 (M-M9) (VMRD), anti-IgG1-PE. (Southern Biotech), anti-hsp70-FITC (Stressgen Biotechnologies), anti-mouse igG1-FITC (Sigma)

Peripheral blood was collected into 4.5 ml EDTA Vacutainer blood collection tubes from the jugular vein, via venipuncture. All blood samples were kept at room temperature and processed within 2 hr of collection. For heat shock, 0.5 ml samples of whole blood were pre-equilibrated in a 38 °C water bath for 15 min then placed in water baths of temperatures ranging from 42.5°C to 44.5°C for 1 h, followed by a recovery period of 3 h in a 38°C water bath. Control blood samples (0.5 ml) were maintained in the 38°C water bath for the entire 4 h 15 min period.

Whole blood (100μl) aliquots were resuspended in 100μl 1% paraformaldehyde solution and incubated for 10 min at room temperature. Samples were washed by centrifugation (470 x *g* for 3 min; 1450 rpm benchtop centrifuge) in 1 ml PBS supplemented with 1% BSA. Cells were resuspended in 50μl PBS-BSA containing anti-CD14 and incubated for 20 min at room temperature in the dark. Samples were washed in 1 ml PBS-BSA by centrifugation for 3 min at 470 x *g*. Supernatant was poured off and washing step was repeated 50 ul PBS-BSA containing PE-IgG1 secondary antibody was added. Samples were incubated for 20 min at room temperature in the dark. Cells were washed in 1 ml PBS-BSA by centrifugation for 3 min at 470 x *g*. Supernatant was poured off and washing step was repeated 50 ul of 0.6% saponin in PBS-BSA containing anti Hsp70-FITC or FITC-labelled isotype control was added Samples were incubated for 20 min at room temperature in the dark 100μl of 7-8% formalin in PBS was added to each tube of whole blood, Tubes were flicked to mix and let sit for 1-2 min. 1 ml 37°C ddH_2O was added o each tube. Tubes were flicked to mix then placed in a 37°C water bath for 3-4 min. 2 ml PBS-BSA was added to each tube then tubes were centrifuged at 1450 rpm at 4°C for 5 min. Supernatant was poured off and tubes were flicked. 1 ml PBS-BSA was added to each tube then tubes were centrifuged at 1450 rpm at 4°C for 5 min. Supernatant was poured off and tubes were flicked 200μl 1% parformaldehyde was added to resuspend cells. Samples were covered and stored at 4°C until flow cytometric analysis was performed on a BD-FACS Vantage with Cell Quest software (V3.1f). This analysis was completed within 24 hr of staining.

Protocol 2: Determination of leukocyte phenotype following cryopreservation of whole blood samples

Materials & Methods

Materials

4.5 ml EDTA vacutainer blood collection tubes (BD Biosciences)
Paraformaldehyde (1 % in ddH_2O)
5 ml Falcon polystyrene tubes (BD Biosciences)
PBA – PBS + BSA + Azide (Final conc. 0.5% BSA 0.1% sodium azide)
0.6% saponin in PBS/BSA
Paraformaldehyde (1 % in PBS)
7 – 8% formalin in PBS

Antibodies

Anti-CD4 (17D) Basel IM, anti-bovine CD4 (MCA834) Serotec, anti-CD8 (SBUT8 38.65) CAB, anti-WC1 (SBU-T19).CAB, anti-bovine WC1 (CC15) Serotec, anti-BB2 (BB2) VMRD, anti-CD14 (M-M9) VMRD, anti-CD45 (1.28). CAB, anti-CD45 (1.11.32). CAB, anti-IgG1-FITC. Caltag, anti-IgG2-PE. Southern Biotech, anti-IgM-FITC Caltag, anti-dexamethasone FITC Southern Biotech, anti-mouse igG1-FITC (Sigma).

Peripheral blood was collected into 4.5 ml EDTA Vacutainer blood collection tubes from the jugular vein, via venipuncture. All blood samples were kept at room temperature and processed within 2 hr of collection. Blood samples were mixed for 4 min. Aliquots (100μl) were mixed with 500μl 1% PFA. Samples were left at room temperature for 10 – 15 min. Samples were frozen at -80°C for 7 days. Samples were thawed rapidly in a 38°C water bath. Samples were transferred to polystyrene falcon tubes and washed twice by centrifugation (1700 rpm for 5 min) in 2 ml PBS supplemented with 0.5% BSA and 0.1% NaN_3). Cells were resuspended in 50μl PBS-BSA containing appropriate extracellular marker antibodies and incubated for 20 min at 4°C in the dark. Samples were washed in 2 ml PBS-BSA by centrifugation for 5 min at 1700rpm. Supernatant was poured off and washing step was repeated. 50 μl PBS-BSA containing PE/FITC labeled secondary Abs was added and samples were incubated for 20 min at 4°C in the dark. Cells were washed in 1 ml PBS-BSA by centrifugation for 5 min at 1700rpm. Supernatant was poured off and washing step was repeated. Tubes 5 and 6 proceeded through steps 9-10 for intracellular staining. Tubes 1 – 4 omitted these steps and proceeded straight to erythrocyte lysis steps.
For intracellular staining 50 ul of 0.6% saponin in PBS-BSA containing anti dexamethasone-FITC or FITC labeled isotype control was added to the appropriate tubes. Samples were incubated for 20 min at 4°C in the dark.

To lyse erythrocytes 100μl of 7-8% formalin in PBS was added to each tube of whole blood, Tubes were flicked to mix and let sit for 2 min.. 1 ml 38°C ddH_2O was added to each tube. Tubes were flicked to mix then placed in a 37°C water bath for 5 min, 2 ml PBS-BSA was added to each tube then tubes were centrifuged at 1700 rpm at 4°C for 5 min. Supernatant was poured off and tubes were flicked. 2 ml PBS-BSA was added to each tube then tubes were centrifuged at 1700 rpm at 4°C for 5 min. Supernatant was poured off and tubes were flicked 200μl 1% paraformaldehyde was added to resuspend cells. Samples were covered and store at 4°C until flow cytometric analysis was performed on a BD-FACS Vantage with Cell Quest software (V3.1f). This analysis was completed within 24 hr of staining.

Protocol 3: Use of a cryopreservation system for immunophenotyping livestock blood samples

Materials

4.5 ml EDTA vacutainer blood collection tubes (BD Biosciences)
4.5 ml CytoChex® blood collection tubes (Streck)
BD FACS Lysing solution (BD Biosciences)
BD FACSPerm-2 (BD Biosciences)
PBA – PBS + BSA + Sodium azide (Final conc. 0.5% BSA 0.1% Azide).
Paraformaldehyde (1 % in ddH_2O) (Sigma)
5 ml Falcon polystyrene tubes (BD Biosciences)

Antibodies
Anti-CD4 (17D) Basel IM, anti-bovine CD4 (MCA834) Serotec, anti-CD8 (SBUT8 38.65) CAB, anti-WC1 (SBU-T19).CAB, anti-bovine WC1 (CC15) Serotec, anti-BB2 (BB2) VMRD, anti-CD62L (BAQ92A) VMRD, anti-CD14 (M-M9) VMRD, anti-CD45 (1.28). CAB, anti-CD45 (1.11.32). CAB, anti-IgG1-FITC. Caltag, anti-IgG2-PE. Southern Biotech, anti-IgM-FITC Caltag, anti-dexamethasone FITC Southern Biotech, anti-mouse igG1-FITC (Sigma).

Method
Peripheral blood samples were collected from 3 Merino wether weaners and 3 Holstein bull calves into an EDTA BCT and a CytoChex BCT. EDTA samples were stained fresh and the CytoChex samples were stored at room temperature for 7 days in the dark prior to staining. Staining panels used were: CD4/CD45, CD8/CD45, WC1/CD45, B-B2/CD45, CD62L/CD45, CD14/GR. Flow cytometric analysis was completed within 24 h of staining using a BD FACSVantage with CellQuest software. Haematology was performed on each sample using an Abbott CellDyn haematology analyzer... Statistical analysis was performed using a paired Student's t-test.

Protocol 4: Measurement of glucocorticoid receptor in leukocyte subpopulations

Materials

4.5 ml EDTA vacutainer blood collection tubes (BD Biosciences)
5 ml Falcon polystyrene tubes (BD Biosciences)
PBA – PBS + BSA + Sodium azide (Final conc. 0.5% BSA 0.1% sodium azide)
0.6% saponin in PBS/BSA
Paraformaldehyde (1 % in PBS)
7 – 8% formalin in PBS
BD FACS Lysing solution (BD Biosciences)
BD Perm-2 (BD Biosciences)

Antibodies
Anti-CD4 (17D) Basel IM, anti-bovine CD4 (MCA834) Serotec, anti-CD8 (SBUT8 38.65) CAB, anti-WC1 (SBU-T19).CAB, anti-bovine WC1 (CC15) Serotec, anti-BB2 (BB2) VMRD, anti-CD62L (BAQ92A) VMRD, anti-CD14 (M-M9) VMRD, anti-CD45 (1.28). CAB, anti-CD45 (1.11.32). CAB, anti-IgG1-FITC. Caltag, anti-IgG2-PE. Southern Biotech, anti-IgM-FITC Caltag, anti-dexamethasone FITC Southern Biotech, anti-mouse igG1-FITC (Sigma).

Method
Peripheral blood was collected into 4.5 ml EDTA Vacutainer blood collection tubes from the jugular vein, via venipuncture. All blood samples were kept at room temperature and processed within 2 hr of collection.

Whole blood (100μl) aliquots were resuspended in 100μl 1% paraformaldehyde solution and incubated for 10 min at room temperature. Samples were washed by centrifugation (470 x *g* for 3 min; 1450 rpm benchtop centrifuge) in 1 ml PBS supplemented with 1% BSA. Cells were resuspended in 50μl PBS-BSA containing anti-CD14 and incubated for 20 min at room temperature in the dark. Samples were washed in 1 ml PBS-BSA by centrifugation for 3 min at 470 x *g*. Supernatant was poured off and washing step was repeated 50 ul PBS-BSA containing PE-IgG1 secondary antibody was added. Samples were incubated for 20 min at room temperature in the dark. Cells were washed in 1 ml PBS-BSA by centrifugation for 3 min at 470 x *g*. Supernatant was poured off and washing step was repeated 50 ul of 0.6% saponin in PBS-BSA containing anti dexamethasone-FITC or FITC-labelled isotype control was added Samples were incubated for 20 min at room temperature in the dark 100μl of 7-8% formalin in PBS was added to each tube of whole blood, Tubes were flicked to mix and let sit for 1-2 min. 1 ml 37°C ddH_2O was added o each tube. Tubes were flicked to mix then placed in a 37°C water bath for 3-4 min. 2 ml PBS-BSA was added to each tube then tubes were centrifuged at 1450 rpm at 4°C for 5 min. Supernatant was poured off and tubes were flicked. 1 ml PBS-BSA was added to each tube then tubes were centrifuged at 1450 rpm at 4°C for 5 min. Supernatant was poured off and tubes were flicked 200μl 1% parformaldehyde was added to resuspend cells. Samples were covered and stored at 4°C until flow cytometric analysis was performed on a BD-FACS Vantage with Cell Quest software (V3.1f). This analysis was completed within 24 hr of staining.

Protocol 5: Modification of staining techniques for flow cytometry to suit a 96-well plate format

Below is an example of the basic protocol developed to suit the staining requirements for sheep and cattle blood samples.

96-well Intracellular and Surface Staining Protocol

Materials

Vee-bottomed 96-well microplate (Sarstedt)
Adhesive plate sealers (Sarstedt)
BD FACS Lysing solution (BD Biosciences)
BD Perm-2 (BD Biosciences)
FACSwash solution – PBS + BSA + Sodium azide (Final conc. 0.5% BSA 0.1% sodium azide)
Paraformaldehyde (1 % in PBS)

Method

Whole blood samples (100μl) were aliquoted into wells of a v-bottomed 96-well microplate. Primary surface marker antibodies (50 μl) were added to the appropriate wells. Plates were incubated for 30 minutes on ice in the dark. Plates were washed 2X with FACS wash solution (90 μl) to wells, Plates were centrifuged at 400 x g for 3minutes. Supernatant was discarded by flicking. Fluorochrome-labelled secondary antibody (50μl) was added to surface marker antibody diluted in FACS wash solution. Plates were incubated for 30 minutes on ice in the dark. Plates were washed 1X with FACS wash solution as described above. FACS Lysing Solution (200μl) was added to cells in wells. Plates were incubated at room temp in the dark for 10 minutes. Plates were centrifuged as above and supernatant was discarded. FACS Perm Solution 2 (100μl) was added to cells. Samples were mixed well and incubated for 10 minutes at room temperature in the dark. FACS wash solution (90μl) was added to the wells. Plates were washed 1X as described above. Flurochrome-conjugated intracellular antibody (50 μl) was added. Plates were incubated for 30 minutes on ice in the dark. Plates were washed 3X with FACS wash solution. Cells were fixed with 1% paraformaldehyde, (100 μl) and stored at 4°C until flow cytometric analysis.

Protocol 6: Detection of hsp70 by Western immunoblot

Blood collection

Peripheral blood samples were collected from two sheep and two cows by venipuncture into EDTA vacutainers for analysis of leukocyte protein synthesis.

Isolation of lymphocytes

Approximately 20 ml of whole blood was used for the lymphocyte harvest. Lymphocytes were isolated from blood using the Ficoll-Paque® Plus (Amersham-Pharmacia, Baulkham Hills, Sydney, NSW) gradient centrifugation procedure as previously described (B∅yum, 1968). Briefly, 15 ml RPMI medium without methionine was added to 20 ml of whole blood in a 50 ml centrifuge tube, and gently mixed. Ficoll-Paque Plus (10 ml) was carefully underlaid taking care to maintain a perfect interface between blood/media and the Ficoll. Tubes were centrifuged at 400 x *g* for 30 min without acceleration or braking. Lymphocytes were aspirated and mixed with RPMI medium without methionine and re-centrifuged at 400 x *g* for 8 min with a 5 min brake. The supernatant was discarded and the pellet was resuspended in 10 ml RPMI medium without methionine. Tubes were centrifuged at 400 x *g* for 8 min with 5 min acceleration and 5 min braking. The supernatant was discarded and the lymphocyte fraction was resuspended in RPMI medium without methionine and supplemented with 10% foetal bovine serum.

Stress treatment
Heat shock

For heat shock, the lymphocytes were placed in a 42.5°C water bath for 1 hr, followed by a recovery period of 3 hr in a 37°C water bath. Control cells were maintained in the 37°C water bath for the entire 4 hr period.

Preparation of leukocyte smears for immunohistochemistry

100μl aliquots were removed from each sample and dispensed into the wells of a cytospin for preparation of cell smears as per protocol 2 - Immunohistochemistry.

Analysis of protein synthesis
Protein extraction

Heat shock and control samples were centrifuged at 900 x *g* for 3 min, after which the supernatant was removed and the pellet resuspended in 800 μl RPMI to remove excess label. The suspension was centrifuged again for 3 min at 900 x *g*. The supernatant was removed and the pellet resuspended in 50 μl protein extraction buffer [10 μl 10% v/v Triton X-100, 100 μl 1000 mM-KCl, 8 μl 1000 mM-$MgCl_2 \cdot 6H_2O$, 150 μl 1000 mM-NaCl, 20 μl 1000 mM-Tris-HCl pH 7.4, 10 μl 100 mM-PMSF, 702 μl ddH_2O]. Samples were frozen overnight at -20°C. The following day, samples were thawed over ice with intermittent vortexing, then centrifuged at 900 x *g* for 3 min. The supernatant was transferred into fresh microfuge tubes and frozen at -80°C until the time of assay.

Protein assay

Protein concentration of samples was determined using the Pierce Protein Assay Kit (Pierce Biotechnology) following the manufacturer's instructions. Briefly, a two-fold serial dilution of BSA standard was performed (250μg/ml – 5 μg/ml). Aliquots (10μl of standards or samples) were pipetted into the wells (in duplicate) of a 96-well microplate (Sarstedt). 200μl of working reagent (50 parts of reagent A to 1 part reagent B) was added to each well. Plate was mixed on a plate rocker for 30 seconds. Plate was incubated at 37°C for 30 min. Absorbance was read at 562 nm. To construct a standard curve, the average of absorbances for the blank samples was subtracted from the average of each standard absorbance. Protein concentration of unknown samples was interpolated from the standard curve,

Electrophoresis

Protein samples (10 μg) and molecular weight standards (10 μl) were separated by 1D-SDS-PAGE (as described by Laemmli, 1970) on 10% iGels (NuSep). Electrophoresis was carried out at room temperature at 150v- 200 v for approximately 60 min or until Electrophoresis was discontinued once the bromophenol blue loading dye had migrated to the end of the gel.

Transfer of protein to membranes

Following electrophoresis, gels were immersed in continuous transfer buffer [39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, 20% methanol] for 5 minutes. Proteins were transferred onto Hybond C super nitrocellulose membrane (Amersham) that had been pre-equilibrated in transfer buffer. The transfer was performed using BioRad mini-protean system for wet transfer….. Transfer was carried out at room temperature, with a constant current of 100 mA for 60 min. The membrane was blocked overnight in a cool room in a blocking solution [80 mM-Na_2HPO_4, 20 mM-NaH_2PO_4, 100 mM-NaCl, 0.1% (w/v) Tween, pH 7.4 with 5% (w/v) skim milk powder].

Detection of bound antibody

Response to specific antibodies was detected using the ECL™ western blotting detection kit (Amersham) in accordance with the manufacturer's instructions. After non-specific binding sites were blocked overnight, membranes were rinsed twice in PBS-T [PBS, 0.1% (w/v) Tween-20], followed by 15 min in PBS-T and 2 x 5 min in PBS-T, all with gentle rocking. Membranes were incubated with the appropriate antibody (anti-hsp70 1:1000, anti-β-actin 1:3000) in PBS-T with 5% (w/v) skim milk powder in a sealed plastic bag, for 1 hr at room temperature with gentle rocking. Primary antibodies were used at the concentrations listed in Table 2.2. The membranes were rinsed twice in PBS-T [PBS, 0.1% (w/v) Tween-20], followed by 15 min in PBS-T and 2 x 5 min in PBS-T, all with gentle rocking. After washing, the membranes were incubated with a secondary antibody (anti-mouse) at a concentration of 1:1000 in PBS-T with 5% (w/v) skim milk powder in a sealed plastic bag, for 1 h at room temperature with gentle rocking. The membranes were rinsed twice in PBS-T [PBS, 0.1% (w/v) Tween-20], followed by 15 min in PBS-T and 2 x 5 min in PBS-T, all with gentle rocking. The membranes were incubated with ECL™ detection reagents for 1 min, drained, then wrapped in cling-wrap and exposed to ECL™ Hyperfilm film (Amersham) for periods of between 30 sec and 3 min. Films were developed by immersion in Kodak liquid X-Ray developer for 30 sec, rinsed in water, followed by immersion in Kodak liquid X-Ray fixer for 30 sec and finally rinsed in water.

Stripping of membranes

Prior to reprobing, Western immunoblot membranes were stripped using a previously described method (Liao *et al.,* 2000). Membranes were incubated in 100 ml stripping buffer [100 mM-2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM-Tris-HCl pH 6.7] at 50°C for 30 min with occasional agitation. Membranes were washed twice in 100 ml PBS-T [PBS, 0.1% (w/v) Tween-20] for 10 min with gentle rocking at room temperature. Membranes were then blocked for 1 hr at room temperature in blocking solution [80 mM-Na_2HPO_4, 20 mM-NaH_2PO_4, 100 mM-NaCl, 0.1% (w/v) Tween-20, pH 7.4 with 5% (w/v) skim milk powder].

Densitometry

Western immunoblots can be analyzed using a gel documentation and analysis system and densitometry software package. The ratio of respective band intensities relative to that of actin in corresponding control and heat shock lanes is calculated and used to quanitate the particular proteins of interest. The relative increase in protein synthesis is expressed as a ratio of normalized pixel intensity with respect to β-actin.

Protocol 7: Immunohistochemistry

Sample preparation
PBMC smears
PBMC smears were prepared using a standard protocol for cell preparation by cytocentrifuge using a Shandon Elliott, “Cytospin” (Cheshire, UK).

Tissue sections
Ovine and bovine tissue samples were collected from testis, mammary gland and jejunum. Tissue sections were prepared using standard protocols. Tissue sections were frozen at -80°C until use.

Slides were thawed at room temperature for several hours prior to staining. A PAP (wax) pen was used to draw around cell smears and tissue sections.. Slides were placed in Coplin jars containing MDF fixative (30% formaldehyde, 15% ethanol, 5% glacial acetic acid, 50% milliQ watrer v/v) for 2 min. Fixative was tipped off into waste bottle and slides were soaked in milliQ x 2 then washed with PBS two times then soaked in PBS-T (PBS with 0.05% Tween 20) for 10 min. Slides were removed from jar one at a time and placed in a humid chamber. PBS-BSA (0.5 % BSA) was pipetted carefully onto each cell smear and slides were incubated for 30 min. PBS-BSA was tipped of and edge of slide blotted, one at a time, primary antibodies (diluted in PBS-BSA) were added to cover cell smear. Slides were incubated in the dark for 45 min. Slides were washed, one at a time, by holding over a sink and carefully rinsing with PBS-T in a wash bottle, being careful not to aim the stream directly at the cell smear. Slides were then placed into a fresh jar of PBS-T. Slides were removed from the jar, one at a time, blotted dry, placed in the humid chamber and secondary antibodies (diluted in PBS-BSA were added as above). Slides were incubated at room temperature, in the dark for 45 min. Slides were washed as above and placed into a jar containing PBS-T. Slides were removed from the jar, one at a time, blotted dry, and ProLong Gold antifade reagent with DAPI (Molecular Probes) was applied to the centre of each cell smear. Coverslip was applied and tapped down gently with forceps to remove any air bubbles. A small dab of nail varnish was applied to one corner of the coverslip to anchor it to the slide. Slides were placed in a clean, dry slide box and protected from the light. Slide box was loosely covered with aluminium foil and left on the bench overnight to air dry. The following day, nail polish was applied to the edge of the coverslip. Slides were examined using an Axio Imager Z1 microscope with Colibri illumination (Carl Zeiss Imaging Solutions, Germany) Images were photographed and analysed using AxioVision (version 4.6.1) software (Carl Zeiss Imaging Solutions, Germany).

Anitibodies
Anti-hsp70-FITC (1:50) (Assay Designs)
Dexamethasone-FITC (1:500) (Molecular probes)
Anti mouse IgG1-FITC (1:20) (Sigma-Aldrich)

Protocol 8: Immunoassay for measuring hsp70 in plasma

Materials & Methods

The Surveyor™ IC human/mouse/rat total hsp70 immunoassay kit (R & D Systems, Minneapolis, USA) was used, following the manufacturer's instructions, to detect and quantitate levels of hsp70 in sheep and cattle plasma. This ELISA is a quantitative sandwich immunoassay that uses an immunoassay plate pre-coated with an antibody that is specific for hsp70. The immobilized antibody captures hsp70 and is detected with an hsp70 specific biotinylated detection antibody using a streptavidin-horseradish peroxidase conjugate. The assay is developed with tetramethylbenzidine substrate and colour development is stopped with an acid stop solution. Briefly, 100 µl of hsp70 standard solutions of varying concentrations, blank solutions and plasma samples (diluted with sample diluent) were pipetted into a 96-well microplate that had been pre-coated with anti-hsp70. All standards, blanks and samples were run in duplicate. The microplate was incubated for 2 h at room temperature. The contents of each well were aspirated and 400 µl wash buffer was added to each well. This was then aspirated and the washing steps repeated a further two times. The microplate was patted dry with paper towel then 100 µl biotinylated detection antibody was added to each well. The microplate was covered and incubated for 2 h at room temperature. The washing steps were repeated as above. The microplate was patted dry with paper towel, then 100 µl streptavidin-horesradish peroxidase conjugate was added to each well. The microplate was covered and incubated for 20 min at room temperature. The washing steps were repeated as above. The microplate was patted dry with paper towel then 100 µl tetramethylbenzidine substrate was added to each well. The microplate was covered and incubated for 20 min at room temperature in the dark. Finally, 50 µl acid stop solution was added to each well and the colour intensity was read at 450 nm with a correction of 570 nm in an Emax™ Precision Microplate Reader (Molecular Devices, Sunnyvale, USA). A standard curve was constructed using a calibrated hsp70 protein standard and sample concentrations of hsp70 were quantitated by interpolation of absorbances from the standard curve using the SOFTmax (v 2.35) software package (Molecular Devices, Sunnyvale, USA).

Protocol 9: qRT-PCR detection of hsp70

Sample preparation

Bovine tissue biopsies were collected and mRNA was isolated from the tissue samples using standard protocols.

Primer Details

Primers were designed based on advice from Dr RJ Collier (personal communication). Primers were obtained from Sigma-Aldrich (Sydney, Australia)

Table 2. Primers used in qRT-PCR.

Target Gene	Species	Sequence (5' - 3')[a]
Hsp70 1A	Bovine	Fwd- GGGGCCTTACTTTTTGTCTGTCT Rev- GTGGCAGTGTTGACTCACTTGAA
Hsp70 1B	Bovine	Fwd- CCTGGATTGCTCATGTTTGTTAT Rev- AAAGGGACAGTTCAACATCTCAA
Hsp70 inducible	Bovine	Fwd- CGTGCTCATCTTTGATCTGG Rev- TGGCTGATGTCCTTCTTGTG
Beta-actin	Bovine	Fwd- AGCAAGCAGGAGTACGATGAGT Rev- ATCCAACCGACTGCTGTCA
Rp-LPO	Bovine	Fwd- CAACCCTGAAGTGCTTGACAT Rev- AGGCAGATGGATCAGCCA

[a] Fwd = forward primer, Rev = reverse primer.

qRT-PCR Protocol

For this study, components for qRT-PCR were provided in a QuantiTect SYBR Green RT-PCR Kit (Qiagen). This kit includes SYBR Green RT-PCR Master Mix together with a QuantiTect RT mix to allow both RT and PCR reactions to take place in a single tube (one-step qRT-PCR). Advantages of one-step qRT-PCR over the two-step alternative (where the RT and PCR steps are separated) lies in the reduced experimental variation associated with the one-step technique along with the obvious time saving benefit (Wong and Medrano, 2005). Disadvantages of the one-step approach include the limitation of no longer being able to conduct several real-time PCR assays on a single cDNA synthesis sample, and the observed increase in primer dimmer (PD) accumulation inherent with this technique. In one-step qRT-PCR, the DNA-dependant polymerase activity of reverse transcriptase on RNA:DNA hybrids is thought to drive the initial formation of PDs which are subsequently amplified in the PCR process (Vandesomple et al. 2002). Details of qRT-PCR reaction components are listed in Table 3.

RT-PCR assays were performed on a Rotor-Gene™ 3000 Real-time Multiplexing System (Corbett Research) fitted with a 72-well rotor using the following cycling protocol:

50°C	30 Minutes	(RT step)
95°C	15 Minutes	(Activates HotStar Taq and inactivates RTases)

Followed by 45 cycles of:-

94°C	15 Seconds	(Denaturation step)
58°C	30 Seconds	(Annealing step)
72°C	30 Seconds	(Extension step)
78°C	15 Seconds	(Extra data acquisition step)

At completion of cycling:-

72°C 10 Minutes
65-99°C Ramped in 1°C increments (Generation of melt curve data)

Based on the knowledge that one-step qRT-PCR inherently leads to PD accumulation, an extra data acquisition step was added to the cycling profile. This meant data was acquired at both the 'extension' and the 'extra data acquisition' cycling steps. The extra data acquisition step at 78°C, a temperature above the T_m of PDs but below the T_m of specific amplification products, allows the overall fluorescent signal measured to be biased against that generated by PD and toward that generated by specific amplification products. The cycling protocol listed above remained the same for all primer sets used in this study, as did the qRT-PCR components listed in Table 3. Details of the primers used in qRT-PCR are outlined in Table 2.

Table 3. qRT-PCR reaction components.

Component	Volume (µl)/Reaction	Final Concentration
2x QuantiTect SYBR Green RT-PCR Master Mix	12.5	1x
Forward Primer (25µM)	0.5	0.5µM
Reverse Primer (25µM)	0.5	0.5µM
Quantitect RT Mix	0.25	1x
RNase-free Water	6.25	-
RNA template* (0.5-10ng/ul)	5.0	2.5-50ng/reaction
Total Volume	25.0	

* DNase-Treated.

Amplification products were purified using a QIAquick PCR purification kit (Qiagen) which removes nucleotides <100bp in length (such as PD) and carry over contaminants from the PCR reaction. An aliquot of purified product (diluted in DNA/RNA sample loading buffer) was then loaded onto a 2% TAE Nu-Sieve 3:1 agarose gel containing 0.5µg/ml Ethidium Bromide along with a quantified 100bp ladder (Promega) and run at 90V for 1.5 hours. The presence of a single sharp band representing a product of expected molecular weight was evidence of high primer specificity (Figure 1). The presence of low molecular weight bands (<50bp) prior to purification indicate the presence of PD.

The concentration of purified amplification products were estimated using a combination of UV spectroscopy and electrophoresis results and products were then sequenced.. Sequencing reactions were performed using a CEQ Dye Teminator Cycle sequencing(DTCS) Kit (Beckman Coulter) as per the manufacturer's protocol with the single exception that the final reaction volume was reduced to 10µl. Reaction components consisted of 2.0µl of primer (1.6µM), 4.0µl DTCS Master Mix and 4.0µl of template (containing 2-6ng of PCR product or 40-70ng of plasmid). When sequencing plasmid DNA templates, samples underwent pre-heat treatment for 5 minutes at 86°C prior to the sequencing reaction to improve reaction efficiency. Following the sequencing reaction, samples were ethanol

precipitated, vacuum dried for 40 minutes in a SpeediVac Concentrator (Savant) and resuspended in sample loading solution prior to loading into the CEQ™ 8000 Genetic Analysis System (Beckman Coulter) for sequence determination. Sequence data was analysed using CEQ™ 8000 analysis software (Beckman Coulter) and analysed sequences aligned with known mRNA sequences to verify the identity of amplification products.

Protocol 10: Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation, before and after oxidative stress, was measured by the determination of malondialdehyde (MDA) in plasma, which reacts with thiobarbituric acid to form a TBA-MDA complex (Buege & Aust, 1978). Plasma samples were thawed over ice with intermittent vortexing. For measurements before oxidative stress, aliquots of 300 μl were combined with 1ml of a solution containing 15% (w/v) TCA and 0.375% (w/v) TBA in 0.25 M HCl. The reaction mixture was heated for 15 min in a boiling water bath, then cooled, on ice, to room temperature.

Samples were then centrifuged at 15,000 x *g* for 3 min, after which optical densities of the supernatant were read at 535 nm against a TCA-TBA-HCl blank. The MDA concentration of the sample was calculated using the molar extinction coefficient of 1.56×10^5 mol/L/cm^{-1} (Wills, 1969).For measurements after oxidative stress, 300 μl plasma aliquots were combined with an equal volume of 100 mM AAPH [2,2-azobis(2-amidinopropane) dihydrochloride] in PBS, pH 7.4 and were incubated in a 37°C water bath for 3 hr. After incubation, 300 μl aliquots were withdrawn from the suspension and combined with 1 ml of a solution containing 15% (w/v) TCA and 0.375% (w/v) TBA in 0.25M HCl. The reaction mixture was heated for 15 min in a boiling water bath, then cooled on ice, to room temperature. Samples were then centrifuged at 15,000 x *g* for 3 min, after which optical densities of the supernatant were read at 535 nm against a TCA-TBA-HCl blank. The MDA concentration of the sample was calculated using the molar extinction coefficient of 1.56×10^5 mol/L/cm^{-1} (Wills, 1969).

Protocol 11: Protein Carbonyl Assay

Protein Assay

Protein concentration of samples was determined using the Pierce Protein Assay Kit (Pierce Biotechnology) following the manufacturer's instructions. Briefly, a two-fold serial dilution of BSA standard was performed (250μg/ml – 5 μg/ml). Aliquots (10μl of standards or samples) were pipetted into the wells (in duplicate) of a 96-well microplate (Sarstedt). 200μl of working reagent (50 parts of reagent A to 1 part reagent B) was added to each well. Plate was mixed on a plate rocker for 30 seconds. Plate was incubated at 37°C for 30 min. Absorbance was read at 562 nm. To construct a standard curve, the average of absorbances for the blank samples was subtracted from the average of each standard absorbance. Protein concentration of unknown samples was interpolated from the standard curve.

Protein Oxidation

Protein oxidation was estimated by measuring protein carbonyls using a previously described method (Reznick *et al.,* 1994). Briefly, plasma samples were thawed over ice with intermittent vortexing. 100 μl aliquots of plasma were incubated with 600 μl 10 mM 2,4-DNPH [0.0495 g 2,4-DNPH in 25 ml 2 M HCl] at room temperature, in a dark cupboard for 1 hr, with vortexing every 15 min. Blank samples were incubated with 600 μl 2 M HCl at room temperature, in a dark cupboard for 1 hr, with vortexing every 15 min. 600 μl 20% TCA was then added to each of the tubes. Samples were vortexed then centrifuged at 15,000 x *g* for 3 min. Supernatant was discarded and the remaining pellet was washed three times in 1 ml ethanol-ethyl acetate (1:1 w/v) with 10 min incubation over ice followed by centrifugation at 15,000 x *g* for 3 min. After washing, pellets were resuspended in 1 ml 6 M guanidine hydrochloride in 20 mM KH_2PO_4 [14.3295 g guanidine hydrochloride; 0.068 g KH_2PO_4, ddH_2O to 25 ml; pH to 2.3 with HCl]. Tubes were vortexed then incubated in a 37°C water bath for 1 hr. Following incubation, tubes were vortexed then centrifuged at 15,000 x *g* for 5 min. The optical density of the supernatant from sample tubes was read at 370 nm against supernatant from blank tubes. Carbonyl content was calculated using the molar extinction coefficient of 22,000 mol/L/cm^{-1} (Levine *et al.,* 1990) and was expressed as μmol carbonyl/mg protein.

9.2 Appendix 2

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Development of a method of measuring cellular stress in cattle and sheep

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Abstract

In the current studies, flow cytometric methods were used to demonstrate that heat shock protein (hsp) 70 is constitutively expressed in ovine and bovine leukocytes but that the level of expression varies considerably between different leukocyte types and between species. We also show that expression of hsp70 is upregulated in response to an *in vitro* heat shock treatment. The optimal temperature for heat shock of leukocytes from sheep and cattle is 43.5 °C. In sheep and cattle, the relative susceptibility of leukocyte type to upregulation of hsp70 expression, as assayed as percent positive cells, by *in vitro* heat shock was cell type specific. Best results were obtained from fresh samples; after storage at room temperature for 24 h upregulation was highly variable between animals and less than in fresh samples. These studies demonstrate that evaluation of leukocyte hsp70 expression by flow cytometry is a robust, reproducible method for use in the evaluation of cellular stress responses in cattle and sheep. The application of the methods described may be a valuable tool in assessing *in vivo* stress responses in livestock species.
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Keywords: Heat shock protein 70; Leukocyte; Flow cytometry

1. Introduction

The cellular stress response was first observed in *Drosophila busckii* salivary glands that had been exposed to an increased temperature (Ritossa, 1962). The increase in transcriptional activity of a set of genes on a particular locus was evidenced by a novel pattern of chromosomal puffing. The proteins encoded by these genes were later named heat shock proteins (hsp), due to their inducibility by increases in temperature (Tissières et al., 1974).

Hsps or stress proteins as they are also known are a group of highly conserved proteins that represent between 2% and 15% of total cellular protein and are expressed by every living organism (Morimoto et al., 1994). As the name implies they are expressed when cells are exposed to stressful stimuli such as: free radical attack, UV light, ozone or fever; however they are also expressed constitutively in the unstressed cell (Welch, 1992). These proteins allow cells to adapt to gradual changes in their environment. The main functions of hsps are to regulate apoptosis and to act as intracellular molecular chaperones that facilitate protein folding, biogenesis and assembly (Morimoto et al., 1994).

Some hsps are implicated in the induction of humoral, cytotoxic T-lymphocyte (CTL), and natural killer (NK) cell responses against viruses, tumours and other infectious disease agents (Brenner and Wainberg, 1999). This property has increased speculation that a potential role exists for hsp-peptide complexes as constituents for viral and tumour vaccines (Srivastava

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and Maki, 1991; Mizzen, 1998; Brenner and Wainberg, 1999). Immunogenic mechanisms include, and may not be limited to, hsps acting as classic antigens, hsps' ability to redistribute to cell surface membranes following infection or transformation, hsps' adjuvant properties, and hsps' ability to be integrally involved in antigen presentation (Brenner and Wainberg, 1999).

The stress proteins belong to a multi-gene family and range in size from 8 to 150 kDa. Hsps are classified according to their molecular weight, for example the 70 kDa protein is named hsp70, and each hsp species has a unique mechanism of action (Craig et al., 1993; Whitely et al., 1999). Hsp70 is the most widely studied of all the heat shock proteins and is reported to have a number of important chaperoning functions including aiding in the folding of new proteins, refolding of incorrectly folded proteins, reducing protein aggregates, presenting proteins in a conformation suitable for degradation by the proteasome, and presenting steroid receptors in a ligand-binding conformation (reviewed in Fink, 1999).

Four hsp70 genes have been identified in cattle. Two, localized to chromosome 23 and identified as *hsp70-1* and *hsp70-2* (Grosz et al., 1992) have since been confirmed to be orthologous to human HSPA1A and HSPA1B, respectively. The third, *hsp70-3*, is localized to chromosome 10 (Grosz et al., 1992) and has been identified as being orthologous to human HSPIL. The fourth, *hsp70-4*, has been localized to chromosome 3 (Gallagher et al., 1993). In cattle, hsp70 mRNA and protein expression have been reported in a wide range of tissues and cell types including brain, heart, kidney, liver, lung, skeletal muscle, spleen and testis (Gutierrez and Guierriero, 1995). In addition, hsp70 has also been detected in bovine thymus (Ziemienowicz et al., 1995), smooth muscle (Brophy et al., 1998), lenses (Bahn et al., 2003), cardiac muscle (Lakshmikuttyamma et al., 2004), plasma (Kristensen et al., 2004), spermatozoa (Kammarudin et al., 2004) and in cardiac endothelial cells (Rylander et al., 2005). It has also been shown that hsp70 mRNA is expressed in bovine oocytes and embryos under control conditions, and that early stage embryos can respond to heat stress by transcribing hsp70 mRNA (Kawarsky and King, 2001).

The hsp70 genes are not as well characterized in sheep. The presence of one hsp70 gene on the *Ovar-Mhc* region of chromosome 20 has recently been reported (Dukkipati et al., 2006). In sheep, hsp70 mRNA and protein expression has been reported in lung epithelial cells (Kramer et al., 2002), myocardium (Scharte et al., 2001), brain (Andrews and Matthews, 2000) and in uterine tissue (Wu et al., 1996).

Guierriero and Raynes (1990) reported that lymphocytes from a range of livestock species, including sheep and cattle, displayed an upregulation of hsp70 and hsp90 following an *in vitro* heat shock. Furthermore, in cattle, breed differences have been reported in lymphocyte responses to increased temperature at both protein and mRNA levels (Kamwanja et al., 1994; Lacetera et al., 2006).

In human studies, it has been reported that flow cytometry is a rapid, easy and quantitative method of determining intracellular hsp70 expression, in individual cells from a heterogeneous population such as peripheral blood mononuclear cells (PBMC) (Bachelet et al., 1998). Flow cytometric methods have also demonstrated that hsp70 is constitutively expressed in human leukocytes but that the level of expression varies considerably between different cell types (Oehler et al., 2001). Flow cytometry holds several advantages over the more traditional methods of determining levels of heat shock protein expression, such as Western immunoblot, 1D-SDS-PAGE and ELISA. These advantages include the specific information about which cells are expressing the protein and the significantly smaller volume of blood required to conduct flow cytometric analyses.

To the best of our knowledge, there are no reports of patterns of hsp70 expression in leukocyte subpopulations from sheep and cattle; therefore, the aim of the current study was to determine the optimal experimental conditions for the measurement of leukocyte hsp70 in cattle and sheep leukocyte subpopulations and for induction of hsp70 in response to heat shock.

2. Materials and methods

2.1. Animals and samples

Blood samples were collected from Merino wether weaners and Holstein bull calves aged between 6 and 9 months. Blood collection procedures were performed with appropriate animal ethics committee approval. Peripheral blood was collected into 4.5 ml EDTA Vacutainer blood collection tubes from the jugular vein, via venipuncture. All blood samples were kept at room temperature and processed within 2 h of collection.

2.2. In vitro stress treatment

For heat shock, 1 ml samples of whole blood were pre-equilibrated in a 38 °C thermostatically controlled water bath for 15 min then placed in thermostatically controlled water baths at temperatures ranging from 42.5 to 44.5 °C for 1 h, followed by a recovery period of

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Table 1
Antibodies used for cell labelling

Specificity	Identity	Source	Isotype	Dilution	References
Ovine CD4	17D	McClure	IgG1	1:5	Mackay et al. (1988)
CD8	SBUT8 38:65	CAB	IgG2a	1:100	Maddox et al. (1985)
WC1	CC15	Serotec	IgG2a	1:200	Clevers et al. (1990)
B cells	B-B2	VMRD	IgM	1:50	Hein et al. (1989)
CD14	M-M9	VMRD	IgG1	1:50	Brodersen et al. (1998)
Granulocytes	G1 CH138A	VMRD	IgM	1:50	Davis et al. (1987)
Goat anti-mouse	IgG1-RPE	Southern Biotech		1:200	
Goat anti-mouse	IgG2a-RPE	Southern Biotech		1:200	
Goat anti-mouse	IgM-RPE	Caltag		1:200	
Human hsp70	hsp70-FITC (clone C92F3A-5)	Stressgen	IgG1	1:50	Welch and Feramisco (1984)
Isotype control	Mouse IgG1-FITC	Sigma	IgG1	1:20	

3 h in a 38 °C water bath. Control blood samples (1 ml) were maintained in the 38 °C thermostatically controlled water bath for the entire 4 h 15 min period.

2.3. Cell labelling

Following the heat shock, whole blood (100 μl) aliquots were resuspended in 100 μl 1% paraformaldehyde solution and incubated for 10 min at room temperature. Samples were washed by centrifugation (470 × *g* for 3 min; Beckman GS-6R benchtop centrifuge) in 1 ml PBA (PBS supplemented with 0.5% BSA and 0.1% NaN_3). Cells were resuspended in 50 μl PBA containing the appropriate leukocyte extracellular marker mAbs as detailed in Table 1 and incubated for 20 min at room temperature in the dark. Samples were washed in 1 ml PBA by centrifugation for 3 min at 470 × *g*. Supernatant was poured off and washing step was repeated. PBA (50 μl) containing the appropriate fluorochrome-labelled secondary antibody (Table 1) was added. Samples were incubated for 20 min at room temperature in the dark. Cells were washed in 1 ml PBA by centrifugation for 3 min at 470 × *g*. Supernatant was poured off and the washing step was repeated. Saponin (0.6%) in PBA (50 μl) containing anti Hsp70-FITC or FITC-labelled isotype control (Table 1) was added. Samples were incubated for 20 min at room temperature in the dark then 100 μl of 7–8% formalin in PBS was added to each tube. Tubes were flicked to mix and let sit for 1–2 min then 1 ml 38 °C ddH_2O was added to each tube. Tubes were flicked to mix then placed in a 38 °C water bath for 3–4 min. 2 ml PBA was added to each tube then tubes were centrifuged at 470 × *g* at 4 °C for 5 min. Supernatant was poured off and tubes were flicked. 1 ml PBA was added to each tube then tubes were centrifuged at 470 × *g* at 4 °C for 5 min. Supernatant was poured off and tubes were flicked and 200 μl 1% parformaldehyde was added to resuspend cells. Samples were covered and stored at 4 °C until flow cytometric analysis was performed.

2.4. Alternative cell labelling technique using commercially available reagents

Samples (100 μl) of whole blood were added to a FACS tube containing the appropriate amounts and combinations of leukocyte extracellular marker antibodies (Table 1). The tubes were gently vortexed then incubated for 12–15 min. PBA (2 ml, phosphate buffered saline + 0.5% BSA + 0.1% sodium azide) was added to the tubes which were centrifuged at 470 × *g* for 7 min (Beckman GS-6R benchtop centrifuge). The supernatant was poured off. PBA (50 μl) containing the appropriate fluorochrome-labelled secondary antibody (Table 1) was added and samples were incubated for 15 min in the dark. FACSLyse (2 ml) (Becton-Dickinson) was added and the tubes were vortexed gently then incubated for 10 min in the dark. The tubes were centrifuged at 470 × *g* for 7 min. The supernatant was poured off, and 500 μl FACSPerm (Becton-Dickinson) was added. The tubes were gently vortexed and incubated for 10 min in the dark. PBA (2 ml) was added to the tubes which were centrifuged at 470 × *g* for 7 min. The supernatant was poured off. PBA (50 μl) containing anti Hsp70-FITC or FITC-labelled isotype control (Table 1) was added to the tubes which were vortexed gently then incubated in the dark for 30 min. PBA (2 ml) was added to the tubes, which were centrifuged at 470 × *g* for 7 min. The supernatant was poured off and 200 μl 1% paraformaldehyde was added to the tubes. The tubes were stored at 4 °C until flow cytometric analysis was performed.

2.5. Flow cytometry

Samples were acquired on a single laser (argon-ion 488 nm) FACS Vantage flow cytometer (Becton

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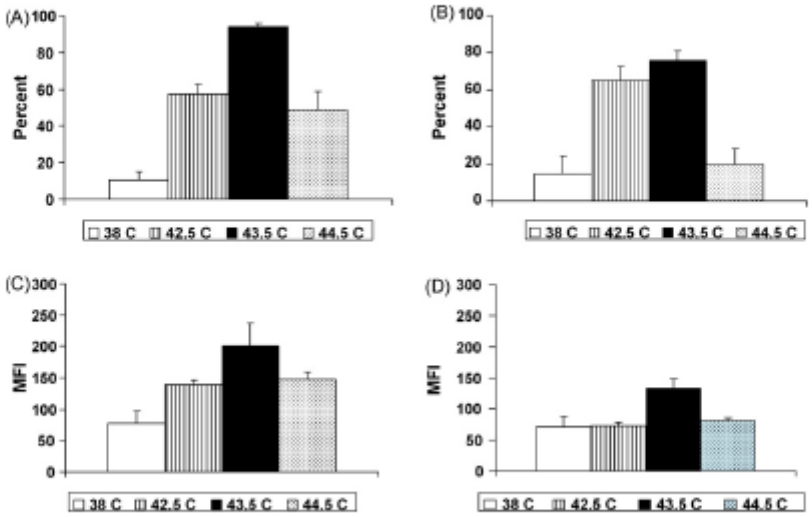

Fig. 1. (A) Temperature dependant ovine hsp70-positive CD14+ leukocytes. (B) Temperature dependant bovine hsp70-positive CD14+ leukocytes. (C) Temperature dependant ovine hsp70 mean fluorescence intensity in CD14+ leukocytes. (D) Temperature dependant bovine hsp70 mean fluorescence intensity in CD14+ leukocytes. Values are means ± S.E.M. ($n = 4$).

Dickinson) within 24 h of staining. Following calibration of the instrument for 3-colour analysis using calibrate beads (Becton Dickinson), forward scatter (FSC) and side scatter (SSC) data were collected in conjunction with fluorescence emission data. Data from a minimum of 10,000 cells were acquired for each sample. Results were analysed using Cell Quest software V3.1f. (Becton Dickinson).

2.6. Data analysis

Lymphoid cells were selected by gating on FSC versus SSC. Further gating was applied to differentiate between positive and negative cell populations based on staining with leukocyte extracellular marker mAbs. Histograms of fluorescence for hsp70-specific labelling were constructed and percentages of positive and negative cells were determined using isotype control gating. The intensity of the labelling was determined as mean fluorescence intensity (MFI).

3. Results

3.1. Temperature dependant hsp70 expression

All animals displayed low basal hsp70 expression in CD14+ monocytes and upregulated expression patterns following heat shock. A series of experiments was conducted to establish the appropriate heat shock temperature for optimal induction of hsp70 in ovine and bovine leukocytes. Fig. 1A and B are representative results for the percentage of ovine and bovine CD14+ leukocytes expressing hsp70 over a range of temperatures. Fig. 1C and D illustrate the MFI of hsp70 expression in ovine and bovine CD14+ leukocytes. These results indicated that the optimum temperature for heat shock induction of hsp70 in leukocytes is 43.5 °C for both sheep and cattle as heat shock temperatures of 42.5 and 44.5 °C displayed a decrease in both the percentage of cells expressing hsp70 and the mean fluorescence intensity of hsp70.

3.2. 24 h storage of blood

A series of experiments was conducted to determine whether leukocyte hsp70 analysis could be conducted on stored blood samples. Blood samples were kept at room temperature in the dark for 24 h prior to staining for leukocyte hsp70 analysis. The results demonstrated that when compared to staining patterns of fresh samples from the same animals, those stored for 24 h showed a reduction in inducibility of hsp70 following heat shock and greater variation in individual responses was evident as denoted by greater standard errors (results not shown). The marked changes that occur in the pattern of leukocyte hsp70 expression following a 24 h delay in staining indicate that fresh blood samples are required for optimal hsp70 expression profiles.

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3.3. Protocol comparison

Variability in the methodology of intracellular staining protocols for flow cytometry may potentially influence experimental results. Two protocols for detection of leukocyte hsp70 expression by flow cytometry were compared. The first protocol utilized standard reagents, whilst the second protocol made use of commercially available lysing and permeablizing solutions. The results as illustrated in Fig. 2 demonstrated that the use of commercially available reagents and the use of alternative methods not requiring such reagents yielded similar results for leukocyte hsp70 expression.

3.4. Anticoagulant comparison

Heparinized blood samples are routinely used for flow cytometric analysis of hsp70 expression in human leukocytes (Oehler et al., 2001). Use of the anticoagulant EDTA, however, would allow both haematologic and flow cytometric analysis to be performed on the same sample. A comparison of staining patterns for hsp70 in leukocytes collected in vacutainers containing EDTA and lithium heparin anticoagulants was performed. The results demonstrated that EDTA could be routinely used for this assay as there was no significant difference between results obtained for samples collected in EDTA or lithium heparin (results not shown).

3.5. Hsp70 in leukocyte subpopulations

A panel of mAbs recognizing cell surface antigens on CD4+ T cells, CD8+ T cells, WC1+ γδ T cells, B cells, monocytes and neutrophils was used to examine hsp70 expression in leukocyte subpopulations. The representative profiles of hsp70 expression in cattle and sheep leukocyte subpopulations are shown as percentage of cells expressing hsp70 in Fig. 3A and B. Following an *in vitro* heat shock the proportions of cells expressing hsp70 was cell type specific. In sheep the order of magnitude observed was CD14+ cells > WC1+ γδ T cells > neutrophils > B cells > CD4+ T cells = CD8+ T cells. A slightly different apparent ranking was observed in cattle with CD14+ cells > CD8+ cells > CD4+ T cells > WC1+ γδ T cells = neutrophils = B cells. Fig. 3C and D illustrate the subsequent fold-increase in hsp70 expressed as a ratio of heat shock values relative to control values.

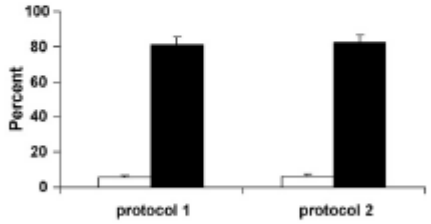

Fig. 2. Comparison of staining protocols for CD14+ leukocyte hsp70 expression. Protocol 1 utilized standard reagents. Protocol 2 made use of commercially available lysing and permeabilizing solutions. Values are means ± S.E.M. ($n = 6$). (□) Denotes cells incubated at 38 °C; (■) denotes cells incubated at 43.5 °C.

4. Discussion

The application of a mild, transient, non-lethal heat shock to cells has been widely reported to induce the expression of a number of hsps. In the current studies, flow cytometric methods were used to investigate the expression of hsp70 in ovine and bovine leukocytes. To our knowledge this is the first report of differential hsp70 expression in livestock leukocyte populations.

We have determined that the optimum temperature for heat shock of leukocytes from sheep and cattle is 43.5 °C as this temperature provided the greatest upregulation, observed as both percent positive cells and mean fluorescence intensity, in hsp70 in CD14+ leukocytes. In human leukocytes the appropriate heat shock temperature has been reported to be 42 °C with temperatures above 43 °C resulting in a decrease in the percentage of hsp70-positive cells and a concomitant increase in apoptotic cells (Dressel and Gunther, 1999). It is possible that the difference in optimum heat shock temperature between species may be a reflection of differences in the core body temperatures of these species as the mean core body temperature of sheep is 39.5 °C and of cattle is 38.9 °C (Piccione and Refinetti, 2003), compared with 37 °C for humans.

We have also determined that the best results are obtained from fresh blood samples. In blood samples stored at room temperature for 24 h the patterns of hsp70 expression following heat shock were not consistent with the results obtained from the fresh blood samples, suggesting that storage of blood prior to staining for hsp70 will not provide an accurate indication of the stress responsiveness of these cells. The current studies also revealed that EDTA is a suitable anticoagulant for use in blood collections for flow cytometric analysis of hsp70 in leukocytes. The benefit of being able to stain cells for flow cytometric analysis of hsp70 expression from blood collected in tubes containing EDTA is that it avoids the necessity of collecting more than one blood sample per animal.

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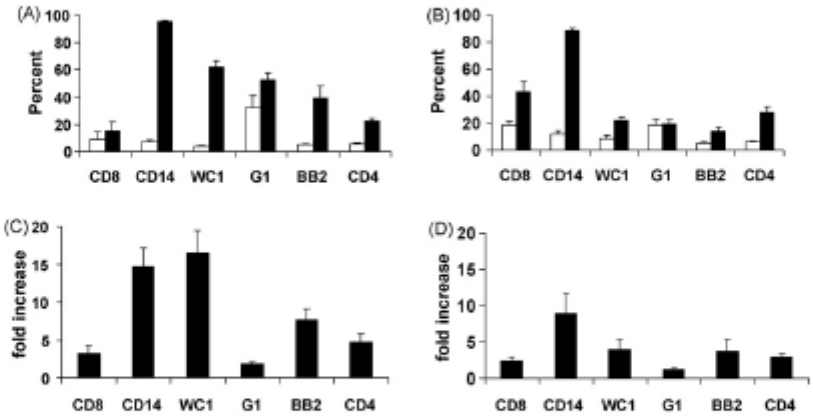

Fig. 3. (A) Ovine hsp70 expression in leukocyte subpopulations. (□) Denotes cells incubated at 38 °C; (■) denotes cells incubated at 43.5 °C. (B) Bovine hsp70 expression in leukocyte subpopulations. (□) Denotes cells incubated at 38 °C; (■) denotes cells incubated at 43.5 °C. (C) Fold-increase in ovine hsp70 expression. (D) Fold-increase in bovine hsp70 expression. Values are means ± S.E.M. ($n = 4$).

The results of the current studies revealed that hsp70 was expressed in a differential manner, both constitutively and following heat shock, by ovine and bovine leukocyte sub-populations, confirming and extending previously reported findings in human leukocytes (Oehler et al., 2001). *In situ* hybridization studies in rodent and human lymphocytes have also demonstrated cellular variability in hsp induction. This variability was shown to occur at the single-cell level in both protein and mRNA expression, with involvement of *hsp70-1* and *hsp70-2* genes implicated (Dressel and Gunther, 1999). It has also been postulated that differences in hsp70 expression in monocytes and lymphocytes may be a function of monocyte protection against self-produced toxic metabolites upon activation (Jäättelä and Wissing, 1993). Since hsp70 plays a role in important cellular functions both in the unstressed cell and following a stress imposition, these results suggest that cell function and activity such as the activation state of the cell, the presence of denatured proteins within the cell, the protein synthetic activity of the cell and the presence of reactive oxygen species, are factors influencing constitutive hsp70 expression as well as induced hsp70 expression patterns.

In the current studies we also observed species differences in constitutive and induced hsp70 expression patterns across the leukocyte subpopulations examined, confirming and extending previously reported differences in lymphocyte hsp70 expression in livestock species (Guierriero and Raynes, 1990). It has been suggested that species differences in hsp expression may not only be a result of regulatory variation in the heat shock response but also a reflection of the physiological environment of the cell (Krebs, 1999). It is also possible that differences in hsp70 expression may be a reflection of species differences in thermotolerance. Given the complexity of hsp regulation, the mechanisms underlying species differences in hsp70 expression require further research.

The application of a mild, transient, non-lethal, *in vitro* heat shock is a technique that allows the researcher to examine the cellular stress response which is a highly conserved defence mechanism. *In vitro* studies in rodent and human lymphocytes have shown that the kinetics of hsp70 expression undergo a rapid upregulation 1–2 h post-heat shock with a decline by 4–6 h (Pahlavani et al., 1996; Bratton et al., 1997). It has also been demonstrated that hsp70 expression is induced *in vivo* in a range of cell types in response to a variety of stressors including increases in body temperature (>1.5 °C), ether anaesthesia, surgery, restraint stress, water immersion stress, ischemia, trauma, hemodynamic overload and exercise (reviewed in Rokutan et al., 1998). As such, leukocyte hsp70 expression may be a useful indicator of an organism's adaptation to environmental or physiological stress.

The ratio of hsp70 expression after heat shock as compared with that under basal conditions, described as fold-increase, is an indicator of the stress responsiveness of cells. There is an increasing body of evidence suggesting that hsps play key roles in antigen presentation to induce humoral and cellular immune responses (Zugel and Kaufmann, 1999; Basu and Srivastava, 2000). Furthermore, the involvement of

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altered hsp expression in a number of disease states has emphasized the important role of these highly conserved proteins in the modulation of the immune response (Srivastava, 2002). In addition, there is well documented evidence that increased production of hsps protects cells against subsequent lethal stress induced by a number of conditions including oxidative stress, cytotoxins, heat stress and cellular damage after ischaemia or sepsis-induced injury (Jolly and Morimoto, 2000). Therefore, hsp70 expression may be modified by disease progression and as such may be an indicator of immune response. In the current study, it is likely that the animals used may well have had parasitic or low level bacterial infections that may have influenced the pattern of hsp70 observed. It is possible that the presence of an underlying infectious agent may have the ability to modulate the impact of the heat shock response of leukocytes. Further studies will be required before a definitive statement can be made regarding the expected patterns of hsp70 from each species.

5. Conclusion

Taken together the results of these recent studies have demonstrated that evaluation of leukocyte hsp70 expression by flow cytometry is a robust, reproducible method for use with cattle and sheep blood. The application of the methods described provides an assessment of the cellular stress responses of leukocytes and may be a useful tool to apply to physiological and psychological models of stress in livestock species.

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