Section I: General introduction

Literature review

Research objectives

Chapter 1 Literature review

1.1 Animal welfare concerns

Animal welfare is a social issue that has ethical, political, scientific and aesthetic properties and is concerned with how an animal "feels" as well as its physiological and behavioural status (Swanson, 1995). Welfare legislation sets a minimum standard and tends to be primarily driven by public knowledge, empathy and activism. It is at this point where technical evaluation of the production systems is required and hence the role of science is to measure the "well-being" of the animal/s (Swanson, 1995; Hartung, 2000, Webster 2005), so that a more informed legislation can be developed.

The public focus on animal welfare in the livestock industries is becoming more apparent as consumer awareness of the management practices that animals are subjected to increases. Producers are bound by legislation; however, public perception plays a large part in what is acceptable in terms of animal welfare. With the public desiring cheaper products, the producer is required to meet these demands and as a consequence producers are increasing the size of their production enterprises and reducing their unit costs of production. Often increased confinement of animals, increased reliance on technology and reduced human labour inputs accompany these changes (Hoberg *et al.,* 2005). Although the public are requesting that products are cheaper they are still demanding that an animal's welfare is not compromised. Therefore, the producer has to increase production without compromising animal welfare (Appleby, 2005). The European Union (EU) has led the way in developing legislation related to animal welfare and livestock production and although the majority of the animal welfare concerns have been focused in other western countries, Australian consumers are becoming increasingly aware of these issues and are beginning to drive changes in the way in which livestock are produced.

Animal welfare is assessed both physiologically (absence of disease and/or injury) and psychologically (range of species typical behaviours, freedom from distress and the ability to adapt and cope with environmental changes) (Swanson, 1995). It is the psychological response of animal welfare that is difficult to measure as it is assessing the mental state of the animal. Mental assessment of an animal generally includes evaluating the animal's perception, what motivates it, the emotional state of the animal and cognitive processes (Swanson, 1995). Welfare measures are based on productivity, health, behaviour and the physiology of an animal.

Scientific research is critical if we are to understand the impact of animal management during phases of the production system. Measuring the stress response of animals undergoing various aspects of animal production will help scientists, the community and industry to make informed decisions about the management practice and thus consequently improve the "well-being" of the animals. This thesis evaluated the impact of management practices (weaning, transportation, re-grouping and intensive management) on the acute phase protein haptoglobin in Australian beef cattle.

1.2 The stress response

Stress causes disruption to the homeostatic environment of an animal and therefore can be threatening to the well-being of an animal. Stress results in a number of biological, chemical and physical changes, which can result in the restoration of homeostasis but can also result in failure of restoration and entry into a pre pathological state as defined by Moberg (1985a). Homeostasis is achieved by a series of feedback mechanisms (both positive and negative), which lead to activation of behavioural and physiological responses.

If we evaluate stress in terms of signs and symptoms then stress effects can result in changes such as weight loss, increased glucocorticoids, increased concentrations of cytokines, changes in acute phase proteins, altered behaviour, changes in concentrations of haematological variables, changed heart rate, vascular resistance and poor feed conversion (Moberg, 1985a; Elasser *et al.,* 1997; Colditz, 2002). The extent of these changes depends largely on the initial cause of the stress, the duration of exposure and whether or not the animal has had time to adapt to these changes.

In 1985, Moberg stated that there was a crucial need for a reliable and acceptable measure of stress. Twenty years on this statement still holds true and it is probably more crucial than ever to find a reliable and acceptable measure of stress particularly for the livestock industries where the production practices are being scrutinised by the general public as animal welfare becomes an issue of public debate. In the past 20 years progress has been made on understanding the stress response but it appears that there is still no one reliable measure of stress and that the complexity of the stress response suggests that this may be impossible. There are a number of variables that might be used; for example Cole *et al.* (1997) reported that stress leads to adrenaline release which causes changes in the leukogram (neutrophilia, lymphopenia, monocytopenia and absence of eosinophils). Adrenaline also causes an

increase in blood pressure and splenic contraction. Thus a leukogram can be used to measure stress and tends to be associated with conditions that do not involve serious inflammation. Changes in cortisol, catecholamines, adrenocorticotrophic hormone (ACTH) and glucocorticoids have also been reported to alter in concentration as a result of stress (Golub and Gershwin 1985; Kelly, 1985; Levine, 1985) and have become widely used indicators of stress in many species.

The most common stress indicators are endocrine measures such as corticosteroids. These were originally believed to increase in response to all stressful stimuli, but, Mason (1968a and 1968b) showed that in monkeys the physical discomforts of feed deprivation did not have an increase in corticosteroids when provided with a diversion. This is also consistent for other measures; therefore, there is no one stress indicator for measuring the impact of all stressors on animals.

The stress response involves a cascade of events which alters the physiological and behavioural responses of an animal (Figure 1.1). The most 'cost effective' response to a stressful stimulus is to initiate behaviour that removes an individual from the stressful situation (Moberg, 1985a). Alternatively the Hypothalmic-Pituaitry-Adrenal (HPA) axis can be activated to alter physiological states. Activation of the autonomic nervous system and/or activation of the neuroendocrine system are a response to the appropriate stress exposure.

The autonomic nervous system (flight or fight response) mobilizes stored body reserves by the release of catecholamines. Other hormones are also released leading to changes in blood flow, respiration and immune activation (Moberg, 1985a). To continue activation, ACTH is released from the pituitary gland to increase the concentration of cortisol, which acts to counteract the effects of the stressor. In reality the neuroendocrine system regulates virtually all physiological systems (Moberg, 1985a) including the immune system.

Amadori *et al.* (1997) reported that stress (following transport in cattle) caused a reduction in immuno-competency which increased an animal's susceptibility to environmental pathogens. Stress also leads to changes in the partitioning of nutrients from production of muscle, milk and wool to the liver to support synthesis of acute phase proteins (Colditz, 2002). Elasser *et al.* (1997) suggested that the priority for nutrient partitioning was according to need with tissue accretion only occurring after the basal metabolic needs are met (Figure 1.2).

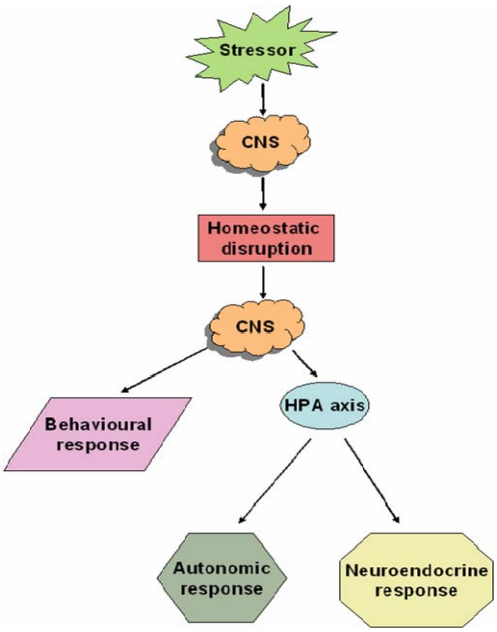

Figure 1.1: The biological response for coping with stress.

Stress not only impacts on nutrient partitioning but also on immune competence and stressed animals are more susceptible to disease (Colditz, 2002). The following section will examine in greater detail how stress and the immune system are interrelated

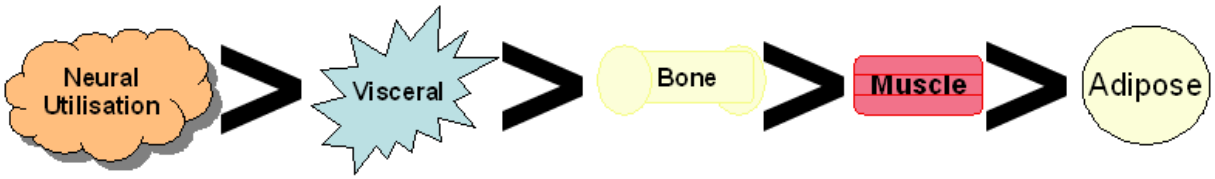

Figure 1.2: Nutrient partitioning

1.3 The immune system and its role in restoring homeostasis following stress

The immune system is triggered into activation by physical and/or psychological factors. Activation of the immune response is not only caused by micro-organisms but also by perceived threats (transportation, weaning, social re-grouping (Phillips *et al.,* 1989; Mench *et al.,* 1990; Zavy *et al.,* 1992; Arthington *et al.,* 2003)). Padget and Glaser (2003) in their review on how stress influences the immune response have shown that psychological stress does influence the immune system and consequently can impact on the health of the individual by altering the pathophysiology of infection. The way in which an animal copes with each of these factors depends largely on previous experience as to whether or not, and the degree to which, the immune system will be activated. There are two stages in activation of the immune response, the primary or innate response and the secondary response, also

known as acquired immunity, which leads to antibody formation and generation of antigen specific effector cells such as cytotoxic T cells.

Of the immune response it is innate immunity that is of interest as it can be activated by stress. Stress in itself does not lead to antibody formation but can make the animal more susceptible to pathogens due to the innate immune response trying to stabilise the homeostatic environment.

Phillips *et al.* (1989), through their research evaluating stress in beef cattle suggest that "stress may be accumulative and … could serve to sensitize the animal to stress" and therefore the immune response will differ. There have been a number of studies reporting the difference in immune expression to the same stressor between different genotypes (Phillips *et al.,* 1989; Zavy *et al.,* 1992).

1.3.1 Innate immunity

Innate immunity is the first arm of defence that an organism has against an antigen and leads to activation of leukocytes, cytokines, glucocorticoids and acute phase proteins. The role of these cells, proteins and hormones is to firstly contain and remove the antigen and secondly remove damaged tissue in order to restore homeostasis (Kushner *et al.,* 1981).

The cells of the innate immune response include macrophages and monocytes, polymorphonuclear granulocytes and dendritic cells (Knox *et al.,* 1994a). The role of macrophages and monocytes in immune defence is to engulf and digest dead and damaged cells. These actions are modulated by low molecular weight protein messengers of the immune system called cytokines. They can be found at the site of both chronic and acute inflammation (Knox *et al.,* 1994a). Polymorphonuclear granulocytes consist of neutrophils, eosinophils, basophils and mast cells. Neutrophils and eosinophils are phagocytic cells and basophils and mast cells are involved in acute inflammation and allergies. Dendritic cells present the antigens to T and B cells enabling activation of acquired immunity. However it is the role of cytokines and certain hormones that are of interest here as they are involved in activation of acute phase proteins following an acute phase response.

1.4 Acute phase response

The acute phase response is "the early and immediate set of reactions that are induced" as a result of inflammation (Baumann and Gauldie, 1994) and stress (Murata *et al.,* 2004), predates (on an evolutionary scale) acquired immunity (Eckersall, 2000) and acts in restoring homeostasis and preventing microbial growth (Gruys *et al.,* 1994). Eckersall (2000), defines the acute phase response as the

> "re-distribution of nutrients in peripheral tissue such that the deposition of triglyceride in the adipose tissue is reduced while lipolysis is maintained at the same or higher rates, with a net increase in the release of lipid from fat stores. Similarly, there is a reduction in the uptake of amino acids and the formation of muscle protein - so that with continuing proteolysis in the normal turnover of muscle protein there is a net loss of muscle mass that results in the poor growth rates."

The systemic response of an acute phase reaction in addition to the above changes in lipid and protein metabolism and re-distribution includes fever, neutrophilia, a reduction of iron and zinc in serum, increased gluconeogenesis, hormonal changes and activation of positive acute phase proteins (Moshage, 1997).

Activation of the acute phase response is due to the site of inflammation sending signals which activate the hypothalamic-pituitary-adrenal axis which leads to a cascade of events that activate cytokines and in turn the acute phase response and consequently the acute phase proteins (Gabay and Kushner, 1999; Murata *et al.,* 2004) (Figure 1.3). The acute phase response is regulated by a series of feedback mechanisms which control the inhibitory factors (cytokines) (Gabay and Kushner, 1999; Murata *et al.,* 2004).

1.5 Cytokines

Cytokines play an important role in communication between leukocytes and in influencing the class of immune response such as Th-1 and Th-2 response (Basset *et al.,* 2003). They have both local and systemic effects (Basset *et al.,* 2003) and are large hydrophilic molecules often between 10,000 and 20,000 Daltons molecular weight (Dantzer *et al.,* 2000). There are a number of different cytokines and these include the interferon (IFN) family, tumour necrosis

factor (TNF) family and the interleukin (IL) family (Basset *et al.,* 2003). The pro-inflammatory cytokines include TNF-α, IL-1 and IL-6 (Basset *et al.,* 2003).

Cytokines are produced by immune cells and epithelial and endothelial cells (Basset *et al.,* 2003). IL-6 is also produced by kupffer cells, keratinocytes, the pituitary and mucosal epithelium (Murata *et al.,* 2004). Pro-inflammatory cytokines produced during disease have been shown to impact on synthesis of products of commercial value such as meat, wool and milk through effects on protein accretion (Colditz, 2002).

Cytokines are involved in the regulation of acute phase proteins. Horadagoda *et al.* (1994 as reported by Eckersall, 2000) showed that TNF-α had a transient peak prior to an increase in the acute phase proteins serum amyloid A and haptoglobin. Alsemgeest *et al.* (1996), in their *in vitro* studies evaluating the induction of serum amyloid A and haptoglobin revealed that serum amyloid A was up-regulated by IL-6 and/or TNF-α, whereas, haptoglobin required both IL-6 and TNF-α to increase in concentration. Moshage (1997) reported that IL-6 is able to induce all Type II acute phase proteins with the remainder of the IL-like cytokine family being able to induce a subset of Type II acute phase proteins.

1.6 Acute phase proteins

Proteins that are up or down regulated during an acute phase response are collectively known as acute phase proteins (APP's). During the past 10 years acute phase proteins have come to the forefront of animal welfare research due to their potential as veterinary tools for the diagnosis and monitoring of disease development and progression. The potential use for these proteins as an indicator of psychological stress is still in the early stages.

Acute phase proteins are primarily produced in the liver but can also be produced in non hepatic sites including the mammary glands, intestinal epithelium and the lung (Eckersall, 2004). Acute phase proteins are classified as proteins that have a 25% change (increase or decrease) as a result of inflammation (Gabay and Kushner, 1999) and can be classified into two groups based on the cytokines that are required to induce a response (Moshage, 1997) (Table 1.1). Type I acute phase proteins are induced by IL-1 family of cytokines and require the presence of the IL-6 family, whereas, the Type II proteins are induced by the IL-6 family (Moshage, 1997). IL-1 has either no effect or acts as an inhibitor on the Type II proteins (Moshage, 1997).

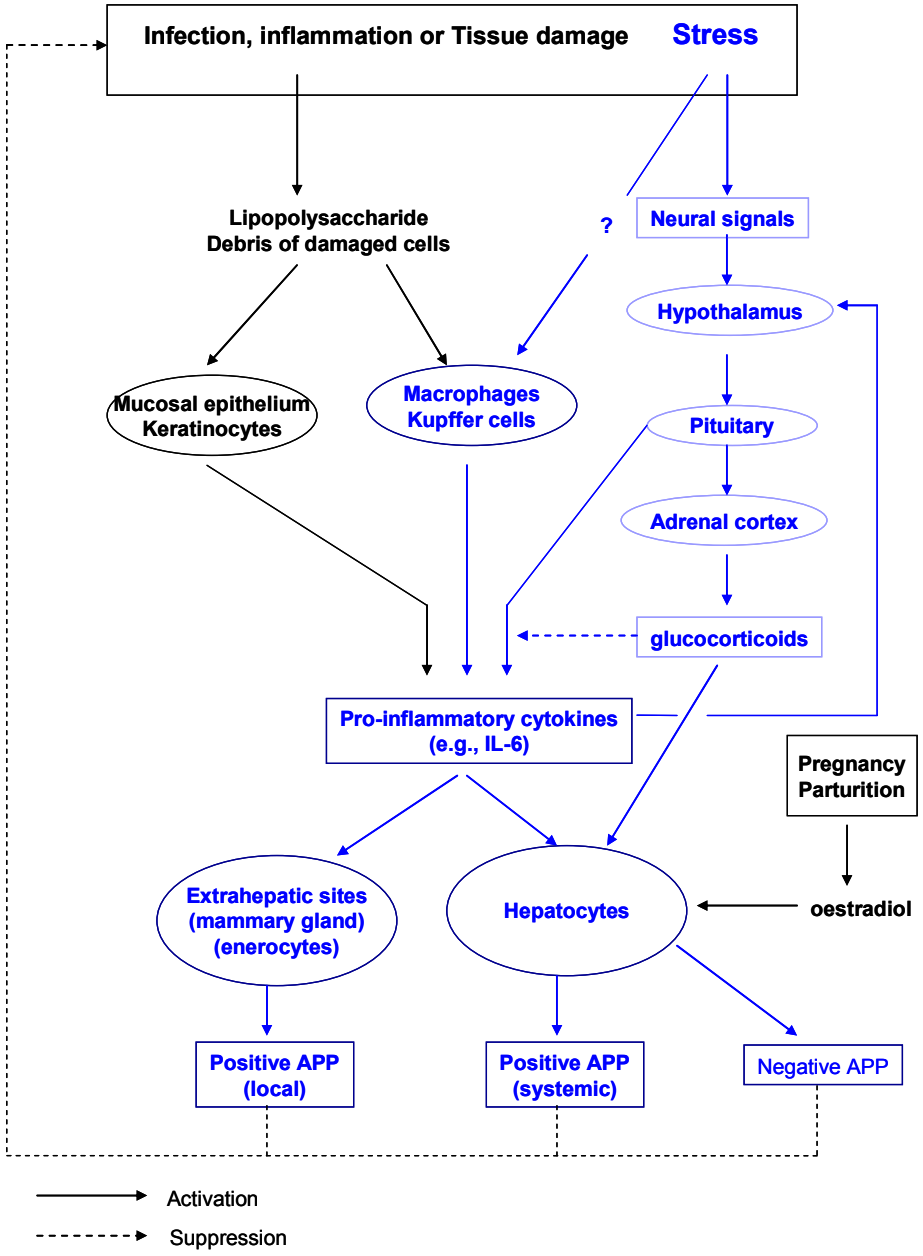

Figure 1.3: "Induction and regulation network of acute phase protein (APP) synthesis in animals subjected to internal or external challenge", modified from Murata *et al*. 2004.

The acute phase proteins that decrease as a result of an acute phase response are known as negative acute phase proteins and include pre-albumin, albumin and transferrin. Whereas, the positive acute phase proteins increase in concentration as a result of the acute phase response and can be subdivided into three broad groups according to the degree of expression based on normal plasma concentrations and their response to inflammation (Table 1.2). However, species differences do occur in the expression of acute phase proteins. For example, in man, haptoglobin is considered to be a normal serum protein but in cattle it is a major acute phase protein, the reverse is true for C-reactive protein. Table 1.3 provides a list of acute phase

proteins and their classification according to their basal concentration and acute phase response. As can be seen from Table 1.3 there are species differences for the majority of acute phase proteins.

Table 1.1: Type I and Type II acute phase proteins and the cytokines that induce a response. Adapted from Moshage, 1997.

Type	APP	Induced by	Cytokines
Type I	SAA	IL-1 family	IL-1α
	human-CRP	IL-6 family	IL-1β
	Complement C3		TNF- α
	rat-Hp		TNF- β
	α_1-AGP		IL-6
Type II	Fibrinogen	IL-6 family	IL-6
	human-Hp		Leukaemia inhibitory factor
	α_1-antichymotrypsin		IL-11
	α_1-antitrypsin		Oncostatin M
	rat- α_2-macroglobulin		Ciliary neutrophic factor
			Cardiotrophin- 1

Table 1.2: Criteria for classifying the positive acute phase proteins in to each subdivision

APP Subdivisions	Plasma concentration in healthy individuals	Plasma concentration during an immune response
Major	Present in low concentrations	100× increase
	<0.01 mg/mL	1-2 mg/mL
Moderate	Present in plasma	2-3 fold increase
Minor	Present in plasma	50% increase

Eckersall (2000) pointed out that the functions of the acute phase proteins are not well defined however they are involved in reducing inflammation. From the research that has been undertaken on acute phase proteins it appears that they have a role in repair, recovery, preventing further tissue loss, acting as scavengers, binding bacterial components and more importantly aiding in the restoration of homeostasis (Gruys *et al.,* 1994; Eckersall, 2000). There are two ways in which the acute phase proteins can be measured. The first is using biochemical assays which are often not species-specific and the second method is using immunological assays which are usually species-specific (Eckersall *et al.,* 1999).

Table 1.3: Major, moderate and minor acute phase proteins in each species (adapted and modified from Murata *et al.,* 2004)

APP	Man	Chicken	Dog	Horse	Pig	Ruminants	Cattle
Acid glycoprotein	Δ-○	○	○	Δ-○	○	○	○
Ceruloplasmin	○	○	○	Δ	Δ	Δ	☺
C-reactive protein	☺	NC	☺	○	☺	○	○
Fibrinogen	☺	Δ	○	Δ-○	Δ-○	○	☺
Haptoglobin	○	NC	○	○	○	☺	☺
Protease inhibitor	☺	NC	NC	Δ	NC	Δ-○	Δ
Serum amyloid-A	☺	○	○	☺	☺	○-☺	☺
Transferrin	NC	○	NC	▼	▼	▼	▼

Classification of APP: ☺- major; ○- moderate; Δ- minor (increase); ▼- minor (decrease); NC- not confirmed

The acute phase response can be artificially induced by injecting an animal with an irritant such as turpentine which causes a local inflammatory response. The acute phase proteins released into circulation are haptoglobin, fibrinogen, seromucoid, ceruloplasmin and α1-antitrypsin in cattle. These proteins peaked 4 to 7 days post-injection and returned to normal 11 to 17 days post-injection (Conner *et al.,* 1988). Conner *et al.* (1988) showed that the concentration of total protein and albumin decreased by the second day post injection and did not return to normal until the seventh day. The total protein concentrations increased five percent above normal on the fourth and fifth days before returning to normal. They also showed that as the dose of turpentine increased so too does the intensity and duration of the haptoglobin and seromucoid response; with haptoglobin peaking 24 hours prior to the seromucoid.

Very little research has been undertaken to determine if psychological stress can induce an acute phase response. From the little information in the literature it would appear that it is possible. Alsemgeest *et al.* (1995) evaluated the effect of physical stress in calves by using two different floor types. As a result of this stress serum amyloid-A increased but haptoglobin did not. Cortisol concentrations also did not differ between the two different treatment groups. Thus Alsemgeest *et al.* (1995) said that the "stressor was mild and more likely to be physical than neuro-endocrine in nature." Deak *et al.* (1997) in there study evaluating stress on acute phase proteins reported that haptoglobin concentrations increased in rats following an electrical shock. Nukina *et al.* (2001) reported that restraining germ-free mice led to elevated IL-6 concentrations. From the studies that have looked at the effect of

induction of acute phase proteins by IL-6 and other cytokines then it is plausible that psychological stress can induce acute phase proteins. A general description of several commonly analysed acute phase proteins is discussed below.

With the recent advances in acute phase protein research into veterinary diagnosis and monitoring it would appear that there is a possibility that these proteins (in particular haptoglobin and serum amyloid-A based on their expression profile during a non-inflammatory response, (Alsemgeest *et al.,* 1995) have the potential to quantify a non-inflammatory stress response. If the acute phase proteins are proven to be accurate and reliable measures of stress they have one major advantage over the current methods for measuring stress like cortisol in that their expression profile is not as transient as cortisol and therefore provides a larger window of opportunity in which to obtain a measure.

Due to acute phase proteins having proven their ability in veterinary diagnosis and monitoring (Gruys, *et al.*, 1994; Eckersall, 2000) and their potential to measure psychological stress, they may be beneficial for measurement of the welfare of animals under different production environments. However, due to the complex nature of the stress response it is unlikely that any one measure will be an accurate indicator. Therefore, to obtain an acute measure of stress will probably require use of several measures. Before we can conclude that acute phase proteins are useful in the measurement of psychological stress in beef cattle, research needs to be undertaken to determine if acute phase proteins are induced during non-inflammatory production practices such as weaning, transportation and social re-grouping.

1.6.1 Serum amyloid A

Serum amyloid A is a hydrophobic protein that is found in association with high density lipoprotein and has a molecular weight of 11 to 14 kD (Eckersall, 2000) and is thought to be the precursor of amyloid protein A (Eckersall and Conner, 1988). It is produced not only by the liver but also by intestinal epithelium and mammary tissue (McDonald *et al.,* 2001). Its role is to act as a cholesterol transporter (Eckersall, 2000) and it is under regulation by IL-1 (Jain, 1993).

1.6.2 Haptoglobin

Haptoglobin is a tetrameric quaternary protein consisting of two alpha and two beta subunits. It has a molecular weight greater than 100kD and is produced by Kupffer cells of the liver

(Eckersall, 2000). Its role is to bind to and remove free haemoglobin that has been converted into methaemoglobin forming a haemoglobin-haptoglobin complex. This complex is then transported back to the liver where it is broken down. Haptoglobin has a plasma half-life of 2-4 days (Jain, 1993)

Additional information on haptoglobin can be found in Chapter 2, page 28.

1.6.3 C-reactive protein

C-reactive protein consists of 5 subunits of 20kD each and belongs to the pentraxin family of proteins (Eckersall, 2000). Its role is to assist in the clearance and repair of damaged tissue by binding to exposed phosphoryl choline due to its Ca^{2+} dependent affinity (Volonakis and Kaplan, 1971 as reported by Eckersall and Conner, 1988) and nucleic acid due to having a binding affinity for chromatin (Robey *et al.*, 1985 as reported by Eckersall and Conner, 1988). C-reactive protein obtained its name due to binding to the polysaccharide C fraction from pneumococci (Eckersall and Conner, 1988) in 1941; 11 years after it was first described (Tietz 1986a).

1.6.4 α_1- acid glycoprotein

α_1-Acid glycoprotein is a highly glycosylated protein and is the main protein component in seromucoid (Eckersall, 2000) and is made up of up to 45% carbohydrates (Laurel, 1985). It is highly resistant to acid precipitation and binds to a number of pharmacological agents and therefore can affect drug efficacy during an acute phase response (Eckersall, 2000; Murata *et al.,* 2004). The role of α_1-acid glycoprotein is undefined but is known to suppress PHA-induced lymphocyte transformations in culture (Laurell, 1985) and inhibits natural killer cell activity (Murata *et al.,* 2004). α_1-Acid glycoprotein is also known as seromucoid or orosomucoid (Eckersall and Conner, 1988).

1.6.5 α_1- antitrypsin

α_1-Antitrypsin is a proteinase inhibitor with its primary function to inhibit polymorphonuclear elastase (Beatty *et al.*, 1980 as reported by Eckersall and Conner, 1988) and was first isolated and described in 1955 (Tietz, 1986a). α_1-Antitrypsin has a molecular weight of 55kD and contains 10-12% carbohydrates (Tietz, 1986a). This size enables it to move between the tissue fluid and capillaries with ease even when bound to protease (Tietz, 1986a). Due to its

role as a proteinase inhibitor it is also known as α_1- proteinase inhibitor (α_1-PI) (Eckersall and Conner, 1988).

Mattila *et al.,* (1985 as reported by Eckersall and Conner, 1988) showed that there was a correlation between α_1-antitrypsin and mastitis in dairy cows.

1.6.6 Ceruloplasmin

There have been several possible roles for ceruloplasmin proposed. The first is that it may act as a transporter of copper into the cells for synthesis of cytochrom oxidase due to its affinity for copper (Laurell, 1985). Ceruloplasmin has been shown to be a major extracellular inhibitor of auto-oxidation of plasma lipids and thus is believed to be an antioxidant and is also a scavenger of oxygen derived free radicals (Laurell, 1985). It is also an anti-inflammatory due to reducing the number of neutrophils that attach to the endothelium (Murata *et al.,* 2004).

1.7 Australian cattle industry

The Australian cattle industry began in 1788 with the arrival of the first fleet from England. Since then the cattle industry has grown substantially with beef cattle being produced in all states and mainland territories and environments. This industry represents 47.1% of the Australian agricultural income and was worth $6,918 million (Au) in 2003/2004 (Jeong *et al.,* 2004). Australian beef is exported to over 100 countries (Jeong *et al.,* 2004) with the majority of beef (90%) being exported to Asia and the USA (Pearse, 2004). With Australia being one of the largest exporters of beef, welfare issues need to be addressed to ensure that Australian beef is not rejected from the international (and domestic) markets.

Over the past few years the Australian cattle industry (along with all sectors of agriculture) has been consistently influenced by drought and has seen the national herd number decline from 27.9 million in 2002 to 23.3 million in 2003/2004 (Jeong *et al.,* 2004). However, long term forecasts such as those of the Australian Bureau of Agricultural and Resource Economics suggest that by June 2009 the national herd will reach 28.2 million head (Jeong *et al.,* 2004).

The management practices of beef cattle vary between northern and southern Australia, with some northern beef cattle only coming into contact with humans as little as once a year compared to southern beef cattle which may have some form of human contact on a weekly

basis. Additionally, the genetics of the cattle produced in northern and southern Australia differ. Northern beef cattle contain some *Bos indicus* which tends not to be found in southern beef which are from the *Bos taurus* line.

In addition to the different production practices that occur between northern and southern Australia, there are also marked differences in beef cattle production between Australia and the rest of the world (particularly Europe and Northern America). These differences in production practices are apparent in intensive management situations and transportation. In Australian intensive management refers to lot feeding cattle in open pens rather than the confines of sheds/barns that are used to house cattle during the winter months in Europe and Northern America.

Differences in transportation occur as a result of the need to consistently transport cattle long distances for example from inland Australia to the livestock centres located around the coastline of Australia. Depending on where the cattle originate from and where they are destined for, transportation time can exceed 36 hours, especially during transcontinental trips. The current transport code in Australia for mature cattle (non-pregnant and/or lactating) is that cattle can not be off feed and water for more than 36 hours. However, if the cattle can reach their final destination within 48 hours (and are travelling well) then the total time off feed and water can be extended to 48 hours.

The breed and temperament of cattle play a part in how they cope with the production practices imposed by humans. There are a number of different temperament measures and include but are not limited to flight time, crush score and hair whorl characteristics. Burrow and Dillon (1997) and Voisinet *et al.* (1997) showed that flight time was related to weight gain with cattle that had a slower flight speed had a greater weight gain whilst in the feedlot. Fell *et al.* (1999) reported a correlation between flight time and cortisol; that is, the faster the flight time the higher the plasma cortisol concentration. Both Randle (1997) and Lanier *et al.* (2001) showed a link between hair whorl characteristics and temperament. Consequently temperament measures are being used to select suitable animals for breeding as it has been shown by Burrow (1998) that temperament is hereditary. As a result of this, Australian cattle producers have been selecting and breeding cattle with calmer temperaments for their production system.

The codes of practices that have been developed for the European Union for cattle production may not be relevant for the Australian beef cattle industry. That is not to say that the scientific research previously undertaken is irrelevant for the Australian industry but many of the recommendations need to be evaluated because of differences in Australian cattle and the Australian environment. It is possible that Australia will require substantially different codes of practices even within the country due to different production practices and genotypes in the northern and southern beef industries.

Irrespective of country, climate or breed of cattle, producing quality and consistent beef may place production stressors on the animals throughout the course of their lives. These production stressors include, but are not limited to, handling, castration, weaning, transport, branding, de-horning, social interactions, diet change and environmental changes (housing, shelter and weather exposure).

1.8 Management Practices

Management practices can be divided into several groups. The first are those that result in tissue trauma and include castration, branding and dehorning; the second are those that cause physiological stress and include transportation and flooring; and the third group are those practices that induce a psychological stress including weaning and social re-grouping. Finally the last group are those that can lead to infection. As most management practices try and avoid stressors that result in infection by ensuring that animals are vaccinated against common diseases and that the environment that the animals are in will not be considered as part of this thesis. However it is important to understand that infection can be the result of tissue trauma and physical and psychological stress and that these management practices can cause both inflammatory and non-inflammatory responses.

1.8.1 Handling stress

In cattle production systems, handling stress is unavoidable due to the need to manage cattle to maintain the welfare of the herd and the individual animal. However, the degree of stress response can be minimised by selecting cattle that have a quiet temperament, by using appropriate handling facilities and by ensuring that stock handlers are experienced and have a good attitude to the stock (West, 2004). The majority of handling stress is thought to result from stock handlers who do not understand cattle behaviour; for example, the reasons for cattle baulking is not always apparent to the handler but if time is taken to identify the cause

of baulking then imposition of considerable stress on the animals may be avoided. A review by Grandin (1997), states that "how an animal is handled early in life will have an effect on its physiological response to stressors later in life". Fordyce (1987) as reported by Grandin (1997) noted that adult cows were calmer and easier to handle when trained as weanling heifer calves, implying that the more positive the early life handling experience the easier it will be to work with the animals and the less stress is likely to result from handling animals in later life. Therefore, when working with cattle the handling of these animals should be such that handling stress is negligible by using trained stock handlers, whom understand cattle behaviour.

1.8.2 Branding, castration, and de-horning

The husbandry procedures of branding, castration and de-horning are performed on cattle to ease later management and the practices normally occur relatively early in life in order to reduce handling stress to both the animal and handlers. All three of these husbandry procedures can induce an inflammatory response due to tissue destruction and/or removal (Lay *et al.,* 1992a; Graf and Senn, 1999; Fisher *et al.,* 2001), and consequently the stress response recorded as a result of these practices may be due to inflammation rather than psychological trauma. Further studies are required to clarify the relative importance of the different stressors to the overall impact on the stress response and consequently production.

Castration of male calves is a common practice and is necessary for the management of male cattle. Fisher *et al.* (2001) evaluated the stress of surgical and banding castration in bulls and found that those bulls that were surgically castrated had better subsequent growth but also higher acute stress responses as indicated by increases haptoglobin concentrations when compared to the banded bulls. Both methods caused a stress response and haptoglobin increased after surgical castration but the concentrations had returned to normal after 4 days and these animals also exhibited greater behavioural changes (leg stamping and tail swishing). An earlier study by Fisher *et al.* (1997b) revealed that surgically castrated steers had increased cortisol concentrations from 15 minutes after castration and these remained elevated for several hours. Both studies indicate that the stress response is a result of inflammation due to tissue damage/removal which is accompanied by increased haptoglobin concentrations, which has been reported as a measure of inflammation (Conner *et al.,* 1988).

De-horning is a procedure that occurs in cattle to reduce the risk of injury to herd mates and stock handlers particularly when working with animals in confined spaces. The usual method for dehorning is by cauterization using a heated iron. Less commonly used methods involve the use of caustic agents and surgery. De-horning has been shown, predominately through the measurement of cortisol, to induce transient stress and pain (Graf and Seen, 1999). Graf and Senn (1999) assessed the response of cattle undergoing de-horning with and without anaesthetic. The anaesthetic reportedly reduced the pain response in the first two hours but three to four hours post-dehorning the anaesthetised calves had elevated cortisol and increased head shaking suggesting that there was residual pain once the anaesthetic wore off. In this case it would appear that pain rather than inflammation may be the cause of stress.

Branding results in a permanent mark on the skin/hide of the animal and is used by producers to identify their cattle. There are two methods of branding: hot-iron which results in the formation of scar tissue on the skin and secondly freeze branding causing the pigment-producing cells and/or hair follicles to be destroyed (Lay *et al.,* 1992b). Several studies evaluating the two methods of branding report that freeze branding is the preferable method based on behavioural and cortisol data (Lay *et al.,* 1992a; Lay *et al.,* 1992b; Schwartzkopf-Genswein *et al.,* 1997).

Measuring the psychological stress immediately following these procedures is difficult due to the inflammatory response that occurs as a result of tissue damage and/or removal. The psychological stress may not be apparent until the subsequent time that these animals are handled (often a minimum of several weeks to allow any inflammation to resolve). Due to not knowing the extent of the psychological stress induced by these processes, it is often difficult to ascertain whether the stress response is due to previous handling experiences or some other recent (unidentified) stressor. This thesis is concerned with non-inflammatory stressors; and therefore, inflammatory procedures will not be addressed as such as they have been well evaluated in previous studies.

1.8.3 Weaning and transport

Weaning and transportation are known to be stressful events in the life of an animal (Crookshank *et al.,* 1979; Phillips *et al.,* 1989; Zavy *et al.,* 1992; Mackenzie *et al.,* 1997; Steinhardt and Thielscher, 2001). Weaning beef cattle is generally a forced procedure that occurs before the natural time of weaning. Likewise at some stage in their lives the majority

of beef cattle will undergo transportation and this presents another stressful situation that the animals need to cope with.

There is a considerable body of literature that has evaluated the effects of transport stress and this has often been done on animals that have recently been weaned or weaned onto the trucks to replicate the production practice of many producers (Crookshank *et al.,* 1979; Phillips *et al.,* 1989; Zavy *et al.,* 1992; Mackenzie *et al.,* 1997; Steinhardt and Thielscher, 2001). Such experimental designs have the advantage that they allow the effects of the two stressors to be differentiated. There are a number of biological changes that take place as a result of the stress from transport and/or weaning including a change in weight, body temperature, heart rate, cortisol, haptoglobin, glucose, serum urea nitrogen, fibrinogen, and antibody titres (Crookshank *et al.,* 1979; Phillips *et al.,* 1987; Phillips *et al.,* 1989; Murata and Miyamoto, 1993; Lefcourt and Elasser, 1995; Mackenzie *et al.,* 1997).

1.8.3.1 Weaning

Weaning is stressful (Price *et al.,* 2003), as it disrupts the social bond between the calf and the dam and also leads to a change in diet and/or environment. There are several methods of weaning used in the Australian beef industry including: total separation, yard weaning, fence line separation and half weaning.

The total separation method involves drafting calves from their dams and returning the cows and calves to separate paddocks, often as far apart as possible so as to minimize vocal communication between the two groups. In some cases this will involve transport of calves. Behavioural observations (Hickey *et al.,* 2003; Price *et al.,* 2003) suggest that it takes 7 to 10 days for the calves to adjust to the separation in this system.

Yard weaning is identical to total separation with the exception that the calves remain in and/or around the yards for a period of 5 to 10 days. The benefit of this form of weaning is that social interactions are encouraged and the confinement allows the calves to habituate to humans, dogs, horses, motorbikes and vehicles and also to supplementary feed (Fell *et al.,* 1998; Pettiford, 2005). In Australia this method is widely used for cattle that are destined for feedlots where the animals need to habituate to handling and feeding in a feedlot environment. Reports suggest that there is reduced morbidity and mortality in yard weaned calves compared to calves that undergo traditionally weaning before entering a feedlot. Weaned calves also

have been shown to have a significantly greater weight gain in a feedlot than paddock weaned calves (Fell *et al.,* 1998).

In Northern Australia yard weaning practises are modified slightly with calves held in a paddock near the yards and worked daily by exposure to horses and dogs and handling. During this period of time they are also supplementary fed. As with the other method of yard weaning this procedure may continue for around 10 days.

The fence line separation method of weaning allows social contact between the cow and calf whilst preventing the calf from suckling and tends to mimic what happens in a natural weaning process. Price *et al.* (2003) have shown that this method reduces the behavioural distress, which has the benefit of improving weight gain when compared to total separation. Grazing, walking and resting patterns exhibited by these calves were identical to that of unweaned calves but the calves were less vocal than the calves that have undergone total separation (Price *et al.,* 2003).

The least common weaning technique is half weaning. This involves a two-step process; at the start of weaning half of the dams are removed from the herd then approximately a week later, the remaining cows are removed from the calves. The basis of this technique is that the first group of calves that are weaned are comforted by the remaining cows and their calves. When the final group of cows are removed, the previously weaned calves provide the social "support for the newly weaned calves." Whether this procedure reduces the stress of weaning is yet to be scientifically quantified.

Evaluating all weaning practices at the same time has not been undertaken. Although there have been several comparisons between these methods with abrupt weaning (Price *et al.,* 2003). The stress response that results needs to be further evaluated to determine the duration and intensity for each weaning method and which is the least stressful.

According to Blezinger (2003) the stress of weaning can be minimized by careful management prior to or post weaning. The pre-weaning practises that should be considered are marking (castration, ear tagging and branding) and vaccinations. Bagley (1997) recommended that such procedures, if not done prior to weaning, should be delayed until 30 days post weaning to reduce the compounded effect of weaning and marking stress. Ideally

these procedures should be performed pre-weaning as this appears to minimize their impact (Bagley, 1997).

Immune compromise is common during the period of weaning and consequently a number of health issues such as bovine respiratory diseases (pneumonia, shipping fever) in addition to coccidiosis and digestive problems regularly occur (Bagley, 1997). Mackenzie *et al.* (1997) showed that weaning affects the humoral immune response of calves and the suppression of the immune system appears to persist for several weeks. Therefore, when weaning occurs producers need to be aware of these problems and have management strategies in place to firstly reduce their incidence and secondly provide treatment, if these problems should occur.

1.8.3.2 Transportation

The stress from transportation is multifaceted in that there is the stress from loading and unloading, the actual journey, close encounters with herd mates, and feed and water deprivation all of which can cause a number of biological and physiological changes in cattle. These changes usually cause an increase in cortisol, a reduced ability to produce antibodies, weight loss, and changes in the concentration of blood leukocytes and glucose. Transport is therefore considered to be an acute stressor (Crookshank *et al.,* 1979; Mackenzie *et al.,* 1997; Earley *et al.,* 2004a). The weight loss of cattle following transport is most likely the result of a combination of feed and water deprivation, excretion, dehydration and metabolic changes. Phillips *et al.* (1987), suggest that 60% of the weight loss is due to excretion and fasting.

Zavy *et al.* (1992) reported that there were breed differences in cortisol response in cattle undergoing transportation. Cattle with a *Bos indicus* genetic component (Brahman × Angus or Brahman × Hereford) tended to have a higher plasma cortisol baseline than calves that were pure *Bos taurus* (Hereford × Angus). However, these authors were unclear as to whether the *Bos indicus* calves have a true high basal cortisol concentration or that they have not habituated to the repeated handling for blood collection.

Mackenzie *et al.* (1997) reported that for short transportation, the majority of the transport stress is due to handling at loading and unloading rather than the process of transit. Longer haulage can expose cattle to other stressors including thermal stress, dehydration, exhaustion and crowding (Mackenzie *et al.,* 1997; Earley *et al.,* 2004a).

The multifaceted nature of transport stress and the possibility of genetic differences influencing how animals cope with the stress raises many questions with regards to road transportation in Australia. These issues include: the pre-transport preparation with regards to feed and water deprivation, the condition of cattle, the loading and unloading of cattle and the stocking densities and its impact on space available during transportation. Therefore, we can ask how stressful is transportation? What is the maximum duration of transit that is acceptable? Is a rest period required and for how long and should feed and water be made available? And, how long should cattle be rested following transportation? In order to understand and answer these questions there is a need to be able to measure the intensity and duration of the stress response under various experimental conditions.

1.8.4 Social interactions

Social interactions are likely to occur as a result of an established hierarchy when animals are competing for essential resources like food, water and shelter. Additionally, interactions associated with social hierarchy occur when animals are confined to small spaces during yarding, transportation and housing. A review by Bøe and Færevik (2003) on social re-grouping noted that social stressors lead to behaviour and physiological changes during the establishment of a hierarchy. Age was shown to be important factor in determining whether or not an animal will be dominant within the social group (Kondo and Hurnik, 1990; Hasegawa *et al.,* 1997; Bach *et al.,* 2005) and older animals tend to be more dominant.

However, "estimation of social hierarchy using observation data generated shortly after group formation may be inaccurate, as the estimate obtained during its formation could differ significantly from that obtained after social stabilization" (Kondo and Hurnik, 1990). Thus the stress measured during establishment may not reflect accurately the stress in a socially stable herd. The majority of the work undertaken on social interactions has relied on behavioural changes of individual animals and the herd to determine when the herd dynamics are stable. Behavioural observations are informative but it takes a considerable amount of time and effort to observe animals within the herd over an extended period of time. Several studies have evaluated cortisol and acute phase proteins and reported that sub-ordinates had increased concentrations of both (Mench *et al.,* 1990; Gupta *et al.,* 2005).

Producing beef is stressful for cattle at various stages of the production cycle. Understanding what stress is, how it is activated, what are the consequences of being stressed are and how it

can be measured are significant to understanding methods of prevention and or reducing the impact that stress has on cattle production.

1.9 Conclusion

Measuring stress in animals is difficult as there is no one reliable, acceptable measure. Measuring psychological stress is even harder as we are not able to determine how an animal feels about the stressor. Previous methods for determining if an animal is stressed have relied on behavioural and physiological changes (reduced body condition, haematology and cortisol). It is unlikely that we will have only one measure of stress as the stress response (irrespective of the stressor) is complex and multi-faceted. However, what we do need is a way of distinguishing whether an animal is undergoing psychological stress. Previous studies use preferential testing to determine what an animal prefers; however, it is possible that neither or both situations are stressful. It is also possible that one of the stressors is just slightly less stressful than the other and will be favoured by the animals. Thus does this mean that this preference is better for the animal? Therefore if we can evaluate the different production systems that we expose livestock to then it may be possible to determine the optimal management system to produce animals.

With consumers becoming more aware of animal welfare the focus is now turning to livestock production and as a consequence the European Union has developed a set of welfare standards for livestock production for its member states. These welfare standards may or may not have relevance in the Australian environment due to its diversity and consequently the livestock practices that are undertaken. In addition, Australian producers have been selecting and breeding animals to suit a specific environment (tropical, temperate or mediterranean). Thus it is necessary to evaluate the production practices that occur within Australia rather than relying on previous experimentation that has occurred internationally.

As humans we often project our emotions onto animals when attempting to evaluate how the animal feels under specific circumstances. Thus what we might perceive to be in the best interest of the animal may not be so. Therefore, finding and evaluating alternative measures of stress is important as not only will it improve animal welfare but also productivity and profit.

Although there are established metabolites for measuring stress, stress is a complex and multifaceted process. Therefore, an increase in one metabolite associated with a certain stressor may not increase as a result of another stressor as shown by Moberg (1985a) and presents the question, is the second stressor stressful?

Recent research has indicated that acute phase proteins may be useful in measuring stress due to their response during inflammation and not being as transient as previous measures for stress (e.g. cortisol), thus allowing a wider window for measurement. Currently, acute phase proteins are used to aid in diagnosing and monitoring disease and inflammation and thus this is where the research on acute phase proteins in animals has primarily focused. Upon reviewing the literature there is a possibility that the major acute phase proteins (in particular) may be induced as a consequence of psychological stress (Murata and Miyamoto, 1993) and may also be an indicator of animal performance (Eurell *et al.,* 1992).

There are two major acute phase proteins that could be used to measure the non-inflammatory stress response in beef cattle undergoing normal production practices. These are serum amyloid-A and haptoglobin. The reason for considering these two proteins is that in addition to being major acute phase proteins in cattle they have also been shown by Alsemgeest *et al.* (1995), Deak *et al.* (1995) and Nukina *et al.* (2001) to possibly increase due to non-inflammatory stressors.

It was decided to initially focus the research on only one of the acute phase proteins due to the need to establish an in-house assay for measuring the protein and if time permitted to evaluate the second acute phase protein. It was decided that the acute phase protein haptoglobin would be our initial focus for measuring the psychological response to stress and as a potential measure of beef cattle performance rather than using serum amyloid-A. The latter was not chosen as:

1. the literature suggests that serum amyloid-A is most likely a better measure of disease diagnosis and progression and that it increases in all cases of inflammation where haptoglobin does not (Horadagoda *et al.,* 1999).
2. Serum amyloid-A has greater sensitivity to inflammation than haptoglobin and thus can provide an alternative if haptoglobin can not be measured (Gruys *et al.,* 1994).
3. Serum amyloid-A can be used to assess the physical welfare of cattle (Alsemgeest *et al.,* 1995).

4. Serum amyloid-A is difficult to measure (Murata *et al.,* 2003) as it requires antibodies for determination unlike haptoglobin which can be measured with a biochemical assay. As a result, measurement of serum amyloid-A is time consuming.
5. Nukina *et al.* (1999), in their study in mice showed an increase in IL-6 in response to a handling restraint. IL-6 has been shown to lead to the induction of haptoglobin. Serum amyloid-A on the other hand requires IL-1 to induce a response (Alsemgeest *et al.,* 1996).
6. Haptoglobin has been shown to increase in response to conditions that are not generally associated with inflammation (starvation, fatty liver syndrome, transport stress, weaning and feed deprivation) (Murata and Miyamoto, 1993; Nakagawa *et al.,* 1997; Katoh *et al.,* 2002; Hickey *et al.,* 2003; Murata *et al.,* 2003; Earley *et al.,* 2004a; Earley *et al.,* 2004b).
7. Haptoglobin has been reported to be correlated with weight gain in pigs at seven weeks of age (Eurell *et al.*, 1992).
8. Haptoglobin can be expressed in much higher concentrations (mg/mL rather than ng/mL). Thus changes in plasma concentration are easier to detect.

For these reasons haptoglobin was chosen for examination of the acute phase response to non-inflammatory management practices of beef cattle in this thesis.

The study hypothesis is that the acute phase protein haptoglobin has the potential to be used as an indicator of the stress imposed on cattle undergoing normal management practises.

1.10 Research objectives

In view of the substantial contribution to Australian agriculture made by the beef cattle industry, the diversity of the production practices that we undertake in producing quality beef for both domestic and export markets, and growing consumer awareness of animal welfare, it is time to evaluate haptoglobin as an alternative measure of stress with the aim of improving animal welfare standards in Australia. Therefore the experiments that follow will evaluate haptoglobin as measure of production stress in beef cattle. The specific objectives are to

1. Develop an 'in-house' assay for measuring haptoglobin based on its binding capacity with haemoglobin.
2. Determine if haematological differences occur between sampling sites (jugular vein versus the coccygeal vessels) in particular for haptoglobin.
3. Evaluate the response of haptoglobin to weaning, transportation, intensive management and social re-grouping.
4. Profile the intensity and duration of response to these stressors

As a result of this the overall hypotheses developed for this thesis were:

1. That known non-inflammatory management stressors can induce a haptoglobin response in beef cattle
2. That flight time (a measure of temperament) can be used as a predictor of the acute phase response to the management practices that are thought to impose a psychological stressor on cattle

It has been well established that haptoglobin is a useful measure of inflammation; therefore, management practices that result in inflammation (branding/castration/dehorning) were not investigated as part of this thesis. The non-inflammatory management stressors weaning, transportation, intensive management and social re-grouping were investigated.

Section II: Assay development

Development of a colorimetric assay for measuring haptoglobin.

A colorimetric assay for measuring haemoglobin.

Chapter 2: Development of a colorimetric assay for measuring haptoglobin

2.1 Introduction

Haptoglobin is a positive acute phase protein that belongs to the family of glycoproteins and was first discovered and named in 1940 by Polonovski and Jayle (as reported in Bremner, 1964). An understanding of the binding relationship of haptoglobin and haemoglobin dates back to its discovery (Polonovski and Jayle, 1940 as reported by Bremner, 1964). Haptoglobin has a molecular weight of 80K to 160K and consists of a 4-polypeptide chain, has a plasma half-life of 2 to 4 days and is synthesized by the liver (Jain, 1993). It is involved in the innate immune response and is the first arm of defence for removing free haemoglobin (after being converted into methaemoglobin) from circulation (Owen *et al.,* 1960; Jain, 1993). Haptoglobin removes methaemoglobin from circulation by binding in a 1 to 1 ratio forming a stable complex that has preserved peroxidase activity (Jain, 1993).

The haemoglobin-haptoglobin (Hb-Hp) complex, once formed travels back to the liver where it is removed by the Kupffer cells (Jain, 1993). The rate at which the Hb-Hp complex is removed from circulation is species dependent (Jain, 1993). Removal of free haemoglobin is important as it prevents the bacterial use of haeme (Guyton, 1971). Partially reduced haptoglobin as shown by Morimatsu *et al.* (1991) is still able to bind haemoglobin. The Hb-Hp complex has a high functional affinity to the macrophage CD163 (Horn *et al.,* 2003), thus enabling the Hb-Hp complex to be engulfed by CD163 aiding in its removal. CD163 is a mediator of intracellular signalling and regulates inflammation (Horn *et al.,* 2003).

Haptoglobin is induced as part of the innate immune response and is regulated by the cytokines IL-1, IL-6 and TNF-α (Alsemgeest *et al.,* 1996; Yoshioka *et al.,* 2002). *In vitro* studies by Yoshioka *et al.* (2002) showed that bovine hepatocytes could be induced to secrete haptoglobin (9 fold) by adding IL-6. They also showed that a 2-fold increase of haptoglobin occurred when TNF-α and IL-1β were added. IFN-γ was also evaluated and found to have no or minimal effect in inducing a haptoglobin response. When IL-6 was coupled with IFN-γ the secretion of haptoglobin was inhibited. However, when IL-6 was coupled with TNF-α the secretion of haptoglobin was enhanced implying that IL-6 stimulates haptoglobin synthesis in bovine hepatocytes. This is also supported by the findings of Alsemgeest *et al.* (1996) who evaluated IL-6 and TNF-α on haptoglobin and serum amyloid-A secretion *in vitro*.

A haptoglobin response can be artificially induced by injecting the animal with turpentine which results in a localised inflammatory response. Conner and Eckersall (1988) revealed that injection of calves with turpentine caused an increase in haptoglobin concentration the day after the injection. The magnitude and longevity of the response was dose dependent with a greater volume of injected turpentine resulting in a longer and greater haptoglobin response (Conner and Eckersall, 1988). In adrenalectomized sheep, activation of the haptoglobin response via turpentine injection showed minimal or no response (Jarrett, 1972). Injection of ACTH in the adrenalectomized sheep failed to induce a response, thus implying that the adrenal gland has a role in activation of the haptoglobin response (Jarrett, 1972).

Species differences occur in the expression of haptoglobin in normal healthy individuals and during an acute phase response (Table 1.3). This can and does cause differences in physiology and immuno-reactivity as seen when comparing human and bovine haptoglobin (Eckersall and Conner, 1988). Porcine and human haptoglobin are biochemically similar and will cross react immunologically with human haptoglobin (Eurell *et al.,* 1992). However, Busby and Travis (1978, as reported in Morimatsu *et al.,* 1991) showed that antisera to bovine haptoglobin did not cross react with porcine or human haptoglobin. However cross-reactivity has been reported for haptoglobin from sheep, goats, elk, bison and deer with goat antiserum for haptoglobin, but, did not cross react with monkey, human, rat, horse, pig, rabbit, dog or cat haptoglobin (Travis and Saunders, 1972 as reported by Eckersall and Conner, 1988).

Several methods have been used to determine the quantity of haptoglobin in circulation. These include using radial immunodiffusion (Morimatsu *et al.,* 1992); ELISA based methods (Young *et al.*, 1995; Gray *et al.,* 1998; Nakagawa *et al.*, 1997), high performance chromatography (Salonen *et al.,* 1996), electrophoresis (Bremner, 1964) and colorimetric assays based on the peroxidase activity of the haemoglobin-haptoglobin complex (Owen *et al.,* 1960; Bremner, 1964; Eckersall *et al.,* 1999a).

The method described in this chapter is a modification of the method of Jones and Mould (1984), who based their assay on Owen *et al.* (1960). The assay uses the peroxidase activity of the haemoglobin-haptoglobin complex to determine the concentration of haptoglobin in the blood. The advantage of the peroxidase method over other methods described for measuring haptoglobin is that it is not species specific like those that use antibodies and is not as time consuming as the immunological methods. However, the technique does have one major

limitation in that haemolysed samples give false positive results due to the peroxidase activity of methaemoglobin, which Owen *et al.* (1960) reported contributes 10% of the peroxidase activity of the haemoglobin-haptoglobin complex. Both Eckersall *et al.* (1999a) and the commercial haptoglobin kit produced by *Tridelta* state that haemolysed samples should not be analysed using this method. Although it is recommended that haemolysed samples are not used in the assay, *Tridelta* does not define what degree of haemolysis can cause accuracy problems, while Eckersall *et al.* (1999a) indicates that grossly haemolysed samples should be avoided as they lead to a decrease in the apparent haptoglobin concentration.

For the collection of blood samples from large numbers of cattle, venepuncture of the coccygeal vessels is often practised. Variable degrees of haemolysis are frequently found in samples collected from this site. Therefore the aim of the study reported below was to firstly develop a haptoglobin method based on the method of Jones and Mould (1984) that has high repeatability; and secondly to test if multiple freeze-thaw cycles affect the haptoglobin concentration in samples and thirdly, to assess the effect of haemolysis (caused at the time of sampling) on the samples and if necessary develop a method to correct for haemolysis errors.

Part 1 – Development of a colorimetric assay for measuring haptoglobin

2.2 Materials and methods

The method described in this chapter is based on the method by Jones and Mould (1984). This method uses the peroxidase nature of the haemoglobin-haptoglobin bond. This bond in the presence of oxygen (provided by hydrogen peroxide) and the chromogen guaiacol causes a redox reaction which results in a colour change of the chromogen that can be spectrophotometrically measured at 450nm.

The method of Jones and Mould (1984) was modified in the current study, the test was examined for its repeatability, and the effect of repeated freeze thaw cycles on haptoglobin measurements were determined.

Reagents

Guaiacol solution

Guaiacol, 12.28mL (Sigma Aldrich: Guaiacol Product No. G502) and 366mL of 1M glacial acetic acid were made to a one litre working solution by addition of Milli Q water and the pH adjusted to 4.0 with 1M NaOH.

Hydrogen peroxide

A 0.02M H_2O_2 solution was made by adding 2.27mL H_2O_2 (AnalaR: Hydrogen peroxide 30%w/v Product No. 10366) to 997.73mL H_2O.

Bovine haemoglobin

Bovine methaemoglobin (Sigma Aldrich: Methemoglobin (Bovine) Product No. M9250) or bovine haemoglobin (Sigma: Haemoglobin (Bovine) Product No. H2500) stock solution was made up to 20mg/mL by adding the lyophilized powder to Milli Q water and frozen at -20ºC in aliquots until required. The stock solution was diluted to give a 0.12mg/mL solution by adding 0.15M NaCl.

Standard and controls

Serum with a high haptoglobin concentration was obtained from sheep infected with blowfly larvae and standardized using a commercial Hp Kit (Tridelta Development Ltd., Wicklow, Ireland). This serum reported a haptoglobin concentration of 2.9mg/mL, and was then aliquoted and stored at -20ºC. The working standard was diluted to 150ng/mL in 1×PBS pH=7.2 and serially diluted to give standards of 75, 37.5, 18.75 and 9.375ng/mL. A 0ng/mL standard was also included by using 1×PBS pH=7.2.

High, medium and low controls samples were made from a bulk plasma sample (bovine) that had a high haptoglobin concentration. The pooled sample was diluted in 1×PBS pH=7.2 to give three high, medium and a low dilutions of approximately 100ng/mL, 50ng/mL and 10ng/mL for measuring between plate variation.

Plasma samples

Bovine blood was collected in vacutainers using 18 gauge 1-inch needles containing EDTA, centrifuged at 1200g (2500rpm) for 15 minutes. The plasma was then removed and frozen at -20°C until analysis. Dilutions were made in 1×PBS where required.

Assay procedure

The guaiacol solution and Milli Q water (used for diluting the hydrogen peroxide) were placed in a 30°C water bath until required. Fifty micro-litres of appropriately diluted samples (serum or plasma), standards, controls and blank solutions were added in triplicate to wells of flat-bottom microtitre plates (Sarstedt: Microtest plate 96 flat well Product No. 82.9923.148),

followed by the addition of 50µL of 0.12mg/mL haemoglobin solution. The plates were then gently tapped to ensure mixing and left to incubate for 10 minutes at room temperature. 0.02M H_2O_2 solution was made using the pre-warmed Milli Q water and then added to the guaiacol solution in the ratio of 2 parts H_2O_2 solution to 3 parts guaiacol solution. A 100µL of this mix was then dispensed to all wells. The plates were then incubated at room temperature for 3 minutes and then immediately read at 450nm using the plate reader (Softmax Version 2.35, Molecular Devices Corp, 1993) and then again at 12 minutes. The 12 minute reading, although not used for analysis, allowed enough time for extremely low concentrations of haptoglobin to develop the chromogen. This provided information on samples that had low haptoglobin concentrations by indicating whether or not a less diluted sample would be more appropriate for analysis where possible.

Testing for repeatability of the haptoglobin assay

Plasma samples from 22 sheep were analyzed for haptoglobin twice on day one and then twice on day two for analysis of repeatability across plates and time. The results were then analyzed using a repeated measures model in the statistical package ASReml (Gilmour *et al.,* 2002).

Freeze thaw cycles

Frozen plasma samples from 10 sheep were thawed, mixed and aliquoted to give 8 replicates for each animal. The samples were then returned to the freezer where they were subjected to the freeze thawing process based on their aliquoted number (a sample with aliquot number 5 was thawed 5 times compared to an aliquot number of 8 which was thawed 8 times). Thawing of samples occurred at room temperature. The samples were then assayed for haptoglobin using the assay procedure described previously. Statistical analysis of the data were performed using a repeated measures model in ASReml.

2.3 Results

The repeatability of the haptoglobin assay was found to be 92.26% across plates and 94.78% between assays. There was no significant effect of multiple freeze thaw cycles on haptoglobin concentrations ($F=0.03$, $P>0.05$) (Figure 2.1).

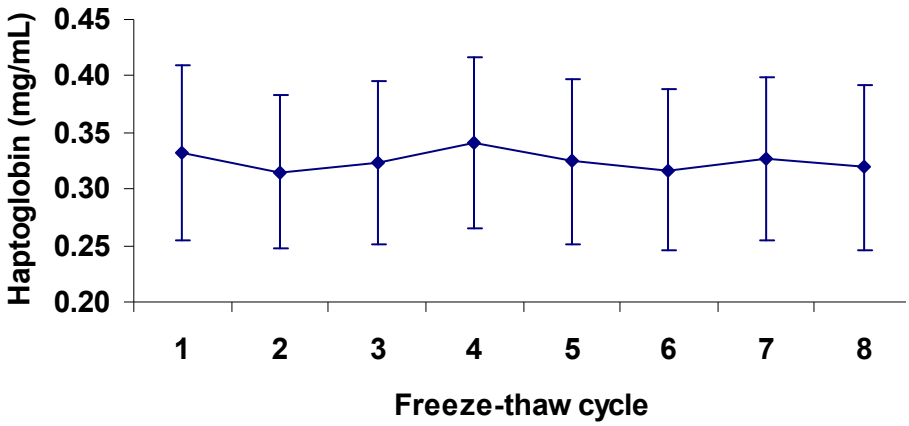

Figure 2.1: The effect of multiple freeze-thaw cycles on haptoglobin (n=12; mean ± se).

Part 2 – Determining the point at which haemolysis interferes with the haptoglobin assay

2.4 Introduction

The issue of haemolysis did not present itself until analysis of plasma samples obtained from a coccygeal blood vessel and a jugular vein at the same time point were analysed for haptoglobin concentration (see Chapter 4 for further details on this comparison). The results from the preliminary analysis of the data indicated that haptoglobin concentration in the coccygeal blood vessel tended to be higher. This difference in blood variables collected from different sampling sites is not uncommon (Parker and Blowey, 1974; Sears *et al.,* 1978; Morrow-Tesch and Whitehead, 1998; Kováč *et al.*, 2001). To understand what may be occurring in the coccygeal blood vessels to account for this difference, the paired plasma samples were compared visually. It was evident that when the sample from the coccygeal blood vessel had a higher haptoglobin concentration it generally also had a greater degree of haemolysis.

Eckersall *et al.* (1999a) and *Tridelta* advise that haemolysed samples should not be assayed but neither source identifies the degree of haemolysis that interferes with the assay. Thus a study was undertaken to establish when samples needed to be discarded due to haemolysis.

2.5 Materials and methods

Ten heifers were jugular bled into five 10 mL EDTA vacutainers. Upon returning to the lab the blood from each individual animal was pooled and mixed before being aliquoted in 3mL volumes to test-tubes. Milli Q water was added according to Table 2.1 and the total volume

made up to 3500µL with saline. The water was added prior to the saline with mixing occurring in between (Table 2.1).

The blood was centrifuged at 1200g (2500rpm) for 15 minutes and the plasma pipetted off and stored at -20˚C until analysis for haptoglobin using the method described above. The data were analyzed statistically using the statistical package 'R' (R-version 1.9, https://www.r_project.org/) to define the point at which haemolysis began to impact on the haptoglobin concentrations ($P<0.05$).

The procedure was repeated on 30 heifers and 20 steer calves to validate the preliminary findings. Haemolysis was induced as in Table 2.1 but tubes 2, 10, 11 and 12 were omitted.

Table 2.1: The volume of water added to induce haemolysis in the blood and the volume of saline added to each tube to bring the total volume to 3500µL.

Tube	H_2O added (µL)	Saline added (µL)	% water added
1	0	500	0.000
2	3	497	0.086
3	6	494	0.171
4	9	491	0.257
5	12	488	0.343
6	15	485	0.429
7	18	482	0.514
8	21	479	0.600
9	30	470	0.857
10	50	450	1.429
11	100	400	2.857
12	500	0	14.29

The results were analysed using paired t-tests comparing the non-haemolysed sample (tube 1) with the haemolysed samples (tubes 2-12). Photographs were taken of the tubes to be used as a guide for determining samples that had unacceptable degree of haemolysis.

The paired samples comparing haptoglobin from a coccygeal blood vessel and a jugular vein were visually graded according to the results of haemolysis test to identify samples that were unacceptable due to haemolysis.

2.6 Results

As the degree of haemolysis increased, the apparent concentration of haptoglobin within the plasma samples also increased (Figure 2.2). The relationship between the amount of water added and absorbance by the chromogen was linear ($y = 0.120x + 0.081$; $r = 0.917$). It was identified that haemolysis caused a significant increase in absorbance ($P<0.05$) when greater than 15µL water was added to 3mL whole blood (equating to 0.43% water added to induce haemolysis).

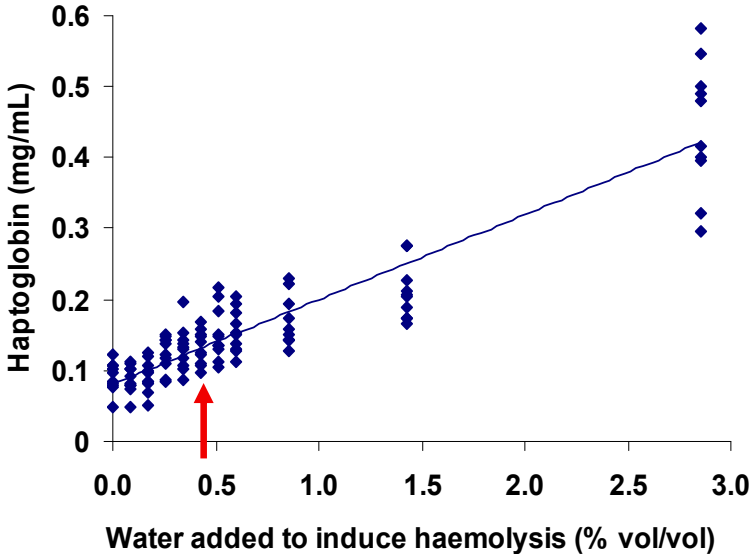

Figure 2.2: The relationship between the percentage of water added to induce haemolysis and apparent haptoglobin ($y = 0.120x + 0.081$; $r=0.917$). The red arrow indicates the point at which haemolysis starts having a significant ($P<0.05$) influence on the haptoglobin value.

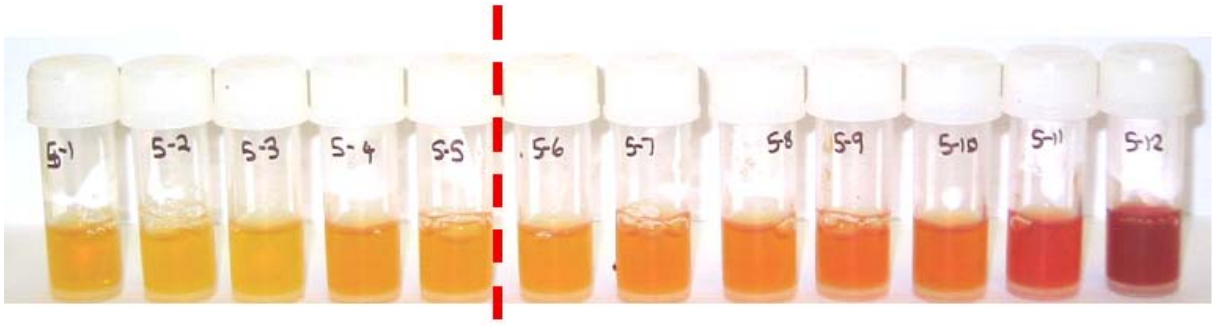

Figure 2.3: Bovine plasma samples with increasing degrees of artificially induced haemolysis as in Table 2.1.

- - - represents the point at which haemolysis significantly impacts on the haptoglobin assay.

Using the colour scale (Figure 2.3) to determine which samples (from the paired analysis) had an unacceptable degree of haemolysis, it was revealed that 40.8% of the 120 paired samples would need to be removed from the analysis due to unacceptable visual levels. Of the 120 coccygeal plasma

samples 54.2% had unacceptable visual levels of haemolysis with jugular plasma having only 27.5% unacceptable visual levels of haemolysis.

Part 3 – The components of haemolysed plasma that contribute to the apparent haptoglobin activity

2.7 Introduction

The findings in Part 2 that a minimum 27.5% of samples would be removed from analysis due to haemolysis posed an experimental and an ethical dilemma. More animals would need to be included in experiments (the ethical dilemma as all animals need to be accounted for) for statistical robustness. Therefore, it was proposed that analysis of the major component/s of haemolysed samples and any other plasma products that may interfere with the determination of haptoglobin may enable the development of a method to use haemolysed samples and thereby reduce the need to repeat or increase the number of experimental animals used in order to obtain sufficient data for statistical analysis.

2.8 Material and methods

Various components of the haptoglobin assay (methaemoglobin, haptoglobin and guaiacol solution) were added together in various combinations and spectrophotometrically scanned from 200nm to 800nm using a scanning spectrophotometer (Cary 50 UV-visible spectrophotometer, Varian Inc). In addition, haemolysed plasma samples were also scanned to analyse and identify the various components of haemolysis.

2.9 Results

The spectrophotometric scans revealed peaks at 414 and 570nm in plasma samples with artificially induced haemolysis (Figure 2.4a). The peaks correspond to the various forms of haemoglobin, with the peak at 414nm corresponding to methaemoglobin (Figure 2.4b) and the peak at 570nm corresponding to oxyhaemoglobin (Fairbanks *et al.,* 1992) and not haptoglobin (Figure 2.4d). The scan of the components of the haptoglobin assay (individually and in various combinations) (Figure 2.4e) show that it is possible to determine the haptoglobin concentration by measuring the chromogen after it has been reduced between the approximate wavelengths of 350nm to 600nm.

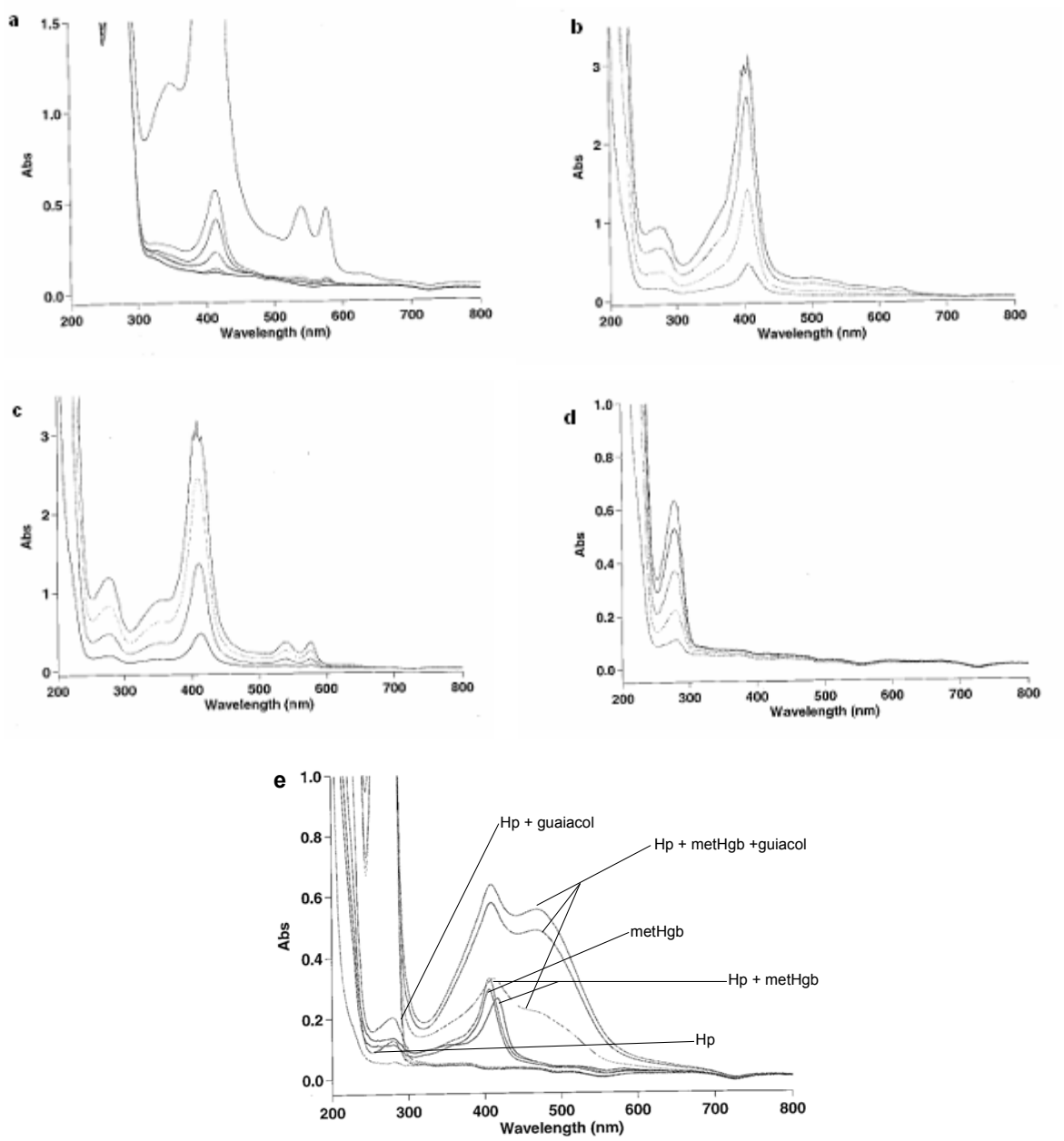

Figure 2.4: Spectrophotometric scans of: a) plasma sample with increasing degree of artificially induced haemolysis; b) methaemoglobin increasing in concentration; c) haemoglobin increasing in concentration; d) haptoglobin increasing in concentration; e) permutations of haptoglobin assay components (the large broad peaks represent the response of haptoglobin (Hp) + haemoglobin (metHb) +chromogen solution (guaiacol)).

Part 4 – Using an alternative wavelength to measure the colour product of the chromogen

2.10 Introduction

From the above findings we reported that the main product of haemolysis is methaemoglobin. This stands to reason as haemolysis is caused when erythrocytes rupture spilling their cellular content into the blood. We also know that the colour product of the chromogen can be detected between the wavelengths of 350nm to 600nm, and that, when the chromogen peak is

overlaid with the peaks of haemoglobin, there are points where negligible interference occurs from haemoglobin. This suggests that measuring the reaction product at an alternative wavelength may overcome the problem of interference by free haemoglobin.

2.11 Material and methods

The material and methods described in 2.2 were used with the alternative wavelengths of 490nm and 570nm chosen as both of these wavelengths were available on the plate reader and according to the photospectric scans there is minimal interference of haemoglobin at these points.

2.12 Results

As can be seen in Figure 2.5 the alternative wavelengths did not overcome the problem of haemolysis interfering with the haptoglobin assay. Although the absorbance values differed at these wavelengths there was a trend for the haemolysed samples to have greater absorbance than non haemolysed samples and the gradient with increasing haemolysis did not remain at zero.

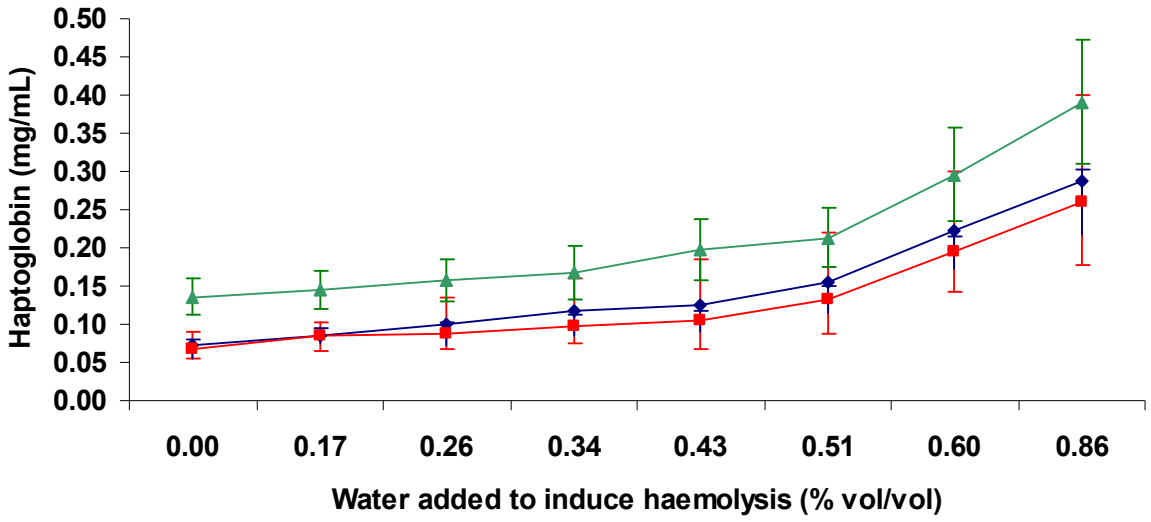

Figure 2.5: Effect of wavelength on the determination of haptoglobin in plasma samples with varying degrees of haemolysis (mean ±se) Wavelength: ♦ 450nm; ■ 490nm; ▲ 570nm

Part 5 – Understanding the relationship between haptoglobin and haemoglobin

2.13 Introduction

Using an alternative wavelength did not address the problem of haemolysis and led to re-evaluating the principles behind the assay; in particular, the relationship between haptoglobin and haemoglobin and its peroxidase activity when these two proteins are coupled.

2.14 Materials and methods

The relationship between haptoglobin and free haemoglobin was examined by "spiking" plasma samples with haemoglobin in 0.1mg/mL increments ranging from 0.1mg/mL to 1mg/mL (Table 2.2). The "spiked" samples were rested overnight at 4°C before assay for haptoglobin. The results were graphed and a linear regression analysis was performed to provide the relationship between the haemoglobin added and the apparent haptoglobin value.

Table 2.2: The volume of haemoglobin added to the samples to achieve a degree of haemolysis.

Hb (mg/mL)	Tube	Sample (µL)	Hb (µL)	PBS (µL)	Total (µL)
0.0	1	200	0.00	400.00	600
0.1	2	200	1.01	398.99	600
0.2	3	200	2.02	397.98	600
0.3	4	200	3.04	396.96	600
0.4	5	200	4.05	395.95	600
0.5	6	200	5.06	394.94	600
0.6	7	200	6.07	393.93	600
0.7	8	200	7.08	392.92	600
0.8	9	200	8.09	391.91	600
0.9	10	200	9.11	390.89	600
1.0	11	200	10.12	389.88	600

Individual sample blanks were included to examine endogenous peroxidase activity in the haptoglobin assay. This was achieved by substituting the methaemoglobin solution with saline. To calculate the haptoglobin value due to endogenous peroxidase activity in the sample, the endogenous activity was subtracted from the raw haptoglobin value before being multiplied by the dilution factor where appropriate.

2.15 Results

The relationship between added haemoglobin and apparent haptoglobin as measured in the haptoglobin assay was found to be linear ($y=0.338x + 0.044$, $r^2=0.983$) (Figure 2.5). These same samples were assessed for endogenous activity and when adjusted for this activity, the relationship remained linear ($y=0.118x + 0.015$, $r^2=0.946$) (Figure 2.5) but with a reduced slope. Thus implying endogenous activity is interfering within the assay, but, does not account for all of the interference (Figure 2.6).

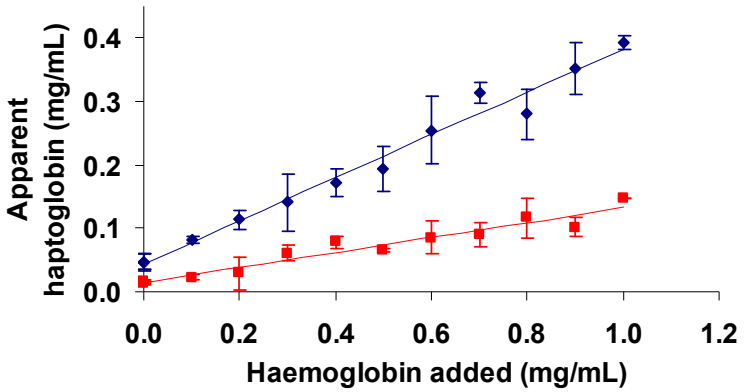

Figure 2.6: Effect of added haemoglobin on the determination of haptoglobin in plasma samples (mean ± sd). ♦ Apparent Hp (y=0.338x+0.044, r^2=0.983); ■ Apparent Hp minus endogenous peroxidase activity (y=0.118x+0.015, r^2=0.946).

Part 6 – Correcting for endogenous activity and interference caused by haemolysis

2.16 Introduction

The findings in part 5 implied that both endogenous activity and haemolysis are interfering within the assay and therefore need to be accounted for. Accounting for the endogenous activity is relatively simple as individual sample blanks can be included, while accounting for interference from haemoglobin would require the use of the linear relationship between haemoglobin and haptoglobin.

2.17 Materials and methods

Plasma samples were spiked with known amounts of haptoglobin (ranging from 0.00 to 0.77 mg/mL increasing in 0.17mg/mL increments) and haemoglobin (ranging from 0.00 to 1.00 mg/mL increasing in 0.1mg/mL steps) in various combinations. The reason behind spiking the samples with known amounts of haptoglobin was so that when the correction equation was applied to the haemolysed samples we could determine if the equations were working.

Three equations were used as possible ways of dealing with the endogenous activity and/or haemolysis interference and the samples from part 5 were used to assess these.

The first equation developed was: $Hp_{corrected} = Hp_{raw} - Hp_{endogenous\ activity,}$

The second equation developed was: $Hp_{corrected} = Hp_{raw} - Hp_{due\ to\ Hb,}$

And the third equation developed was: $Hp_{corrected} = Hp_{raw} - Hp_{endogenous\ activity} - Hp_{due\ to\ Hb;}$

Where $Hp_{due\ to\ Hb} = 0.118 \times Hb_{free} + 0.015$

The data for the various correction methods was statistically analysed using paired t-tests to determine if any of the methods were suitable for correcting for haemolysis. The statistical package used for the analysis was Systat version 9 (SPSS Inc, Chicago IL, USA).

2.18 Results

The artificially "spiked" plasma samples were processed for an apparent haptoglobin value based on the above equations and were graphed (Figure 2.7). The analysis of the data revealed that there was a significant difference in haptoglobin concentration (P=0.00) when equation 1 or equation 2 were used to correct for haemolysis and endogenous activity. However, there was no significant difference between the samples when corrected using equation 3 (P=0.327) (Table 2.3).

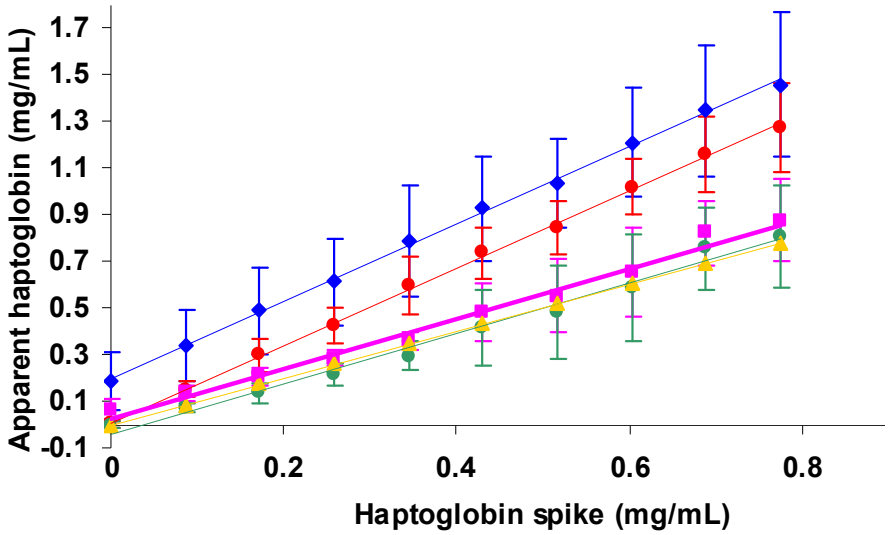

Figure 2.7: Apparent haptoglobin corrected for haemolysis (mean ± sd). ▲ Unhaemolysed sample; ♦ No correction; ■ Equation 1; ● Equation 2; ● Equation 3

Table 2.3: Statistical results of the various correction factors

Correction of sample	sample mean (unhaemolysed)	sample mean (haemolysed)	sd difference	t-value	P-value
No correction	0.347	0.771	0.267	-22.50	0.000
Equation 1	0.347	0.403	0.117	-6.839	0.000
Equation 2	0.347	0.583	0.207	-16.10	0.000
Equation 3	0.347	0.347	0.338	0.982	0.327

2.19 Overall discussion

A number of methods have been developed for measuring haptoglobin. A cheap and widely used assay principle is to measure the peroxidase activity of haptoglobin when complexed to methaemoglobin. In the assay by Jones and Mould (1984), peroxidase activity of the

haemoglobin-haptoglobin complex is used to oxidise guaiacol to a coloured product that can be measured at 450nm. Haemolysis is reported to interfere with the measurement of haptoglobin in this assay. In the current chapter modification of this method required re-evaluation of the repeatability of the assay, the effect of freeze-thaw cycles on haptoglobin and to assess the effect of haemolysed samples on the assay.

This assay reported good repeatability and the analyte was resistant to multiple freeze-thaw cycles. The results from the freeze thaw cycles reflect those from the study by Peterson *et al.,* (2001) in that there was no effect on the plasma concentration of haptoglobin. The current results thus confirm that the haptoglobin protein is able to withstand multiple freeze-thaw cycles, and the assay method is highly repeatable and robust.

Having developed a repeatable and robust assay the effect that haemolysed samples have on the assay was evaluated. The issue of haemolysed samples was important to address not only due to the warnings of Eckersall *et al.* (1999a) and those in the *Tridelta* kit, but because collection of blood from the coccygeal blood vessels is more convenient when dealing with large numbers of cattle as they can be placed and have blood harvested whilst in a raceway and therefore multiple collectors can be used. Collection of blood from this site generally results in a haemolysed sample and therefore, it was important to assess the degree to which haemolysis interferes with the assay.

This assay has very little tolerance for haemolysis as can be seen in Figures 2.1 and 2.4. Unlike Eckersall *et al.* (1999a), we found that as the degree of haemolysis increased so too did the apparent haptoglobin concentration. A plausible reason for this is that the study evaluated haemolysis caused at the time of blood collection rather than haemolysis induced through disease or trauma. In our samples as the blood was removed from the animal it can suffer mild haemolysis when entering the tube. This allows the free haemoglobin released at sampling to bind to any haptoglobin that is present in the blood. Due to measuring the haptoglobin concentration by its binding capacity to added haemoglobin the already bound haptoglobin will also contribute to the redox reaction of the chromogen and thus greater colour intensity is produced, therefore reporting a higher haptoglobin value. If the blood is haemolysed due to disease or trauma before blood collection one would expect there initially to be reduced haptoglobin concentration due to the body removing the haemoglobin-haptoglobin complex from circulation once formed. Later, haptoglobin concentrations might

rise as part of the acute phase response. This would account for the findings of Eckersall *et al.* (1999a).

The original idea was to use a colour scale to identify and remove haemolysed samples. However, this technique was very subjective particularly for cattle plasma where the colour is greatly influenced by plant proteins (Jain, 1993) which can cause the colour of unhaemolysed plasma to range from almost opaque to a rich golden yellow. In addition, the number of plasma samples that would have been removed was in the order of 25 percent for samples collected from a jugular vein and 47 percent of samples collected from a coccygeal blood vessel. The large number of samples that would be removed from analysis due to haemolysis posed both an ethical and statistical dilemma. As part of the ethical approval process that is required for scientific research on animals in Australia, we are required to justify the number of animals that we use in an experiment and to minimise numbers needed to test a hypothesis. The development of a method to adjust values for the effect of haemolysis let us fulfil these requirements.

To gain additional insight into the assay, haemolysed samples and the various components were spectrophotometrically scanned. The result of the spectrophotometric scan indicated that the main product of haemolysis is free haemoglobin. Literature on haemoglobin (Chapter 3, page 42) reveals that haemoglobin has pseudo-peroxidase activity that can be a measured. Thus the interference with the haptoglobin assay is possibly due to 3 factors: 1) endogenous activity including the pseudo-peroxidase activity of haemoglobin; 2) the colour product of haemoglobin being absorbed at this wavelength, as analysis of haemoglobin at 450nm revealed that methaemoglobin had some absorbance at this point; and 3) the formation of haemoglobin-haptoglobin complex as a result of the presence of free haemoglobin caused by haemolysis. From the results of the scan on the various components of the assay it was determined that haemoglobin does not react with the chromogen guaiacol.

Evaluating the endogenous activity takes into account the colour product of free-haemoglobin and the by products of the oxidation of ferrohaemoglobins to methaemoglobin and any other proteins that may contribute to the endogenous activity. This led to the development of Equation 1. From the results we know that this does not account for the total increase in apparent haptoglobin, which led to the evaluation of the third possibility that interference is based on the formation of the haemoglobin-haptoglobin complex.

The spectrophotometric scan revealed that the majority of free haemoglobin was in the form of methaemoglobin (the form that is required for the formation of the haemoglobin-haptoglobin complex). Thus, there is a real possibility that this excess haemoglobin is binding to haptoglobin in the whole blood (after collection) and plasma and causing the apparent increase. Therefore, measuring the haemoglobin concentrations in the plasma sample used for the analysis allows for adjustment of the extra methaemoglobin present in the sample. By knowing the amount of haemoglobin in the plasma sample allows us to determine the haptoglobin concentrations due to the binding ratio. As the excess haemoglobin in the sample contributes to the colour and leads to an increase in haptoglobin. Therefore, the measured haemoglobin can be converted into an apparent haptoglobin value based on the relationship between haptoglobin and haemoglobin and then can be subtracted from the raw haptoglobin value (Equation 2).

Equations 1 and 2 on their own do not account for the net increase in haptoglobin as a result of haemolysis. However, combining the two equations led to the development of the third equation which accounts for the net influence of haemolysis on the haptoglobin values.

This laboratory is also interested in measuring haptoglobin in sheep and therefore sheep plasma was also subjected to testing for the effects of haemolysis on apparent haptoglobin. The blood from sheep was collected from a jugular vein using 18 gauge needles which typically results in very little haemolysis. Samples were subjected to haemolysis as described above and analysed for haptoglobin. The results revealed that haemolysis does not impact on the haptoglobin values in sheep in the same way that it does in cattle (results not shown); that is it does not cause the dramatic increase seen in haemolysed cattle plasma. Correction in sheep only required the inclusion of individual sample blanks to adjust for any endogenous activity present in the sample.

Although it is recommended that haemolysed samples be avoided where possible for the haptoglobin assay, it is not always feasible hence the development of the method to correct for haemolysis caused at the time of sampling cattle. The effect of haemolysis was also evaluated for sheep haptoglobin. This led to the development of the correction equations for cattle and sheep; with the cattle correction equation being slightly more complex than that for sheep due to the requirement to measure haemoglobin.

The cattle correction equation is: $\mathbf{Hp_{corrected} = Hp_{raw} - Hp_{endogenous\ activity} - Hp_{due\ to\ Hb};}$
Where $\mathbf{Hp_{due\ to\ Hb} = 0.118 \times Hb_{free} + 0.015,}$
And the sheep correction equation is: $\mathbf{Hp_{corrected} = Hp_{raw} - Hp_{endogenous\ activity}}$

Whether this correction factor would work for plasma samples that have haemolysis induce by haemolytic disease and or trauma is not clear. Therefore, it is recommended that these correction equations only be used in blood samples that have haemolysis induced at the time of sampling. The correction equations have only been evaluated and tested using bovine and ovine blood. Given the difference in the response between ovine and bovine blood, testing the relationship in other species is important as it is unclear how reliable this relationship is in other species.

As can be seen from equation 3, it is important to determine the amount of free haemoglobin in the plasma sample. This finding led to the development of a haemoglobin assay based on the pseudo-peroxidase nature of haemoglobin (Chapter 3).

Chapter 3 A colorimetric assay for measuring plasma haemoglobin

3.1 Introduction

Haemoglobin is an important molecule for multicellular organisms as it binds to and transports oxygen to the cells. It is necessary as the diffusion of oxygen from the atmosphere into the cells is near impossible in complex metazoan organisms with the exception of the top few cell layers. In vertebrates, haemoglobin is carried and transported around the body via red blood cells and makes up approximately 95% (dry weight) of a red blood cell (Lewis, 1974; Metzler, 1977; Jain, 1993). The haemoglobin molecule consists of four haeme units and two alpha and two beta globulin chains (one attached to each of the haeme molecules). Some additional characteristics of haemoglobin are listed in Table 3.1.

Table 3.1: Facts about haemoglobin

Fact	Value	Reference:
Oxygen-combining capacity of blood	1.34mL O_2 per g haemoglobin	Lewis, 1974; Jain, 1993
Molecular weight	64,000 to 68,000MW	Guyton, 1971; Lewis, 1974; Jain, 1993
Iron Content :Haemoglobin	100g Haemoglobin ≡ 0.347g iron	Lewis, 1974
Molar absorptivity	44.0 mmol is pH dependent	Lewis, 1974
Unit Mole	15g/dl = 9.3mmol/l	Lewis, 1974
One molecule of haemoglobin	Carries four iron atoms and therefore can carry four oxygen molecules	Guyton, 1971
%Weight of RBC	95% dry weight	Jain, 1993

There are four main types of haemoglobin found in circulation; oxyhaemoglobin, deoxyhaemoglobin, carboxyhaemoglobin and methaemoglobin. The first three types are able to bind oxygen and collectively belong to the group known as ferrohaemoglobin. The fourth form of haemoglobin, methaemoglobin is oxidised haemoglobin and belongs to the group ferrihaemoglobin. The characteristics of the four types of haemoglobin are summarised in Table 3.2. Other forms of haemoglobin include sulphaemoglobin and cyanmethaemoglobin. Lewis (1974) reported that all forms of haemoglobin can be converted into cyanmethaemoglobin with the exception of sulphaemoglobin. He also reported that there is a sex difference in the expression of haemoglobin with females (humans) having a higher concentration. This sex difference is also reported in this thesis (Chapter 6, page 93). The reason for this is not completely understood but is most likely due to hormonal influences. The age of the subject can also influence the haemoglobin concentration (Lewis, 1974).

Table 3.2: Types of haemoglobin

Haemoglobin	Characteristics	Reference
Oxyhaemoglobin[a] (HbO_2)	Delivers oxygen to the tissues then resumes its native form	Jain, 1993
Deoxyhaemoglobin[a] (Hb)	Does not contain bound oxygen	
Methaemoglobin (ferrihaemoglobin)	Oxidised haemoglobin Can not bind oxygen Contains iron (Fe) in the +3 oxidation state	
Carboxyhaemoglobin[a] (HbCO)	Binds irreversibly to carbon monoxide due to the affinity for CO being 325 times greater than O_2	Guyton, 1971

[a]Reduced forms of haemoglobin also known as ferrohaemoglobin. Haemoglobin must be in one of these states to bind oxygen.

Free haemoglobin (non-protein bound) when present in plasma and/or urine is an indicator of haemolytic disease and/or trauma. It can cause physiological problems if not removed from circulation as excess haemoglobin precipitates in the kidney tubules, crystallizing and eventually causing renal failure (Guyton, 1971). However, it was noted by Wood *et al.* (2001) that "as long as the protein-binding capacity for haemoglobin released from erythrocytes has not been exceeded, the danger to organs such as the kidney is minimal".

The body has two mechanisms to enable removal of free haemoglobin. The first is by using the acute phase protein haptoglobin, which binds to methaemoglobin in a one to one ratio forming a haemoglobin-haptoglobin complex (Guyton, 1971) and the second is via excretion (Guyton, 1971). Excretion occurs when the quantity of free haemoglobin exceeds the capacity of the liver to produce sufficient haptoglobin to capture the excess haemoglobin. Figure 3.1 illustrates the process by which haemoglobin is removed/broken down in circulation. An additional reason why removal of free haemoglobin is important is to prevent bacterial use of haeme as iron is a limiting element for proliferation of many bacterial species.

The ability to measure free circulating haemoglobin from disease or trauma is important for prevention of renal damage (Guyton, 1971). Standefer and Vanderjagt (1977) state that in their "experience physicians are most interested in monitoring haemoglobin values between 100 and 250mg/liter" as it is at these concentrations that free haemoglobin is most likely to do damage unless counteracted.

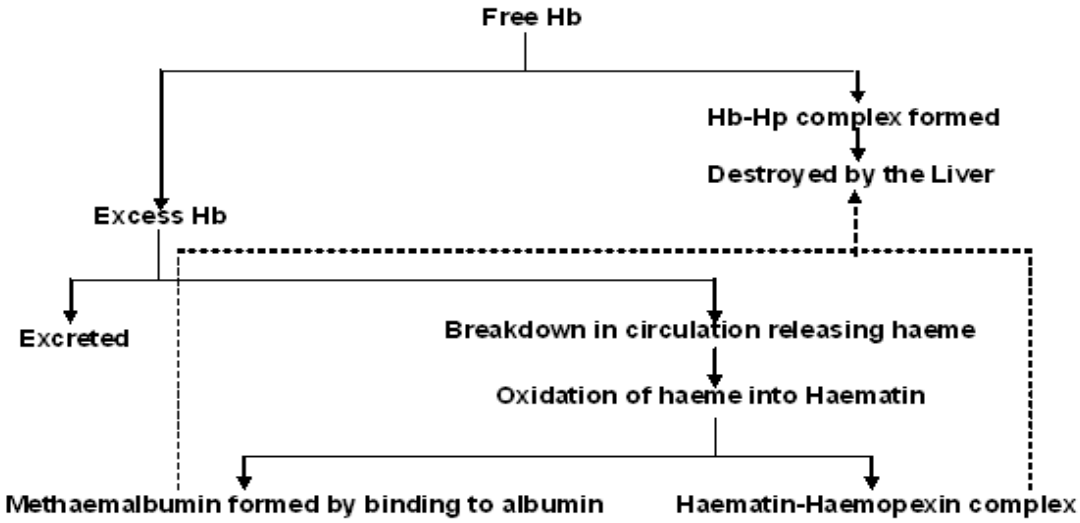

Figure 3.1: The process in which free haemoglobin is removed from circulation.

There are a number of different methods for measuring haemoglobin in plasma and urine based on the extinction coefficient of the various forms of haemoglobin and its pseudo-peroxidase nature. Table 3.3 provides a list of methods, their mode of action and associated advantages and disadvantages. An examination of this table shows that traditional methods are adequate for detecting haemolytic disease and/or trauma when concentrations of free haemoglobin in circulation are high (g/L). However, where it is necessary to measure low amounts of haemoglobin in plasma, none of these methods were considered suitable.

The development of a new and more sensitive method is described in this chapter and it was designed to determine the degree of haemolysis that seems to regularly occur during blood sampling of cattle from the coccygeal blood vessels. This need arose because a routine assay used within the laboratory is affected by haemolysis caused at the time of sampling, giving false positive results. The aim of the present study was to develop a method for detecting low concentrations of haemoglobin with greater sensitivity by utilising the pseudo-peroxidase nature of haemoglobin to catalyse a redox reaction between the peroxide and tetramethylbenzidine to allow for correction of haemoglobin interference within the haptoglobin assay. Standefer and Vanderjagt (1977) have shown that tetramethylbenzidine is an adequately sensitive method with accuracy for haemoglobin determination.

Table 3.3: Methods for measuring haemoglobin and the associated advantages and disadvantages.

Method	Method Description	Advantages	Disadvantages	Range	References
Reference Method (Cyanmethaemoglobin or (haemoglobincyanide method)	Measures methaemoglobin using spectrophotometric method using the absorption co-efficient for plasma haemoglobin of 682.17cm^2/g	- International reference standard since 1965 - Reliable standard - Converts all forms Hb except sulphaemoglobin - Indirect measure of iron status - Reference preparation is stable	- Several minutes needed for reaction to occur		Lewis, 1974; Gehring *et al.,* 2002; Bhaskaram *et al*., 2003
Oxyhaemoglobin Method (Second derivative method)	Measures the absorbance of oxyhaemoglobin between 570 and 580nm	- Simple and quick	- oxyhaemoglobin standard is not stable - Does not work when carboxy-, met- and/or sulph-haemoglobin present	- >0.04g/L	Lewis, 1974; Fairbanks *et al.,* 1992
Alkaline-haematin Method		- Gives true estimate of total haemoglobin	- More elaborate		Lewis, 1974
Azide-methaemoglobin Method		- Alternative to cyanmethaemoglobin method	- Azide waste is potentially explosive when in contact with metal pipes		Lewis, 1974
HemoCue Method	Photometrically –a modified azide methaemoglobin reaction using microcuvettes	- Cost effective - Easy to use	- over estimates Hb compared to the blood cell Counter - has wide scattering of results	- >0.25g/L	Morris *et al.,* 2001; Gehring *et al.,* 2002; Kapoor *et al.,*2002; Bhaskaram *et al.,* 2003

Method	Method Description	Advantages	Disadvantages	Range	References
Haemoglobin Photometer Method (HemoCue Low) **Haemoglobin Photometer Method continued**		- Takes between 11-54 seconds for measurement - Reliable for low concentrations of haemoglobin		- 0.3 – 30 g/L	Yokoyama *et al.,* 2002
Haemoglobin Colour Scale Method	A drop of blood on white absorbent paper and the colour is then compared to a chart	- cheap - easy to perform - portable	- too inaccurate for general use - subjective	- 4 to 14g/L	Paddle, 2002
High Performance Liquid Chromatography Method	Uses the chromogen tetramethylbenzidine in acetic acid and in the presence of hydrogen peroxide	- Can measure both free and protein bound haemoglobin takes 30minutes to perform from collection			Wood *et al.,* 2001
Sahil's acid haematin Method		- OBSOLETE (still used in India)			Kapoor *et al.,* 2002
Haldane's carboxyhaemoglobin Method		- OBSOLETE			Lewis, 1974
Blood Gas Analyzer Method (BGA)	Spectrophotometric method- total [Hb] is calculated using the sum of the derivatives in the following way; $cHb=cRHb+cO_2Hb+cCOHb+cMetHb$				Gehring *et al.,* 2002
Automated haematology analyser	Based on the reference method	- Faster than the reference method. Takes 30 seconds			Gehring *et al.,* 2002
BD Method (Method of Blakney and Dinwoodie, 19750	Spectrophotometric method- [Hb] is calculated from an absorption curve: $c(Hb)=1.55(\Delta A)$		- Colour reaction is not stable - Inhibited by serum proteins	- >0.1g/L	Fairbanks *et al.,* 1992
Kahn Method	Based on the BD method $cHb=1.55A_{578}-0.861A_{562}-0.689A_{598}$			- >0.1g/L	Fairbanks *et al.,* 1992

Method	Method Description	Advantages	Disadvantages	Range	References
Peroxidase Method	Uses the chromogen tetramethylbenzidine in acetic acid and in the presence of H_2O_2			- 0.02 to 2.5g/L	Fairbanks *et al.,* 1992; Levinson and Goldman, 1982
	Uses 2,2'-Azino-di-(3-ethylbenzthiazoline-6-sulfonic-acid)			- 0.02 to 1.0gL	Takayanagi and Yashiro, 1984
Point-of-care-testing (POCT)	Based on the HemoCue and BGA tests				Gehring *et al.,* 2002
Harboe Method	Haemoglobin is calculated using the following formulae for 11-fold-diluted specimen $c_{Hb}=0.836[2A_{415}-(A_{380}+A_{450})]$ $=1.65A_{415}-0.83A_{380}-0.83A_{450}$				Fairbanks *et al.,* 1992; Bedner and Bayer, 1993
Noe Method	Haemoglobin is calculated using the following formulae for 11-fold-diluted specimen $cHb=1.65A_{415}-0.93A_{380}-0.73A_{470}$				Fairbanks *et al.,* 1992; Bedner and Bayer, 1993
AI Method	Uses the known absorption coefficients of haemoglobin and bilirubin at 415 and 450 nm. The calculation is based on an 11-fold dilution $c_{Hb}=1.58A_{415}-0.95A_{450}-2.91A_{700}$ $c_b=268A_{450}-34.4A_{415}-682A_{700}$				Fairbanks *et al.,* 1992
AII Method	Compensates for hyperbilirubinemia and is based on AI method $c_{Hb}=1.55A_{415}-1.30A_{450}-1.24A_{700}$ $c_b=257A_{450}-49.8A_{415}-364A_{700}$				Fairbanks *et al.,*1992

3.2 Materials and methods

The design of the experiment was based on the pseudo-peroxidase nature of haemoglobin to catalyse a redox reaction between the reducing substance tetramethylbenzidine and hydrogen peroxide. This reaction produces a colour change that can be quantitatively measured.

Reagents

TMB solution

The TMB solution was prepared by dissolving one tetramethylbenzidine (TMB) tablet (1mg/tablet, Sigma 3, 3', 5, 5'- tetramethylbenzidine tablets, Product number T-3405) in 10mL of 0.05M Acetic acid. To help catalyse the reaction, 2µL of hydrogen peroxide was added to the 10mL of TMB solution prior to use.

Standards and controls

Five hundred millilitres of blood was harvested from one heifer via the jugular vein into a blood bag containing 3.8g EDTA dissolved in 10mL of physiological saline. The blood was then haemolysed by adding an equal volume of Milli Q water and then centrifuged at 11000g (8000rpm) (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments) for 30 minutes to remove white blood cells and the stroma from the destroyed red blood cells. The plasma was stripped from the cellular debris and a sample of the plasma was then analysed using a haemocytometer for the presence of red blood cells and or stroma. Due to the large volume of stroma present the plasma was re-spun at 11000g (8000rpm) for 30 minutes and re-checked using the haemocytometer. The haemoglobin concentrations of the plasma were measured using the Cell Dyn 3500R (Abott Diagnostics, North Ryde, NSW, Australia). The Cell Dyn 3500R measures haemoglobin spectrophotometrically by the haemoglobincyanide method and is inaccurate at determining haemoglobin concentrations below 4g/dL. The instrument reported a haemoglobin concentration of 59.35mg/mL. The preparation was aliquoted and stored at -20˚C. The concentrated haemoglobin solution was diluted to 2mg/mL with 1× PBS pH=7.2 and serially diluted to give standards of 1, 0.5, 0.25, 0.125 and 0.0625mg/mL. A 0mg/mL standard was also included by using 1× PBS pH=7.2. High, medium and low controls were also made up from the stock haemoglobin by diluting with 1× PBS.

Test samples

Blood was collected from a coccygeal blood vessel from 80 heifers and was artificially haemolysed by adding an equal volume of Milli Q water. The blood was then centrifuged at 2000g (3500rpm) for 30 minutes and the top fraction of the plasma removed and frozen at -20°C. An additional 9 samples were obtained by combining left over blood from the heifers, thus the total number of different samples analyzed was 89. The haemoglobin in the plasma was measured using the Cell Dyn 3500R to provide a comparative benchmark for the values obtained from the micro plate assay.

Methods

Assay procedure

Ten micro litres of the standards, controls and diluted test samples (n=89) (diluted in 1× PBS) were added to the wells in triplicate, followed by the addition of 150µL of TMB solution to each well. A triplicate of diluent (1×PBS) was also included for a blank, which was subtracted from each well. The plates were then left to incubate at room temperature for 10 minutes before being stopped using 75µL of 1.8M H_2SO_4. The plates were read at 450nm (Softmax Version 2.35, Molecular Devices Corp, 1993). The test samples were multiplied by their dilution factor to obtain the haemoglobin concentration in the plasma samples.

Repeatability of the microplate assay

The microplate assay was performed on the test samples a total of four times to measure the repeatability of the assay. Test 1 and 2 were the same diluted plasma sample but run on consecutive days. Test 3 was a re-dilution of the test samples and test 4 was a re-run of test 3. The results from the four tests were then compared to the results obtained from the Cell Dyn 3500R.

Albumin interference

The effect of endogenous peroxidase activity in albumin was examined by "spiking" the diluted plasma samples with three concentrations (20, 40 and 60 mg/mL) of bovine serum albumin (Sigma, Albumin, Bovine, Product Number A-6793) to simulate the normal concentration of albumin in plasma (35 to 50% of the total serum protein, Jain, 1993). These concentrations were chosen as they represented 3 out of the 5 reported in the study by Levinson and Goldman (1982) who noted the effect of albumin on haemoglobin using TMB as the chromogen. The haemoglobin for each of the four samples from each individual

remained constant for the treatment groups. The treated samples were run on the micro plate assay and the results compared to the control sample that had no albumin added.

The effect of time on albumin interference within the assay

To assess how albumin interference changed with time, 20 plasma samples were run in duplicate. One half of the samples were spiked with 40mg/mL of bovine serum albumin (Sigma, Albumin, Bovine, Product Number A-6793) whilst the other half of the samples were diluted to give the same final haemoglobin concentration within the paired sample. The samples were subjected to the haemoglobin assay described above with triplicate plates to allow for the reaction to be stopped at 5, 10 and 15 minutes.

Statistical analysis

The statistical package used for the analysis of the data was Systat version 9 (SPSS Inc. Chicago IL. USA). Analysis of the raw data revealed a normal distribution. Outliers were identified by using box plots and removed from the analysis when required.

3.3 Results

Microplate assay

The microplate haemoglobin assay exhibited a linear relationship between the optical density readings and the haemoglobin concentration to 3mg/mL (maximum tested) (Figure 3.2) and the correlation coefficient was 0.999. Because the concentration of plasma haemoglobin was low (<1mg/mL data taken from 2500 plasma samples), 2mg/mL was set as the upper limit for the standards that were used for haemoglobin determination.

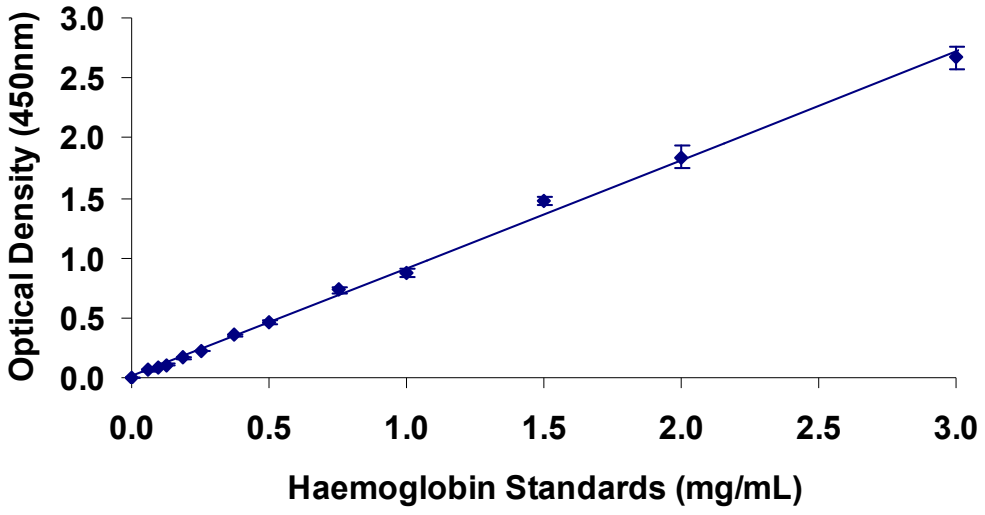

Figure 3.2: Haemoglobin standard versus optical density. $y=0.905\times +0.01$; $r^2=0.999$.

Repeatability of the micro plate assay

To test the reproducibility of the assay the same samples were measured on several occasions (test 1, 2, 3 and 4) and the results were subjected to a general linear model with repeated measures analysis. The results revealed that there was no significant difference in the samples between animals (F=2.347; P=0.129) or between each testing (F=1.219; P=0.303).

Intra and inter assay variation

The intra and inter assay variation for the microplate assay was determined from the control samples across 97 plates and was found that the co-efficient of variation (CV) was below 15%, which was set as the upper limit for accepting a result. Table 3.4 summarises the intra and inter assay variation obtained for the high, medium and low controls.

Table 3.4: The intra and inter assay co-efficient of variation.

Assay CV (%)	High	Medium	Low
Intra	2.27	2.29	3.89
Inter	5.17	11.27	9.39

Comparison of the micro plate assay and the Cell Dyn 3500R

The comparison of the Cell Dyn 3500R and the microplate assay for the 89 grossly haemolysed plasma samples are shown in Table 3.5 where paired t-test revealed conflicting results. Obvious variation in the association between the methods was further examined by graphing the data to look for any trends (Figure 3.3) with the Cell Dyn 3500R haemoglobin values set to zero. Due to the repeatability of the microplate assay the results from test 2 and 4 were graphed to compare with the haemoglobin values obtained from the Cell Dyn 3500R. This revealed that test 2 was more closely associated with the Cell Dyn 3500R results than test 4. However, it is important to note that there was no trend between the Cell Dyn 3500R and the microplate assay in terms of one of the methods reporting a higher or lower haemoglobin value.

Albumin interference

Figure 3.4 shows that the albumin did interfere with the assay as the haemoglobin concentration was estimated to be on average 12.3mg/mL higher in the treated versus non-albumin treated control, a pattern consistent across almost all animals. The average haemoglobin concentration for the 85 samples used was 63.6mg/mL.

Table 3.5: Paired t-test comparing the haemoglobin values from the Cell Dyn 3500R with the micro plate assay (n=89).

Cell Dyn 3500R compared with	t- value	P-value
Test1	-4.282	0.000[a]
Test 2	-5.046	0.000[b]
Test 3	0.807	0.422
Test 4	0.607	0.545[ab]

[a, b] Paired t-test between the various test revealed a significant difference P<0.05. All test used the same grossly haemolysed plasma samples. Test 1 and 2 are a repeat on the same diluted sample. Test 3 and 4 are identical samples but differ from Test 1 and 2 as they are a new dilution of the samples.

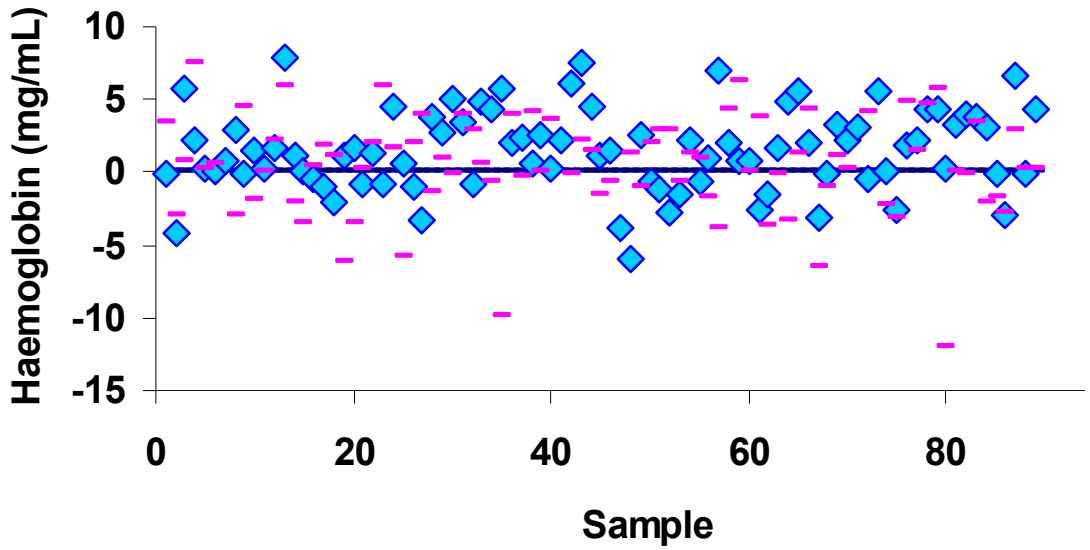

Figure 3.3: Comparison of the haemoglobin values obtained from the Cell Dyn 3500R with the micro plate assay. – The difference in haemoglobin between the Cell Dyn 3500R and test 4 of the micro plate assay; ◊ The difference in haemoglobin between the Cell Dyn 3500R and test 2 of the micro plate assay.

The effect of time on albumin interference within the assay

To understand how time affects the haemoglobin assay the standards were analysed for linearity. From the results (Figure 3.5) the optimal time for stopping the reaction would be 10 minutes due to the 15 minute incubation time leading to a parabolic relationship between the standards and the optical density, whereas the 5 minute incubation time does not allow sufficient time for the very low plasma concentrations of haemoglobin to be distinguished.

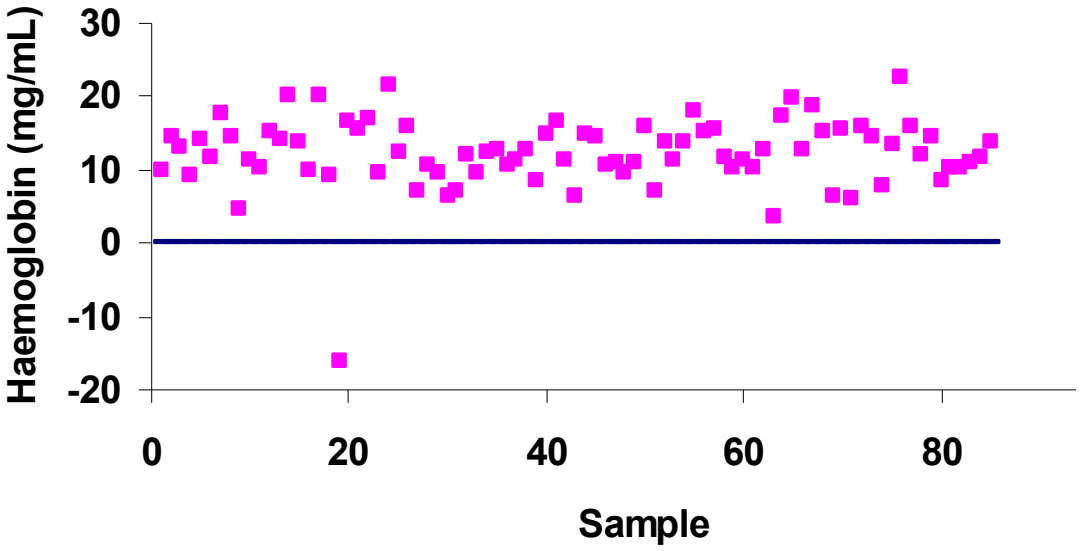

Figure 3.4: Albumin interference on the micro plate assay. The points represent the difference between the control (PBS added) and the average value obtained from the addition of albumin. The control samples were set to zero to display the difference when albumin is present in the sample.

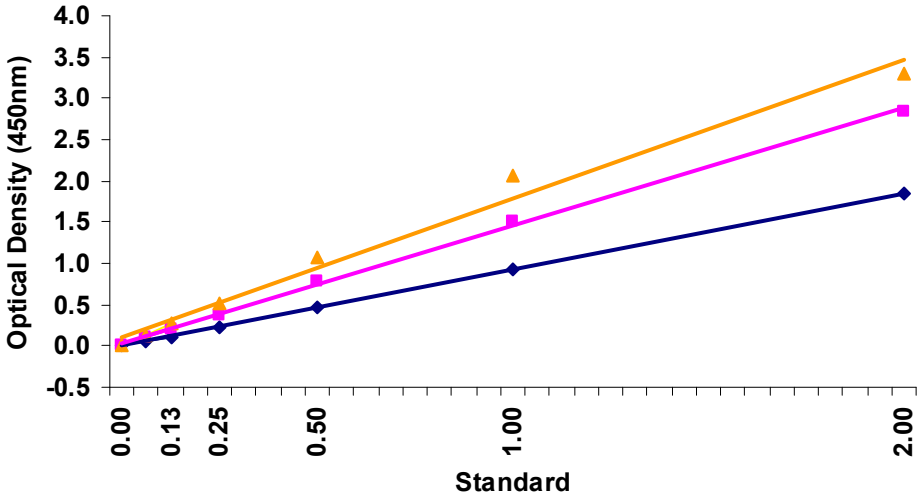

Figure 3.5: The effect of reaction time on the haemoglobin assay standards -♦- 5 minutes, y = 0.0576x - 0.0588 r^2 = 1; -■- 10 minutes, y = 0.0894x - 0.0662 r^2 = 0.999; -▲- 15 minutes y = 0.1047x + 0.005 r^2 = 0.9836

As reported in the above section albumin interferes with the haemoglobin assay and it was thought that testing the incubation time would be valid due to the findings of Levinson and Goldman (1982) and Fairbanks *et al.* (1992). As can be seen from Figure 3.6 increasing the incubation time does help to reduce the impact of albumin interference on the haemoglobin assay. However, if one analyses the effect that incubation time has on plasma samples where

no albumin was added then the reverse is true in that an increase in incubation time leads to an increase in estimated haemoglobin. Using these incubation times there is no cross-over effect where albumin interference equals that of no albumin interference.

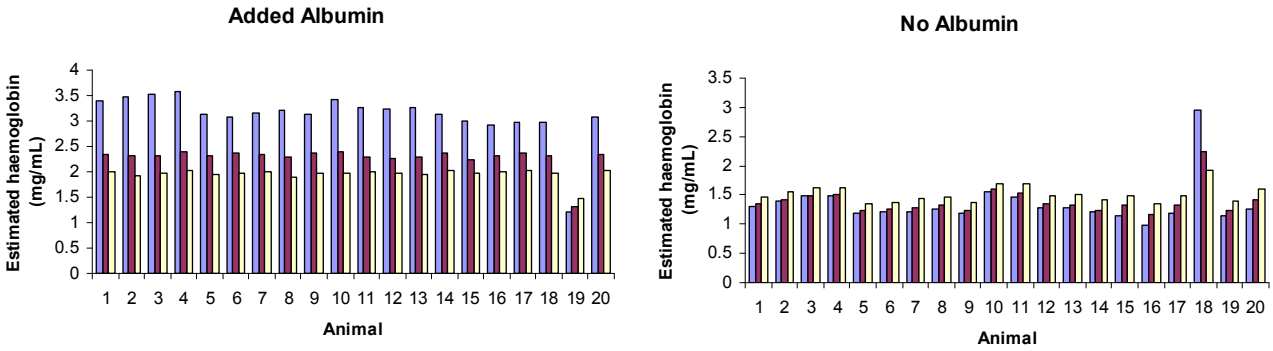

Figure 3.6: The effect of reaction time on albumin interference in estimation of haemoglobin by the microplate assay, 5 minutes; 10 minutes; 15 minutes.

3.4 Discussion

The assay was developed in order to measure low concentrations of haemoglobin in plasma samples "contaminated" by haemolysis during sampling of blood from the coccygeal vessels in cattle. There are a number of different methods for measuring haemoglobin. While sensitivity of these methods is adequate for detecting dangerous concentrations of haemoglobin in plasma/urine during haemolytic disease and/or trauma they are not accurate for measuring low concentrations of haemoglobin. An exception to this was the method by Standefer and Vanderjagt (1977). These authors reported sensitivity of the tetramethylbenzidine method to concentrations of haemoglobin in the range of 5mg/L (normal haemoglobin range in plasma is 3-25mg/L). The automated haematology analyser (Cell Dyn 3500R) used for routine analysis of cattle blood lacks the sensitivity to measure haemoglobin concentrations below 40mg/mL. Therefore, the current experiment examined the utility of the Standfer and Vanderjagt method for measuring low concentrations of bovine haemoglobin that can contaminate samples when blood is collected from the coccygeal vein.

The microplate assay developed here is repeatable ($F=1.219$; $P=0.303$) and is able to detect plasma haemoglobin at low concentrations with accuracy. Thus it has proved useful for detecting the severity of haemolysis caused at the time of blood sampling.

The haemoglobin concentrations in paired samples were estimated using the Cell Dyn 3500R and, after appropriate dilution to be within the assay's dynamic range, in the micro plate

assay. Repeated measures analysis revealed that there was no significant difference (P>0.05) between the Cell Dyn 3500R and the microplate assay across the four tests. Discrepancies between mean estimated values for the two methods within individual tests showed no consistent trend. Dilution of test samples for the micro plate assay may have contributed to the observed difference between methods in tests one and two. In any event, this discrepancy between the two methods for measuring haemoglobin is not unique to this thesis as similar differences in estimated haemoglobin concentrations between the methods are frequently reported (Lewis, 1974; Fairbanks *et al.,* 1992; Gehring *et al.,* 2002).

The presence of albumin was found to contribute to 25% of the reported haemoglobin value. As the current assay was developed for measuring low concentrations of haemoglobin on haptoglobin estimations, it is important to consider the potential impact on the final estimates of haptoglobin. In approximately 2500 plasma samples from beef cattle measured in this thesis, the haemoglobin concentrations have all been below 1mg/mL. Using the conversion equation y=0.1179Hb + 0.0149, for 1 mg/mL of reported haemoglobin assuming albumin interference we obtain a value of 0.133mg haptoglobin/mL. If this same sample had no albumin interference then we would obtain a reported haemoglobin value of 0.8mg/mL (25% removed due to albumin interference) this would give us a converted value of 0.109mg haptoglobin/mL or a difference of 0.024mg/mL. Given the normal range for haptoglobin in cattle being less than 0.05mg/mL and an acute response being greater than 0.1mg/mL then albumin is not likely to have a substantial impact on the acute phase protein assay. Furthermore, albumin is a negative acute phase protein and will decrease in concentration as positive acute proteins such as haptoglobin increase (Rotheschild *et al.,* 1988 as reported by Jain, 1993).

This study evaluated the effect of incubation time on albumin interference and it was found that an increase in incubation time led to a reduction in albumin interference. This supports the findings of Levinson and Goldman (1982) who indicated that there can be a problem in over and/ or underestimating haemoglobin. When they ran their assay at 37ºC, the results indicate that the optimal time to read the samples was at four minutes. Reading prior to this time point led to an over-estimation of haemoglobin with later readings leading to an underestimation. Fairbanks *et al.* (1992) also performed a similar test and found that incubating the samples at 24ºC led to the cross-over in estimated values at 10 minutes. From our results the cross-over in estimated values would require a longer incubation time as the

samples that contained no albumin increased in haemoglobin concentration as the incubation time was extended. The problem with extending the incubation times for the assay developed in this thesis is that the linear relationship between the haemoglobin standards and the optical density (as reported by the plate reader) would lead to a non-linear relationship (parabolic) leading to the increased inaccuracy of measuring the higher plasma concentration of haemoglobin. The interference by albumin arises from its reaction with the methaeme component from haemoglobin. The reaction leads to the formation of methaeme-albumin, thus increasing the peroxide activity through the bond formed between methaeme and albumin. However, Bunn and Jandl (1968) showed that of the species tested (*canine*, *rufus*, *cavia*, *oryctolagus*, monkeys and *bovine*) only primates have the ability to form methaemalbumin, thus the overestimation seen in the current experiment is unlikely to be due to the peroxidase nature of methaemalbumin, but most likely due to albumin being in its unaltered form which can be detected at 450nm.

The method developed here has proven to be beneficial for correcting for the interference of haemoglobin, caused at the time of blood sampling, on the routine assay used within the laboratory for measuring plasma haptoglobin (Chapter 2, Figure 2.6, page 40). Although the presence of albumin in the plasma sample being analysed for haemoglobin is reporting a higher haemoglobin value, this does not pose the problem that one would expect for the correction factor as the haptoglobin assay of which this assay was developed for is also affected by albumin interference (Eckersall *et al.,* 1999a). It would therefore seem that this haemoglobin assay is able to take into account the effect that haemoglobin (as a result of haemolysis at the time of blood sampling) and albumin in the plasma sample have on estimation of haptoglobin with the method described in Chapter 2.

In conclusion, the accuracy and repeatability of the method developed here for measuring low concentrations of haemoglobin proves to be an adequate assay for correcting for haemolysis caused at the time of sampling. The results reported for haemoglobin in this assay may be used as a way to correct for the interference of haemolysis caused at the time of sampling in the haptoglobin assay. Paired samples of blood collected from a coccygeal blood vessel and a jugular vein at the same time point were subjected to this analysis and it was found that when these samples were analysed for haptoglobin and the haemoglobin interference was taken into account there was no significant difference in haptoglobin expression (Chapter 4). Therefore, this method is adequate for measuring low concentrations of haemoglobin (as a result of

haemolysis caused at the time of blood sampling) so that the values obtained can be used to correct for haemolysis interference in the haptoglobin assay (Chapter2).

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Section III: Understanding of haematological variables

Hypothesis 1: Haematological variables vary between blood collected from the jugular veins and the coccygeal blood vessels when samples are collected at the same time point.

Chapter 4: A comparison of blood variables taken from two blood sampling sites – The jugular veins versus the coccygeal vessels in cattle.

Chapter 4: A comparison of blood variables taken from two blood sampling sites - The jugular veins versus the coccygeal vessels of cattle

4.1 Introduction

Differences in blood variables taken from individual animals at different sampling sites in cattle do exist, however there are discrepancies between reports in the literature (Table 4.1). Parker and Blowey (1974) found that there was a significant difference in haematocrit, haemoglobin, albumin, calcium, magnesium, inorganic phosphate and potassium between the jugular veins and/or the coccygeal artery or veins. Their study also revealed that significant differences can occur between the coccygeal artery and the coccygeal vein for haematocrit, glucose and inorganic phosphate.

Table 4.1: Comparison of different blood variables

Blood variable	Jugular vein versus Coccygeal vein	Jugular vein versus Coccygeal artery	Coccygeal vein versus Coccygeal artery
Haematocrit (PCV)	#	*	*
Haemoglobin	*	*	
Albumin	#	*	
Glucose	NS		*
Calcium	#	*	
Magnesium	#	*	
Inorganic phosphate	*	*	*
Potassium	#	*	
White blood cells	#		

* Significant difference; # Conflicting result; NS Non-significant. Sources: Parker and Blowey, 1974; Sears et al., 1978; Morrow-Tesch and Whitehead, 1998; Kováč *et al.,* 2001

Morrow-Tesch and Whitehead (1998) tested a number of blood variables for significant differences between jugular and the tail vein collection sites. Unlike Parker and Blowey (1974), they found that there was no significant difference in albumin, calcium, magnesium, potassium or haptoglobin. It is difficult to compare the findings from the two studies, as only Parker and Blowey (1974) defined how each of the laboratory measurements was determined.

As shown in Table 4.1, there are also conflicting reports on the effect of blood collection sites on haematocrit. Four years after Parker and Blowey's study in 1974, Sears *et al.* (1978) again examined the differences in blood variables between sampling sites and found that there was no significant difference for haematocrit. Parker and Blowey (1974) attributed the difference in haematocrit to "the "plasma skimming" effect in small vessels and the accumulation of a higher density of blood cells towards the central axis of the vessel with increasing blood flow

rate". Plasma skimming is a result of reduced blood flow in the vessel which causes the red blood cells to stick together which increases viscosity and consequently the red blood cells remain in the centre of the vessel (Rogers, 1999). Thus the blood closest to the vessel wall has a lower haematocrit. This insight led Sears *et al.* (1978) to use "the larger iliac vein for sampling where this phenomenon would not be expected".

More extensive comparisons of the effect of blood collection sites on haematological variables are available in mice and rats. Nemzek *et al.* (2001) found that there are significant differences in the counts for white blood cells (neutrophils, monocytes, basophils and eosinophils) red blood cells, platelets and haematocrits between the tail, heart and eye samples of Balb/c mice. Schwabenbauer (1991) examined the effect of blood sampling site in Sprague-Dawley rats and found significant differences occur between the sampling sites of the tail, heart and eye. Differences between sites were found for leukocytes, red blood cells, haemoglobin, protein, glucose, sodium, potassium alkaline phosphatase and creatine kinase.

Both the jugular veins and coccygeal vessels are convenient for collection of blood in cattle but because of the equivocal results reported above, a re-evaluation of blood variables taken from different sampling sites in cattle was conducted. This is important as there are occasions where it is necessary to collect a blood sample from a different site due to difficulties in accessing the sampling site as a result of haematomas forming on/around the blood vessels due to repeated blood collection. Without knowing the impact that sampling site may have on the haematological variable, incorrect conclusions may be drawn.

4.2 Materials and methods

Animals and design

Twelve Angus, Angus cross and Hereford heifers aged 14 months and with an average weight of 285.0kg ± s.e. 7.3kg were used in this trial. The animals were part of an experiment on the effect of candidate vaccines on anaplasmosis and were experimentally infected with *Anaplasma marginale* during the course of the current study. This infection can lead to intravascular lysis of red blood cells.

The heifers were bled on four occasions approximately two weeks apart from the jugular vein followed immediately by sampling the coccygeal blood vessel. This sampling occurred prior to infection with *Anaplasmosis* to obtain base levels for the haematology. The heifers were

then bled a further six times from both sites, on alternate days starting ten days post infection to minimize haematomas forming on/around the coccygeal vessels. Figure 4.1 depicts the timeline of events.

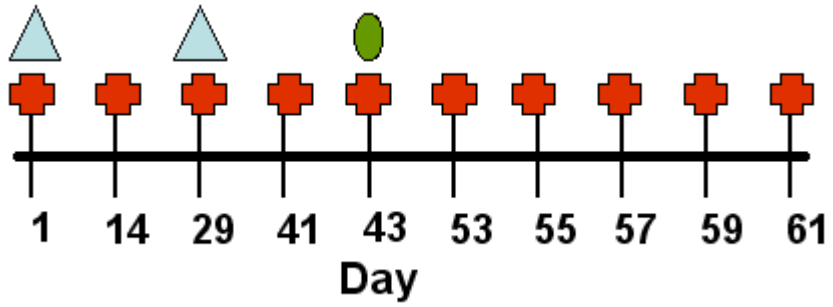

Figure 4.1: Time line of events. Key to time line: + blood sample (jugular and coccygeal vessel); ▲vaccination; ● challenge.

Blood collection and processing

Blood was collected from a jugular vein and a coccygeal blood vessel using an 18 gauge, 1 inch precision glide vacutainer system needle inserted into 5mL BD Vacutainer tubes containing 0.054mL of 15% K_3EDTA.

On arrival in the laboratory the blood was processed for routine haematology. The blood was rotated for four minutes to re-mix the blood before being analysed on a Cell-Dyn 3500R (Abbott Diagnostics, North Ryde, NSW, Australia) calibrated for cattle blood. The analyser measures total and differential white cell numbers, platelets and seven red cell variables. The blood was then centrifuged at 1200 g (2500 rpm) for 12 minutes at 4°C and plasma removed and stored at -20°C until analysed for haptoglobin.

Haptoglobin analysis

The plasma samples were originally analysed for haptoglobin using the method described in Chapter 2, page 28 without the correction factor. This method is based on the pseudo-peroxidase nature of the haemoglobin-haptoglobin complex which leads to a redox reaction between the complex and the chromogen. The results from this study led to the development of the correction factor and thus the samples were re-analysed using the corrected method for measuring haptoglobin.

Statistical analysis

The data were examined for normality of distribution and transformed as required. The following traits were transformed using a natural log scale: neutrophils, monocytes, eosinophils, basophils, mean corpuscular volume, mean corpuscular haemoglobin and haptoglobin. Red blood cells were transformed using a cubed root. Data from all time points were combined and the effect of sampling site on the blood variables was examined by a Student paired t-test (Systat version 9, SPSS Inc).

4.3 Results

Analysis of the data revealed that there was a significant difference ($P<0.05$) in a number of blood variables collected from a jugular vein and a coccygeal blood vessels at the same point in time. Total white blood cell count, lymphocytes, basophils, red blood cells and haematocrit all differed significantly between the sites. The results for the other blood variables (no significant differences between sites) and the correlation co-efficient between the two sampling sites can be seen in Table 4.2.

Table 4.2: Comparison of haematological variables

Haematological trait	**Units**	**Jugular Mean ±SD**	**Tail Mean ±SD**	**Correlation co-efficient**	**P-Value**
Total WBC	$10^3/\mu L$	7.747 ± 2.244	7.339 ± 2.122	0.912	0.000
Lymphocytes	$10^3/\mu L$	3.325 ± 1.425	2.916 ± 1.188	0.705	0.000
Basophils	$10^3/\mu L$	0.080 ± 0.070	0.133 ± 0.321	0.703	0.000*
RBC	$10^6/\mu L$	6.968 ± 2.076	6.847 ± 2.059	0.995	0.000*
Platelets	$10^3/\mu L$	536.43 ± 202.27	488.85 ± 233.42	0.879	0.006
MCH	pg	15.217 ± 1.515	15.426 ± 2.488	0.985	0.230*
Haemoglobin	g/dL	10.394 ± 2.753	10.285 ± 2.761	0.995	0.257
Haematocrit	%	28.368 ± 7.526	28.052 ± 7.628	0.994	0.267
MCHC	g/dL	36.647 ± 0.535	36.719 ± 0.694	0.556	0.290
Haptoglobin[1]	mg/mL	0.031 ± 0.072	0.022 ± 0.063	0.433	0.326*
Eosinophils	$10^3/\mu L$	0.072 ± 0.153	0.072 ± 0.149	0.893	0.596*
Monocytes	$10^3/\mu L$	1.686 ± 1.351	1.668 ± 1.371	0.884	0.625*
MCV	fL	41.547 ± 4.370	41.333 ± 4.973	0.990	0.694
Neutrophils	$10^3/\mu L$	2.585 ± 1.258	2.605 ± 1.284	0.955	0.898*

The raw mean and s.d. presented in this table are the untransformed values. With the P-value representing the data that was analysed (*using the transformed data).

[1]The haptoglobin results presented in this table are those that have undergone the correction for haemolysis interference.

The experimental *Anaplasma* infections introduced on day 43 altered blood variables over time (Figure 4.2). All haematological data with the exclusion of lymphocytes, eosinophils and mean corpuscular haemoglobin concentration were found to significantly differ with time

from day 43 to day 61. However, there were no time by site interactions for any of the variables measured.

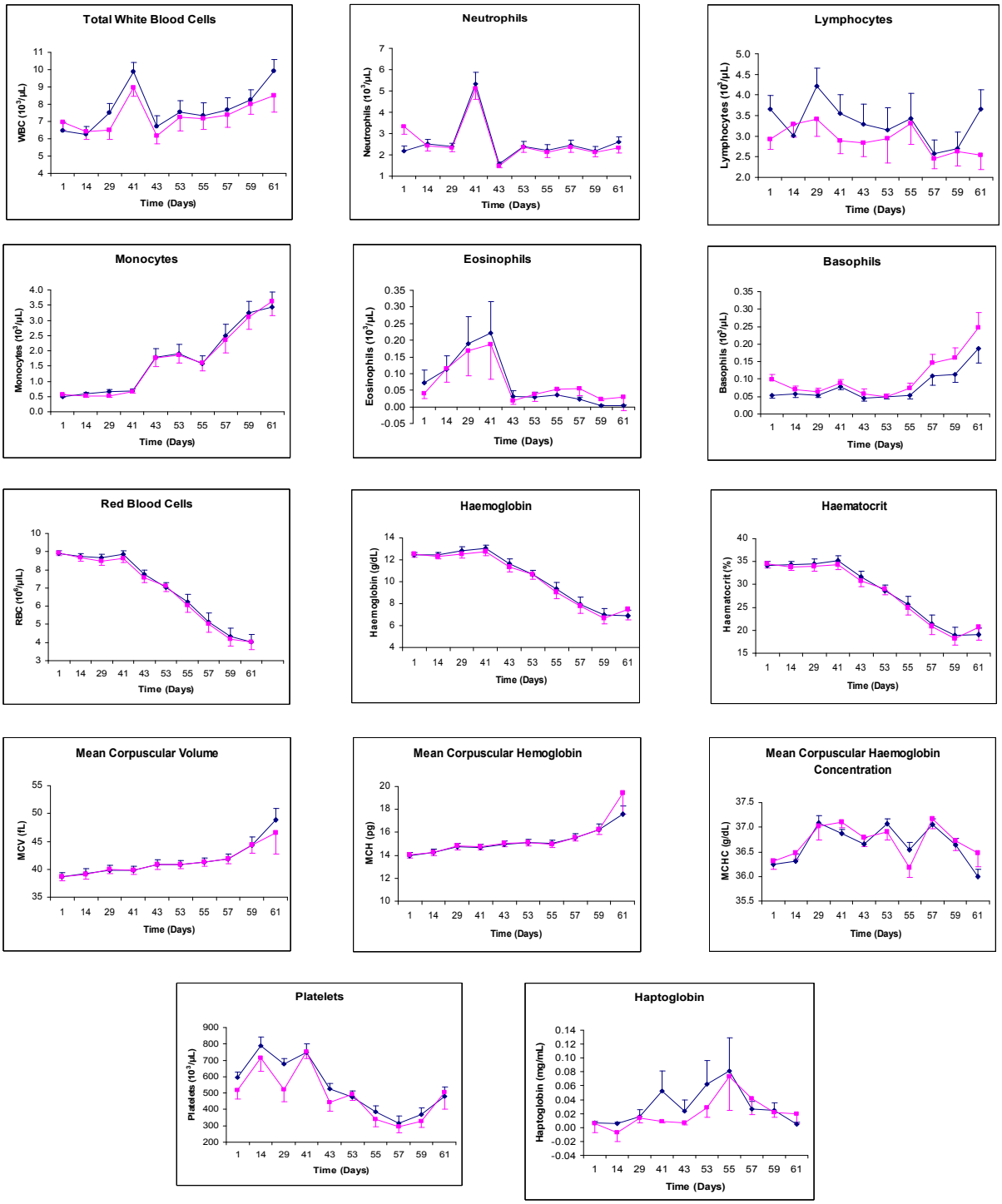

Figure 4.2: Haematological variables across time (raw mean ± se); ♦ jugular vein ■coccygeal blood vessel.

Prior to developing the correction factor for haptoglobin there was a significant difference (t=-5.116, P=0.000) in the plasma haptoglobin concentration with the coccygeal plasma having a higher concentration (42.5% compared to 8.3% for jugular plasma). Figure 4.3 shows the difference in the haptoglobin concentration pre and post correction.

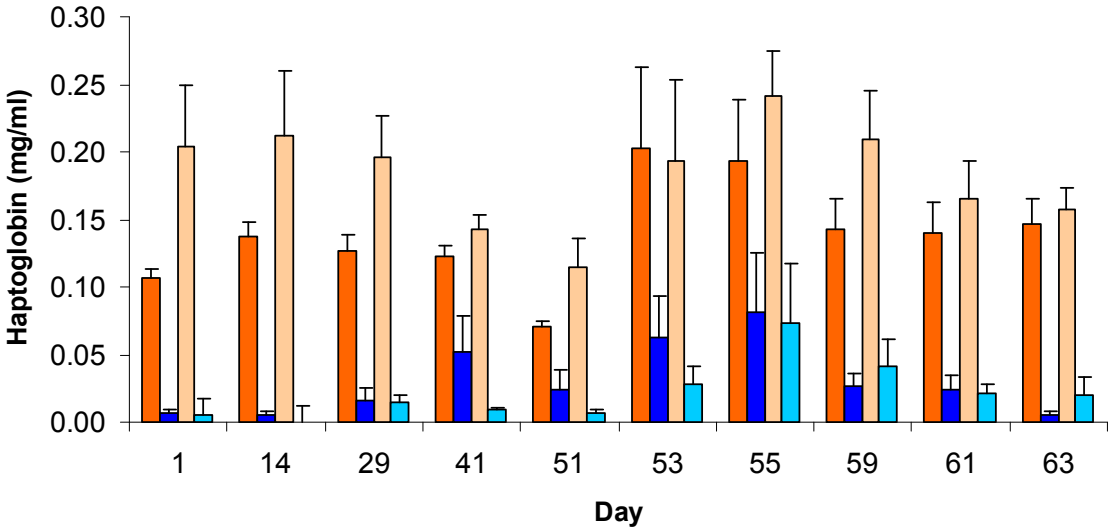

Figure 4.3: Haptoglobin values from blood collected from a jugular vein and a coccygeal blood vessel pre and post correction method (mean ± se). Jugular – pre correction; Coccygeal – pre correction; Jugular – post correction; Coccygeal – post correction.

4.4 Discussion

The present study was undertaken to evaluate if there were any differences in routine blood haematology and haptoglobin concentrations when blood was collected from the different sampling sites (jugular versus coccygeal blood vessels) at the same time in cattle. Previous studies on comparing routine haematology have been performed but there were some discrepancies.

As clinical decisions and testing of experimental hypotheses can depend on haematological values derived from jugular vein and coccygeal blood it is important to understand if the blood variables are comparable at both sites. In the current study it was found that there is a significant difference between the two prime sampling sites in haematological traits including: total white blood cell count, lymphocytes, basophils, red blood cells and platelets. All variables for which there was a significant difference between sites were higher in the jugular than the coccygeal blood with the exception of basophils. These findings differ from previous studies which reported that these haematological variables do not differ significantly between sampling (Parker and Blowey, 1974; Sears *et al.,* 1978; Morrow-Tesch and Whitehead, 1998). However, Kováč *et al.,* 2001 also reported a significant difference for white blood cell counts with jugular vein blood having a higher number. Several factors could contribute to these differences:

1) coccygeal blood may have contained a mixture of arterial and venous blood;
2) haemodynamic differences between the vessels may influence cell populations, and
3) there may be regional differences in the contribution of lymphoid tissues to cell populations in blood. However, it is not immediately apparent from this study what is the cause of the differences.

The jugular vein is the major vessel draining deoxygenated blood from the head and neck of an animal. The vessel collects both venous blood and lymph from the surrounding tissues and lymph from the head contains recirculating lymphocytes, new lymphocytes generated in the germinal centres within tonsils and lymph nodes together with the fluid volume supporting these cells (Ford, 1974). Importantly, lymphatic drainage of the thymus also enters the jugular (Chorfi *et al.,* 2004). It is noteworthy that the thoracic duct contains large numbers of lymphocytes especially those derived from proliferation of cells in the Peyer's patches and mesenteric lymph nodes; however, the thoracic duct joins the anterior vena cava or caudal jugular vein below the blood collection site routinely sampled by jugular venepuncture (Saar and Getty, 1975). These effects may contribute to the lower total white blood cell count in the smaller coccygeal blood vessels.

A different mechanism is likely to underlie the high red cell numbers observed in the jugular blood than that which may contribute to higher leukocyte counts in this vessel. Several possibilities need to be considered. Firstly, haemodynamics of flow in smaller vessels may lead to lower numbers of red cells per unit volume of fluid phase. Secondly, operator difficulty in placing the needle within the coccygeal vessels may result in damage and red cell sludging or lysis within sites of vascular leakage and damage. This seems likely as when the plasma of the blood from each of the sites was compared visually and by measuring the haemoglobin (results not shown) the plasma from the coccygeal blood vessels had a greater degree of haemolysis. Haemolysed samples did not affect the haemoglobin concentrations as determined by the Cell Dyn 3500R, as this instrument first lyses the sample before measuring the haemoglobin concentration. This tissue damage during sampling could also contribute to the lower platelet count in coccygeal blood than in jugular blood. The role of platelets is to form clumps around connective tissue at a wound site to reduce blood loss (Hardisty, 1974).

The results found in the present study support the findings of Sears *et al.* (1978; Table 4.1) who found no difference in haematocrit in blood from different sampling sites in cattle.

However; unlike the previous studies of Parker and Blowey (1974), the present study did not find a significant difference for haemoglobin and haematocrit. In addition this study revealed a significant difference for total white blood cell count, basophil and lymphocyte numbers. The concentration of albumin, glucose, calcium, magnesium, inorganic phosphate and potassium although important were not examined in the current study and could warrant further investigation.

The original results for haptoglobin indicated that there was a significant difference between the two sampling sites, with coccygeal plasma on average having a higher concentration of haptoglobin (8.3% of jugular plasma reported a higher concentration of haptoglobin compared with 42.5% for coccygeal plasma). To determine the possible causes of the difference in concentration visual examination of the paired plasma samples was undertaken. This indicated that the difference was most likely a result of haemolysis due to the plasma sample having a red tinge of colour that could not be attributed to plant proteins. These plant proteins result in unhaemolysed blood ranging in colour from almost opaque to a rich golden yellow in cattle (Jain, 1993). Although these cattle were undergoing *in vivo* lysis of red blood cells from day 43 due to infection we expected to see haemolysed samples. However, this did not account for the haemolysis seen in the samples prior to infection. Therefore, the haemolysis in the plasma samples prior to the infection would suggest that haemolysis was a result of sampling. The personnel involved in the collection of blood from a coccygeal blood vessel and/or a jugular vein were competent operators and had collected blood from these sites from a large subset of animals and on numerous occasions.

As a result of the identification of haemolysis a correction method (Chapter 2, Page 40) was developed. Having developed the correction method these same samples were re-analysed and no-significant difference in haptoglobin concentration between the two sampling sites was identified. This result reflects the findings of Morrow-Tesch and Whitehead (1998) who reported no significant difference between sampling sites for haptoglobin.

Given haptoglobin is involved in the removal of free haemoglobin, the expression profile of haptoglobin following the *Anaplasmosis* infection is as would be expected for a situation where infection results is lysis of red blood cells. As these heifers were vaccinated with candidate vaccines for *Anaplasmosis* it is possible that they may have developed some immunity to the infection and therefore the haptoglobin response was not as great as

potentially possible. However, the haptoglobin response still follows the expected profile with an initial increase in haptoglobin which then declines as the infection is being fought.

The difference in blood variables collected from different sites is not restricted to cattle, rats and mice but is also observed in humans and other species of mammals and reptiles (Maala, 1981; Cuadrado *et al.,* 2003). Therefore, when taking blood samples from any organism for comparison to previous haematological variables the protocol should stipulate that blood should be collected from the same blood vessel each time. Where this is not possible there needs to be previous knowledge of comparative values to allow correct interpretations of haematological data.

Section IV: Non-inflammatory management stressors

Hypothesis 1: That known management stressors causing non-inflammatory responses can induce a haptoglobin response in beef cattle.

Hypothesis 2: That flight time of cattle is related to the extent of acute phase responses of individuals to the management practices that are thought to impose a psychological stress on cattle.

Chapter 5: Comparing cortisol and haptoglobin following social re-grouping of beef cattle in a feedlot environment.

Hypothesis 1: That an increase in cortisol will be paralleled by an increase in haptoglobin.
Hypothesis 2: That the duration of the haptoglobin expression is longer than that for cortisol.
Hypothesis 3: That flight time can predict the magnitude of a response to the psychological stressor of social re-grouping

Chapter 6: Can weaning stress be detected using the acute phase protein haptoglobin?

Hypothesis 1: That haptoglobin concentrations increase in newly weaned calves.
Hypothesis 2: That haptoglobin concentrations following weaning can be used as an indicator of how beef cattle will perform under intensive management situations (feedlot environment).
Hypothesis 3: That weaning is a psychological stressor
Hypothesis 4: That flight time can predict the magnitude of a response to the psychological stressor of weaning

Chapter 7: The effect of road transportation, intensive management and muscle biopsy on haematological variables and haptoglobin.

Hypothesis 1: That haptoglobin concentrations will increase following an acute stressor such as transportation and a muscle biopsy.
Hypothesis 2: That haptoglobin concentrations can be used as an indicator for longer term adaptation to intensive management.

Chapter 5: Comparing cortisol and haptoglobin following social re-grouping of beef cattle in a feedlot environment

5.1 Introduction

The development of a social hierarchy in many species of animals helps maintain stability within that social group. Hierarchies and alliances are found in many social species including primates and animals with a tendency to herd or flock such as elephant, zebra, geese, poultry, sheep and cattle. Social structures are of interest in cattle as hierarchies are usually well established and strong and the reestablishment of a hierarchy after disruption is known to lead to aggression. Subordinates and foreign additions in particular are likely to be stressed by such changes. Upon disruption of the hierarchy within a cattle herd, the herd will attempt to re-establish and maintain the hierarchy, the former via displays of agonistic behaviours (Kondo and Hurnik, 1990;Veissier *et al.,* 2001) and it can take up to two weeks before a stable (maintenance) phase is reached in cattle (Veissier *et al.*, 2001; Bøe and Færevik, 2003).

Hasegawa *et al.* (1997) reported that body weight, chest girth and the age of the cattle are all correlated with dominance order (the ranking of hierarchical position) while Gupta *et al.* (2005) suggest that the starting weight was not related to dominance. A study designed to evaluate the hierarchy of primiparous cows in a multiparous group (Bach *et al., 2005)* reported that primiparous cows tended to be sub-ordinates. It seems that the degree of aggression at establishment increases with age (Mench *et al.,* 1990; Veissier *et al.,* 2001), although Kondo and Hurnik (1990) suggested that a social hierarchy is not only determined by age (and sex) but also by social experience. Dominance is particularly apparent in social groups when necessary resources (food, water and resting spaces) are restricted and aggressive interactions occur in both the establishment and maintenance phases of the hierarchy.

The establishment phase is perceived as the time when the greatest stress is likely to occur as the individuals attempt to re-define their position within the herd. However, Veissier *et al.* (2001) and Gupta *et al.* (2005) have both reported that cattle habituate to repeated re-grouping through decreased levels of aggression. Gupta *et al.* (2005) reported that there was a significant difference in cortisol concentrations between the control animals and those that were re-grouped and re-located only after the first round of re-grouping and relocation. However, Veissier *et al.* (2001) reported in their study that the effects of social re-grouping

had no differential impact on the basal plasma cortisol concentrations of the two treatment groups.

Re-grouping is common place in dairy cows and in feedlot cattle as it usually simplifies management choices. Re-grouping of cattle also occurs in abattoirs, sale yards and during transportation and has been reported to reduce milk production in dairy cattle (Hasegawa *et al.,* 1997). In feedlots it seems likely that re-grouping may have consequences on performance in terms of growth rates and meat quality; however, there appears to have been very little research on the effect of re-grouping in feedlot cattle.

Cortisol is an established measure of stress; however, the response can be very transient (Pineiro *et al.,* 2007) and can increase and decrease very rapidly. Colditz *et al.* (2005) in their study on fly strike in sheep revealed that the initial rise in cortisol was paralleled by haptoglobin, with the haptoglobin response having a longer duration. However, Fisher *et al.* (2001) using castration as a model for stress revealed that surgically castrated bulls had an increase in plasma haptoglobin concentration when compared to the non-castrated bulls, but, plasma cortisol concentrations did not differ between the two groups and highlights the possibility that cortisol and haptoglobin may have different response depending on the stressor applied. It is presently unclear if a social re-grouping stressor will lead to an increase in plasma haptoglobin along with elevated cortisol concentrations.

The experiment reported here was part of a larger project designed to evaluate the effect of social re-grouping on meat quality in feedlot cattle (Colditz *et al.,* 2007). The work presented in this thesis aims to determine 1), if there is a stress response as a result of social re-grouping by measuring cortisol and haptoglobin concentrations; and 2), if there is a stress response then the duration of this response. It was therefore hypothesised firstly, that an increase in cortisol will be parallel with an increase in haptoglobin; secondly, that the duration of the haptoglobin expression is longer than that for cortisol; and finally, that flight time will be correlated to the magnitude of the measures of haptoglobin and cortisol in response to social re-grouping.

5.2 Materials and methods

Animals and design

The research presented in this chapter forms part of larger study evaluating the effects of social re-grouping on meat quality (Colditz *et al.*, 2007). In summary, two cohorts of steers approximately 9 months old were purchased from Goondiwindi, Queensland (22.55S,

150.31E) (Hereford steers, abruptly weaned) and Armidale, New South Wales (30.53S, 151.62E) (Angus steers, yard weaned) and entered the feedlot at 12 months of age. Upon entering the feedlot the average weight of the steers was 306.7kg ± s.e. 1.8kg. The steers remained segregated until re-grouping within feedlot pens occurred. The study took into account the origin/breed and temperament based on flight time (Burrow *et al.,* 1998) and crush scores using the method of Voisinet *et al.,* (1997) for assigning each animal to a treatment group. Each cohort of steers was randomly divided into 3 replicates of 5 groups of 14 (Table 5.1). A total of six pens were used as controls to provide three replicated pens of Hereford only and Angus only steers. These cattle were not mixed throughout the study but at the time of re-grouping were returned to a new pen. The steers were mixed one, two or four weeks prior to slaughter to evaluate the effect of mixing unfamiliar animals on meat quality as well as blood variables.

Table 5.1 Treatment groupings of steers and the number in each replicate cell

Group	Mixing Treatment	Number of Hereford Steers	Number of Angus Steers
1	1 (controls-nil)	14	14
2	2 (4 weeks)	7	7
3	3 (2 weeks)	7	7
4	4 (1 week)	7	7

Mixing of unfamiliar counterparts occurred at day 43 for treatment 2, day 57 for treatment 3 and day 64 for treatment 4 (that is 4, 2 and 1 week prior to the end of feedlot finishing). Blood samples were collected from all animals on days 35, 57, 64 and 71 post feedlot induction. Figure 5.1 provides a schematic representation of the timeline of the events for blood collection and re-grouping.

Blood collection and processing

Blood was collected from a coccygeal artery/vein using an 18 gauge, 1 inch precision glide vacutainer system needle inserted into 10mL BD Vacutainer tubes containing 0.054mL of 15% K_3EDTA and stored on ice and transported to the laboratory.

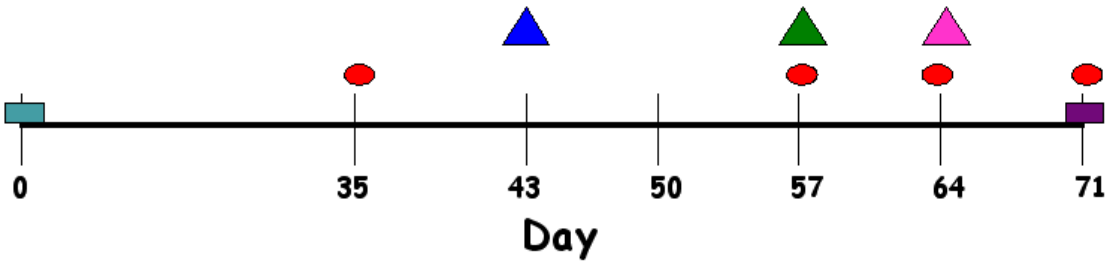

Figure 5.1: Timeline of events for social re-grouping. ■ Feedlot induction; ■ End of study; ● Blood collection; ▲Group 2 mixed; ▲Group 3 mixed; ▲Group 4 mixed.

On arrival in the laboratory blood samples were rotated for 4 minutes to re-mix the blood before being analyzed on the Cell-Dyn 3500R (Abbott Diagnostics, North Ryde, NSW, Australia) to obtain a haemogram. The blood was then centrifuged at 1200g (2500 rpm) for 12 minutes at 4°C to obtain plasma samples. Plasma was stored at -20°C until required for haptoglobin analysis using the method described in Chapter 2, page 28, based on the pseudo-peroxidase nature of the haemoglobin-haptoglobin complex which leads to a redox reaction between the complex and the chromogen. Cortisol concentrations were determined by using a radio-immunoassay with standards, antiserum and [^{125}I] cortisol being supplied by Orion Diagnostica (Espoo, Finland).

Flight times

The flight time was calculated using the method of Burrow *et al.* (1998). It is a measure of the time that it takes each animal to cover a distance of 1.7m after being released from a cattle crush. This measurement was taken on day 35.

Statistical analysis

The statistical package Systat version 9 (SPSS INC. Chicago IL. USA) was used for the analysis of the data. The data were subjected to normality testing and transformed where necessary. Neutrophils, lymphocytes, neutrophil:lymphocyte and haptoglobin were transformed using a natural log scale while flight speed was transformed using a squared root transformation.

Analysis 1: Haptoglobin and cortisol data from the 4 time points, 4, 2, 1 and 0 weeks before exiting the feedlot were analysed by RM ANOVA to examine the effect of breed, treatment, time and their interactions.

Analysis 2: As the individual treatments were imposed at different time points in this time series, the individual treatments were contrasted with values for control animals at the matching time points.

Analysis 3: An ANOVA was performed on day 71 values to examine the effect of breed and treatment and their interaction on day 71 values for the 4 treatments.

Analysis 4: Pearson's correlations were determined for the association between haptoglobin concentration at each time point with flight time or cortisol concentration.

Analysis 5: The issue of whether an increase in haptoglobin was a result of an inflammatory response or a particular response to a psychological stress was determined by evaluating the haptoglobin concentrations that were considered as outliers (haptoglobin>0.3mg/mL).

5.3 Results

Analysis 1: There was a significant effect of time on haptoglobin (F=24.573, P=0.000) (Figure 5.2); and, across the 4 time points, breed was bordering on significance (F=3.707, P=0.057), but there was no significant effect of treatment, or interactions between time and treatment or breed and treatment. Cortisol concentrations also differed between samples (F=11.043, P=0.000) (Figure 5.4) and there was a breed by time interaction (F=2.742, P=0.043) with breed differences being significant at day 64 (F=11.888, P=0.001).

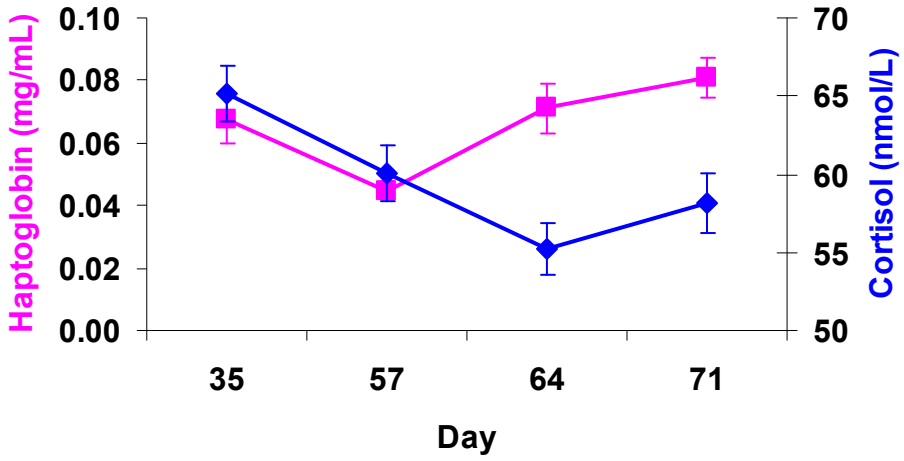

Figure 5.2: The change in haptoglobin and cortisol concentrations (mean ±se).

Analysis 2: Table 5.2 shows that haptoglobin values did not differ significantly between the controls and individual treatment groups, nor was breed or the breed by treatment interaction

significant (Figure 5.3). The cortisol analysis had similar findings to that of haptoglobin with the exception of the effect of breed in animals mixed 4 weeks before feedlot exit (F=4.059, P=0.046) (Table 5.3 and Figure 5.3).

Table 5.2: The effect of treatment, breed and breed×treatment on haptoglobin when controls are contrasted against individual treatment groups.

Controls compared to	Treatment		Breed		Breed×treatment	
	F-value	P-value	F-value	P-value	F-value	P-value
4 weeks	2.745	0.102	2.896	0.093	0.213	0.646
2 weeks	0.867	0.355	3.150	0.080	0.290	0.592
1 week	0.675	0.413	0.015	0.903	0.477	0.491

Table 5.3: The effect of treatment, breed and breed/treatment on cortisol when controls are contrasted against individual treatment groups.

Controls compared to	Treatment		Breed		Breed×treatment	
	F-value	P-value	F-value	P-value	F-value	P-value
4 weeks	2.318	0.126	4.059	***0.046***	0.031	0.860
2 weeks	1.491	0.225	2.530	0.114	0.475	0.492
1 week	0.602	0.439	3.610	0.060	2.329	0.130

Analysis 3: The day 71 samples were analysed to assess the interactions between treatment, breed and breed by treatment and no significant effects where seen for haptoglobin or cortisol (Figure 5.4)

Analysis 4: The correlation between haptoglobin with cortisol and flight time is presented in Table 5.4. There is no significant relationship between flight-time and haptoglobin nor between haptoglobin and cortisol although in the later case the trend tends to be positive suggesting that an increase in cortisol corresponds to an increase in haptoglobin.

Analysis 5: Thirty steers had at least one haptoglobin value greater than 0.4mg/mL and these animals were represented in all treatment groups (Table 5.5). Only one steer in this class of animals with high haptoglobin values had a high total white blood cell count that was attributable to an increase in neutrophils. Thus only one steer is likely to have had an elevated haptoglobin value due to a concurrent inflammatory response. The lymphocyte count for this steer was considered to be in the normal range for cattle.

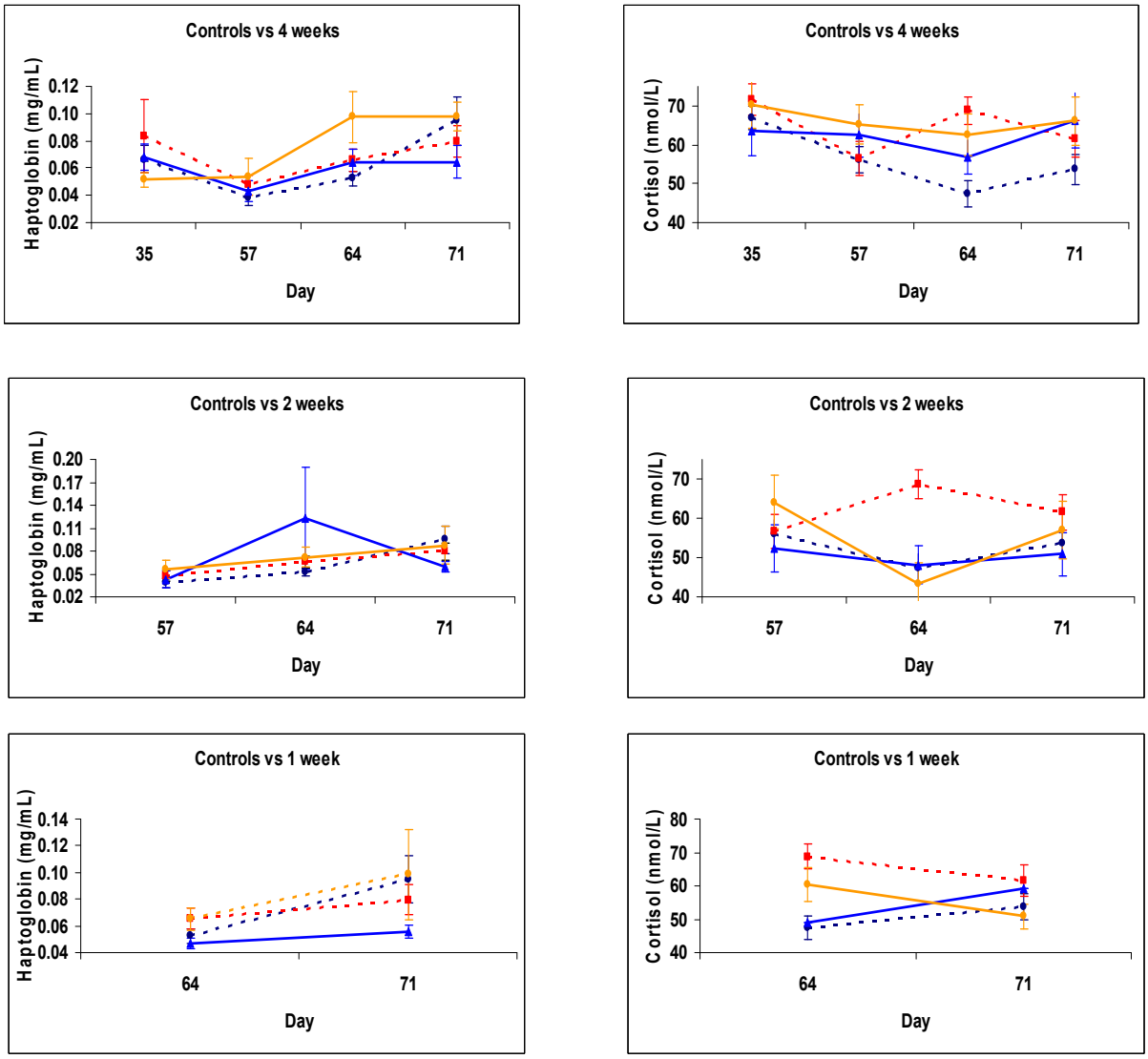

Figure 5.3: A comparison of the controls and individual treatment groups following social re-grouping for haptoglobin and cortisol (mean ±se). --●-- Angus control; --●-- Hereford control; —●— Angus treatment; —●— Hereford treatment.

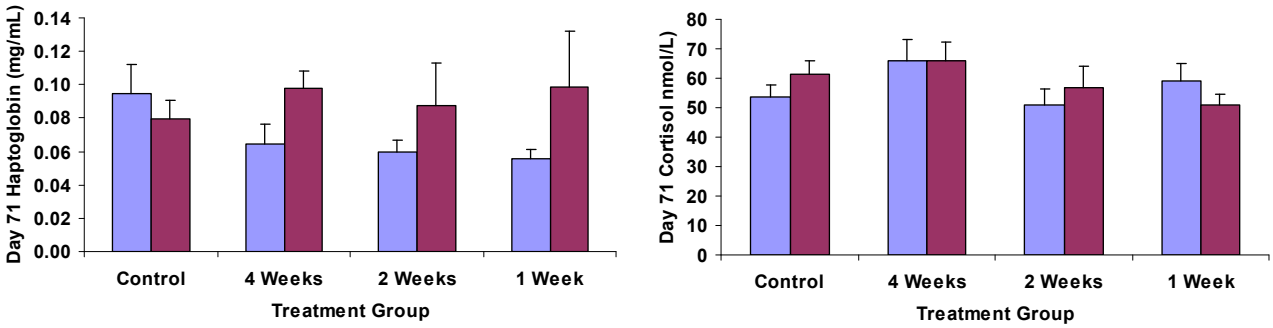

Figure 5.4: Day 71 haptoglobin and cortisol concentrations according to treatment group and breed (mean ±se). ▌ Angus; ▌ Hereford.

Table 5.4: The correlation between haptoglobin, cortisol and flight times.

Correlation		Haptoglobin				Cortisol			
		Day 35	**Day 57**	**Day 64**	**Day 71**	**Day 35**	**Day 57**	**Day 64**	**Day 71**
Flight time	**Day 35**	0.039	0.226	-0.133	-0.007	0.062	0.031	0.122	0.353
Haptoglobin	**Day 35**	1.000	0.084	-0.083	-0.094	0.148	0.078	0.059	0.087
	Day 57		1.000	0.077	0.154	0.234	0.228	0.232	0.148
	Day64			1.000	0.633	-0.117	0.061	0.000	-0.177
	Day 71				1.000	0.228	0.298	0.135	0.019

5.4 Discussion

Re-grouping of unfamiliar cattle normally causes disruption to the social structure and leads to agonistic behaviours as a hierarchy is redefined. This process is thought to place stress on the cattle as it has been reported to be associated with decreased production (Hasegawa *et al.,* 1997; Gupta *et al.,* 2005). The aim of this study was to determine if social regrouping was stressful as measured by cortisol and haptoglobin. It was therefore hypothesised that an increase in cortisol would be paralleled by an increase in haptoglobin; secondly, that the duration of the haptoglobin expression is longer than that for cortisol; and finally, that flight time can predict the magnitude of a response to the psychological stressor of social re-grouping.

Acceptance or rejection of all hypotheses is not as clear cut as one would hope. Examination of the first hypothesis reveals that there is an overall weak positive correlation between plasma cortisol and haptoglobin concentrations. However, evaluation of the individual graphs (Figure 5.3) shows that the relationship is not consistent when comparing the controls with the individual treatment groups, as the correlation between haptoglobin and cortisol can be either positive or negative. Therefore, it is not possible to conclude that an increase in cortisol will be paralleled by an increase in haptoglobin as reported by Colditz *et al.* (2005). This is most likely due to the different stressors as fly-strike results in an inflammatory response, whereas social re-grouping is considered to be a psychological stressor. In the control animals, the haptoglobin concentration tends to mimic the cortisol concentration throughout the study. However, in the re-grouped animals the haptoglobin response tends not to mimic the cortisol response. As a result of this it is therefore not possible to test the second hypothesis. However, observations made from the data would suggest that plasma haptoglobin concentrations increase prior to an increase in cortisol. Further exploration of the relationship between

Table 5.5: Individual steers with high haptoglobin concentrations with corresponding total white blood cell count and neutrophil and lymphocyte counts

Tag	Breed	Mixing Treatment	Haptoglobin (mg/mL)				Total White Blood Cell Count ($\times 10^3/\mu L$)				Neutrophil count ($\times 10^3/\mu L$)				Lymphocyte count ($\times 10^3/\mu L$)			
			Day 35	Day 57	Day 64	Day 71	Day 35	Day 57	Day 64	Day 71	Day 35	Day 57	Day 64	Day 71	Day 35	Day 57	Day 64	Day 71
156	2	1	0.033	0.036	0.074	**2.697**	9.2	7.5	7.7	9.4	3.1	4.2	2.6	4.2	5.1	2.4	4.4	4.1
158	2	1	0.065	0.048	**1.257**	**0.925**	10.7	10.1	**17.0**	5.4	3.2	2.1	**9.8**	1.6	6.5	7.4	6.0	3.2
167	2	1	**1.121**	0.033	0.041	0.067	11.3	7.0	7.2	7.9	3.7	3.0	1.8	1.9	6.4	3.4	4.7	5.2
176	2	1	0.032	0.025	0.094	**2.853**	**13.1**	**13.1**	11.3	1.6	2.6	3.8	3.5	3.7	9.3	8.3	6.8	5.7
183	2	1	0.009	0.036	0.039	**6.099**		4.8	7.4	7.4		1.9	1.8	3.7		2.3	4.9	2.6
199	2	1	**0.519**	0.018		0.008	9.2	7.2		6.9	2.2	3.0		2.3	5.3	3.1		3.7
204	2	1	**1.483**	0.029		0.115	10.7	12.6			3.2	3.8			6.7	8.1		
244	2	1	0.047			**2.733**	7.9	9.0	7.2	6.4	2.6	2.4	2.5	2.7	4.4	5.9	4.1	2.8
13	1	2	**0.415**	**0.329**	0.043	0.052	7.4	10.1	6.0	5.7	2.6	**6.9**	1.9	1.9	3.9	2.3	3.5	3.2
55	1	2		0.158	**0.876**	0.053	11.3	7.9	7.2	9.0	3.5	5.6	2.5	3.3	6.5	1.5	3.6	4.9
74	1	2		**0.361**	0.135	0.037	6.1	9.2	7.1	7.9	1.8	2.0	2.9	1.7	3.6	6.8	3.8	5.7
99	1	2	**0.810**	0.032	0.064	0.036	**13.1**	6.8	9.2	8.0	7.8	3.9	3.3	2.2	3.2	2.1	5.1	4.9
120	1	2	0.026	**0.700**	0.038	0.052	8.8	11.9	13.1	6.7	2.4	4.2	3.7	2.6	5.0	6.5	8.7	3.6
187	2	2	0.030	**1.924**	**1.827**	0.127	6.0	8.7	4.7	4.7	1.9	**6.3**	1.8	2.3	3.5	1.4	2.5	1.8
17	1	3	0.040	**0.343**	0.029	0.052	7.8	7.7	8.8	7.9	2.5	1.8	2.4	1.8	4.4	5.3	5.9	5.6
107	1	3	0.032	0.027	**0.594**	0.028	12.6	10.2	11.3	10.7	3.5	2.8	2.8	4.4	8.1	6.6	7.4	5.5
121	1	3	0.038	**1.317**	**1.434**	0.064	7.8	6.5	7.5	7.6	2.4	4.0	1.2	1.7	4.5	1.9	5.7	5.3
144	2	3	**0.961**	0.043	0.035	0.071	9.8	4.5	6.9	7.3	1.1	1.7	2.2	2.5	6.3	2.5	4.1	4.2
145	2	3	0.133	0.030	**0.389**	0.068	12.3	13.6		8.2	5.9	2.4		2.5	4.5	**10.8**		4.5
159	2	3	0.046	0.031	0.044	**3.119**	8.7	8.1	5.7	7.5	3.5	2.6	2.5	4.5	4.4	5.0	2.7	2.2
208	2	3	0.031	0.048	0.062	**3.454**	11.8	6.0	1.1	8.9	2.5	2.1	1.4	4.0	7.9	3.2	7.9	4.0
52	1	4	**0.433**	0.026	0.057	0.040	9.9	3.9	5.7	5.5	4.2	2.1	1.3	1.9	5.0	1.4	4.0	3.0
66	1	4	0.046	0.014	**0.670**	0.037	6.2	5.5	4.8	6.2	1.6	2.6	1.3	2.8	3.6	2.2	3.0	2.7
153	2	4	0.107	0.026	**2.015**	0.055	6.3	6.7	5.4	6.6	2.3	1.4	1.5	2.5	3.3	4.6	2.8	3.4
162	2	4	0.034	0.086	**1.587**	0.071	8.2	6.2		6.8	1.7	3.5		2.6	5.5	1.8		3.2
175	2	4	0.032	0.034	**2.285**	**0.684**	7.6	8.4	**13.5**	5.8	1.6	1.7	**8.5**	2.0	5.3	6.1	4.0	3.1
198	2	4	0.037	0.031	**0.467**	**1.571**	7.3	8.6	11.2	5.7	1.9	3.4	**6.2**	2.0	4.3	4.3	3.9	3.0
209	2	4	0.082	0.054	0.066	**3.930**	9.7	7.7	7.1	7.7	2.0	1.7	1.6	3.4	7.2	5.7	5.0	3.5
210	2	4	0.052	0.028	**1.235**	0.052		9.5	12.3	9.9		2.0	4.2	2.3		6.8	6.9	6.7
242	2	4	0.036	0.041	**1.083**	0.044	10.2	7.0	9.8	10.8	2.4	2.8	2.8	2.0	6.4	3.2	5.4	7.9

The text in bold are considered to be outside the physiologically "normal" range for healthy cattle. Breed 1 = Angus steers; Breed 2 = Hereford steers

haptoglobin and cortisol is needed. Results from previous studies (Colditz *et al.,* 2005) indicate that there is a relationship between cortisol concentration and haptoglobin expression. However, Fisher *et al.* (2001) have shown that an increase in haptoglobin can occur in the absence of cortisol, which could account for plasma haptoglobin concentrations increasing prior to cortisol in the current study. This would imply that there are at least two different pathways that cause the activation of haptoglobin expression. Whether this is a result of the difference in the pathways that are activated in response to psychological stress or inflammation is yet not clear as we do not have a complete understanding of the processes involved in activation of haptoglobin under different circumstances. Therefore, understanding whether haptoglobin is a useful indicator of psychological stress needs to be determined.

The final hypothesis evaluated whether flight times could be used as a predictor of the magnitude of the stress response. As regrouping did not cause a significant change in either cortisol and/or haptoglobin plasma concentration, there is no evidence of stress associated with regrouping in the current experiment and it is not possibly to reject or accept this hypothesis at this time. The feedlot environment may impose stressors on cattle other than those associated with regrouping. Regression analysis was therefore performed to examine the relationships between flight time, haptoglobin and cortisol. There was a weak positive association of flight time with cortisol. A conclusion can not be drawn on the underlying relationship between haptoglobin and flight times as the association between these two variables changed from positive to negative at various times. Further exploration of this association seems warranted.

Based on the above findings this study would suggest that haptoglobin is not a good measure of the stress response to re-grouping cattle when measured 1, 2 or 4 weeks after re-grouping. The results confirm the earlier findings of Arthington *et al.* (2003) who reported that group mixing of cattle had no significant effect on the acute phase proteins (haptoglobin, fibrinogen, ceruloplasmin, bovine α-acid glycoprotein, and serum amyloid-A). Likewise, Gupta, *et al.* (2005) found no significant difference in haptoglobin expression between cattle in re-grouped and control groups. They suggested that cortisol is probably a better indicator of social stress. However it is notable that re-grouping of cattle in the current study did not elicit a cortisol response as has been previously reported by Veissier *et al.* (2001). There are a number of differences between this current study and the one by Veissier *et al.* (2001); this includes the

age of the cattle involved and also the time when the blood samples were collected for cortisol measurement. Veissier *et al.* (2001) collected blood samples for cortisol analysis after the eighth re-grouping from one calf per pen every 20 minutes. These differences in cortisol concentrations between the controls and re-grouped animals may explain why the current study did not report a significant difference.

This study showed a significant difference ($P<0.05$) in cortisol concentration due to breed, but only on day 64 where Hereford steers had a higher concentration than Angus steers. There are two possible reasons for this breed difference. Firstly, the cattle originated from two different properties and therefore had experienced different management practices and environments before entering the feedlot. The Angus steers underwent yard weaning, whilst the Hereford steers were weaned onto a truck and transported to a new location. Therefore, it might be expected that the Hereford steers would be more susceptible to the general stress of mixing as reflected in the haptoglobin and cortisol concentrations. However it is also possible that there may be genetic differences in the basal cortisol concentrations and the mechanisms used to deal with stress. As breed was confounded with pre-feedlot management in this study, no absolute conclusion can be drawn as to the relative contribution of these two effects. However, there is evidence for differences between breeds in their dominance status (Mench *et al.,* 1990). These authors evaluated the effect of social re-grouping of, Angus, Hereford and Angus × Hereford cows and found that the crossbreed and Angus cows dominated the Herefords.

The change in haptoglobin concentration overtime was not due to the re-grouping treatment. Therefore one possibility for this change was changes in environmental conditions. Between days 48 and 57 approximately 120mm of rain fell at the feedlot causing the pens to become boggy and thus the change in feedlot conditions may be considered as an additional stressor on the steers. This could account for the increase in haptoglobin at day 64 (Figure 5.4) across all groups and may be a response of animals in competition for dryer resting areas.

It is apparent from the literature that it is not clear whether haptoglobin can be used to measure psychological stress; although the study by Nukina *et al.* (2001) where restraining mice led to an increase in IL-6 would suggest that it is highly probable. Studies by Alsemgeest *et al.* (1996) and Moshage (1997) showing that IL-6 can induce an acute phase response also confirm this. In the current study a haemogram was available for each animal at

each time point which together with haptoglobin values allowed examination of whether the increase in haptoglobin was associated with systemic inflammation or possibly with psychological stress. The results are promising in that a significant increase in haptoglobin was rarely accompanied by an increase in white cell counts, and in particular with neutrophils which are a marker of systemic inflammation (Jain, 1993). It seems plausible then that elevated haptoglobin concentrations in these animals were associated with the response to psychological stressors.

From this study haptoglobin concentrations were not affected by regrouping cattle one, two or four weeks before feedlot exit. Notably, nor was cortisol. Thus the treatment may have failed to constitute a stressor. Alternatively, measuring haptoglobin (and cortisol) on a daily basis for the first 14 days following regrouping may have detected transient effects associated with reestablishment of the maintenance phase of social hierarchies in cattle (Veissier *et al.,* 2001, Bøe and Færevik, 2003). The results do reveal the possibility that haptoglobin provides a measure of psychological stress

Chapter 6: Can weaning stress be detected using the acute phase protein haptoglobin?

6.1 Introduction

Abrupt weaning of beef calves is an integral part of the beef cattle production system. In Australia, weaning occurs when calves are between six and nine months of age, several months early than in natural systems which can take greater than 12 months (Stookey, 1998). The timing is based primarily on a target weaning weight of 180-250kg but there are several other factors that may influence this decision. These include when cows are next due to calve; the availability of food to ensure that weaners have the best possible growth rates and/or the likely markets that they are destined for when sold at weaning. The weaning process is known to be a stressful event for the calves due to disruption of the social bond between the cow and calf and also due to changes in diet and/or environmental conditions (Phillips *et al.,* 1989; Crookshank *et al.,* 1979; Lefcourt and Elasser, 1995).

There are several different methods of abrupt weaning that are used in the Australian beef industry. The four most commonly used methods are the traditional method of total separation, yard weaning, fence line separation and finally half weaning. For further information on these methods refer to Chapter 1, page 19.

Artificial weaning of beef cattle is a stressful event for the calves, not only because they are separated from the dam but because it is often accompanied by environmental and feed changes. This can lead to behavioural (increased walking and bellowing) and physiological changes (increased cortisol concentration and total protein) (Table 6.1), which are relatively easy to measure compared to the psychological trauma that weaning might cause under these circumstances.

With such knowledge on the impact of weaning, producers are seeking alternative ways to wean that will increase herd profitability and also increase the well-being of each individual animal. Robust measures of the stress of weaning would greatly facilitate this process. Blood corticosteroid concentrations have been the most commonly used variable for measuring stress (Zavy *et al.*, 1992) but, corticosteroids can be influenced by age, sex, genetics and prior experiences in addition to diurnal rhythms (Zavy *et al.,* 1992). The potential of haptoglobin as a measure of weaning stress is investigated here.

Table 6.1: Biological changes associated with weaning

Biological change	Weaning	References
Cortisol	↑	Crookshank *et al.,* 1995; Lefcourt and Elasser, 1995
Antibodies	Reduced ability to produce	Lefcourt and Elasser, 1995
Leukocyte	↓	Phillips *et al.,* 1989
CPK	↑	Crookshank *et al.,* 1995
LDH	↑	Crookshank *et al.,* 1995
SGOT	↑	Crookshank *et al.,* 1995
SGPT	↑	Crookshank *et al.,* 1995
Noradrenaline	↑	Lefcourt and Elasser, 1995
Adrenaline	↑	Lefcourt and Elasser, 1995
Glucose	Breed dependant	Phillips *et al.,* 1987
Serum urea nitrogen	↑	Phillips *et al.,* 1987
Total protein	↑	Phillips *et al.,* 1987
Eosinophils	↓	Phillips *et al.,* 1989
Basophils	↑	Phillips *et al.,* 1989
Urea	↑	Phillips *et al.,* 1989

Haptoglobin, an acute phase protein is a stress indicator that is currently under examination by a number of groups for its potential role as a stress indicator in cattle. To date, results have been equivocal when measuring the stress of weaning. Hickey *et al*. (2003) reported that sampling time, weaning and sex had no effect on haptoglobin concentration. Stookey (1998) reported a significant difference in haptoglobin expression in a study over two years comparing fence line separation from total separation; however, the trend differed for each year. Hence, there was no advantage or disadvantage in either of the weaning methods as assessed by impact on haptoglobin concentrations.

These equivocal results suggest that further examination of the association between stress and haptoglobin concentrations is justified in cattle. This led to the development of four hypotheses:

1. that haptoglobin concentrations increase in newly weaned calves;
2. that haptoglobin concentrations following weaning can be used as an indicator on how beef cattle will perform under intensive management situations;
3. that weaning is a psychological stressor;
4. that flight times can predict the magnitude of a psychological stressor using haptoglobin as the indicator of the stress response.

The weaning experiments presented in this chapter used two methods of weaning. The first used the practice of half weaning and the second used the northern practices of yard weaning. In experiment one ‘effects of weaning on plasma haptoglobin’ the design addressed whether or not weaning increases the concentration of plasma haptoglobin. The second experiment

'association of weaning stress with feedlot performance' evaluated whether or not the change in haptoglobin at the time of weaning is associated with subsequent growth rate and feedlot performance. In addition, it also enabled some preliminary assessment of the effects of genotype on the expression of haptoglobin in calves.

6.2 Materials and methods

Experiment one: Effects of weaning on plasma haptoglobin

Animals

Thirty-eight Polled Shorthorn calves of mixed sex (n♀=21, n♂=17) aged approximately 9 to 10 months and with an average weight of 232.7kg ± s.e. 5.9kg were used for this study. The animals were from a commercial herd located at Elaine in Central Victoria, Australia (-37.77S, 144.02E) and remained the sole property of the owner throughout the duration of the experiment. The dams were maiden cows approximately three years of age.

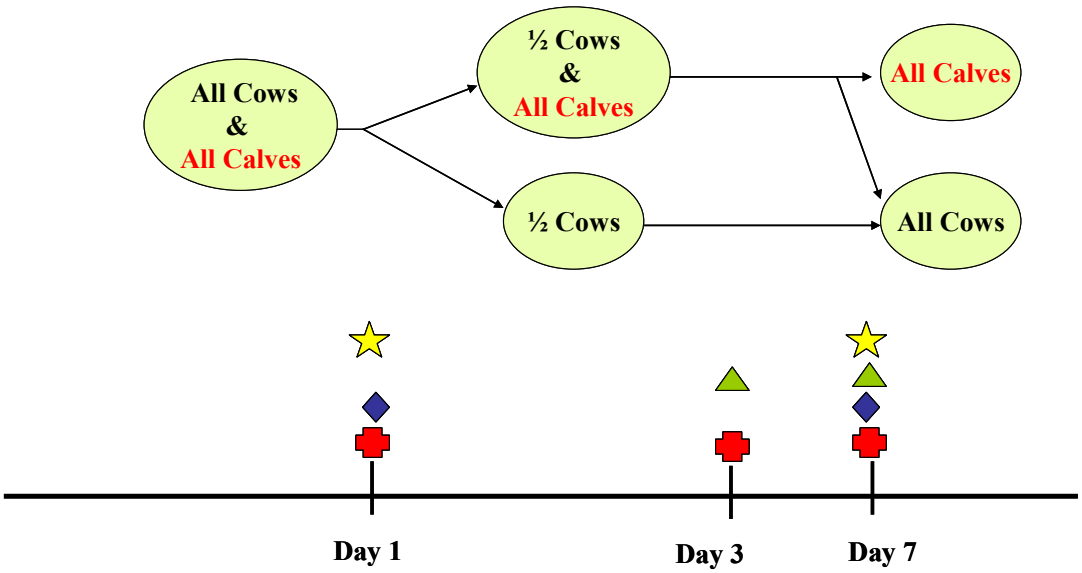

Figure 6.1: Experimental design to examine the impact of half weaning of calves on haematological variables. ✚ blood sample; ▲ Flight times; ◆ weighed; ☆ weaned

Design

The calves were divided into two groups, weaned and unweaned (controls) and remained as one group for the duration of the experiment (Table 6.2). All calves had a blood sample taken on days 1, 3 and 7. In addition, weight and flight times (Burrow *et al.,* 1998; a measure of the time it takes for an animal to cross a set distance of 1.7m after exiting a cattle crush into an open space) were recorded on days 3 and 7. On day 1, the calves from the weaned group had their dams removed after the blood sampling had concluded. The control calves were weaned

at the end of the experiment on day 7 after they exited the experiment (Figure 6.1). In addition, hair whorl location, placement, shape and number were recorded as per Grandin *et al*. (1995), Figure 6.2. Grandin *et al.* (1995) have proposed that hair whorl characteristics are correlated with temperament.

Blood collection and processing

Blood was collected from the coccygeal artery/vein using an 18 gauge; 1 inch precision glide vacutainer system needle inserted into 5mL BD Vacutainer tubes containing 0.054mL of 15% K_3EDTA. The blood was chilled and transported to a laboratory for processing within six hours.

Table 6.2: Number of steer and heifer calves in each treatment group

Treatment Group	Steers	Heifers	Total
Weaned	9	9	**18**
Unweaned (controls)	8	12	**20**
Total	**17**	**21**	**38**

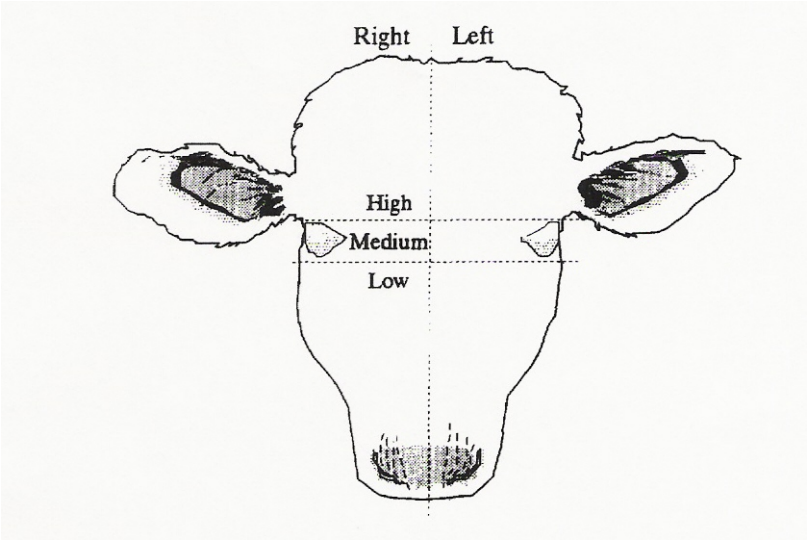

Figure 6.2: "Method for determining the location of the hair whorls on an animal's face. The centre of the hair whorl was used as the reference point" (Grandin *et al.,* 1995).

On arrival in the laboratory blood was processed for routine haematology. Vacutainers were rotated for 4 minutes to re-mix the blood before analysis on a Cell-Dyn 3700 automated haematology analyser (Abbott Diagnostics, North Ryde, NSW, Australia) for routine haematology (white blood cell counts, red blood cell counts and packed cell volume). Blood

was centrifuged at 1200g (2500 rpm) for 12 minutes at 4°C; plasma was collected and then stored at -20°C until required for haptoglobin analysis as described in Chapter 2.

Experiment two: Association of weaning stress with feedlot performance

Animals

Belmont Red (n=85), Belmont Red composite (n=35) and Brahman (n=125) steers calves aged around 6 months and with an average weight of 201.2kg ± s.e. 1.9kg were used in this experiment. Calves were located at the CSIRO Livestock Industries Belmont Research Facility, Rockhamptom, Queensland, Australia (-23.38S, 150.50E) and originated from three herds as indicated in Table 6.3.

Table 6.3: The number of steers of each breed represented in each herd

Breed / Herd	Belmont Red	Brahman	Belmont Red composite	Total
Herd 1	53	23	21	**97**
Herd 2	23	84	9	**116**
Herd 3	9	18	5	**32**
Total	**85**	**125**	**35**	**245**

Design

The steer calves were weaned over three consecutive days with herd one weaned on day 1, herd two on day 2 and herd three on day 3 representing 3 cohorts of animals. A blood sample was taken from all steers before weaning with a final blood sample taken 7 days after weaning of the first group (97 steers), 6 days after weaning group 2 (116 calves) and 5 days after weaning group 3 (32 calves; Figure 6.3). Twelve months post weaning, these steers were relocated to the 'Co-operative Research Centre for Beef and Cattle Quality' research feedlot "Tullimba" located west of Armidale, NSW Australia (-30.52S, 151.67E) for feedlot performance measurements. Blood samples were not collected during the feedlot stage; however, data on growth traits throughout this period were available for analysis as were flight times and crush scores (both indicators of temperament) (Figure 6.4).

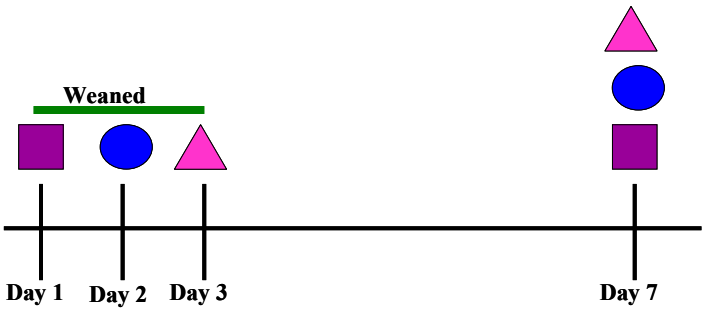

Figure 6.3: Pictorial representation when each group of steers were weaned and the time points at which a blood sample was taken. ■ Herd 1; ● Herd 2; ▲ Herd 3

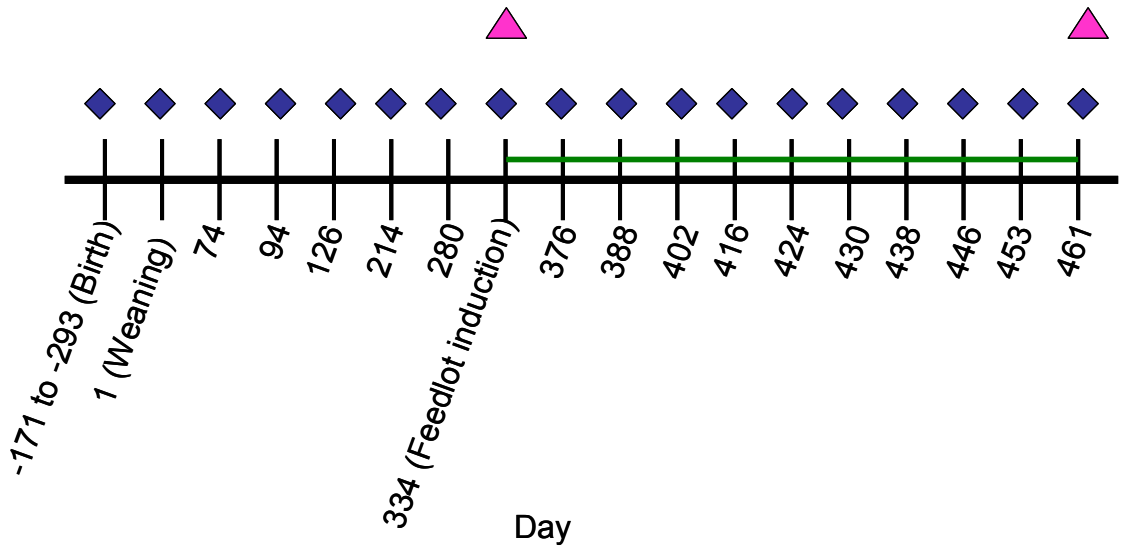

Figure 6.4: Timeline of the events for the steers throughout the course of their lives.— Feedlot duration; ♦ Weight; ▲ Growth variables and temperament measures.

Blood collection and processing

Blood was collected from the coccygeal artery/vein on day 1, with the subsequent blood sample being taken from the jugular vein when haematomas were noted on and around the tail vessels preventing further blood sampling from this sight. A previous study had shown that there was no significant difference in the haptoglobin concentration between the jugular vein and the coccygeal blood vessels (Chapter 4, page 66). All blood samples were collected using an 18 gauge, 1 inch precision glide vacutainer system needle inserted into 5mL BD Vacutainer tubes containing 0.054mL of 15% K_3EDTA. After transportation to the laboratory, the blood was centrifuged at 1200g (2500 rpm) for 12 minutes at 4°C to obtain plasma samples. The plasma was then stored at -20°C until required for haptoglobin analysis.

Statistical Analysis

The data from both studies was analysed using Systat version 9 (SPSS INC. Chicago IL. USA) and were subjected to normality testing and transformed where required.

Experiment one required natural log transformation for total white blood cell counts, neutrophils, lymphocytes, monocytes, eosinophils, basophils and haptoglobin. The data were then analysed to examine the impact of weaning on the haematological variables and also on flight times, weight gain and any association with the incidence of hair whorls. The fixed effects of weaning, time, sex and hair whorl location and first order interactions on the haematological variables were examined using repeated measures in a general linear model. Correlations with weight and flight times were also examined.

In experiment 2, with the exception of haptoglobin (log transformed) the data were normally distributed. Repeated measures in a general linear model were performed to examine the interactions between herds, breed nested in herd and time for haptoglobin. Correlations with haptoglobin and feedlot performance (weight gain and fat coverage) and flight times were also evaluated.

6.3 Results

Experiment one: Effects of weaning on plasma haptoglobin

The effect of weaning treatment, time and sex and their interactions on the haematological variables were examined (Table 6.4). There was a significant effect of sex on several red blood cell variables including haemoglobin, haematocrit and mean corpuscular haemoglobin (Table 6.4). Of the variables examined, only haptoglobin was significantly affected by weaning treatment ($F=4.227$, $P=0.048$).

A repeated measures model was used to analyse the change in variables across time (Table 6.4). Lymphocytes, platelets and haptoglobin concentration did not change significantly with time. However, lymphocyte and eosinophil counts differed for the treatment groups over time. Hypothesis testing revealed that there was a significant difference between weaned and unweaned calves for lymphocytes and eosinophils on day 7 (Figure 6.5).

There was no effect of the placement of hair whorl (high, middle or low on the forehead; left, right or central on the midline; or normality of the hair whorl) on the haematological variables

with the exception of mean corpuscular haemoglobin concentration (F=6.148; F=0.019) when testing for interactions between the haematological variables and the characteristics of the hair whorl.

There was no effect of weaning on weight change over the seven days of the experiment. There was however substantial variation within groups in weight change during the course of the experiment. The weight change varied from minus 18.5kg to plus 7kg. The average change in weight across all calves was -2.4kg (-3.3kg for weaned calves and -1.4kg for control calves (F=7.508, P=0.09) (Figure 6.5). All weight change was within ±10% with the average weight change being -0.9%.

Flight times were also analysed to evaluate if "temperament" was affected by weaning and no significant differences were found across time or between groups although there was a trend for flight times to decrease with time (Figure 6.5). The relationship between flight time (at day 3 or the difference between day 3 and day 7) and the change in weight or haptoglobin (before and after weaning and the change in haptoglobin concentrations) were examined and found to be non-significant.

Correlations between haptoglobin and the other variables measured are presented in Table 6.5. In general, correlations were not significant although a correlation of greater than ±0.4 was seen between haptoglobin and white blood cell count, lymphocytes, monocytes, haemoglobin, haematocrit and platelets. The relationships were not consistent across time (Table 6.5).

Table 6.6 shows those animals that had an increase in haptoglobin greater than 0.1mg/mL. Of these nine calves only one animal whose haptoglobin was greater than 0.1mg/mL had an increase in total and differential white blood cell counts.

Table 6.4: First order and full interaction models of weaned, sex and/or time on the haematological variables measured.

Blood variable	Value	First order interaction model				Full interaction model
		weaned	sex	Time	Time× weaned	Time×sex× weaned
White blood cells	**f-value**	0.187	0.121	7.440	2.595	2.335
	p-value	*0.669*	*0.730*	***0.001***	*0.082*	*0.105*
Neutrophils	**f-value**	0.723	0.824	17.051	0.427	1.124
	p-value	*0.401*	*0.370*	***0.000***	*0.654*	*0.331*
Lymphocytes	**f-value**	1.083	1.123	1.101	4.156	1.813
	p-value	*0.305*	*0.297*	*0.338*	***0.020***	*0.171*
Monocytes	**f-value**	0.152	0.004	6.910	2.073	1.039
	p-value	*0.699*	*0.950*	***0.002***	*0.134*	*0.360*
Eosinophils	**f-value**	0.725	0.649	10.766	4.821	0.662
	p-value	*0.400*	*0.426*	***0.000***	***0.011***	*0.519*
Basophils	**f-value**	1.073	1.500	3.025	1.594	0.100
	p-value	*0.308*	*0.229*	*0.055*	*0.211*	*0.905*
Red Blood Cells	**f-value**	1.501	1.184	36.682	2.261	1.448
	p-value	*0.229*	*0.284*	***0.000***	*0.112*	*0.243*
Haemoglobin	**f-value**	0.199	18.333	4.051	1.851	0.914
	p-value	*0.658*	***0.000***	***0.022***	*0.165*	*0.406*
Haematocrit	**f-value**	0.486	21013	27.580	2.491	1.119
	p-value	*0.490*	***0.000***	***0.000***	*0.090*	*0.333*
Mean corpuscular volume	**f-value**	0.542	3.161	16.402	0.721	2.416
	p-value	*0.466*	*0.084*	***0.000***	*0.490*	*0.097*
Mean haemoglobin concentration	**f-value**	0.319	4.431	258.488	0.944	0.843
	p-value	*0.576*	***0.043***	***0.000***	*0.393*	*0.435*
Mean corpuscular haemoglobin concentration	**f-value**	1.514	3.142	154.419	1.383	0.025
	p-value	*0.227*	*0.086*	***0.000***	*0.258*	*0.975*
Platelets	**f-value**	0.271	2.881	0.364	0.494	0.635
	p-value	*0.606*	*0.099*	*0.696*	*0.612*	*0.533*
Haptoglobin	**f-value**	4.227	0.533	1.405	0.660	0.192
	p-value	***0.048***	*0.471*	*0.253*	*0.521*	*0.826*

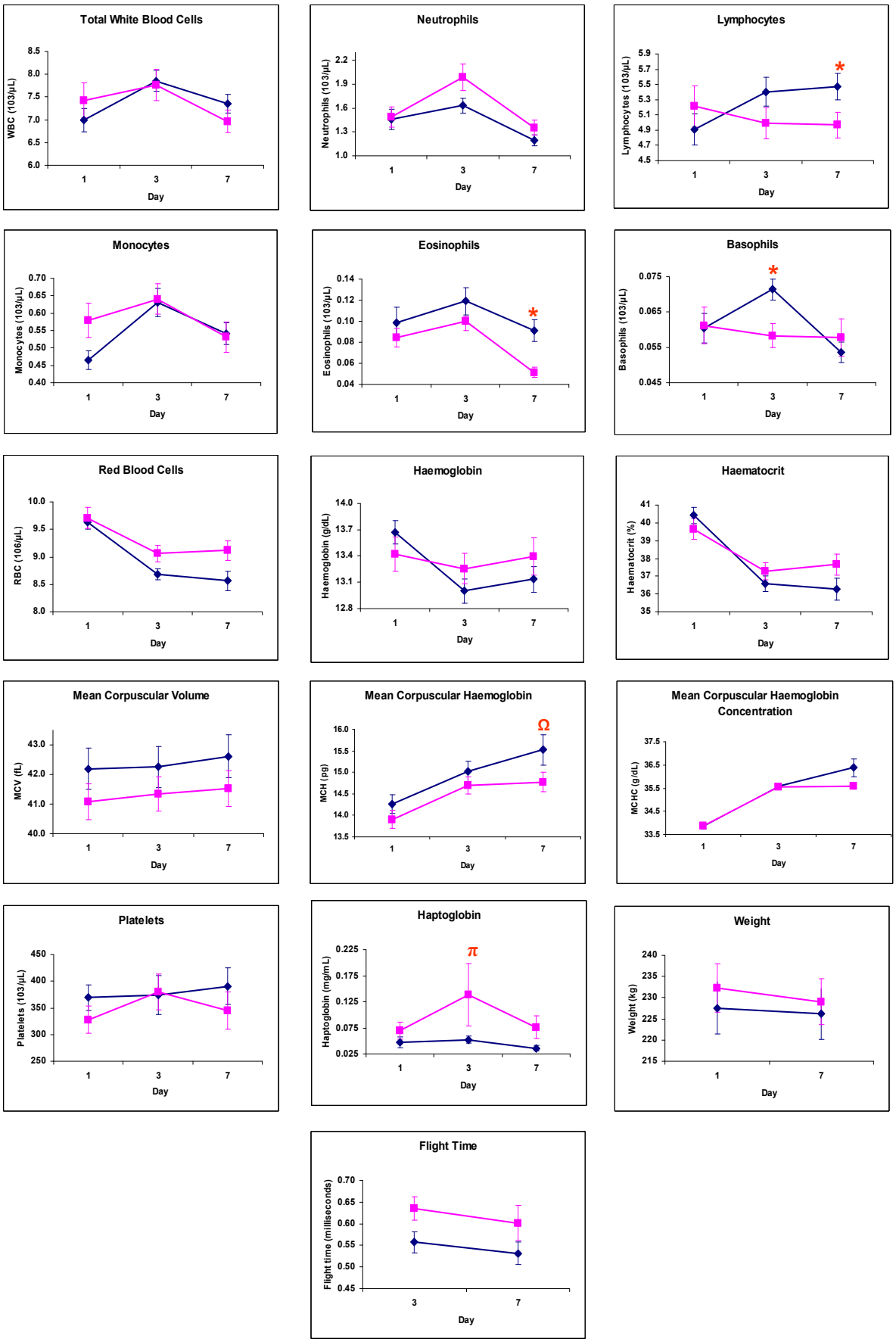

Figure 6.5: Change in variables over time in weaned and unweaned calves (mean ± se). ♦ controls; ■ weaned. Hypothesis test results: * P<0.05; Ω 0.1<P<0.05; π 0.15<P<0.1

Table 6.5: Correlations between haptoglobin and the other variables measured.

Variable Measured	Day	Haptoglobin Day 1	Haptoglobin Day 3	Haptoglobin Day 7	Haptoglobin Day 3 -Day 1
Weight	1	0.139	-0.021	0.045	0.064
	3	0.180	-0.018	0.019	0.064
	Gain	0.219	0.029	-0.184	-0.022
Whorls	Placement	0.031	-0.154	0.106	-0.142
	Centreline	0.060	-0.085	**0.293**	-0.053
	Number of	0.117	-0.129	0.157	-0.058
	Shape	0.031	-0.022	0.044	-0.122
Flight time	3	-0.176	0.174	0.080	0.198
	7	**-0.251**	0.098	**0.217**	0.050
	Change in	-0.141	-0.040	0.187	-0.118
White blood cells	1	-0.036	-0.186	**0.530**	-0.172
	3	-0.140	-0.149	**0.594**	-0.160
	7	0.034	**-0.285**	**0.221**	**-0.225**
Neutrophils	1	-0.091	-0.149	**0.302**	-0.129
	3	-0.140	0.072	**0.344**	0.105
	7	-0.148	-0.161	**0.210**	-0.012
Lymphocytes	1	0.030	-0.151	**0.538**	-0.147
	3	-0.093	**-0.286**	**0.588**	**-0.321**
	7	0.059	**-0.264**	0.147	**-0.231**
Monocytes	1	0.036	-0.126	**0.424**	-0.143
	3	0.055	0.026	**0.223**	**-0.300**
	7	0.191	-0.125	0.094	**-0.201**
Eosinophils	1	-0.197	-0.028	0.153	0.145
	3	-0.194	-0.014	0.139	0.099
	7	**-0.258**	**-0.328**	**0.240**	-0.133
Basophils	1	-0.180	-0.190	**0.291**	-0.069
	3	0.096	-0.160	**0.219**	**-0.272**
	7	0.104	-0.147	**0.309**	**-0.373**
Red blood cells	1	0.157	0.207	-0.096	0.032
	3	0.041	**0.360**	0.046	**0.312**
	7	**0.220**	**0.240**	-0.184	0.104
Haemoglobin	1	**0.413**	**0.206**	-0.078	-0.113
	3	**0.235**	**0.424**	0.092	**0.246**
	7	**0.388**	**0.328**	**-0.250**	0.031
Haematocrit	1	**0.379**	**0.252**	-0.097	-0.067
	3	**0.245**	**0.405**	0.060	**0.235**
	7	**0.431**	**0.272**	**-0.280**	0.016
Mean corpuscular volume	1	0.139	0.010	0.006	-0.082
	3	0.147	0.014	0.000	-0.087
	7	0.137	-0.010	0.011	-0.112
Mean corpuscular haemoglobin	1	0.150	-0.038	0.031	-0.121
	3	0.140	0.017	0.025	-0.093
	7	-0.006	-0.003	0.053	-0.077
Mean corpuscular haemoglobin concentration	1	-0.016	**-0.317**	0.123	-0.173
	3	-0.130	0.025	0.158	0.011
	7	**-0.252**	0.005	0.081	0.019
Platelets	1	0.023	-0.026	0.043	-0.175
	3	**-0.340**	-0.115	**0.354**	-0.170
	7	-0.125	0.151	**-0.420**	**0.355**

$0.2 \leq |r| < 0.4$; $|r| \geq \pm 0.4$

Table 6.6: Individual steers with high haptoglobin concentrations with corresponding total white blood cell count, neutrophil and lymphocyte counts.

Tag	Sex	Treatment	Haptoglobin (mg/mL)			Total White Blood Cell Count ($\times 10^3/\mu L$)			Neutrophil Count ($\times 10^3/\mu L$)			Lymphocyte Count ($\times 10^3/\mu L$)		
			Day 1	Day 3	Day 7	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
33	heifer	control	0.04	**0.11**	0.02	6.23	na	5.26	2.18	na	0.85	3.23	na	3.85
1	steer	control	0.03	**0.13**	0.02	4.80	7.23	5.81	0.56	1.87	1.04	3.98	4.27	4.31
9	steer	control	0.02	**0.11**	**0.11**	5.77	9.93	6.88	0.62	1.36	0.66	4.68	7.70	5.53
10	steer	control	0.02	0.05	**0.10**	6.49	7.67	6.23	1.29	1.64	1.24	4.80	5.46	4.48
323	heifer	weaned	**0.15**	**0.39**	0.03	6.27	6.62	6.21	1.17	1.33	0.68	4.57	4.77	5.17
3	steer	weaned	0.00	0.03	**0.13**	6.15	7.12	6.91	0.56	1.11	0.95	5.09	5.49	5.45
11	steer	weaned	0.03	0.02	**0.48**	**13.88**	**13.94**	10.21	2.35	**4.61**	2.39	**10.05**	**7.79**	7.00
16	steer	weaned	0.05	**1.48**	0.02	6.05	6.58	6.45	0.97	2.00	1.52	4.46	3.67	4.36
13	steer	weaned	0.02	**0.12**	**0.33**	8.87	8.76	7.26	1.49	1.40	0.98	6.22	6.60	5.59

The text in bold are considered to be outside the "normal" range for healthy cattle

Experiment two: Association of weaning stress with feedlot performance

Haptoglobin concentrations in plasma were measured before and after weaning for 3 cohorts of animals, herein called herd. The intervals between blood sampling for these 3 cohorts were 5, 6 and 7 days. There was no significant difference between herd (F=0.535, P=0.586) and breed nested within herd (F=1.027, P=0.408) for haptoglobin values, but there was a significant difference in the values before and after weaning (F=10.733, P=0.001). The timing of collection of the blood sample between herds was also found to be significant (F=3.892, P=0.022); implying that timing of collection is important.

Experiment one revealed correlations between haptoglobin and some haematological variables indicating that weaning was stressful to calves. In Experiment two, the potential for long term consequences of weaning stress on feedlot performance was investigated by examining correlations between haptoglobin and measures of feedlot growth (Table 6.7).

Of particular interest was whether or not there was a correlation between haptoglobin concentrations at weaning and individual flight times. The results in Table 6.7 indicate that if there was a relationship between these two variables then it is a very weak positive correlation. The other measure of temperament used in this study was crush score. The results from this would appear to be more promising as the association between change in haptoglobin at weaning and crush score taken at feedlot entry was a positive correlation (r=0.267). The relationship between pre-weaning haptoglobin and crush score was negative correlation (r=-0.264).

Table 6.7: Correlations between the variables measured and haptoglobin.

Variable	Date	Haptoglobin		
		Before Weaning (19/20/21May 2003)	**After Weaning (25 May, 2003)**	**Change in haptoglobin**
Crush score	Day 334 (Feedlot induction)	**-0.264**	0.030	**0.267**
	Day 461 (End of trial)	0.013	-0.123	0.052
Flight time	Day 334 (Feedlot induction)	0.101	0.081	-0.023
	Day 461 (End of trial)	0.033	0.147	0.177
Weight	Day -171 to -293 (Birth)	-0.027	0.144	0.034
	Day 1 (Weaning)	0.123	0.138	0.056
	Day 74	0.071	0.187	0.101
	Day 94	0.131	0.126	0.040
	Day 126	0.134	0.115	0.040
	Day 214	0.127	0.060	0.016
	Day 280	0.120	0.091	0.026
	Day 334 (Feedlot induction)	0.126	0.066	0.019
	Day 310	0.089	-0.013	-0.032
	Day 388	0.151	-0.029	-0.086
	Day 402	0.114	-0.006	-0.052
	Day 416	0.134	-0.019	-0.063
	Day 424	0.121	-0.011	-0.060
	Day 430	0.129	0.026	-0.048
	Day 438	0.132	0.017	-0.055
	Day 446	0.112	0.026	-0.036
	Day 453	0.142	0.017	-0.054
	Day 461 (End of trial)	0.143	0.028	-0.061

$|r| > 0.2$

6.4 Discussion

The experiments in this chapter were designed to evaluate whether the acute phase protein haptoglobin could potentially be used as an indicator of the stress caused by weaning. An additional aim was to determine whether concentrations of this protein at weaning could be used to predict how individual animals will perform in a feedlot environment. It was therefore hypothesised that firstly haptoglobin concentrations increase in newly weaned calves (Experiment 1); secondly that the haptoglobin concentrations following weaning can be used as an indicator of how beef cattle will perform under intensive management situations (Experiment 2); thirdly, that weaning is a psychological stressor (Experiment 1); and fourthly, that flight time can be used to predict the magnitude of a response to a psychological stressor (Experiment 1 and 2).

Experiment 1: Effects of weaning on plasma haptoglobin

This experiment was designed to primarily address the first hypothesis: does weaning cause an increase in haptoglobin plasma concentrations. The haematological variables measured

during the experiment indicate that at the time of the study there was no underlying illness that would influence the haptoglobin concentrations, as haematological variables fell within the "normal" range for healthy cattle. This experiment was similar to that conducted by Hickey *et al.* (2003), except that they maintained the weaned calves separate from the control calves. Unlike Hickey *et al.* (2003), this study reported a significant difference in haptoglobin expression between weaned and unweaned calves (P=0.048), with weaned calves reporting a higher haptoglobin expression. The haptoglobin concentrations increased in both groups of calves following weaning (unweaned calves increased by 0.005mg/mL compared to weaned calves (0.069mg/mL) which had returned to normal for both groups of calves by day 7. This small non-significant increase in haptoglobin concentration in the unweaned calves could be a result of three things: 1. handling stress; 2. inflammatory response due to be ear-tagged on day 1; and 3. normal physiological changes. Firstly, the stress of handling; it has been shown by Zavy *et al.* (1992) that handling can have a greater impact than weaning and as the calves in the present experiment were not habituated to handling it is possible that this increase reflects the stress of handling associated with blood sampling. This effect could also help to explain at least some of the difference in the results between this study and that of Hickey *et al.* (2003) where the calves were habituated to handling prior to weaning. The second potential reason for the initial haptoglobin increase is the fact that all calves were electronically ear-tagged on day one and that there was a short term inflammatory response which may be reflected in the elevated haptoglobin concentrations. Results from another study (Chapter 7) where muscle biopsy was taken showed that this resulted in a small increase in haptoglobin concentrations 48 hours after the muscle biopsy; therefore, it is possible that this slight increase may be a result of being tagged. However, because the increase was small it is also possible that it is due to normal physiological changes. Without habituating the animals to handling pre weaning or putting in a control for ear-tagging it is not possible to conclude if this slight increase was a result of these two perceived stressors. However, these two additional stressors were applied uniformly across both treatment groups and therefore the significant increase in haptoglobin in the weaned calves on day 3 is a result of weaning. Therefore, the first hypothesis is accepted.

The literature states that an animal is under going an acute phase response when the haptoglobin concentration exceeds 0.1mg/mL (*Tridelta*). Therefore to either accept or reject the third hypothesis, that weaning is a psychological stressor as determined by haptoglobin expression, calves which had haptoglobin concentrations greater than 0.1mg/mL were

compared with their haematological data to determine if the increase in haptoglobin was due to immune activation. As can be seen in Table 6.6, there is equal numbers of weaned an unweaned calves in which haptoglobin concentration exceed 0.1mg/mL. Therefore it is not possible to conclude that weaning is a psychological stressor. However, the increase in haptoglobin, may have been as a result of handling and/or from ear-tagging the animal.

Of the animals included in this study, one animal (tag 11) had greater haptoglobin expression which may have been due to an increase in total white blood cell counts rather than the stress of weaning. The higher than "normal" white blood cell count was due to an increase in both neutrophils and lymphocytes implying that the animal was undergoing a heightened immune response. For the remaining eight calves their haematological variables were considered to be within the "normal" physiological range for cattle (Jain, 1993). Therefore, what caused haptoglobin to increase? Is it the stress of being handled? Without undertaking an experiment to determine the effect of handling on haptoglobin concentrations it is not possible to conclude whether this led to the increase in plasma haptoglobin. It is unlikely that the increase was a result of being ear-tagged as only 7% of steers had a haptoglobin concentration greater than 0.1mg/mL following the muscle biopsy compared to 24% involved in this study.

The average weight loss of 1.5% for the weaned calves was less than expected as previous studies by Phillips *et al.*(1987) and Phillips *et al.*(1989) reported weight loss for calves weaned in a similar manner to be 7%. The most likely reason for this difference is the age of the calves at weaning; the calves in the present study were older and quite possibly undergoing a natural weaning process at the time of the experiment. However, there was some evidence noted by the owner, in that on returning the calves to the yards on the eve of their second blood sample, the weaned calves separated themselves from the herd and headed towards the paddock their dams were located in. This behaviour suggests that the maternal bond had not been severed prior to weaning.

Experiment two: association of weaning stress with feedlot performance

This experiment was designed to address the second hypothesis which states that the haptoglobin concentrations following weaning can be used as an indicator of how beef cattle will perform under intensive management situations (in this case in a feedlot environment). The feedlot environment exposes cattle to potential stressors (Fell et al. 1998). Exposure of pigs to stressors is also seen in commercial pig finishing environments. Eurell *et al.* (1992)

showed that haptoglobin concentration in pigs could be used as an indicator for weight gain. It was therefore proposed that sensitivity to stressors at the time of weaning may be related to sensitivity to stressors during feedlot finishing, thereby influencing growth in the feedlot environment. Analyses revealed that the haptoglobin expression of the steers at weaning could not be used as an indicator for beef cattle performance in a feedlot, as there was a lack of correlation between haptoglobin and the measures of performance whilst in the feedlot. Arthington *et al.* (2005), examined the effect of early weaning on feedlot performance and found that haptoglobin concentrations did increase following feedlot entry with the normal weaned calves having a greater haptoglobin concentration ($P<0.05$) than early weaned calves suggesting a negative correlation between haptoglobin concentration at weaning and average daily gain whilst in the feedlot. The difference between their study and the one presented here is that their weaned calves entered the feedlot immediately following weaning (a high stress state) unlike the steers in this study which did not enter the feedlot until 10 months after weaning. Thus from this study it seems unlikely that the measurement of plasma haptoglobin at weaning may be used as an indicator of feedlot performance.

Evaluation of temperament measures with haptoglobin

The fourth and final hypothesis that was tested was to determine whether flight time can predict the magnitude of a response to the psychological stressor of weaning. The results from both experiments were used to test the hypothesis. Flight time has previously been associated with feedlot performance where it was found that animals with a slow flight time tended to grow faster than animals with fast flight times (Fell *et al.,* 1999; Colditz *et al.,* 2007). Furthermore Fell *et al.* (1999) noted that animals with poor temperament, as assessed by flight time at weaning, exhibited a greater stress response during the first few days of feedlot finishing than cattle with a good temperament. In the current study flight time was examined for association with haptoglobin and weight response to weaning. In the first experiment flight times were measured on day 3 and day 7 to assess whether the magnitude of the change in flight times was related to stress response to weaning. It would have been beneficial to have recorded individual flight times on day 1; however, technical reasons prevented this from happening. Although the flight time for some calves did increase (that is the animal took longer to cross the 1.7m distance) on average it decreased. This could suggest two things. Firstly that the calves were not habituating to the handling and/or secondly that they had learnt that the release of the crush meant freedom. In the second experiment flight times were also recorded and it was found that there was no relationship

between flight times in the feedlot and plasma haptoglobin concentrations at weaning. From both of these studies it would appear that there is no relationship between flight times and haptoglobin or weight changes during weaning.

Both studies evaluated an alternative measure of temperament (hair whorl characteristics – experiment 1 and crush score – experiment 2) and the relationship between these measures and the haptoglobin values. Grandin *et al.* (1995) reported a linear relationship between temperament and location of facial hair whorls, with cattle that had a hair whorl located above the eye line having a more excitable temperament. Additionally Grandin *et al.* (1995) reported that those cattle with abnormal hair whorls (particularly below the eye level) had unpredictable behaviour. In the present study there was no significant association between hair whorls and haptoglobin but there may be some association between lymphocytes and whorl placement. Given there are no previous studies evaluating the association between hair whorls in cattle and haematological data additional studies using larger data sets may be justified. The subjective nature of measuring hair whorls does create some methodological difficulties and if this variable is to be further evaluated development of photographic techniques may be warranted.

Crush score measures the agitation displayed by an animal held within a crush for a set period of time. The results from this study would indicate that the change in haptoglobin concentration before and after weaning is associated with cattle behaviour whilst in the crush as there was a weak positive relationship between these variables. Given the results on the relationship between haptoglobin and the various measures of temperament it would be valid to further explore the relationship between the change in haptoglobin at weaning and crush score.

The results from these two experiments evaluating haptoglobin expression, following weaning indicates four main findings:

1. that haptoglobin concentrations increase following weaning;
2. that it is unlikely that haptoglobin values following weaning can be used as an indicator for how cattle will perform whilst in the feedlot;
3. that haptoglobin has the potential to be a measure of psychological stress;
4. that flight times are not a valid predictor of the magnitude of a haptoglobin response to a psychological stressor.

However, further research is required on the effect of weaning on haptoglobin using a larger number of animals to confirm the findings of Experiment one. If the findings are confirmed then it would be worthwhile designing an experiment that would allow comparison of the various weaning methods. This may provide additional information on which weaning practice is less stressful for the calves.

Chapter 7: The effect of road transportation, intensive management and muscle biopsy on haematological variables and haptoglobin

7.1 Introduction

Cattle production exposes animals to a number of stressors throughout their life. This chapter evaluates the stress of transport, intensive management and muscle biopsy by examining the response in total and differential white blood cells, red blood cells and the acute phase protein haptoglobin to these stressors.

Intensive management in Australia differs from other parts of the world, in particular Europe and Northern America, as Australian cattle are not housed during the winter months. Intensive management of cattle in Australia is focused on feedlots. These feedlots are designed and regulated to ensure that animal welfare is not compromised but even so the intensive nature of the production system is likely to contribute to some stressful situations. Feedlotting is becoming common practice in Australia due to demands from domestic and export markets for consistent meat in terms of texture, flavour, tenderness and marbling. In addition, during drought feedlotting provides an alternative for finishing cattle.

Measuring the degree of stress in a feedlot is important as it is well documented that stress leads to immuno-suppression (Mackenzie *et al.,* 1997) causing the animals to perform below their unstressed counterparts and to be more susceptible to diseases such as bovine respiratory disease. Berry *et al.,* (2004) have suggested measurement of haptoglobin as a diagnostic tool "to make management decisions regarding treatment protocols for calves with bovine respiratory disease".

Transportation causes a number of biological and physiological changes in cattle. These changes are summarized in Table 7.1 and include changes in weight, cortisol, leukocytes and glucose. As transport causes increases in cortisol concentrations and a reduced ability to produce antibodies, it is considered to be an acute stressor (Crookshank *et al.,* 1979; Mackenzie *et al.,* 1997; Earley *et al.,* 2004a).

The majority of studies that have investigated transport stress have used animals that have recently been weaned or weaned onto trucks to mimic a common production practice world wide (Crookshank *et al.,* 1979; Phillips *et al.,* 1989; Zavy *et al.,* 1992; Mackenzie *et al.,* 1997; Steinhardt and Thielscher, 2001). In these studies transport duration varies considerably (two

hours to two days) as does distance travelled (344km to 1400km) (Crookshank *et al.,* 1979; Murata and Miyamoto, 1993; Mackenzie *et al.,* 1997; Arthington *et al.,* 2003), with the longer duration of transit including rest periods to provide water. In such studies animals consistently undergo weight loss following transportation irrespective of journey time, distance and rest periods (Crookshank *et al.,* 1979; Arthington *et al.,* 2003; Earley *et al.,* 2004a; Earley *et al.,* 2004b).

Table 7.1: Biological and physiological changes as a result of transportation.

Variable	Change	References
Temperature	↑ 1ºC	Murata and Miyamoto, 1993
Cortisol	↑↑↑	Crookshank *et al.,* 1979
Antibodies	Reduced ability to produce	Mackenzie *et al.,* 1997
IgG	↑	Mackenzie *et al.,* 1997
Leukocytes	↑	Phillips *et al.,* 1989, Earley *et al.,* 2004b
Weight	↓ 7%	Crookshank *et al.,* 1979; Earley *et al.,* 2004a
Glucose	↑	Phillips *et al.,* 1987
Serum Urea nitrogen	↑↑	Phillips *et al.,* 1987
Eosinophils	↓	Phillips *et al.,* 1989
Basophils	↓	Phillips *et al.,* 1989
Neutrophils	↑	Phillips *et al.,* 1989
Haptoglobin	↑	Earley *et al.,* 2004a
Fibrinogen	↑	Phillips *et al.,* 1989; Earley *et al.,* 2004a
Albumin	↑	Earley *et al.,* 2004a
Red blood cells	↑	Earley *et al.,* 2004a

Weight loss of cattle following transport is probably a result of a combination of feed and water deprivation, excretion, dehydration and metabolic changes. Phillips *et al.,* (1987) suggest that 60% of the weight loss is due to excretion and fasting.

Mackenzie *et al.,* (1997) report that for short transportation, the majority of the stress is due to the handling at loading and unloading rather than the process of transit. Longer haulage of cattle is associated with other stressors including thermal stress, dehydration, exhaustion and crowding (Mackenzie *et al.,* 1997; Earley *et al.,* 2004a). The response to such stressors may vary with genetic background of the animals, for instance Zavy *et al.* (1992) reported that there were breed differences in cortisol response in cattle undergoing transportation. In that study, calves with a *Bos indicus* genetic component (Brahman × Angus or Brahman ×

Hereford) tended to have a higher plasma cortisol baseline concentration than calves that were pure *Bos taurus* (Hereford × Angus).

A number of transport studies (Arthington *et al.,* 2003; Earley *et al.,* 2004a; Earley *et al.,* 2004b) comparing transported and control groups of cattle have shown that haptoglobin increases as a result of transportation.

The experiments to follow aimed to expand our understanding of the blood haematology changes associated with stressful events and in particular the response of haptoglobin to these short and longer term stressors. The specific hypotheses to be tested were that haptoglobin concentrations will increase following an acute stressor such as transportation and a muscle biopsy; and secondly, that haptoglobin concentrations can be used as an indicator for longer term adaptation to intensive management.

7.2 Materials and methods

The steers used in this study were from the New South Wales Department of Primary Industry 'Net Feed Efficiency' herd located at Trangie, NSW (-32.4S, 148.0E) and were entering the feedlot to be used in a trial to determine individual feed efficiency. The steers were progeny from two lines selected for feed efficiency (selection line 1 had a low net feed intake (NFI) (high efficiency) (n=75) and selection line 2 were from the high NFI selection line (n=35)). Due to the design of the feed efficiency study, constraints were placed on the study described in this chapter in particular there was no control group of animals to compare the haematological data following exposure to the stressors. However, the design allowed samples to be taken from each animal prior to the application of the stressor to provide a within-animal control. These within-animal controls provided a baseline value for haptoglobin for each animal.

Prior to the commencement of the study the steers were transported from the field station at Trangie to the New South Wales Department of Primary Industry Field Station "Shannon Vale" located at Glen Innes in New South Wales (-29.44S, 151.44E) where they were grown out on pasture whilst awaiting entry into the feedlot. This present study commenced at 'Shannon Vale" the day before transportation to the feedlot.

Animals and design

The Angus steers (n=110) used were approximately 20 months of age with an average weight of 461.9kg ± s.e. 3.8kg. To evaluate the stress of transport on the haematological variables, the steers were blood sampled on 4 occasions, the day prior to transportation, immediately after transportation and on days 6 and 10 (Figure 7.1). The steers were transported by truck for 90 minutes from "Shannon Vale" to "Tullimba" research feedlot owned by the University of New England, west of Armidale, New South Wales (-30.53S, 151.62E). On arrival at "Tullimba", the steers were placed on pasture and were brought into the yards each morning on the days when a blood sample was required. On completion of the transport study the steers remained at pasture for one month before entering the feedlot where their individual feed efficiency was determined.

At feedlot induction these 110 steers were bled, weighed and fat and muscle tissues were scanned by ultrasonography. Thereafter the steers were blood sampled a further three times (monthly) while in the feedlot. At the conclusion of their last set of measurements the steers underwent a muscle biopsy immediately after blood sampling on day 139. The procedure used to obtain the muscle biopsy has been reported by Gardner *et al.* (2001). Hair over the biopsy site was clipped and then the skin was surgically scrubbed using a Betadine solution, before local anaesthetic was administered to the site. A 1 cm 'stab' incision was made before the biopsy needle attached to a purpose built biopsy drill was passed into the muscle with a vacuum in place to hold the sample. The Betadine solution was used to wash the wound site before topical application of Chloromide surface disinfectant. Forty-eight hours after the biopsy was taken an additional blood sample was collected from each steer to evaluate the haematological response to the stress induced by this procedure. Figure 7.1 provides a schematic timeline of the major events that the steers underwent.

Blood collection and processing

Blood was collected from the coccygeal artery/vein using an 18 gauge; 1 inch precision glide vacutainer system needle inserted into 5mL BD Vacutainer tubes containing 0.054mL of 15% K_3EDTA. The blood samples were placed on ice until arrival at the laboratory where they were processed for routine haematology (within 7 hours of the first blood sample). The blood was rotated for 4 minutes to re-mix the blood before being analyzed on the Cell-Dyn 3500R (Abbott Diagnostics) calibrated for cattle blood. The analyser measures total and differential white cell numbers, platelets and seven red cell variables. The blood was then centrifuged at

1200g (2500 rpm) for 12 minutes at 4°C to obtain plasma samples. The plasma was then stored at -20°C until required for haptoglobin analysis.

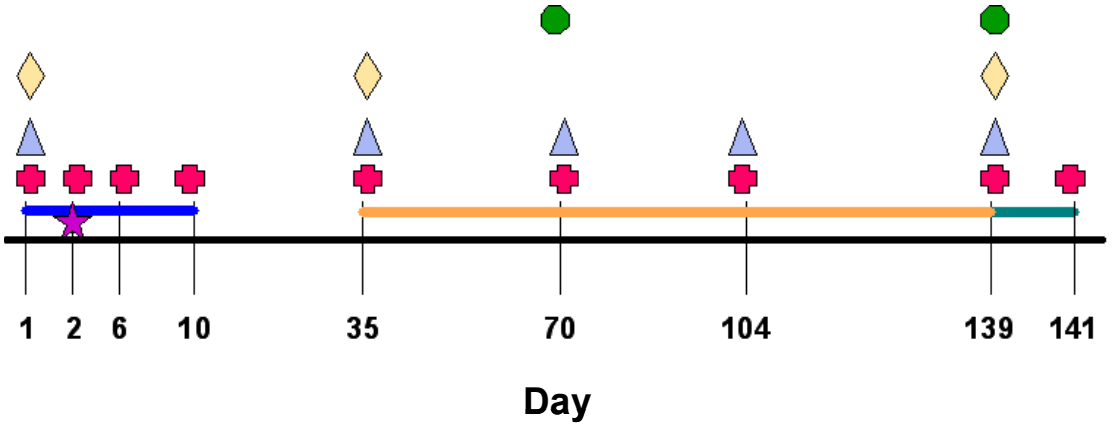

Figure 7.1: Timeline for the steers during the study. ─ Transport stress; ─ Feedlot stress; ─ Biopsy stress; * Transported; ▲ Weight recorded; ♦ Scan; + Blood sample; ● Flight time

Haptoglobin analysis was performed as outlined in Chapter 2, page 28. This method is based on the pseudo-peroxidase nature of the haemoglobin-haptoglobin complex which leads to a redox reaction between the complex and the chromogen.

Statistical analysis

The data were analysed using the statistical package Systat version 9 (SPSS INC. Chicago IL. USA) after an initial evaluation for normality, transformations were performed where necessary. Flight times, haptoglobin, basophils, eosinophils, lymphocytes and neutrophils data were natural log transformed and platelets and net-feed intake data were transformed using a cube root transformation. Outliers were identified using box plots and removed before analysing the data.

A repeated measures model with sire selection line fitted as a fixed effect was performed on all haematological data including haptoglobin to evaluate the response of each variable across time for each stressor. T-tests (paired and grouped) were then used to evaluate the effects of production stressors, with the paired t-tests used to identify how the variable changed across time and the grouped t-test used to identify if the paternal selection lines differed significantly in means for each variable. A Pearson's correlation analysis was used to determine correlations between feedlot performance indicators (average daily gain, net-feed intake, feed

conversion ratio, weight and fat coverage), and haptoglobin immediately following transport (day 2) and on day 6.

7.3 Results

All haematological variables measured changed throughout the course of the study (Figure 7.2). Transportation led to a significant ($P<0.05$) increase in total white blood cell count, neutrophils, red blood cells, haemoglobin, haematocrit and platelets immediately following transportation but all measures, with the exception of platelets, began decreasing through days 6 to 10. Lymphocytes, basophils, eosinophils and mean cell haemoglobin concentration declined significantly ($P<0.05$) after transportation. Elevation of haptoglobin was detected four days after transport and remained elevated at day10. Transport had no immediate effect on monocyte count although an increase was detected between day 6 and day 10.

Intensive management (feedlotting) of the steers led to a significant ($P<0.05$) increase in basophils, mean cell volume, mean cell haemoglobin; mean cell haemoglobin concentration and haptoglobin one month after being introduced to the feedlot and remained elevated above this baseline value (at the subsequent sampling points) whilst the animals were in the feedlot environment (Figure 7.2). A significant ($P<0.05$) decrease was found in total white blood cell count, lymphocytes, monocytes, red blood cells, haematocrit and platelets one month after feedlot induction (Figure 7.2).

The muscle biopsy led to a significant ($P<0.05$) increase in total white blood cell count, neutrophils, lymphocytes, monocytes, mean cell volume, mean cell haemoglobin and a significant decrease in basophils. There was no significant change in haematocrit, eosinophils, haemoglobin, mean cell haemoglobin concentration or haptoglobin concentration (Figure 7.2).

Comparison of the sire selection lines showed significant differences in mean cell haemoglobin, mean cell volume and red blood cells (except at day 6 and 10) (Table 7.2) with the low NFI selection line having a greater response for mean cell volume and mean cell haemoglobin. The reverse was true for the red blood cells with the high NFI selection line having the higher concentration. Selection line had a significant influence on haptoglobin at day 2 and day 104 (Table 7.2) but not in a consistent direction.

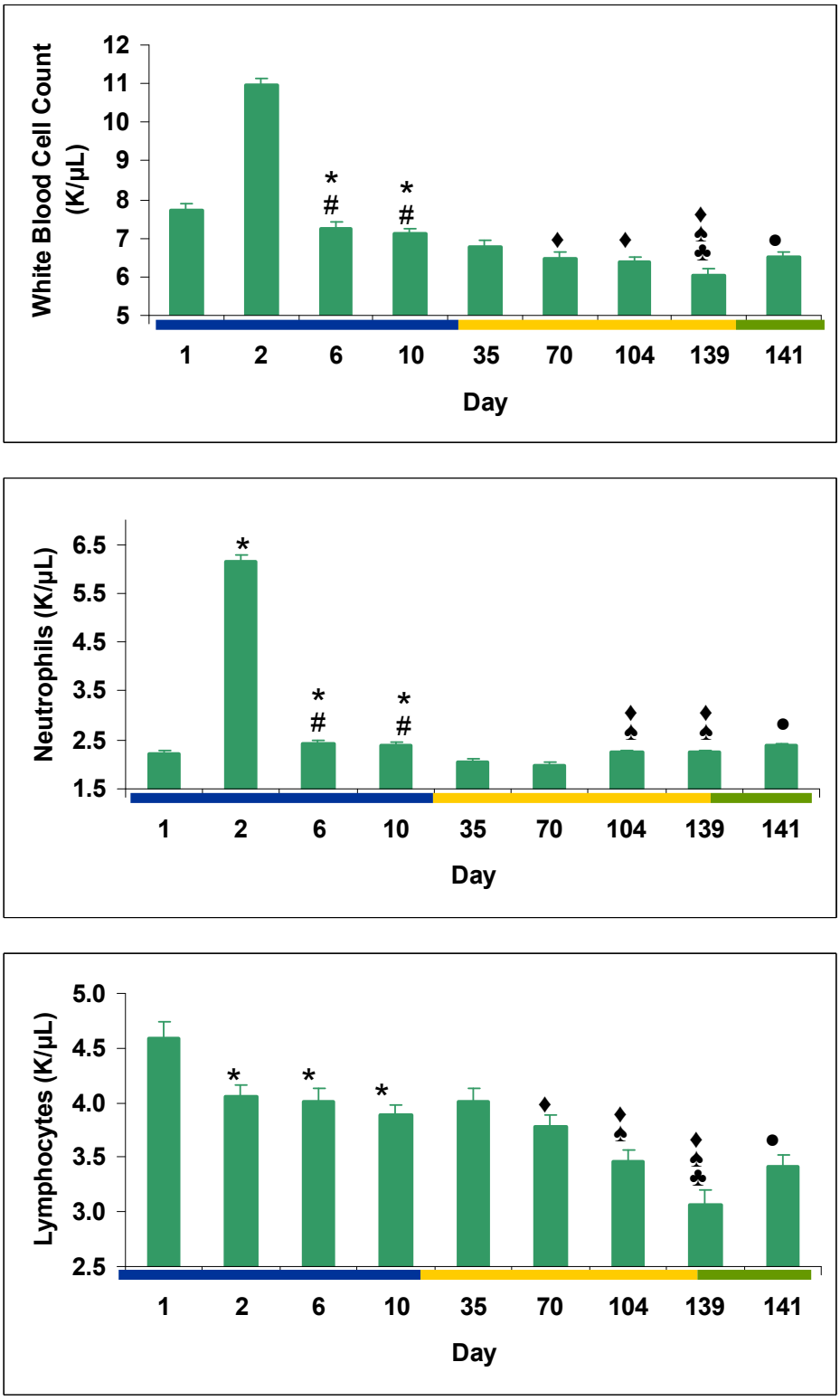

Figure 7.2: Haematological variables and the average response to the production stressors (mean ± se). Duration of each stressor: – Transport stressor**;** – Intensive management**;** – Biopsy

Transport stress (paired t-test): * significantly different (P<0.05) from day 1: # significantly different (P<0.05) from day 2; & significantly different (P<0.05) from day 6; Intensive management stress (paired t-test): ♦significantly different (P<0.05) from day 35; ♠ significantly different (P<0.05) from day 70; ♣ significantly different (P<0.05) from day 104; Muscle biopsy (paired t-test): ● significantly different (P<0.05) from day 139.

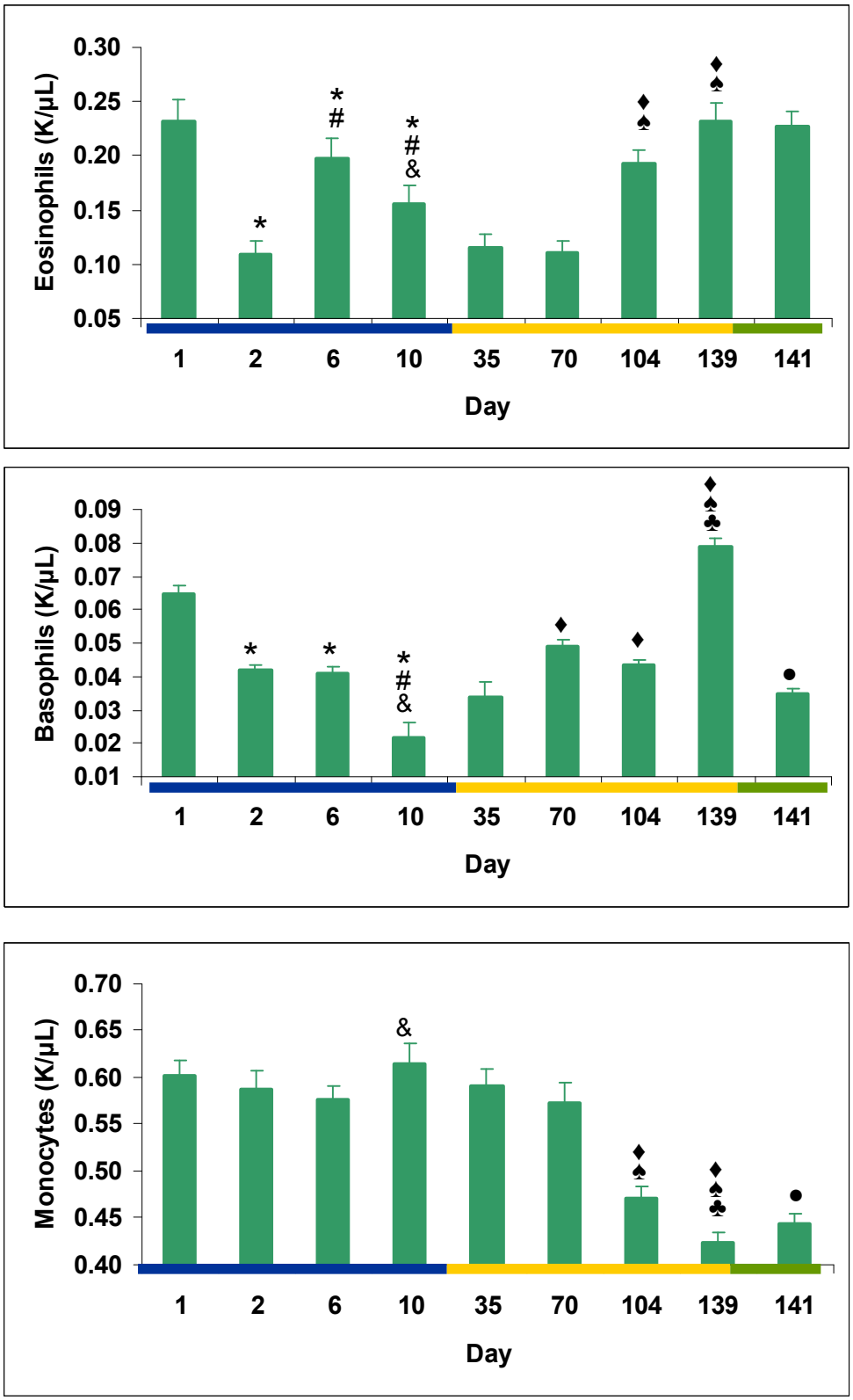

Figure 7.2 continued: Haematological variables and the average response to the production stressors (mean ± se). Duration of each stressor: – Transport stressor**;** – Intensive management**;** – Biopsy

Transport stress (paired t-test): * significantly different (P<0.05) from day 1: # significantly different (P<0.05) from day 2; & significantly different (P<0.05) from day 6; Intensive management stress (paired t-test): ♦significantly different (P<0.05) from day 35; ♠ significantly different (P<0.05) from day 70; ♣ significantly different (P<0.05) from day 104; Muscle biopsy (paired t-test): ● significantly different (P<0.05) from day 139.

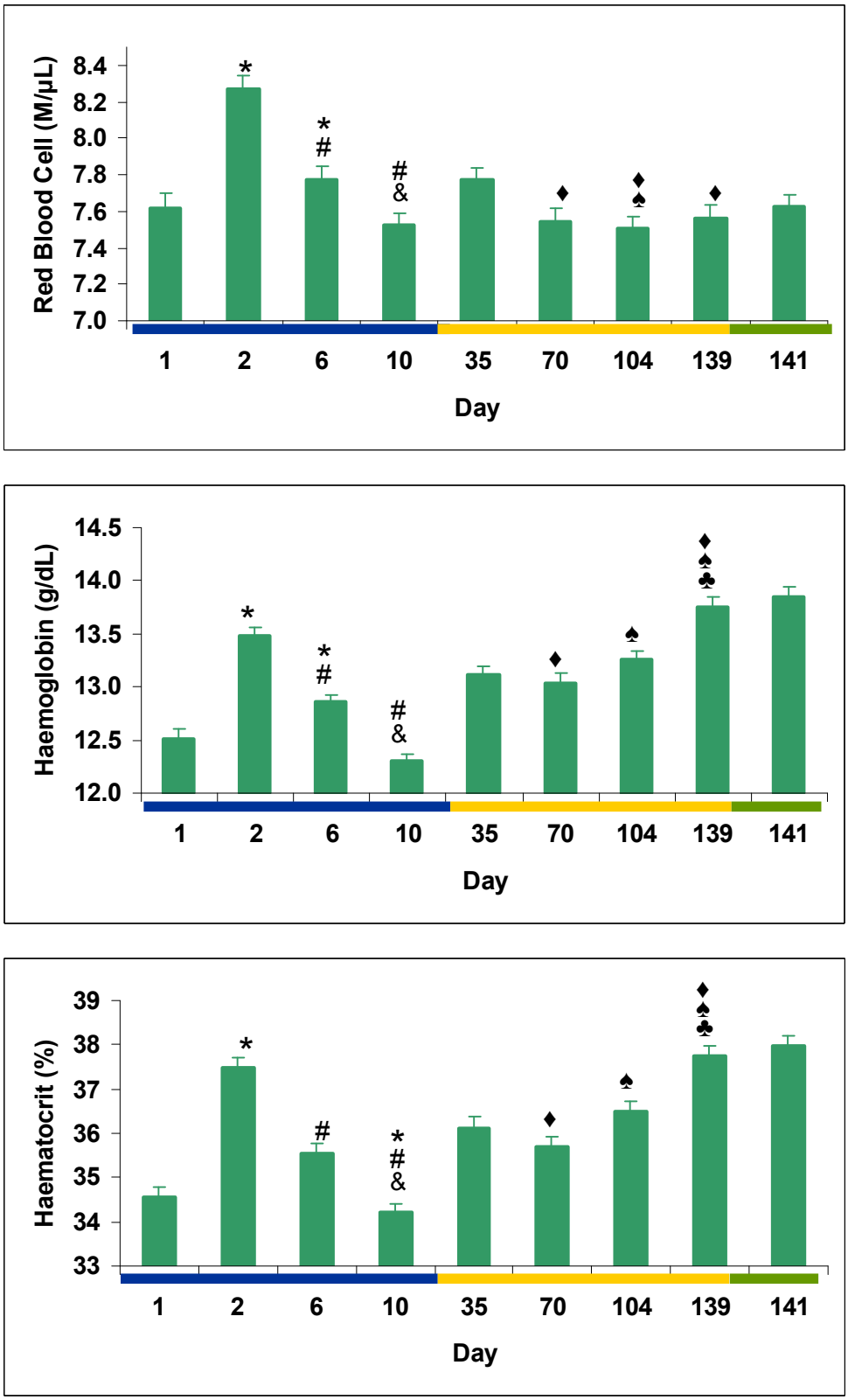

Figure 7.2 continued: Haematological variables and the average response to the production stressors (mean ± se). Duration of each stressor: – Transport stressor**;** – Intensive management**;** – Biopsy

Transport stress (paired t-test): * significantly different (P<0.05) from day 1: # significantly different (P<0.05) from day 2; & significantly different (P<0.05) from day 6; Intensive management stress (paired t-test): ♦significantly different (P<0.05) from day 35; ♠ significantly different (P<0.05) from day 70; ♣ significantly different (P<0.05) from day 104; Muscle biopsy (paired t-test): ● significantly different (P<0.05) from day 139.

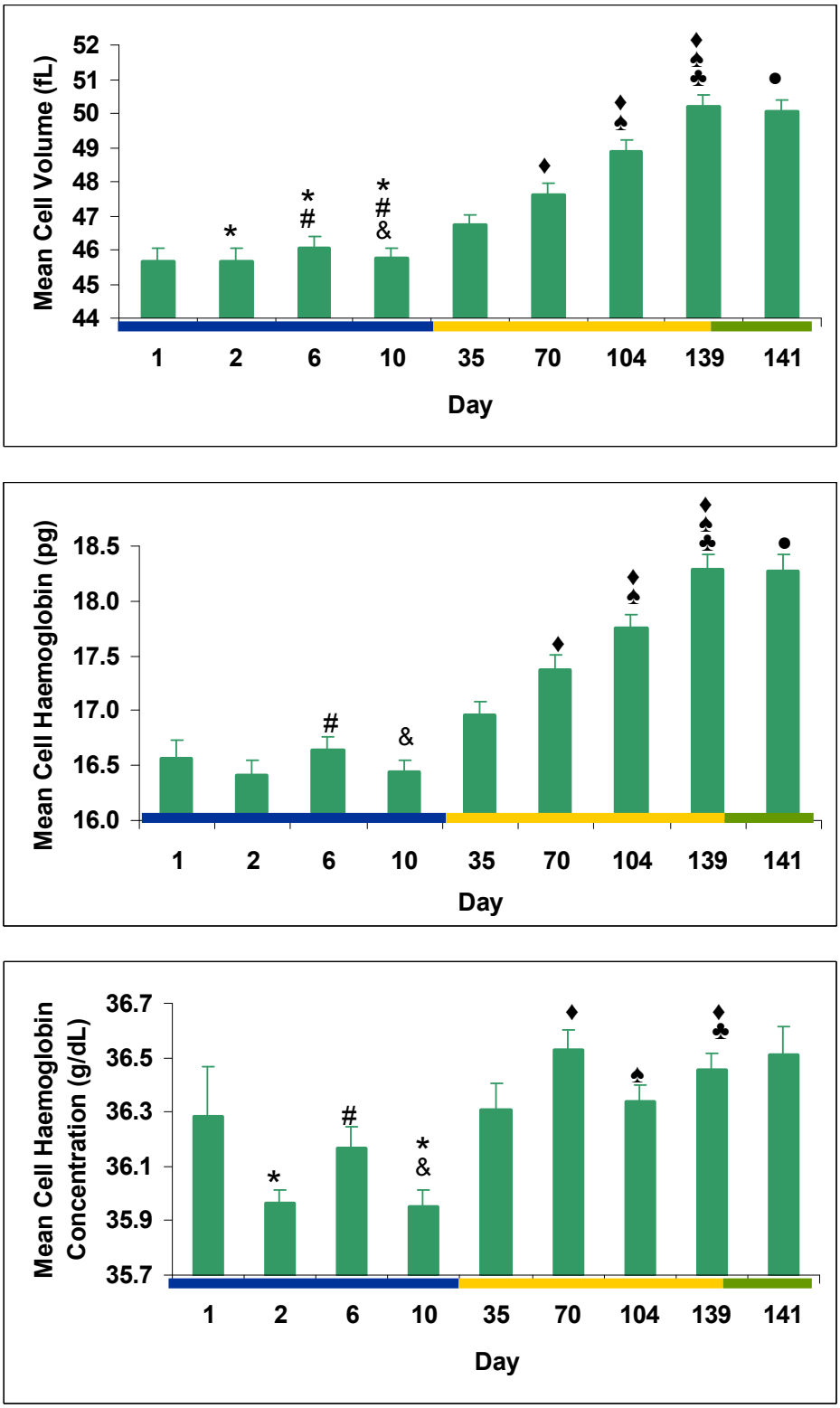

Figure 7.2 continued: Haematological variables and the average response to the production stressors (mean ± se). Duration of each stressor: – Transport stressor**;** – Intensive management**;** – Biopsy

Transport stress (paired t-test): * significantly different (P<0.05) from day 1: # significantly different (P<0.05) from day 2; & significantly different (P<0.05) from day 6; Intensive management stress (paired t-test): ♦significantly different (P<0.05) from day 35; ♠ significantly different (P<0.05) from day 70; ♣ significantly different (P<0.05) from day 104; Muscle biopsy (paired t-test): ● significantly different (P<0.05) from day 139.

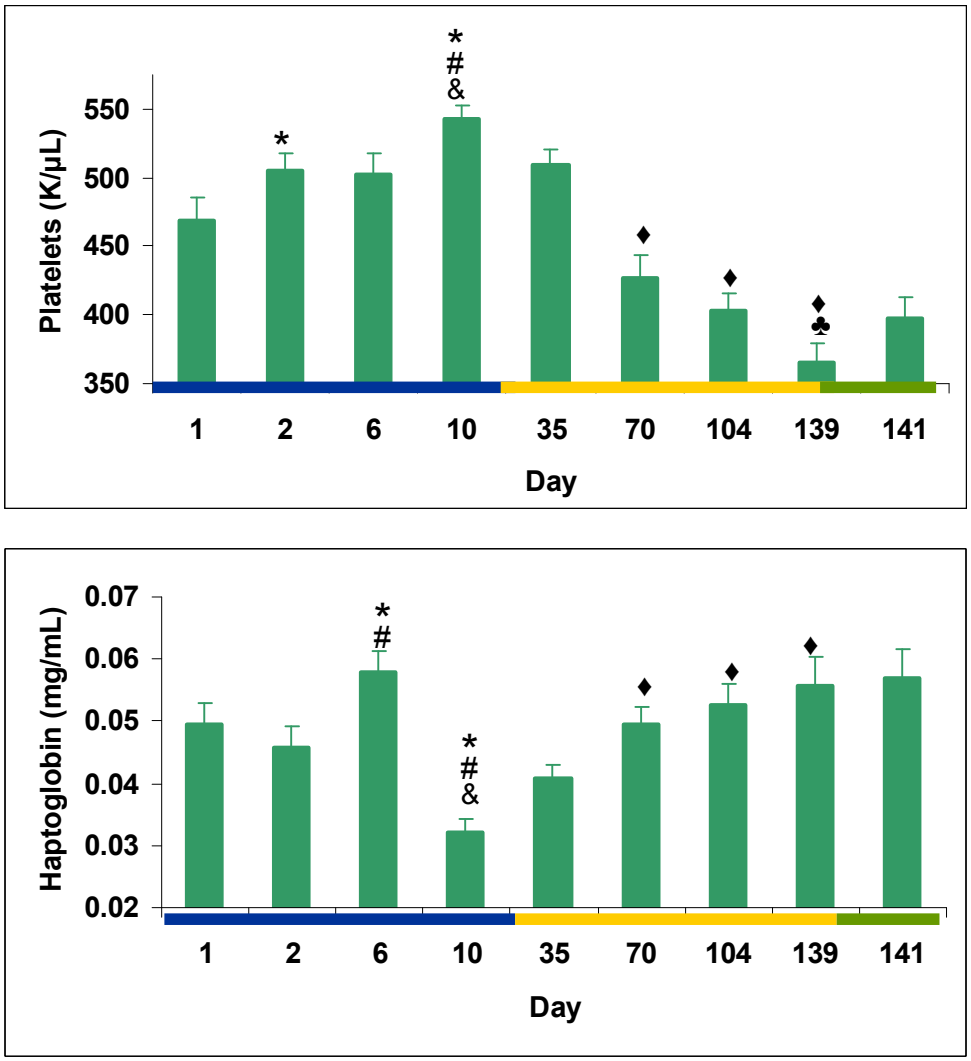

Figure 7.2 continued: Haematological variables and the average response to the production stressors (mean ± se). Duration of each stressor: – Transport stressor**;** – Intensive management**;** – Biopsy
Transport stress (paired t-test): * significantly different (P<0.05) from day 1: # significantly different (P<0.05) from day 2; & significantly different (P<0.05) from day 6; Intensive management stress (paired t-test): ♦significantly different (P<0.05) from day 35; ♠ significantly different (P<0.05) from day 70; ♣ significantly different (P<0.05) from day 104; Muscle biopsy (paired t-test): ● significantly different (P<0.05) from day 139.

Haptoglobin concentrations determined from blood collected on day 2 were found to have a strong association with average daily gain (r= -0,381); net feed intake (r=0.594) and feed conversion rate (r=0.545) whilst measured in the feedlot. The haptoglobin concentrations measured on day 6 had strong correlation with average daily gain (r=-0.445), P8 fat (day 139) (r=0.356) and feed conversion rate (r=0.391).

Transportation is likely to result in both physical and psychological stress and therefore it is not possible to ascertain whether the increase in haptoglobin was a result of psychological stress. However, Table 7.3 shows that in 21 animals there was an increase in haptoglobin

concentration following transit that exceeded 0.1mg/mL. The haemograms of 20 of the21 steers was considered to be in the "normal" physiological range for cattle.

Four of the 21 steers had haptoglobin concentrations greater than 0.1 mg/mL whilst being intensively managed. An additional 18 animals had haptoglobin concentrations greater than 0.1 mg/mL (Table 7.4) while in the feedlot. All of these steers had white blood cell counts that were within the normal physiological range for cattle (Jain, 1993), thus these animals did not have haematological values indicative of an immune response at the time of blood collection.

Following the muscle biopsy, there were eight steers that had haptoglobin concentrations greater than 0.1 mg/mL (Table 7.5). Their total white and differential blood cell count were considered to be in the normal physiological range of cattle.

Table 7.2: Haematological variables that significantly differed between sire lines.

Variable	Stressor	Day	Low NFI Sires mean ± se	High NFI Sires mean ± se	t-value	P-value
Haptoglobin	Transport	2	0.041 ± 0.002	0.056 ± 0.009	-2.578	0.013
	Feedlot	104	0.059 ± 0.005	0.041 ± 0.002	2.796	0.007
Mean Corpuscular Haemoglobin	Transport	1	16.336 ± 0.205	17.105 ± 0.210	-3.029	0.003
		2	16.134 ± 0.159	17.019 ± 0.212	-3.338	0.001
		6	16.368 ± 0.154	17.226 ± 0.200	-3.403	0.001
		10	16.189 ± 0.136	16.891 ± 0.187	-2.907	0.005
	Feedlot	35	16.560 ± 0.126	17.618 ± 0.250	-3.711	0.000
		70	16.930 ± 0.134	18.240 ± 0.215	-4.797	0.000
		104	17.368 ± 0.135	18.507 ± 0.198	-4.338	0.000
		139	17.855 ± 0.140	19.194 ± 0.233	-4.469	0.000
	Biopsy	141	17.807 ± 0.135	19.219 ± 0.289	-4.214	0.000
Mean Corpuscular Volume	Transport	1	45.059 ± 0.480	47.132 ±0.565	-2.675	0.009
		2	44.882 ± 0.466	47.323 ± 0.580	-3.272	0.002
		6	45.336 ± 0.455	47.550 ± 0.581	-3.001	0.004
		10	45.058 ± 0.403	46.982 ± 0.526	-2.774	0.007
	Feedlot	35	45.885 ± 0.377	48.232 ± 0.517	-3.499	0.001
		70	46.417 ± 0.368	49.845 ± 0.630	-4.359	0.000
		104	47.791 ± 0.377	50.984 ± 0.592	-4.156	0.000
		139	49.068 ± 0.385	52.464 ± 0.634	-4.148	0.000
	Biopsy	141	48.945 ± 0.389	52.294 ± 0.633	-4.143	0.000
Platelets	Transport	2	521.257 ± 15.622	472.371 ± 20.278	2.016	0.048
Red Blood cells	Transport	1	7.729 ± 0.101	7.359 ± 0.130	2.136	0.036
		2	8.395 ± 0.098	7.997 ± 0.120	2557	0.012
		6	7.864 ± 0.875	7.574 ± 0.116	1986	*0.051*
		10	7.616 ± 0.080	7.346 ± 0.115	1.844	*0.069*
	Feedlot	35	7.896 ± 0.082	7.541 ±0.116	2.385	0.020
		70	7.735 ±0.082	7.171 ± 0.130	3.399	0.001
		104	7.656 ± 0.081	7.206 ± 0.107	3.059	0.003
		139	7.733 ± 0.082	7.217 ± 0.121	3.181	0.002
	Biopsy	141	7.779 ± 0.073	7.311 ± 0.126	2.944	0.005

Table 7.3: Individual steers with high haptoglobin concentrations following transportation with corresponding total white blood cell count and neutrophil and lymphocyte counts.

Tag	Sire Line	Haptoglobin (mg/mL)				Total White Blood Cell Count ($\times 10^3/\mu L$)				Neutrophil count ($\times 10^3/\mu L$)				Lymphocyte Count ($\times 10^3/\mu L$)			
		Day 1	Day 2	Day 6	Day 10	Day 1	Day 2	Day 6	Day 10	Day 1	Day 2	Day 6	Day 10	Day 1	Day 2	Day 6	Day 10
113	1	**0.101**	0.050	**0.117**	na	6.58	8.82	6.05	na	2.23	**4.68**	2.07	na	3.58	3.50	3.20	na
128	1	0.045	**0.150**	0.055	na	8.36	11.98	8.04	na	2.86	**7.66**	3.18	na	3.94	3.01	3.71	na
152	1	0.032	0.039	**0.236**	0.018	7.69	11.00	5.98	7.90	3.14	**6.74**	2.55	2.80	4.07	3.75	2.89	4.00
154	*1*	*0.044*	***0.142***	*0.054*	*0.028*	*8.88*	*10.47*	*8.06*	*7.50*	*1.44*	***5.26***	*1.68*	*1.90*	*6.57*	*4.29*	*5.79*	*4.90*
237	1	0.035	**0.248**	0.043	0.007	5.83	8.19	5.71	6.30	2.04	**4.78**	1.79	2.00	3.10	2.51	3.35	3.60
242	*1*	*0.028*	***0.219***	***0.100***	*0.033*	*7.98*	*6.67*	*6.13*	*6.50*	*1.94*	*3.85*	*2.07*	*2.00*	*5.28*	*2.23*	*3.31*	*3.70*
265	1	**0.128**	0.038	**0.141**	0.018	4.99	11.73	6.45	7.00	1.81	**6.49**	3.14	2.70	2.27	4.59	2.33	3.40
317	*1*	***0.829***	***0.222***	***0.135***	*0.043*	*8.65*	*9.70*	*6.34*	*7.60*	*2.47*	***4.61***	*1.98*	*2.50*	*4.64*	*3.87*	*3.49*	*3.50*
322	1	0.068	0.054	**0.130**	0.025	5.57	9.00	6.15	6.50	1.72	**4.16**	2.04	1.90	3.23	4.30	3.46	4.10
356	1	0.056	0.026	**0.149**	na	8.27	12.09	7.94	na	2.32	**6.33**	2.87	na	4.76	4.91	4.13	na
379	*1*	*0.038*	*0.049*	***0.127***	*0.028*	*5.65*	*9.20*	*5.5*	*4.90*	*0.88*	***6.28***	*1.81*	*1.90*	*4.27*	*2.24*	*3.24*	*2.60*
469	*1*	*0.043*	*0.043*	***0.120***	*0.026*	*5.59*	*7.60*	*3.14*	*5.60*	*1.19*	***4.43***	*1.13*	*1.70*	*3.72*	*2.55*	*1.59*	*3.40*
45	2	0.034	**0.126**	0.028	**0.147**	8.3	11.27	8.37	4.50	2.55	**7.34**	3.3	2.10	4.68	2.90	3.89	1.60
93	2	0.062	0.034	**0.133**	0.034	6.26	10.11	5.37	6.80	1.99	**5.91**	1.96	2.60	3.52	3.78	2.72	3.60
151	2	0.027	0.065	**0.147**	0.048	7.37	9.21	5.76	5.40	1.73	**5.51**	1.9	1.90	5.16	3.05	3.10	2.90
181	2	0.060	**0.351**	-0.009	0.030	5.53	9.04	5.94	8.40	2.63	**6.19**	2.59	2.90	2.07	2.06	2.66	4.40
214	2	0.054	0.060	**0.172**	0.013	8.07	8.48	8.30	7.30	2.77	**4.76**	3.25	2.10	4.35	2.86	3.91	4.60
276	2	0.055	**0.128**	0.068	0.027	8.62	9.26	8.19	7.70	2.56	**5.39**	2.58	2.50	5.04	3.30	4.71	4.30
294	2	0.026	0.065	**0.565**	**0.148**	7.49	9.14	6.02	7.00	1.27	**4.87**	1.79	1.70	5.49	3.94	3.66	5.00
360	2	0.037	0.033	0.052	**2.610**	5.6	11.09	5.81	7.30	1.81	**7.33**	2.4	3.50	2.74	2.35	2.61	2.40
394	2	0.055	**0.239**	0.082	0.019	na	**14.85**	7.98	10.00	na	**9.00**	2.92	2.90	na	4.36	3.84	5.90

The text in bold are considered to be outside the "normal" range for healthy cattle. Text in italics are animals who have haptoglobin concentrations >0.1mg/mL during feedlot finishing and/or following the muscle biopsy. Sire Line 1 = high efficiency line and Sire Line 2 = low efficiency line

Table 7.4: Individual steers with high haptoglobin concentrations undergoing intensive management with corresponding total white blood cell count and neutrophil and lymphocyte counts.

Tag	Sire line	Haptoglobin (mg/mL)				Total White Blood Cell Count ($\times 10^3/\mu L$)				Neutrophil Count ($\times 10^3/\mu L$)				Lymphocyte Count ($\times 10^3/\mu L$)			
		Day 35	Day 70	Day 104	Day 139	Day 35	Day 70	Day 104	Day 139	Day 35	Day 70	Day 104	Day 139	Day 35	Day 70	Day 104	Day 139
19	1	0.033	0.021	0.050	**0.137**	5.50	5.12	4.84	6.13	2.00	1.57	1.89	1.57	3.10	3.12	2.41	3.90
40	*1*	*0.026*	*0.036*	*0.053*	***0.134***	*5.80*	*5.15*	*4.59*	*4.11*	*1.80*	*1.33*	*1.72*	*1.80*	*3.30*	*3.24*	*2.20*	*1.56*
51	1	**0.107**	0.049	**0.853**	0.043	na	6.03	7.08	6.50	na	1.06	3.23	1.77	na	4.39	3.11	4.09
107	1	0.028	0.034	0.043	**0.123**	6.40	7.79	7.61	8.92	2.10	3.30	2.87	2.10	3.70	3.60	4.07	5.98
154	*1*	*0.020*	*0.052*	***0.213***	*0.063*	*6.30*	*7.75*	*7.61*	*5.24*	*1.10*	*3.04*	*2.65*	*2.44*	*4.70*	*4.04*	*4.09*	*2.28*
227	1	0.034	0.035	**0.249**	0.056	8.60	8.01		9.16	1.90	2.05		2.36	5.90	5.23	na	6.02
242	*1*	*0.035*	*0.054*	***0.249***	*0.033*	*8.50*	*7.90*	*5.84*	*5.43*	*2.00*	*1.58*	*2.11*	*1.67*	*5.90*	*5.67*	*2.94*	*2.87*
264	1	0.032	**0.261**	0.036	0.029	7.50	5.63	5.63	5.80	2.60	1.47	1.89	2.49	4.10	3.35	2.88	2.34
277	1	0.017	0.038	**0.764**	0.051	5.10	4.10	5.89	4.21	2.10	0.93	2.10	1.93	2.60	2.07	3.03	1.10
317	*1*	*0.032*	***0.177***	*0.047*	*0.042*	*8.60*	*6.43*	*7.83*	*8.73*	*1.80*	*2.05*	*2.30*	*2.17*	*5.90*	*3.31*	*4.78*	*5.82*
350	*1*	*0.039*	*0.032*	***0.121***	***0.136***	*7.40*	*7.56*	*5.67*	*4.80*	*2.00*	*2.03*	*1.93*	*2.06*	*4.80*	*4.89*	*3.09*	*2.25*
368	*1*	*0.068*	*0.046*	*0.067*	***0.140***	*5.70*	*6.19*	*5.80*	*4.64*	*1.60*	*1.93*	*2.15*	*1.57*	*3.40*	*3.45*	*3.05*	*2.35*
379	*1*	*0.031*	***0.160***	*0.057*	*0.032*	*4.20*	*na*	*3.45*	*5.50*	*1.40*	*na*	*1.34*	*1.78*	*2.10*	*na*	*1.71*	*3.24*
390	1	0.020	0.040	**0.154**	0.026	7.40	7.10	6.62	5.25	1.80	1.68	2.26	2.17	5.10	4.93	3.58	2.49
393	*1*	*0.051*	***0.163***	*0.042*	*0.034*	*4.10*	*na*	*5.42*	*4.46*	*1.50*	*na*	*1.87*	*1.58*	*2.00*	*na*	*2.84*	*2.16*
398	1	0.036	**0.238**	0.050	0.040	8.30	6.92	8.09	5.96	1.80	2.15	2.14	2.35	5.90	4.1	5.38	2.74
413	1	0.049	0.034	**0.130**	0.055	6.00	6.12	7.12	6.42	2.20	1.91	3.29	3.53	2.80	2.33	3.00	2.09
42	2	0.038	0.033	0.034	**0.418**	9.10	9.84	10.43	7.87	2.70	2.38	2.96	3.14	5.30	6.20	6.33	3.15
76	2	0.063	0.046	0.058	**0.232**	10.10	8.83	8.18	9.26	2.40	2.07	1.79	3.55	6.90	5.84	5.63	4.64
88	2	0.037	0.051	0.044	**0.165**	7.40	7.56	7.22	4.81	1.50	1.72	2.64	2.20	5.30	5.00	3.73	2.21
202	2	0.043	**0.165**	0.048	0.076	7.10	5.54	7.01	5.18	2.30	1.96	2.44	2.41	3.90	2.73	3.81	1.88
295	2	0.018	**0.111**	0.028	0.079	10.30	10.76	10.32	7.27	2.30	2.37	2.71	2.34	7.10	7.55	6.81	3.93

The text in old are considered to be outside the "normal" range for healthy cattle. Text in italics are animals who have haptoglobin concentrations >0.1mg/mL during transport and/or following the muscle biopsy. Sire Line 1 = high efficiency line and Sire Line 2 = low efficiency line

Table 7.5: Individual steers with high haptoglobin concentrations following a muscle biopsy with corresponding total white blood cell count and neutrophil and lymphocyte counts.

Tag	Sire line	Haptoglobin (mg/mL)		Total White Blood Cell Count ($\times 10^3/\mu L$)		Neutrophil Count ($\times 10^3/\mu L$)		Lymphocyte Count ($\times 10^3/\mu L$)	
		Day 139	Day 141	Day 139	Day 141	Day 139	Day 141	Day 139	Day 141
51	1	0.043	**0.143**	6.50	4.46	1.77	1.74	4.09	1.99
102	1	0.032	**0.116**	5.56	6	3.13	3.31	1.94	2.02
149	1	na	**0.483**	na	6.55	na	2.66	na	3.52
183	1	0.040	**0.221**	5.20	5.72	2.82	2.58	1.81	2.48
190	1	0.093	**0.179**	6.55	5.01	2.20	2.29	3.74	2.12
195	1	0.063	**0.263**	4.74	5.17	1.85	1.96	2.25	2.61
368	*1*	***0.140***	***0.472***	*4.64*	*5.22*	*1.57*	*1.89*	*2.35*	*2.6*
469	*1*	*0.050*	***0.137***	*4.67*	*3.13*	*1.53*	*1.31*	*2.73*	*1.26*

The text in bold are considered to be outside the "normal" range for healthy cattle. Text in italics are animals who have haptoglobin concentrations >0.1mg/mL during transport and/or feedlot finishing. Sire Line 1 = high efficiency line and Sire Line 2 = low efficiency line

7.4 Discussion

Stress can cause physiological and behavioural changes in an animal and understanding and measuring these is important in determining the impact a stressor may have on an animal. At the time of this study little was known of the impact that psychological stressors would have on blood variables including haptoglobin concentrations in cattle. This study evaluated haptoglobin in addition to a range of haematological changes in cattle undergoing transportation and intensive management (feedlot) and additional measurements were also undertaken following a muscle biopsy to measure the response of haptoglobin to local surgical trauma of a tissue. It was therefore hypothesised, firstly, haptoglobin concentrations increase following transportation and after a muscle biopsy; and secondly, that haptoglobin concentrations can be used as an indicator for the longer term adaptation to intensive management.

The first hypothesis, that haptoglobin concentrations increase following transportation and after a muscle biopsy were addressed in two parts. In accord with the findings of a number of other studies, haptoglobin concentrations were elevated 5 days post transport (Day 6) and decreased to pre trial concentrations by Day 10. For instance, Earley *et al.* (2004a) reported a significant increase in haptoglobin concentrations for 6 days following transport. It is possible that a similar duration of elevated haptoglobin concentrations occurred in the current study; however, samples were not as frequently taken with samples being collected only immediately following transportation and five (day 6) and nine (day 10) days post transportation. It is noteworthy that approximately 12 months prior to this current study, the steers had been transported for a longer duration and it is possible that the previous transport

experience may have reduced the intensity and duration of the stress response to the current transport challenge.

The second part of the first hypothesis, that an increase in haptoglobin will be associated with a muscle biopsy is possible. Although an increase in haptoglobin was detected 48 hours following the biopsy it was not significant. It is clear from the literature that the haptoglobin expression for an inflammatory response is dependent on the size of the inflammation (based on turpentine injection (Conner *et al.,* 1988)); that is the larger the lesion the greater the haptoglobin response. These findings also support the findings in Chapter 6 where the calves in the first experiment were electronically ear-tagged at the start of the study and had slightly elevated haptoglobin concentrations.

The second hypothesis examines whether haptoglobin concentrations provide an indication of adaptation to the feedlot. We can examine this possibility two ways. Firstly, does the sensitivity of animals to the stress of transport provide a predictor of their sensitivity to the stressors associated with the feedlot environment, which in turn affects their performance. The correlations of performance indicators with haptoglobin concentrations on day 6 do not impact on the over all weight gain as there was no correlation with this variable. However, haptoglobin is negatively associated with average daily gain and positively associated with P8 fat coverage measured on day 139. Average daily gain was also negatively correlated with haptoglobin concentrations immediately following transport. These correlations would suggest that haptoglobin following transport has the potential to be used as an indicator of the average daily gain of beef cattle whilst in the feedlot and is worthy of further investigation. An ability to predict the average daily gain of individual cattle might enable their growth to be better targets to specific market specifications.

Secondly, are haptoglobin concentrations during feedlot finishing associated with performance. The reason for addressing this question is that Eurell *et al.* (1992) showed that haptoglobin concentrations could be used to predict how pigs will grow. The results from this study would indicate that haptoglobin concentrations whilst in the feedlot are not a good indicator of how animals will perform as the haptoglobin concentrations continued to increase whilst in the feedlot, thus suggesting that these animals might not be adapting to the feedlot. Further examination of the haematological data indicates that haemoglobin concentrations increased significantly whilst these cattle were in the feedlot due principally to an increase in

mean cell haemoglobin concentration. Given that the role of haptoglobin is to bind to free haemoglobin than it is possible that the increase in haptoglobin is due to the increase in mean cell haemoglobin concentration. The question then arises as to why there is an increase in red blood cell numbers during feedlot finishing. Personal observations of sheep housed in an animal house and fed a pelleted feed mix with chaff increased their red blood cell numbers over time. This raises the question as to whether or not the increase in red blood cell numbers and the increase in haptoglobin concentrations, whilst these steers were in the feedlot is a result of dietary intake rather than stress. Further studies need to be undertaken before this hypothesis can be accepted or rejected. This could be addressed by measuring the haptoglobin concentrations pre and post a red blood cell transfusion to determine if an increase in red blood cell numbers leads to an increase in haptoglobin in the absence of stress.

This study also confirmed that transportation results in an immediate increase in neutrophil count that returns to normal at most by day 6. This increase is most likely due to the effects of glucocorticoids on the marginal neutrophil pool as neutrophils provide the first line of defence against bacterial attack on the host (Vincent, 1974). The dramatic increase in neutrophils possibly gives the cattle a better chance to fight off infection in the post transport period. A similar short term response has been previously reported by Earley *et al.* (2004a). If this rapid increase in neutrophils is a repeatable response to transport stress it maybe a useful measure of the disease susceptibility of animals exposed to transport.

The remaining white blood cell variables decreased immediately after transportation, with the exception of total white blood cell count which increased in line with the neutrophilia as was also reported by Earley *et al.,* 2004a and Earley *et al.,* 2004b.

The majority of red blood cell variables increased immediately after transportation (with the exception of mean cell haemoglobin concentration which decreased and mean cell haemoglobin which did not differ significantly after transport). Similar changes were also reported by Earley *et al*. (2004a). There are several possible reasons for this increase in the red blood cell variables. Firstly, dehydration would increase the number of red blood cells per unit volume of blood, secondly splenic contraction would cause a release of red blood cells from the marginal pool, and thirdly, an increase in red blood cells and haemoglobin may have resulted from homeostatic responses driven by altered blood chemistry caused by shallow fast breathing, which may hinder the exchange of carbon dioxide with oxygen. Although the red

blood cell numbers had declined by day 6 they were still elevated to a degree and therefore it is unlikely that the increase in red blood cells was due to dehydration, as the haematocrit concentrations of these steers were still considered to be in the normal range; but, was more probably a lingering consequence of splenic contraction. By day 10, concentrations had returned to pre-transport counts.

Intensive penning in the feedlot environment is thought to be potentially stressful due to increased social interactions (Bøe and Færevik, 2003; Gupta *et al.,* 2005) and competition for feed, water, and rest spaces. Such increases in population density could possibly lead to an increased risk of disease outbreak due to the close confines that the animals are in. The haptoglobin values significantly increased during the feedlot stage of this study but were still considered to be within an acceptable range for the majority of the animals (<0.1 mg/ml, *Tridelta*). This increase in haptoglobin also corresponds to an increase in haemoglobin. Several animals at different time points reported an increase in haptoglobin expression beyond 0.1mg/mL with the highest value obtained for one steer being 0.418mg/mL.

These steers were progeny of selection lines bred for high and low net feed efficiency. The associations between haematological variables and net feed efficiency have been previously examined in these selection lines (Richardson *et al.,* 2002; Richardson and Herd, 2004). The current results confirm previous findings where there are significant differences in fat coverage (P8, Rib fat and intra-muscular), feed intake, red blood cell counts, mean cell haemoglobin, and mean cell volume between the selection lines. The present experiment extends those findings by showing that haptoglobin concentrations immediately following transportation were negatively correlated with average daily gain, and positively correlated with feed conversion ratio and net feed intake. Likewise there was a significant difference in red blood cell numbers with the low NFI sire selection line having a higher count. However haemoglobin concentration did not differ significantly between the two lines probably as a result of a lower mean cell volume and mean cell haemoglobin.

This study was part of a larger study that was designed to evaluate two selection lines for feed efficiency and consequently there was no control group to compare the findings of the transportation study. However, it has been well documented that haptoglobin concentrations increase following transportation (Murata and Miyamoto, 1993; Earley *et al.,* 2004a; Earley *et al.,* 2004b) and individual haptoglobin concentrations were taken prior to transit to provide a baseline to compare the values against. This study aimed to determine if the stressor of

transport could be used to indicate how animals will subsequently perform in the feedlot. If this relationship proves to be successful then it will be possible to determine the best management practice for individual animals.

In conclusion, the results of this study would suggests that transportation and feedlot finishing are mildly stressful on steers as reflected in a significant increase in haptoglobin from the baseline measures even though the mean values remained less than the minimum value that is indicative of an acute phase response (0.1mg/mL). Determining which of the stressors is more severe is difficult due to the duration of each event and in part due to not having a larger dataset. An important observation is that the increase in haptoglobin concentrations seen in the feedlot may be a physiological adaptation to the increase in mean cell haemoglobin (absolute amount of haemoglobin in the average cell) that occurs as animals gained weight. Thus haptoglobin concentrations may increase to cope with an increase in haemoglobin disposal and processing in the absence of a stress driving the process. In addition, mild tissue trauma associated with muscle biopsy did not provide a strong stimulus for haptoglobin production.

Section V: General discussion

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Chapter 8: Discussion

This thesis was designed to evaluate the impact of some management stressors on the acute phase protein haptoglobin in beef cattle as there was little information on whether non-inflammatory stressors activate a haptoglobin response. The findings suggest that:

1. Haptoglobin can not be used as a stand alone measure of stress in beef cattle; and
2. Haptoglobin has the potential to be used as an indicator of the impact of some management stressors in beef cattle, in particular weaning and transport.

As with any stress measure, haptoglobin can not be used on its own to determine if animals are stressed. The results presented in this thesis show that haptoglobin concentration can increase as a result of non-inflammatory stress and of the stressors applied only one (social re-grouping) did not result in an increase in haptoglobin although the latter is perceived by humans to be stressful. These results are in agreement with Arthington *et al.* (2003) and Gupta *et al.* (2005).

Although there was no increase in haptoglobin (or cortisol) concentration in some situations, this does not mean that the animals were not stressed. Alsemgeest *et al.* (1995) in their study which examined if acute phase proteins increased as a response to a physical stressor (flooring) showed that haptoglobin concentrations did not increase under these circumstances whereas an increase in serum amyloid-A was detected, which is a more sensitive measure of the acute phase response. Although, haptoglobin is useful for evaluating inflammatory and non-inflammatory stressors it can not be used as a stand alone measure of stress. Horadagoda *et al.* (1999) showed that haptoglobin and serum amyloid-A responses vary between individual animals for similar stressors. Therefore, what one animal perceives to be stressful may not be stressful for another animal. The difference in response between stressors is not limited to haptoglobin but also occurs for corticosteroids as Mason (1968a and 1968b) showed in rhesus monkeys. The difference in responses could be due in part to learnt behaviours implying that low stress handling of cattle may help to alleviate handling stress which could in turn reduce the impact of the stressor. Such a situation may explain why the haptoglobin concentrations for the transported steers in Chapter 7 were not as high as those previously reported by Arthington *et al.* (2003) and Earley *et al.* (2004a); as the steers had already undergone transportation 12 months prior to the study.

The results from this thesis indicate that haptoglobin has the potential to be used to measure the impact of some management stressors. Haptoglobin concentrations increased following weaning and therefore could be used in conjunction with other measures of stress to determine which weaning method has the least impact on animals and on potential performance. The calves used in Experiment 1 to evaluate the haptoglobin response following weaning (Chapter 6, page 85) were 9-10 months of age and it is likely that they had begun the natural weaning process which poses the question as to whether younger calves, if used in this study, would have had a greater haptoglobin response to the weaning process. The mean increase in haptoglobin observed here was equivalent to that of Arthington *et al.* (2005) whose calves were around the same age when weaned. Katoh *et al.* (2002) showed that feed deprivation leads to an increase in haptoglobin and if calves are weaned prior to commencement of the natural weaning process, or whilst obtaining the majority of nutrients from their mothers' milk, then it is plausible that a greater increase in haptoglobin concentration may be seen due to reduced feed intake. Further exploration on the impact that weaning has on haptoglobin concentration is required and should include age and method of weaning design components.

It is well documented that haptoglobin concentrations increase following transportation but can the haptoglobin response be used to determine the relative level of impact of longer transit journeys? Conner *et al.* (1988) have shown that the haptoglobin response is dependent on the size of the eliciting inflammation lesion, which suggests that it might also be useful to indicate relative impacts. The reason for this is that in conjunction with the findings in Chapter 7 and Earley *et al.* (2004a) would indicate that as transit journey increases from 1.5 hours (this thesis) to 23 hours (Earley *et al.,* 2004a) so to does the intensity (0.07 mg/mL compared to 2.42mg/mL) and duration (6 to 10 days compared to greater than 11 days) of the haptoglobin response. Thus further investigation is warranted.

It may be criticised that the study in Chapter 7 which evaluated the effect of transportation, intensive management and the muscle biopsy should have had a control group. However, there is enough research data available that states that haptoglobin concentrations do increase following transportation (Arthington *et al.,* 2003 and Earley *et al.,* 2004a). Having a comparison of pre and post stressor values within individuals enables the assessment of the impact of stressors on individual animals. Ideally the control group of animals should be identical in all ways possible with the exception of the treatment that is being tested.

However, no two animals are identical as it has been shown by Mason (1978) that the way in which the stress response reacts is dependent on learnt behaviour; therefore, trying to construct two groups of animals that are as similar as possible is the only option.

This study was designed to determine if the haptoglobin concentrations increased following transportation and if so does the sensitivity of an individual to the stress of transport (as determined by haptoglobin response) provide a predictor of the sensitivity to the (potential) stressors in the feedlot environment as assessed by haptoglobin, or in addition as assessed by their growth rate in view of Eurell *et al.* (1992) findings and the underlying biology of cytokines, acute phase proteins and nutrient partitioning). Therefore, having a non-transported group of animals would have provided no more information other than to say that the increase in haptoglobin was a result of transportation. These steers had a pre-transport measure and this was used as a baseline to compare the effect of transportation on haptoglobin values. For this study it would have been beneficial to have a control group of animals to compare haptoglobin concentrations with biopsied animals, as although it is likely that the increase in haptoglobin was a result of the biopsy there is some doubt as it may have been due to the stress of intensive handling. However, as alluded to in the discussion of Chapter 7 the rate of increase in haptoglobin within 48 hours is unlikely to have been to the feedlot environment.

Undertaking a comparison of blood collected at the same time point from a jugular vein and a coccygeal blood vessel revealed that the accuracy of the haptoglobin assay developed in this thesis is very susceptible to haemolysis (caused a the time of blood sampling) of cattle plasma. Having undertaken a detailed analysis of this effect it was determined that haemoglobin is the main contributor to the false positive result with some of the activity being contributed to by the endogenous peroxidase activity within the plasma. This led to the development of a correction factor which takes into account the amount of free haemoglobin within the plasma sample as well as the endogenous activity. Once the haptoglobin assay was refined it enabled analysis of the plasma samples (from the various experiments) that were collected whilst the haptoglobin assay problems were being resolved.

As a result of this thesis several areas for further investigation have been identified. These are

1. The need for a complete haptoglobin profile for cattle undergoing a non-inflammatory response.

2. Further exploration of the influence of breed on haptoglobin expression.
3. Evaluation of the various methods of weaning using haptoglobin as a measure of stress needs to be undertaken to determine the optimum method and also to determine if the age of the calves at weaning impacts on the haptoglobin response.
4. Evaluation if the haptoglobin response following transit increases with intensity and duration of the transit journey.
5. Determine if social re-grouping elicits a haptoglobin response and if this occurs during the establishment and/or maintenance phase.
6. Determine if the haptoglobin response reaches a plateau when animals are managed intensively.
7. Determine if non-stressors induce a haptoglobin response?
8. Determine how sensitive is haptoglobin is as a measure of inflammatory response? And, why was only a small non-significant increase detected following the muscle biopsy?
9. Further evaluate if haptoglobin is a good measure of psychological stress.

Each of these areas will be discussed independently to clarify the need for further studies.

A complete profile for haptoglobin during a non-inflammatory response would be beneficial to measure both the intensity and duration of the stressor. The reason for this is that the current profile for haptoglobin that was used as a reference in this thesis, to determine if the animals were undergoing an acute phase response, was developed as a result of an inflammatory response following a subcutaneous injection of turpentine (*Conner et al.,* 1988). What is clear from this thesis is that the inflammatory profile for haptoglobin is not an accurate guide for a non-inflammatory response; thus the need to develop a profile for haptoglobin following a non-inflammatory stressor.

As to whether individual haptoglobin profiles need to be developed for each non-inflammatory stress is not clear; however, they would be beneficial as the way in which animals react to the individual non-inflammatory stressors appears to differ. Whether this is due to using different groups of animals undergoing different production practices to evaluate the haptoglobin response is unclear. This may also lead to the identification of haptoglobin concentrations that indicate the presence of a non-inflammatory stressor; that is it may be less than 0.1mg/mL. From the results presented in this thesis and those from the literature

evaluating the impact of similar stressors on haptoglobin, it is apparent that the results from the feedlot and social re-grouping are similar and this also applies to a lesser extent to weaning. However, differences between studies in haptoglobin concentration following transport do occur. As already alluded to this may have been a result of the cattle used in this thesis already having had previous transport experience. The results of this study indicate that the stress imposed by these non-inflammatory management practices may not induce a haptoglobin response as is the case for inflammation. The point at which an animal is considered to be undergoing an acute phase response for haptoglobin has been previously based on the results of inflammation, however, this may need to be re-evaluated in the light of this research which implies that these non-inflammatory management practices although stressful do not lead to high concentrations of expression for haptoglobin.

The results from this thesis are not clear as to breed differences in haptoglobin responses to stress; with differences being non-significant (Chapter 6, second experiment, results not shown), borderline (Chapter 5. page 78) and significant at several time points (Chapter 7, page 114). The immune response of Angus and Simmental cattle have been shown previously to differ by Engle *et al.,* 1999 and differences between other breeds of cattle (Blecha *et al.,* 1984; Muggli *et al.,* 1987) and also breeds of pigs (Rotheschild *et al.,* 1984) have been identified. A recent study by Clapperton *et al.* (2005) reported that there was a difference in haptoglobin expression between Meishan and Large White pigs. These pigs were of similar age and were apparently healthy and not experimentally challenged throughout the study.

The study on weaning showed marked variation in haptoglobin concentrations for different treatments and it may be possible to use haptoglobin to evaluate the different methods of weaning thus determining which practice has the least impact on welfare. For this to be achieved, a profile of the haptoglobin response following weaning needs to be determined.

Having determined that haptoglobin increased as a result of transport, one question arose and that is does the intensity and duration of the haptoglobin response increase with transit times? Therefore, a complete profile of haptoglobin following different transit times needs to be undertaken.

The issue of whether haptoglobin changes as a result of social re-grouping is still open for debate. The findings of previous studies such as Arthington *et al,* (2003) and Gupta *et al,*

(2005) as well as the one presented in this thesis suggest that haptoglobin is not a sensitive measure of social instability. However, it is possible that an increase in haptoglobin did occur and was not detected as sampling did not occur daily during the establishment phase of the social hierarchy and changes were not identified. Alternatively the steers may have already established a hierarchy within each group prior to re-grouping.

Steers in the social re-grouping study showed that control steers and those mixed 4 weeks before the conclusion of the study generally had an increase in haptoglobin from day 64. When these observations are combined with those of the steers in chapter 7 (whose mean haptoglobin concentrations continued to increase whilst in the feedlot) it seems that maintenance of the hierarchy may be more stressful than the establishment phase. However, environmental conditions need to be considered as they can contribute to the increased stress levels. From these results further evaluation of the haptoglobin response during establishment and maintenance of the hierarchy need to be undertaken.

The question of whether haptoglobin concentration would continue to increase or plateau whilst in the feedlot was raised from the intensive management experiment. One would assume that the steers would habituate to the feedlot environment given that they were under feedlot conditions for over 100 days. The results presented here would indicate that the steers had not adapted to the feedlot; however, these animals appeared to be content whilst in the feedlot as flight times (a temperament measure) increased whilst the animals were in the feedlot suggesting that the animals were adapting to intensive management systems. If this is true, why the increase in haptoglobin? Observations from haemograms taken from sheep in the animal house show a trend for higher red blood cell counts which can be associated to diet; but, the haemogram results from the steers in this study do not show a similar trend although haemoglobin, mean cell volume and mean cell haemoglobin did increase. Thus, it is possible that the increase in haptoglobin was due to the increase in haemoglobin as a result of diet. If we assume that red blood cell destruction occurs at the same rate whilst in the feedlot; then if on average each red blood cell contained more haemoglobin then there would be more haemoglobin released into the blood stream following degradation of red blood cells which would elicit a haptoglobin response due to haptoglobin being the primary method for removal of free haemoglobin from circulation (Guyton, 1973). If this is the case then one would expect that there would be an increase in haptoglobin synthesis to deal with the increase in haemoglobin. Chapter 7 showed that the haptoglobin concentrations increased at each time

point whilst in the feedlot and were just higher than the "normal" physiological range for unstressed cattle which corresponded to an increase in haemoglobin.

Haptoglobin is a known indicator of an inflammatory response (*Conner et al.,* 1988) but following the muscle biopsy there was only a slight and non significant increase in mean haptoglobin concentration. There are several possible reasons for this; firstly, that the wound site was not large enough to induce a significant increase in haptoglobin. Conner and Eckersall's (1988) study on subcutaneous injection of turpentine reported that the more turpentine injected the greater the intensity and duration of the haptoglobin response. Secondly, possibly the haptoglobin peak was not identified as it may have occurred before the 48 hour sampling time point; however, given the half life of haptoglobin is 2-4 days (Jain, 1993) this would appear to be unlikely. Finally, it may also be possible that the local anaesthetic interfered with the haptoglobin response and delayed the onset of the acute phase response.

The results evaluating the haptoglobin response as a measure of psychological stress look promising (Chapter 5, page 81 and Chapter 6, Page 95). However, before any conclusions can be drawn a comprehensive study needs to be undertaken.

This thesis evaluated the haptoglobin response to several productions stressors using different groups of beef cattle. It would have been beneficial to use the same group of cattle for each production stressor but practically this was not realistic. Such a design would allow a more accurate ranking of the production stressors. However, a preliminary ranking can be made using all available information from the present studies and previous research. The order for the stressors in terms of their haptoglobin response for this thesis from the greatest to least stressful would be:

weaning > transport ≥ feedlot environment >> social re-grouping = muscle biopsy.

If we look at each of these stressors to determine whether they are acute or chronic stressors, it is necessary to consider the time frame of what constitutes a chronic stressor. Understanding this time frame and that haptoglobin is an acute phase reactant may help explain why weaning in this study is seen as the most stressful of the management practices. Weaning is a very abrupt and rapid stressor in that the calves are with their dam one moment and separated from them the next (an acute stressor). Transportation as a stressor could also

be considered acute, as it occurs for no-longer than 36 hours at any one point in time, whereas the feedlot environment would be considered to be an acute stressor that has the potential to develop into a chronic stressor if these animals do not adapt during their several months exposure to the environment. As for social re-grouping this is most likely an acute stressor under the circumstances in this thesis as the animals were defining their social structure for a minimum of one week. However, given that the feedlot environment has the potential to cause chronic stress and that those animals involved in social re-grouping were also re-grouped whilst in the feedlot then it is possible that the acute stress associated with re-grouping may alter become a chronic stressor. The muscle biopsy could be considered to be an acute stressor having been inflicted once. However, it is an invasive procedure and does result in pain and inflammation.

What has become clear from this thesis is that there is a need to develop haptoglobin profiles for non-inflammatory stress. In addition the issue of whether haptoglobin can be induced by a non-stressor needs to be evaluated. From the results in this thesis and other published work, haptoglobin appears to be an indicator of some forms of non-inflammatory stress. However, like all previously identified measures of stress it needs to be used in conjunction with other measures to ensure that the correct conclusion about the stressor (intensity, duration and impact) can be drawn.

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