Physiological, Behavioral, and Biochemical Responses to Restraint Stress in Cattle

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Biochemistry
University of Saskatchewan
Saskatoon
By

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Abstract

Stress has a significant impact on animal health and productivity and public perception of animal stress can influence industry practices. Understanding stress responses in livestock may help reduce stressful management procedures and facilitate the selection of stress-tolerant animals. In this study, behavioral responses (chute entry order, chute behavior, and exit velocity), physiological responses (serum cortisol and neutrophil to lymphocyte (N/L) ratios), and biochemical responses (global patterns of kinase-mediated phosphorylation) were evaluated in cattle (n = 20) subjected to three 5-minute restraint periods, with one week intervals. Correlations among stress responses were assessed across all animals as well as for two sub-groups (n = 4/group) selected on the basis of either high or low serum cortisol levels. Across all animals, both entry order and exit velocity were positively correlated with serum cortisol levels. However, these correlations were not consistently reproducible for the sub-groups of high and low serum cortisol responders. Kinome profiles of peripheral blood mononuclear cells (PBMCs) revealed distinct signaling events in high and low cortisol responders and these pathways were independently validated, confirming changes in glycogen metabolism and apoptosis. Levels of serum glucose, as suggested by kinome data, provided a reliable and inexpensive indicator of serum cortisol levels in healthy calves and was significantly ($p < 0.05$) correlated with other physiological and behavioral responses to restraint. Habituation or sensitization of stress responses during replicate restraint events was also evaluated across all 20 animals as well as within the high and low serum cortisol sub-groups. Serum cortisol levels displayed a pattern consistent with sensitization, while no habituation or sensitization pattern was observed for serum glucose levels, N/L ratios, or behavioral responses. Collectively, this investigation provides insight into correlations among physiological, behavioral, and biochemical responses of cattle subjected to a brief restraint. These responses may provide biomarkers for the selection of stress tolerant animals.

Acknowledgements

I would like to give my deepest thank you to my supervisor, Dr. Scott Napper for providing me such a great opportunity to study in Department of Biochemistry and VIDO. The words you said "Ask the right question in science" and "Don't walk away from the data" are still in my mind. The patience you have when going through tables and figures made a huge difference in my research progress. I also really appreciate that you talked about the sexy part of science, were passionate in teaching, raised the interest of students and the public in science, and continually me up with creative ideas. Prompt replies from to all questions were greatly appreciated. I would never have finished this thesis without your considerable support. Thank you for encouraging me to think broadly and deeply. Thank you for giving support during my courses and providing suggestions before my presentations.

I would also like to give my special thanks to my co-supervisor, Dr. Philip Griebel. I can still remember that you showed me how to make a perfect blood smear, told me how important reading literatures was. And thank you for picking blood samples and myself up in the VIDO farm and sacrificing a wonderful Friday night to stay and isolate blood cells. The passion you have for science is really impressive. Thank you for your patience in editing my writing and always asking questions as I went through my project.

Thank you Erin Scruten for performing kinome experiments for my project. Thank you Susan Cook and Endocrine lab in The Western College of Veterinary Medicine (WCVM) in University of Saskatchewan for obtaining serum cortisol levels. I would also like to thank Dr. David Janz and Lucy Kapronczai in Toxicology Centre for providing technical guides to process hair samples; Dr. Joseph Stookey and Kate Creutzinger in WCVM for providing devices to record behavioral responses. Useful suggestions from Dr. Joseph Stookey are also greatly appreciated. Also, thank you Natasa Arsic for helping isolating PBMCs, Ken Bock, Brock Evans, Jamie Kinzie, and Dr. Don Wilson in VIDO Animal Care for handling the cattle, taking blood and hair samples.

I would also like to thank Dr. Punam for giving me an interview chance before taking the biostatics course, Dr. LuKong for providing detailed explanations for the course, and Dr.

Havele keeping encouraging me in Immunology course. And my advisory committee members, Dr. Jeremy Lee, Dr. Erique LuKong, and Dr. Mirek Cygler for suggestions for my project. Thank you for my external examiner Dr. Fiona Buchanan for reviewing my thesis and providing suggestive information in my defence. It was a great time to share and discuss the importance of selecting animals and stress responses.

Colleagues and friend were appreciated. Thank you so much Pekka for providing useful suggestions when I first started and told me “never give up” in science. Thank you Brenden, Joanna, and Kristen, Akanksha for providing company. The walk to the Health Science building was not too long with the fun you made! Thank you Erin and Kim for maintaining the lab and schedule all lab events towards any celebration and giving me technical tips or holiday tips, you are the light of our lab. Thank you Sumudu, Elisa, and Rahwa, being nice officemates and shared all little details. Thank you Patricia, for showing me qRT-PCR, Español, and generously showing other lab technics. Thank you Ciel, Jennifer, Iris, Young, Cathy, Sophie, Lucian, Ivan, Shan, Kuan, Zhubing, Leo, and Mavis for your support.

Sincere thanks were given to my family and relatives. I can’t finish my thesis without emotional support from my mom. Thank you for being on my side no matter what. My dad, thank you for making me think positive at all times. My uncle and auntie, thank you so much for being careful, positive and optimistic whenever I spent my summer holidays with you.

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List of Abbreviations

ACTH	adrenocorticotropic hormone
ANOVA	Analysis of variance
AP1	activation protein 1
AVP	Arginine vasopressin
BHV-1	Bovine herpesvirus-1
BRD	Bovine respiratory disease
Brij-35	Polyoxyethylene lauryl ether
cAMP	Cyclic adenosine monophosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERKs	extracellular signal-regulated kinases
GAS	general adaptation syndrome
GLUT 4	glucose transporter type 4
GREs	glucocorticoids response elements
GRH	Corticotrophin releasing hormone
GRs	glucocorticoid receptors
GSK3A	Glycogen Synthase Kinase 3 alpha
HPA	hypothalamic-pituitary-adrenal
Hsp 90	heat shock protein 90
IFN-γ	interferon γ
IL-1	interleukin-1
IL-6	interleukin-6
IR	Immunoreactivity
IRF-3	interferon regulatory factor-3
JNKs	c-Jun NH2-terminal kinases
LPL	lipoprotein lipase
LPM	liter per minute
LPS	lipopolysaccharide
MKK1	Mitogen-activated protein kinase kinase 1
N/L	The ratios of neutrophils to lymphocytes
Na_3VO_4	sodium orthovanadate
NaF	sodium fluoride
NFκB	nuclear factor κB
NK	natural killer
NOD	nucleotide-biding oligomerization domain
PBMCs	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PIIKA 2	Platform for Integrated, Intelligent Kinome Analysis 2
PKA	protein kinase A
PMSF	Phenylmethylsulphonyl fluoride
PTSD	Post-traumatic stress disease
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SAM	sympathetic-adrenal-medullary
STK-10	Serine/threonine-protein kinase 10
TLRs	toll-like receptors

TMB	Tetramethylbenzidine
TNF	tumor necrosis factor
TRAF2	TNF receptor-associated factor 2
VIDO	Vaccine and Infectious Disease Organization

Statement

The introduction of the thesis has been published as Yi Chen**,** Ryan Arsenault, Scott Napper, Philip Griebel. (2015). Models and Methods to Investigate Acute Stress Responses in Cattle. *Animals* 5(4): 1268-1295. The sections written solely by the co-authors have been removed from the thesis.

1. Introduction

1. 1 Stress and Animal Health

There is a growing awareness of the negative effects of stress on animal health and production within the livestock industry. Increased susceptibility to disease, decreased milk quality, and reduced growth rates have been associated with stress in cattle (Hodgson *et al*., 2012; Philips and Santurtun, 2013; Almeida *et al*., 2014; Thompson-Crispi *et al*., 2014). Thus, identifying effective strategies to manage stress is becoming a priority. This may include the selection of animals with a greater tolerance to stressful situations as well as optimization of animal management practices. A better understanding of the biological responses to stress in livestock may provide fundamental information required to fulfill these objectives. However, stress responses are influenced by animal-specific and stressor-specific variables. Animal-specific variables include species-specific differences as well as individual-specific differences that include genetic, epigenetic, behavioral conditioning, and environmental factors. Frequency, duration, and intensity of individual stressors will also contribute to stressor-specific variables. Research related to stress in all species has increased almost 5-fold over the last 20 years in PubMed publication entries [**Figure 1.1.A**]. Moreover, research activity related to stress in cattle, stress and disease in cattle, and bovine stress-related behavioral studies [**Figure 1.1B, C, D**] has also increased 4 to 5-fold over the last 20 years. However, the definition of stress and stress responses has changed over time and remains controversial.

1. 2 Defining Stress

Claude Bernard first defined stress as a perturbation of the otherwise constant state of the "milieu interieur" early in the nineteenth century (Goldstein and Kopin, 2007). Canon redefined this concept by using the word "homeostasis" fifty years later (Canon, 1929). "The coordinated physiological processes which maintain a steady state in the organism" was used to define homeostasis. The "fight and flight response" was also proposed by Canon as a major mechanism by which an organism responds to stress or danger (Canon, 1929). The definition of stress was subsequently expanded by Selye to include "the nonspecific response of the body

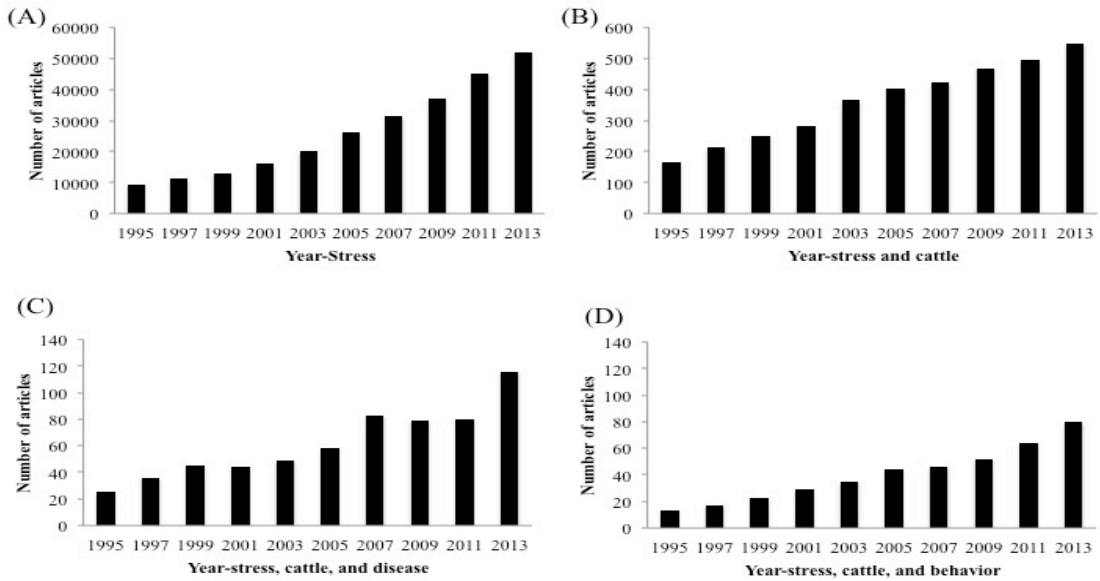

Figure 1.1. Number of publication entries in Medline (PubMed) trend* from 1995-2013. (A) Publication entries searched with query "stress"; (B) Publication entries searched with query "stress and cattle"; (C) Publication entries searched with query "stress, cattle, and disease"; (D) Publication entries searched with query "stress, cattle, and behavior". Medline (PubMed) trend URL: http://dan.corlan.net/medline-trend.html.

to any demand made upon it" (Selye, 1973). According to Selye, three stages of the general adaptation syndrome (GAS) or biological stress syndrome could be identified. The first stage is recognition of a noxious agent. The second stage was defined as the alarm reaction, which is resistance or adapting the body to adjust to the stimuli, including the release of secretory granules from the adrenal cortex into the blood stream that can alter tissue metabolism. The last stage is the stage of exhaustion when the acquired adaption fails and this stage may result in disease (Selye, 1973). Thus, duration of a stress response was incorporated into the definition of stress. The more recent definition of stress was proposed by McEwen and Goldstein, who defined stress as either a consciously or unconsciously sensed threat to homeostasis which is then reflected in a large variety of specific parameters (Goldstein and Kopin, 2007). This definition recognizes that the homeostatic state may shift over time and vary among individuals within a population.

1.3 The Stress Response

Two different signaling axes have been identified by which mammals mount an integrated physiological response to perceived danger [**Figure 1.2**]. The response to danger is initiated at the level of the hypothalamus through the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (VP) (Minton, 1994, Mormede *et al.*, 2007).

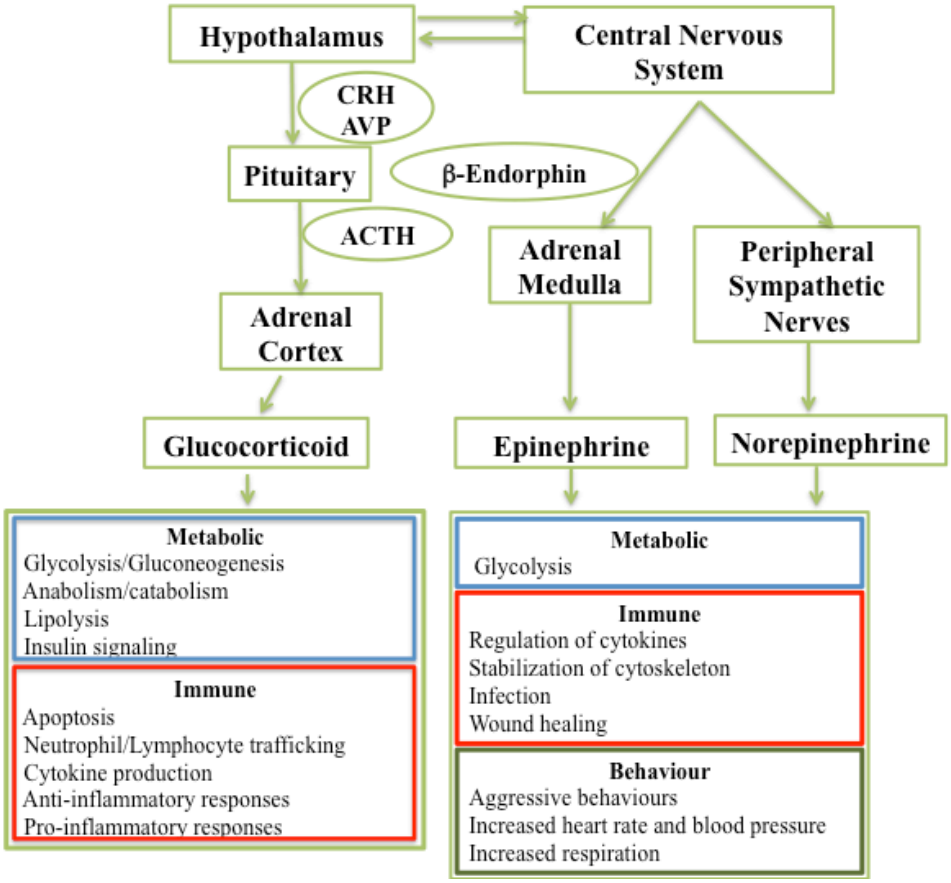

Figure 1.2. Biological functions regulated by the two stress axes. The hypothalamus-pituitary-adrenal (HPA) axis is activated when the body perceives a physical or psychological stressor. Corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) release from hypothalamus results in adrenocorticotropic hormone (ACTH) secretion by the pituitary. ACTH then stimulates release of glucocorticoids from the adrenal cortex. The sympathetic-adrenal-medullary (SAM) axis is a coordinated response to diverse stressors mediated by the release of epinephrine from the adrenal medulla and norepinephrine from the peripheral sympathetic nerves. Cross-talk between the central nervous system and pituitary coordinate HPA and SAM axis activation and release of β-endorphin by the pituitary contributes to this cross-talk. Circulating glucocorticoids and catecholamines interact with a wide variety of cells to alter both metabolic and immune functions.

These hormones transmit a signal to the pituitary gland to initiate release of adrenocorticotropic hormone (ACTH) that targets the adrenal cortex. This hypothalamic-pituitary-adrenal (HPA) axis initiates one arm of the endocrine response to stress, mediated by the release of glucocorticoids from the adrenal cortex [**Figure 1.2**].

The second arm of this response is very rapid and involves the sympathetic-adrenal-medullary (SAM) axis, which culminates in the release of epinephrine from the adrenal medulla and norepinephrine from the peripheral sympathetic nerves [**Figure 1.2**]. The SAM axis initiates the "fight or flight" response (Aich *et al.*, 2007, Wong and Tank, 2007, Huang *et al.*, 2013) that includes an integrated behavioral response to perceived danger or stress as well as metabolic and immune responses [**Figure 1.2**].

1. 4 Measuring Stress Responses

1.4.1 Measurement of Behavioral Responses to Stress

A wide variety of parameters for measuring behavioral responses to stress have been reported [**Table 1.1**]. These include chute entry order (Bristow and Holmes, 2007), chute score (Grandin, 1997), exit velocity (Curley *et al*., 2006), pen score (Curley *et al*., 2006), vocalization (Bristow and Holmes, 2006), time spent lying down (Earley *et al*., 2013), rumination (Bristow and Holmes, 2006), and rope pulling during restraint (Herskin *et al*., 2004) have been quantified. Entry order into a chute system has been used for revealing activation of the HPA axis in anxiety-related behavior. Specifically, a reverse correlation between serum cortisol concentrations and entry order has been reported (Bristow and Holmes, 2007) as well as consistency of entry order across repeated restraint experiments (Andrade *et al*., 2007). Chute score has been defined on a 5-point system to indicate both the frequency and tendency of animals' struggling during restraint (Grandin, 1997). Exit velocity was correlated with serum cortisol levels, which provided a quantitative measurement of temperament in Brahman bulls (Curley *et al*., 2006).

Table 1.1. Measurement of Behavioral Responses to Stress in Cattle

Behavioral Responses to Stress	References
Entry Order	Bristow and Holmes, 2007
Chute Scores	Curley *et al*., 2006
Pen Scores	Curley *et al*., 2006
Exit Velocity	Curley *et al*., 2006
Vocalization	Bristow and Holmes, 2007
Recumbancy	Earley *et al*., 2013
Rumination	Bristow and Holmes, 2007
Rope pulling	Herskin *et al*., 2004

1.4.2 Measuring Physiological Responses to Stress

Quantifying the physiological response to stress is based on the release of bioactive molecules from two stress axes. The activation of HPA axis is frequently monitored through cortisol levels in serum or plasma (Kelley, 1980; Blecha *et al*., 1984; Minton, 1994). Although the release of glucocorticoids from adrenal cortex is rapid and pulsatile, cortisol levels may last for days when animals subject to sustained stress (Hodgson *et al*., 2012). In cattle, the basal cortisol levels are 15-25 nmol/L, which can rapidly increase to 60 to 200 nmol/L, depending on the stressor and individual animal responses (Mormede et al., 2007). Cortisol also displays a circadian rhythm that with peak level usually occurring in the morning (Mormede *et al*., 2007). Thus, it is critical that both the method of sample collection and the time of samples collection be considered when designing stress experiments and interpreting data.

In cattle, Cortisol levels have also been quantified in urine, saliva, fecal metabolites, and milk [**Table 1.2**]. Collection of these body fluids is less invasive than venipuncture and may reduce stress-related responses associated with sample collection. Measuring cortisol in hair has also been suggested as a non-invasive method to monitor the stress response (Macbeth *et al*., 2010). An association between bovine hair cortisol and serum cortisol levels following repeated ACTH injections in Heifers has been reported (Gonzalez-de-la-Vara *et al*., 2011). However, this association may be complicated, since cortisol may enter hair either from blood or through local cortisol production (Russell *et al*., 2012; Stalder *et al*., 2012). Hair cortisol may also be influenced by collection site, hair color, the sex and age of animals.

HPA and SAM axes activity has also been measured by monitoring a diverse range of biological responses in cattle [**Table 1.2**]. For example, total leukocytes and neutrophil to lymphocyte (N/L) ratios in blood may also provide a sensitive prospective to study the effects of stress on leukocyte trafficking (Lynch *et al*., 2010; O'Loughlin *et al*., 2011). Parameters

such as heart rate (Andrade *et al*., 2001), respiratory rate (Andrade *et al*., 2001), acute phase proteins (Earley *et al*., 2013), glucose (McCorkell *et al*., 2013), pyruvate (Mehla *et al*., 2014), epinephrine and norepinephrine (Lefcourt and Elsasser, 1995), body temperature (Allen *et al*., 2015), and body weight (Earley *et al*., 2013) have also been measured as stress responses.

While quantification of cortisol and catecholamines provides an effective starting point for defining the occurrence of stress responses, many points of regulation exist between the release of these signals and the ensuring phenotypic responses. It has been difficult to select one single parameter or biological response that can be effectively used to quantify stress response or discriminate animals that respond differently to the same stressor. As such, there is a considerable value to consider the higher-order responses that are initiated by the stressor. In addition to specific parameters to represent the stress responses, a large number of global omics methods have also been applied including genomics (Mehla *et al*., 2014), transcriptomics (Kolli

Table 1.2. Measurement of Physiological Reponses to Stress in Cattle

Physiological Responses to Stress	References
Cortisol Measurement	
Serum/Plasma	Hulbert *et al*., 2012
Urine	De Clercq *et al*., 2013
Salivary	Loberg *et al*., 2008
Hair	Gonzalez-de-la-Vara *et al*., 2011
Milk	Fukasawa and Tsukada, 2010
Faecal metabolites	Mostl *et al*., 2002
White Blood Cell Counts	
Whole	Hulbert *et al*., 2013
Neutrophil/Lymphocyte	Ishizaki and Kariya, 1999
Heart rate	Andrade *et al*., 2001
Respiratory rate	Andrade *et al*., 2001
Acute Phase Proteins	Earley *et al*., 2013
Glucose	McCorkell *et al*., 2013
Pyruvate	Mehla *et al*., 2014
Epinephrine	Lefcourt and Elsasser, 1995
Norepinephrine	Lefcourt and Elsasser, 1995
Innate Immune Responses	Hulbert *et al*., 2013
Body Temperature	Kolli *et al*., 2014
Body Weight	Earley *et al*., 2013
Serum Proteome	Aich *et al*., 2007
Transcriptional Profiles	Kolli *et al*., 2014

et al., 2014), proteomics (Cruzen *et al*., 2015), kinomics (van Westerloo *et al*., 2011; Napper *et al*., 2014), and metabolomics (Aich *et al*., 2009). These investigations suggest complex nature of stress response as metabolism (including protein, lipid, and carbohydrate metabolism) and immune function (including innate immunity and inflammatory responses) have been revealed.

Collectively, these stress-induced changes can be broadly considered as being either behavioral, physiological or biochemical (molecular). The changes that occur at each of these levels may reflect changes that occur on the other levels. Thus, it is critical to consider the association between the physiological and behavioral response for data interpretation.

1.5 Glucocorticoid-induced Signaling

Glucocorticoid receptors (GRs) present in the cytosol of most cells are the major protein capable of binding free cortisol. Activation of the HPA axis results in the release of free cortisol, which can then bind to GRs (Prager and Johnson, 2009). Following cortisol binding, GRs become activated by disassociating from chaperone protein heat shock protein 90 (Hsp 90) complex. Activated GRs then homodimerize and translocate from the cytosol into the nucleus where they can bind to glucocorticoids response elements (GREs). This interaction with GREs can either activate gene expression by recruiting cofactors, for example SRC-1, which promotes transcription histone-modifying elements to the promoter region or repress gene expression by interacting with transcription proteins such as nuclear factor κB (NF κB), interferon regulatory factor-3 (IRF-3), or activation protein 1 (AP1) (Prager and Johnson, 2009; Liberman *et al*., 2014). Activated GRs may also alter cell function by interacting directly with membrane bound or cytosolic kinases, such as extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38 MAPK (Cruz-Topete and Cidlowski, 2015; Liberman *et al.*, 2014).

1.5.1 Metabolic Functions Activated by Glucocorticoid-induced Signaling

Glucocorticoids and their receptors function as checkpoints for energy homeostasis that mediate many of the stress-related effects on metabolism. A recent study revealed that downregulated genes has been linked to carbohydrate metabolism, such as glycolysis, fructose and pentose phosphate pathways in response to heat stress in bovine peripheral blood leukocytes in transcriptional profiles (Kolli *et al*., 2014). Mehla and colleagues also reported that up-regulated expression of kinase genes that were involved in carbohydrate metabolism,

protein metabolism, as well as down-regulated expression of genes that link to lipid metabolism after exposed to heat stress of cattle (Mehla *et al*., 2014). GRs also play an important role in anabolism and catabolism regulation (Revollo and Cidlowski, 2009). For instance, GRs have been reported to down-regulate the expression of Activating Transcription Factor 4 (ATF 4), which involved in amino acid biosynthetic enzymes (Revollo and Cidlowski, 2009). In addition to anabolism and catabolism metabolism, lipoprotein lipase (LPL) activity was reduced following glucocorticoid treatment that leading to lipolysis in adipose tissues has also been revealed (de Guia *et al*., 2014). Thus, glucocorticoids can regulate a broad range of metabolic functions.

1.5.2 Immune Responses Regulated by Glucocorticoid-induced Signaling

Glucocorticoids influence a broad range of innate and acquired immune responses and are well known for their anti-inflammatory effects. However, recent evidence also demonstrates that glucocorticoids can induce pro-inflammatory responses (Cruz-Topete and Cidlowski, 2015). This is consistent with the results of a recent study with young calves where it was observed that combining the stressors of maternal separation and transportation resulted in elevated cortisol levels in blood and increased interferon (IFN)-γ production following viral infection (Hodgson *et al*., 2012). IFN-γ is a potent pro-inflammatory cytokine and this increased cytokine response was associated with enhanced production of acute-phase proteins. Glucocorticoids may also enhance innate immune response by increasing the expression and signaling by toll-like receptors (TLRs), such as TLR 2 and TLR 4 (Hermoso *et al*., 2004; Cruz-Topete and Cidlowski, 2015). It was demonstrated that individual stressors, such as transportation alone versus combined transportation and weaning, have different effects on lipopolysaccharide (LPS)- induced TLR 4 signaling in bovine blood mononuclear cells (Griebel *et al*., 2014). The increase in LPS-induced secretion of tumor necrosis factor (TNF) was significantly greater in calves experiencing transportation stress alone as compared to calves experiencing the combined stressor of transportation and weaning (Griebel *et al.*, 2014). Thus, despite inducing a similar increase in serum cortisol, specific stressors may have significantly different effects on innate immune responses (Hodgson *et al*., 2012).

Glucocorticoids exert its role in inhibiting the inflammatory responses by interfering the expression of pro-inflammatory genes via interaction with transcription factors such as NF κB

and AF-1 or possibly through the induction of other regulatory proteins (Chinenov and Rogatsky, 2007). However, glucocorticoids also mediate anti-inflammatory responses through other proteins. For example, glucocorticoids resulted in the inhibition of p38 mitogen-activated protein kinase (MAPK), an effect that was mediated by MAPK phosphatase-1, resulting in a negative inflammatory response (Ayroldi *et al*., 2012). Contradictory results between the analysis of glucocorticoid effects in vitro and observations made following elevation of endogenous cortisol during a stress response suggest that the regulation of cell signaling may be much more complex in vivo. Unravelling this contradiction initiated by stress-induced activation of both HPA axis and SAM may be benefit from the global approaches on cellular signaling.

Glucocorticoids have also been reported to have multiple effects on acquired immune responses [**Figure 1.2**]. These wide ranging effects include altered lymphocyte trafficking, apoptosis of naïve lymphocytes, modulated cytokine secretion, decreased ratio of helper/suppressor T cells, and decreased antibody production (Webster Marketon and Glaser, 2008). However, the magnitude and duration of these effects in acquired immune responses remains to be determined. There are mixed results regarding the impact of stress on antibody responses in human and mice following vaccination (Burns, 2004). Antibody responses can be either enhanced or inhibited by specific stressors, depending on the strain of mice, the type of stressor, and the timing of stress relative to vaccination (Burns, 2004). These observations indicate that it is difficult to predict how individual animals will respond and that each stressor, or combination of stressors, may have very different effects on an acquired immune response.

1.6 Catecholamine Signaling

Norepinephrine, and epinephrine are two major catecholamine neurotransmitters released from the SAM axis (Wong and Tank, 2007). These neurotransmitters exert their effects through α and β adrenergic receptors, mainly β_2-adrenergic receptors (Wong and Tank, 2007). A large variety of body systems including heart rate, blood pressure, respiration rate, and gastro-intestinal tract activity are influenced by the catecholamine signaling as a part of the "fight or flight" response (Aich *et al*., 2009). At a cellular level, β_2-adrenergic receptors signals regulate expression of genes relating to immune function via G proteins that catalyze the

synthesis of cyclic adenosine monophosphate (cAMP) to activate cAMP-dependent protein kinase A (PKA) as well as transcription factors (Padgett and Glaser, 2003).

1.6.1 Metabolic Functions Activated by Catecholamine-induced Signaling

Two mechanisms mediated by epinephrine on glucose metabolism have been reported. One mechanism is mediated by activation of adenosine monophosphate activated protein kinase (AMPK), leading to the translocation of glucose transporter type 4 (GLUT 4) to the plasma membrane, which facilitates glucose uptake, and it also increases mitochondrial biosynthesis (Ziegler *et al*., 2012). The second mechanism is mediated by peroxisome proliferator-activated receptor-γ, resulting in the increased amount and translocation of GLUT 4 (Ziegler *et al*., 2012). Catecholamine has also been reported to influence insulin signaling (MacDonald, 1985), lipid metabolism (Vara and Tamarit-Rodriguez, 1991), and thermogenesis (Nguyen *et al*., 2011).

1.6.2 Immune Responses Regulated by Catecholamine-induced Signaling

Catecholamines are also involved in a large spectrum of immune functions [**Figure 1.2**]. For example, norepinephrine has been reported to regulate cytokine secretion (Szelenyi and Vizi, 2007), inhibit wound healing (Stojadinovic *et al*., 2012), and influence host-pathogen interactions (Lyte, 2014). It has been demonstrated that reserpine, an indole alkyloid, reduced circulating norepinephrine levels was associated with increased LPS-induced TNF-α (Szelenyi and Vizi, 2007). Further, a similar relationship between increased circulating norepinephrine and decreased LPS-induced TNF-α were demonstrated (Szelenyi and Vizi, 2007). These observations support the role of norepinephrine in cytokine regulation. Epinephrine has also been demonstrated the ability to influence immune response. For instance, epinephrine releases from keratinocytes in an antocrine fashion and binds to β_2-adrenergic receptors, leading to the activation of AKT signaling pathways, which eventually result in cytoskeleton stabilization and more focal adhesion formation that inhibit keratinocytes migration and impaired would healing (Stojadinovic *et al*., 2012). In addition, epinephrine exerts its role in host-pathogen interactions through influence on expression of virulence related factors in the growth of pathogenic bacteria, which may contribute to enteric infections (Lyte, 2014).

1.7 Stressors Studied in Cattle

A large number of either physical or psychological stressors have been investigated in animal models over the last 50 years [**Table 1.3**]. However, despite the classifications of the stressors, it is difficult to study one isolated stressor because cattle experience many stressors concurrently during normal husbandry practices. Researchers have attempted to design experiments based on simplified one single stressor with controls but noticed that the stress responses might be compromised by other unconsidered stressor or changed by different observers when doing behavioral studies. A large variety of stressors have been investigated in cattle but many of the studies may be limited by sample collection or complicated by combined stressors. The models have been used to mimic stress including thermal stress, feed deprivation, transportation, noise, restraint, weaning, social isolation or mixing.

Table 1.3. Physical and Psychological Stressors Investigated in Animal

Stressor	Cattle	Pigs	Hens	Sheep	Duck	Horse	Mice	Rats
Physical stressors								
Thermal Stressors								
Cold	✔	✔	✔	✔	✔	✔	✔	✔
Heat	✔	✔	✔	✔	✔	✔	✔	✔
Transportation	✔	✔	✔	✔	✗	✔	✔	✔
Feed	✔	✔	✔	✔	✔	✔	✔	✔
Deprivation/restriction								
Noise	✔	✔	✔	✗	✗	✗	✔	✔
Psychological stressors								
Weaning	✔	✔	✗	✔	✗	✔	✔	✔
Social isolation/mixing	✔	✔	✔	✔	✔	✔	✔	✔
Restraint	✔	✔	✔	✔	✔	✔	✔	✔

1.7.1 Physical Stressors

1.7.1.1 Thermal Stress

Thermal stressors, which including both heat stress and cold stress that are experienced throughout the life of livestock. Negative effects on both animal health and productivity induced by heat stress in dairy cows have been documented [**Figure 1.3**]. A reduction in both milk production (West, 2003) and body weight (O'Brien *et al*., 2010) were associated with heat

stress in dairy cows. A more severe outcome mortality has also been reported (Crescio *et al*., 2012). Moreover, heat stress for a one to three weeks during late term of pregnancy can also affect the calves. Significantly shorter gestation, lower body weight, reduced absorption of colostrum, and changes in blood leukocyte function, phenotype and gene expression have been demonstrated in calves born from heat-stressed cows (Tao *et al*., 2012; Strong *et al*., 2015). To understand the mechanisms by which heat stress induces biological responses will be beneficial to animal health and productivity (Carroll *et al*., 2012; Mehla *et al*., 2014).

Gene expression in peripheral blood leukocytes was analyzed following a 4 h heat stress period with heat stress confirmed by measuring elevated skin temperature and respiration rates (Kolli *et al*., 2014). Microarray analysis revealed heat stress had a significant impact on carbohydrate metabolism, such as glycolysis and gluconeogenesis, and also altered expression of immune response genes, including those associated with the TLR and NOD-like receptor signaling pathways. It remains to be determined, however, if heat-induced changes in gene transcription translate into a significant change at the protein level. The effect of cold stress on cattle has not been extensively investigated but cold stress has been reported to cause changes in appetite, digestive function, metabolic function, and death (Young, 1983).

1.7.1.2 Transportation Stress

Transportation is one of the most common stressors experienced by cattle, especially with increased national and international movement of animals. For over 50 years transportation has been reported to increase susceptibility to bovine respiratory disease (Hoerlein and Marsh, 1957; Jensen *et al*., 1976). Many factors may contribute to this stress response, including human-animal interactions immediately prior to and after transportation. A frequent observation following transportation is a transient increase in blood cortisol concentrations [**Figure 1.3**] and associated with this cortisolemia is an increased N/L ratio (Blecha *et al*. 1984). More recently, it has been suggested that natural killer (NK) cells may provide a more sensitive way for measuring stress response of transportation than differential leukocyte counts (Ishizaki and Kariya, 2010). However, it is important to notice that transport

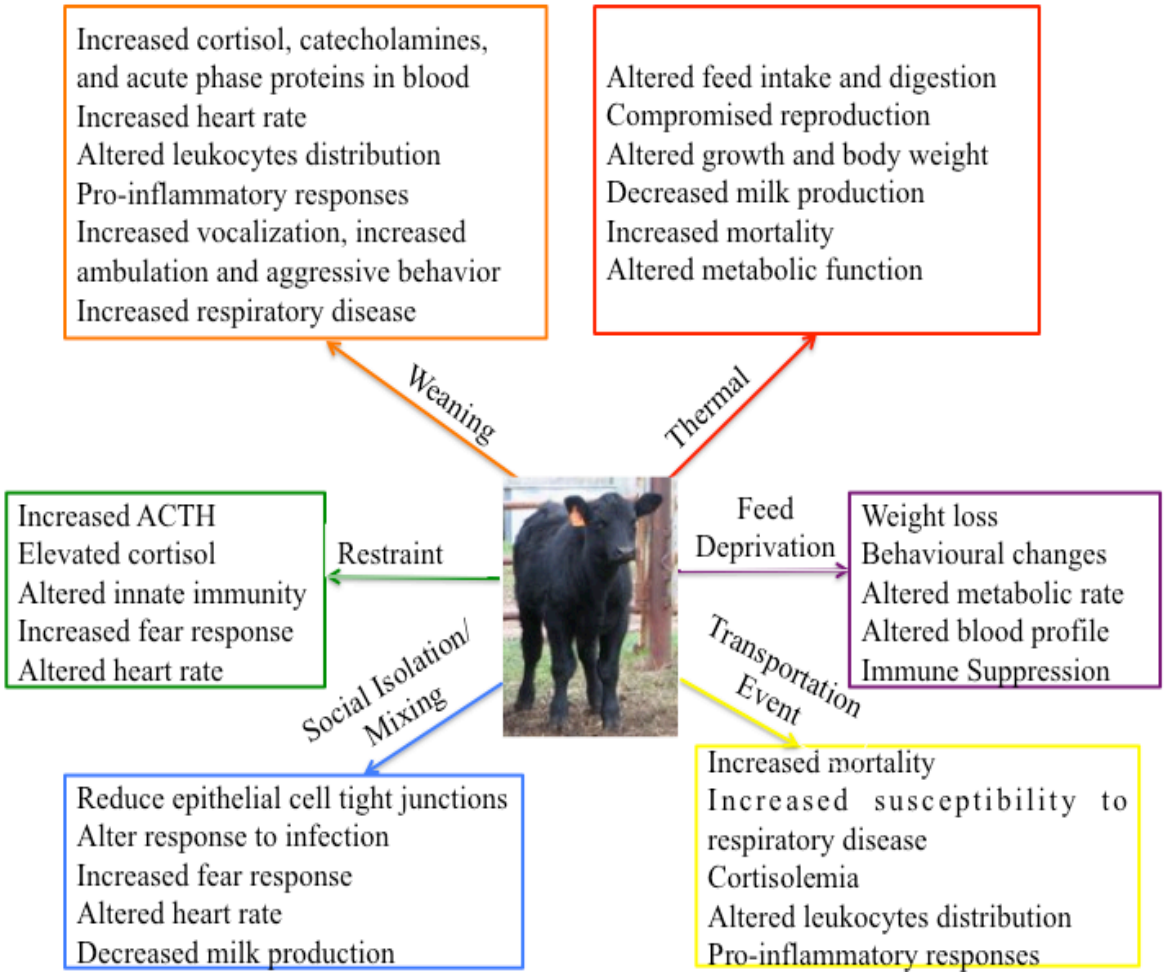

Figure 1.3. Biological functions altered in response to individual stressors. For each stressor investigated in cattle a variety of functions were investigated and evidence presented to demonstrate significant biological or psychological changes. The results from individual studies may be confounded by the presence of more stressors.

is commonly associated with other stressors, such as heat stress and feed deprivation. For example, cattle in sea journey may experience a combination of increased noise, elevated temperatures, and high ammonia concentrations. These factors have been linked to increased alkalosis, pulmonary inflammation and mucosal irritation, as well as an increased incidence of respiratory disease (Philips and Santurtun, 2013). Thus, a broad range of physiological responses has been revealed in transportation studies, including altered immune function, behavioral responses, and changes in muscle physiology (Earley *et al*., 2012). These diverse responses highlight the potential for complex interactions among multiple stressors with each combination of stressors resulting in a unique physiological response.

1.7.1.3 Feed Deprivation

Feed deprivation may occur at many levels, involving specific micronutrients or inadequate intake of protein or energy. It has been demonstrated that controlled protein-energy malnourishment over a 4-week period in newborn calves resulted in altered blood leukocyte counts, decreased lymphocyte function, and reduced antibody responses (Griebel *et al*., 1987). Controlled feed deprivation in Holstein cattle also showed physiological responses and behavioral responses (Bourguet *et al*., 2011). These responses including increased reactivity to stressful events, elevated plasma cortisol levels, and decreased β-hydroxybutyrate levels. Feed deprivation effects on rumen microflora and rumen function have also been investigated (Galyean *et al*., 1981). Feed-deprived steers have been reported to significantly increase rumen pH with a concurrent decrease in rumen bacteria and protozoa (Galyean *et al*., 1981). Also, rumen microbiome recovery was very slow in feed deprived steers and it was concluded that feed deprivation had long-term effects on the rumen capacity for fermentation (Galyean *et al*., 1981). These controlled feed deprivation experiments demonstrate that prolonged malnourishment may have profound effects, especially in young growing animals or lactating cows that have a high metabolic demand. It is also interesting to note, that even relatively brief periods of feed deprivation may have prolonged effect on the gut microbiome. The changes in the gut microbiome may have a direct impact on the HPA axis (Clarke *et al*., 2014). Alterations in the gut microbiome may contribute to stress responses and increase disease susceptibility associated with important dietary transition periods, such as weaning of young calves. Thus, future analysis of detailed profiling of gut microbiome may provide insight into gut-microbiome-brain axis.

1.7.2 Psychological stressors

1.7.2.1 Maternal Separation and Weaning

Maternal separation has been identified as a potent stressor in infants and young animals that may have long-term psychological and physical effects (Faturi *et al*, 2014). Abrupt weaning or separation of suckling calves from their dams is a common husbandry practice in both the beef and dairy industries. In 5 to 6-month old beef calves, abrupt maternal separation results in both the psychological stress of breaking the maternal bond as well as nutritional

changes associated with dietary changes (Haley *et al*., 2005). This separation results in behavioural changes in both calves and cows that may persist for several days, consistent with a more chronic form of stress. For calves, behavioural changes include increased vocalization and ambulation and this activity persists at an elevated level for at least three days after separation (Haley *et al*., 2005). Lefcourt and Elsasser observed a significant increase in norepinephrine and epinephrine concentrations at 24 h after calves were separated from their dams but serum cortisol concentrations did not change significantly (Lefcourt and Elsasser, 1995). Re-uniting cows and calves resulted in a rapid decline in norepinephrine and epinephrine concentrations (Lefcourt and Elsasser, 1995). Studies in rodents have demonstrated that early maternal separation can have long-term effects on the gut-microbiome-brain axis and stress responses adult animals (O'Mahony *et al*., 2011). The long-term consequences of maternal separation in newborn and older calves have yet to be investigated.

The influence of the age of the calf at the time of weaning on the innate immune responses has been studied. Eighty-day old calves had lower TNF, IL-1, and IL-6 responses than 250-day old calves following weaning in an endotoxin study (Carroll *et al*., 2009). In contrast, interferon (IFN) and acute-phase protein responses were greater in early-weaned calves (80 day of age) than normally weaned (250 days of age) calves (Carroll *et al*., 2009). The age-dependent differences were concluded as contributing factors of the ability of the calves to recognize and respond to endotoxin. However, this study did not quantify stress responses to maternal separation which may have contributed to the age-dependent differences in innate immune responses. It is also possible that the earlier weaning may be linked to more profound perturbations in nutrition as calves are more dependent on milk. To differentiate between maternal separation and dependence on maternal nutrition, Haley *et al* (2005) used a model with nose-paddles, demonstrating that dependence on maternal nutrition was an important factor contributing to the psychological responses following maternal separation. These studies revealed the weaning effects on innate immune responses in calves, which may be manifested by nutrition bonds.

1.7.2.2 Social Isolation and Regrouping

Cattle are herd animals that establish social orders with dominant and submissive animals within each group. As such, social isolation or introduction to a new social group

(regrouping) can be significant stressors for an individual animal (Craig, 1986) resulting in diverse behavioral and physiological responses [**Figure 1.3**].

1.7.2.2.1 Social Isolation and Mixing

To evaluate the effects of social isolation, Ninomiya and Sato measured cortisol and Chromagranin A concentrations in saliva to reflect HPA axis activity and SAM axis activity, respectively (Ninomiya and Sato, 2011). Chromogranin A was significantly elevated in socially isolated cows but cortisol concentrations remained unchanged two hours after social isolation (Ninomiya and Sato, 2011). The effect of prolonged social isolation was examined in Friesian cows isolated for a period of either 4 or 8 weeks. Socially isolated cows displayed behavioral changes with increased self-grooming and leaning when compared to control cows but no differences in serum cortisol concentrations were observed (Munksgaard and Simonsen, 1996). Thus, social stressors may not engage both the HPA and SAM axes and behavioral adaptations may occur in response to this type of stressor.

The effect of social isolation on physiological parameters has also been investigated using a respiratory diseases model. Veal calves infected with bovine herpesvirus 1 (BHV-1) were randomly divided into two groups. One group was housed as individual animals while the the other group was housed collectively. Socially isolated animals had lower basal cortisol concentrations and responded to ACTH injection with a decreased cortisol release relative to group-housed animals (van Reenen *et al*., 2000). These studies suggest that social isolation reduced the severity of clinical disease but this observation may have been compounded by a reduction in viral transmission when animals were house individually. A more direct link between serum cortisol and physiological responses to social isolation stress was observed by using serum lactose concentrations as a measure of epithelial cell permeability in the mammary gland (Stelwagen *et al.*, 2000). Cows were first divided into high serum cortisol versus low serum cortisol groups based on measurements during their first lactation. When cows from both groups were then socially isolated for 55 h, epithelial cell permeability was consistently greater in the high cortisol group. The authors concluded that social isolation altered epithelial tight junctions and mammary gland function. Collectively, these observations indicate that social isolation not only alters behavioral responses but may also have an impact on a wide variety of body systems.

1.7.2.2.2 Social Regrouping

Current husbandry practices frequently result in social mixing or regrouping of both young and old cattle. A number of studies have analyzed acute behavioral and biological responses following the introduction of a single animal to an established group. In dairy cows, the introduced animal displayed altered behavior, including reduced allogrooming (which is social grooming between members within species) and decreased feed access, and this was associated with a 24 h reduction in milk production (von Keyserlingk and Weary, 2008). Similarly, the introduction of a single, milk-fed dairy calf to an established group resulted in transient changes in behavior and decreased feed consumption that lasted for a single day (O' Driscou *et al*., 2006). In contrast, co-mingling two groups of 8 dairy cows resulted in behavioral changes and decreased milk production that persisted in all animals for weeks (Philips and Rind, 2001). Veissier and colleagues investigated the effect of repeated regrouping on pairs of male Holstein calves. They observed that calves appeared to habituate to repeated re-grouping but did display an increased adrenal cortex sensitivity to ACTH stimulation (Veissier et al., 2001). Thus, both young and old animals may adapt quickly to social mixing when a single animal is introduced to an established group. In contrast, the co-mingling of larger groups may have much longer term effects on both behavior and physiology, representing a chronic stress response.

1.7.2.3 Restraint Stress

Restraint has been widely used as a stressor in many animal experiments because it is an unavoidable procedure during branding, vaccination, and routine handling. Effects of restraint stress on a variety of species including cattle, pigs, poultry, rats, and mice have been well documented **[Table 1.4]**. Restraint stress has been reported to induce behavioral changes that include locomotion and sitting responses (Jaskulke and Manteuffel, 2011). Physiologically and biochemically, increased natural killer cell cytotoxicity, N/L ratio, lymphocyte lysosomal enzymes, salivary IgA levels, increased plasma concentrations of epinephrine, and norepinephrine resulted from restraint stress (Stojek *et al*., 2006; Muneta *et al*., 2010; Ciepielewski *et al*., 2013). Similar findings were demonstrated in poultry as levels of cortisol, norepinephrine, epinephrine, and heterophil/lymphocyte ratios were increased by restraint (de Jong *et al*., 2002; Hamasu *et al*., 2012; Ericsson *et al*., 2014). A large number of the restraint

stress experiments have been performed with mice and rats. Restraint stress has been linked to decreased locomotor activity, memory, social conflicts, body weight, amounts of splenocyte, chemotactic factors, and glycogen synthesis in mice and rats (Wood *et al*., 2003; Engler *et al*., 2005; Kumari *et al*., 2007). Moreover, restraint stress of rodents also induces plasma concentrations of corticosterone, oxidative damage, lipid peroxidation, apoptosis of splenocyte, and expression of inflammatory response-related genes (Inoue *et al*., 1999; Liu *et al*., 1996; Wang *et al*., 2002; Engler *et al*., 2005; Zfir and Banu, 2009). The animal and tissue specific variability of responses have also been highlighted by studies of restraint effects in rats (Bauer *et al*., 2001; Kitraki *et al*., 2004; Sahin and Gumuslu, 2007).

In cattle, restraint has been reported to increase the ACTH response, elevate serum cortisol concentrations, and alert the immune response (Minton, 1994; Herskin *et al*., 2007). Herskin *et al*. (2007) investigated the effect of restraint or social isolation on adrenocortical and nociceptive responses in dairy cows. While social isolation and restraint both induced hypoalgesia, plasma cortisol levels did not differ between the restrained cows and control cows. It was suggested by the authors that the pretreatment handling might contribute to the minor differences between the two groups (Herskin *et al*., 2007).

Szenci *et al*. (2011) studied the effect of restraint stress on pregnant heifers by measuring plasma cortisol concentrations, progesterone, and bovine pregnancy-associated glycoprotein-1. Plasma cortisol concentrations were significantly elevated after restraint in a squeeze chute for 2 hours on two consecutive days while no differences in progesterone and pregnancy-associated glycoprotein-1 were detected during heifers' pregnancy (Szenci *et al*., 2011). However, when the restraint was repeated on the third day, there was no significant increase in plasma cortisol concentrations compared to the second test-day. These observations suggest that cattle rapidly adapt to some types of repeated stress (Szenci *et al*., 2011).

Andrade *et al*. (2001) also investigated the effect of repeated restraints and the use of a mask in Brahman cattle. Cattle were restrained in a squeeze chute for 10 minutes each day and plasma cortisol concentrations gradually declined over the 19-day period. These results are consistent with Szenci and colleagues, reflecting the habituation of animals to handling and restraint.

Table 1.4. Restraint studies on different species.

Species	Effects of Restraint Stress	References
Cattle	Decreased legs kicking; elevated plasma concentrations of cortisol	Andrade *et al*., 2001; Herskin *et al*., 2007; Szenci *et al*., 2011
Pigs	Increased natural killer cell cytotoxicity, N/L ratio, lymphocyte lysosomal enzymes, salivary IgA levels, plasma concentrations of epinephrine, norepinephrine	Piekarzewska *et al*., 2000; Stojek *et al*., 2006; Muneta *et al*., 2010; Jaskulke and Manteuffel, 2011; Ciepielewski *et al*., 2013.
Poultry	Increased levels of cortisol, dopamine, norepinephrine, epinephrine, and heterophil/lymphocyte ratios	de Jong *et al*., 2002; Hamasu *et al*., 2012; Ericsson *et al*., 2014
Mices and Rats	Reduced memory retention, social conflicts, body weight, amounts of splenocyte, chemotaxis factor, glycogen synthesis; induced glucocorticoid receptors, antioxidant status, oxidative damage, lipid peroxidation, apoptosis of splenocyte, inflammatory response related genes.	Inoue *et al*., 1993; Liu *et al*., 1996; Bauer *et al*., 2002; Wang *et al*., 2002; Wood *et al*., 2003; Kitraki *et al*., 2004; Engler *et al*., 2005; Kumari *et al*., 2007; Zafir and Banu, 2009

The use of a mask by placing it over the face of animals during restraint was conducted 6 months later. Cortisol levels, respiratory rates, heart rates have been reported to decline comparing to control cattle. This indicates the importance of visual information for stress response (Andrade *et al*., 2001). Andrade and colleagues also revealed that consistent orders of entrance in which calves were willing to enter the squeeze chute during the repeated handling, suggesting that the perception of danger may exist prior to the experiment.

Collectively, the investigations of restraint stress effects on cattle are limited. The biological mechanisms mediating the outcomes have yet to be determined (Buynitsky and Mostofsky, 2009). To investigate a more comprehensive profile of the behavioral, physiological, and biochemical responses subjecting to restraint stress will help identifying both the mechanisms and biomarkers associated with these responses. Further, to investigate the cellular signaling pathways mediating physiological changes may help to understand the not

well-characterized mechanisms of these phenotype responses following restraint (Buynitsky and Mostofsky, 2009).

1.8 Hypothesis and Objectives

The hypothesis for the current study is that restraint for five minutes will induce behavioral responses that vary significantly among individual calves and the magnitude of these behavioral responses will correlate with physiological parameters, previously identified as stress-associated responses. Furthermore, physiological parameters should provide a more quantitative measure of stress responses and may provide biomarkers for the identification of animals that consistently differ in their response to restraint stress. To test this hypothesis, four specific objectives were made:

(1) Select 20 animals that had limited prior human contact and were not acclimated to restraint in a chute. Perform three replicate 5-min restraint experiments with one week intervals and obtain physiological information as well as behavioral information for each restraint experiment.

(2) Analyze physiological and behavioral responses for individual animals and stratify calves according to stress responses during the three replicate experiments.

(3) Use kinome arrays to analyze kinase activity in peripheral blood leukocytes collected from a subset of calves selected on the basis of high (n = 4) versus low (n = 4) serum cortisol levels following each restraint episode.

(4) Validate cellular pathways identified on the basis of differentially phosphorylated peptides in high versus low cortisol responders.

2. Materials and Methods

2.1 Animal Subjects

Young (6-8 months) Angus-cross heifers (n = 20) were purchased from the Gaffe Ranch (Maple Creek, Saskatchewan, Canada), weaned and transported for 5 hours to the Vaccine and Infectious Disease Organization (VIDO) research facility. All the calves had previously been restrained when branded and vaccinated at one to two months of age. Calves were assigned individual animal identification numbers upon arrival and housed in a single pen. Animals were adapted to the facility for two weeks prior to conducting the restraint experiment. During the two-week acclimation period human-animal interactions were limited to the morning feeding when 1.5 kg to 2 kg rolled oats with mineral supplement/calf was delivered from outside the fence-line bunk. Throughout the experimental period, calves also had free access to brome-alfalfa hay and water. All procedure used in this study were approved by the University of Saskatchewan Animal Care Committee, following Canadian Council on Animal Care guidelines.

2.2 Restraint Procedure

Following the adaptation period, calves were subjected to three 5-min restraint with a one week interval between each restraint period. A squeeze chute with head gate was used to limit the movement of the calves [**Figure 2.1; Figure 2.2**]. Other potential stressors were minimized throughout the restraint procedure. For example, talking was avoided and the same two individuals handled the animals during the three restraints. Also, isolation stress was minimized by ensuring that two or more calves were always present in the catch pen preceding the chute. This was achieved by returning the third last animal in each experiment to the catch pen while all other animals were returned to the group housing pen immediately after the restraint episode. All restraint procedures were performed between 1:00 to 5:00 pm.

Figure 2.1. A calf restrained in a chute by capturing the head in a head gate. Each calf was restrained for 5 minutes.

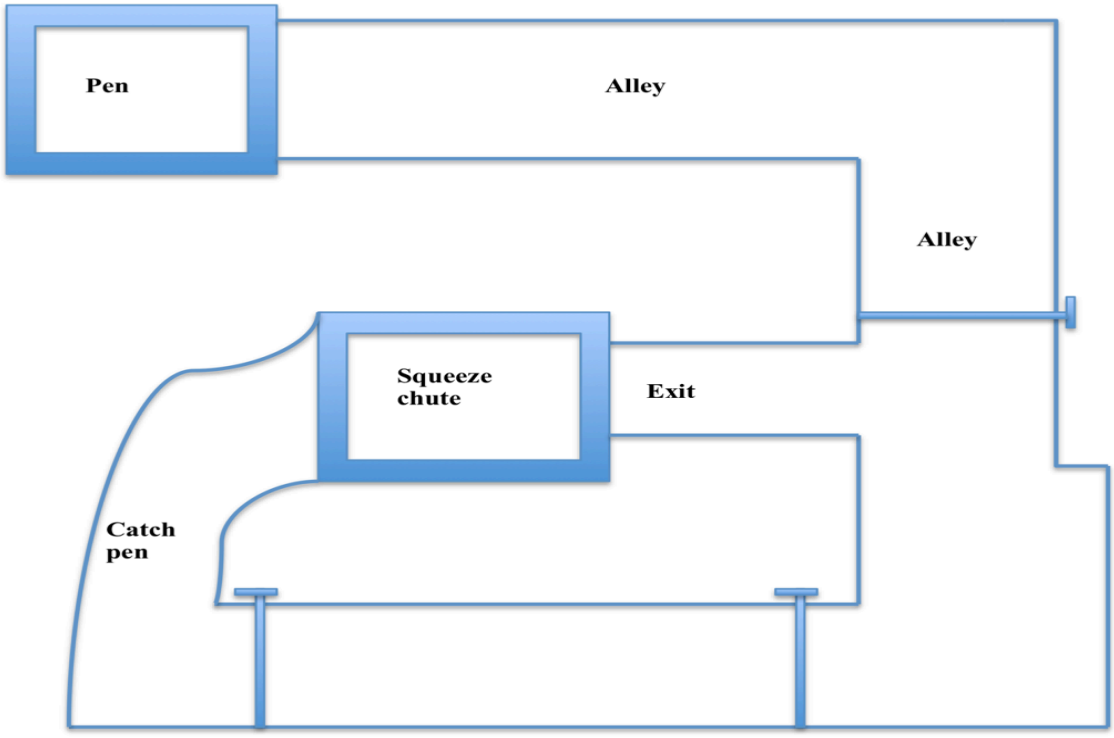

Figure 2.2. Schematic drawing of the restraint facility. Heifers were brought from the feeding pen to the catch pen through the alley. One calf at a time was brought into the squeeze chute. After the 5 minutes restraint procedure, calves were released through the exit door and returned to the feeding pen. The next calf was then brought into the squeeze chute and the restraint procedure was then repeated. The third last calf to be restrained was then returned to the catch pen to provide company with the last calf remaining for the restraint procedure.

2.3 Behavioral Observation

During each restraint period, behavioral responses (chute entry order, chute behavior, and exit velocity) were recorded. Calves standing at the edge of the group closest to the alleyway leading into the squeeze chute were encouraged by the handler to move forward. Thus, animals moving to the back of the group, away from the alleyway, would enter the squeeze chute later in the recorded order. In this way, movement of animals in the catch pen influenced order of entry and the stress of entering the alleyway may have influence subsequent responses during the restraint period. Chute behavioral scores were quantified based on a five-point system (1= calm, no movement; 2= restless shifting; 3= squirming, occasional shaking of squeeze chute; 4= continuous, vigorous movement and shaking of squeeze chute; 5= rearing, twisting, or violent struggling) (Grandin, 1997). Exit frames were recorded by a video camera that captured a distance of 1.63 m beyond the front of the chute [**Figure 2.3**].

2.4 Serum Cortisol

Eight mL/animal blood was collected, via jugular puncture, in BD Vacutainer SST tubes (BD, NJ, USA) immediately following each restraint period, then centrifuged at 2000 X g for 15 minutes after stored at room temperature for 3 hours. Serum aliquots were stored at -20 °C until analyzed. Immulite 1000 Cortisol Analyzer (Siemens Healthcare Global, Erlangen, Germany) was used to analyze 100 µL serum cortisol based on the competitive chemiluminescent enzyme immunoassay (analytical sensitivity =5.5 nmol/L; intra-assay variability =3.6%).

Figure 2.3. A calf exiting after the restraint period. The exit distance measured was from the front of the chute (arrow indicating front of chute) to the door of the restraint facility (arrow indicating end of exit distance). This was a distance of 1.63 m and the number of video frames required to record this distance of travel was used to quantify exit velocity.

2.5 Identification of High and Low Serum Cortisol Sub-Groupings

Based on rank sum scores [**Table 3.1**] four animals representing each extreme of the serum cortisol response were selected to represent high and low serum cortisol responders. Animal 226 was excluded from this subgrouping based on inconsistent serum cortisol responses. To maintain consistent animal numbers within each group, the animal with the lowest summated rankings (animal 227) was also excluded. Animals 208, 210, 211, 212 were identified as high serum cortisol responders while animals 214, 217, 220, and 221 were identified as low serum cortisol responders.

2.6 Differential Counts

Blood smears were prepared and stained using the Hemacolor staining kit (EMD Chemicals, Inc., NJ, USA) and following the manufacturer's instruction. Each blood smear was examined with a light microscope, using the 100 X objective lens with oil immersion (Olympus Canada Inc., Ontario, Canada) and a total of 100 nucleated cells was examined to determine the percentage of lymphocytes, neutrophils, monocytes, and eosinophils. The

neutrophil to lymphocyte ratio (N/L) was calculated by dividing neutrophil numbers to lymphocyte numbers.

2.7 White Blood Cell Counts

Total white blood cell count (WBC)/ml of blood was determined by diluting 40 µL blood in 20 mL Isotone (Beckman Coulter, Inc., Florida, USA), to which Zap-OGLOBIN II Lytic Reagent (Beckman Coulter, Inc., Brea, CA) had been added to lyse the red blood cells. The total white blood cell count per ml of blood was then determined using a Beckman Counter Z series particle counter (Beckman Coulter, Inc., Florida, USA).

2.8 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

A total of 30 mL blood/animal were collected by venipuncture using four 10 mL BD Vacutainer EDTA tubes (BD, NJ, USA). Blood was transferred to 50 mL polypropylene centrifuge tubes (Corning Incorporated, NY, USA) and centrifuged at 1400 X g at 20 °C for 20 minutes. The buffy coat layer was collected into phosphate buffered saline (PBS) (Bio Basic Canada Inc., Ontario, Canada) supplemented with 0.1% EDTA (Bio Basic Canada Inc., Ontario, Canada) and diluted to a final volume of 35 mL before being layered onto 15 mL of 60% isotonic Percoll (GE healthcare, Uppsala, Sweden) in 50 mL polypropylene tubes. This single-step gradient was then centrifuged at 2000 X g at 20 °C for 20 minutes. PBMCs were aspirated from the interface between Percoll and PBS, diluted in a final volume of 50 ml ice-cold PBS supplemented with 0.1% EDTA, and then centrifuged at 300 X g at 4 °C for 8 minutes. PBMCs were washed twice by re-suspending cell pellets with PBS after centrifuging at 300 X g for 7 minutes. The number of PBMCs isolated was determined by counting cells with the Beckman Coulter Z series system particle counter. Aliquots of 10 X 10^6 PBMCS were pelleted and the supernatant removed before cell pellets were either snap-frozen in liquid nitrogen or lysed with one mL Trizol (Sigma Aldrich, St. Louis, MO, USA). All cell pellets were stored at -80 C until used for kinome analysis or RNA extraction.

2.9 Serum Glucose Measurement

A OneTouch Ultra 2 reader and test strips (Life Scan, CA, USA) were used to quantify blood glucose levels by using a 2 µL aliquot of bovine serum/strip and following the manufacturer's instructions.

2.10 Peptide Arrays

Peptide arrays consisting of 1278 peptides were designed based on the following four criteria: (1) Central signaling hubs (Akt; AMPK; PI3K); (2) Innate immune response and adaptive response; (3) Metabolic responses (fatty acid metabolism; proteins metabolism; carbohydrate metabolism); (4) Known stress related pathways. Phosphorylation events for lysates containing 10 X 10^6 PBMCs were assessed using peptide arrays (JPT Peptide Technologies GmbH, Berlin, Germany). A 100 µL cell lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA) (Sigma Aldrich, St. Louis, MO, USA), 1% Trixton-100, 2.5 mM sodium pyrophosphate (Sigma, Aldrich, St. Louis, MO, USA), 1 mM sodium orthovanadate (Na_3VO_4) (Sigma Aldrich, St. Louis, MO, USA), 1 mM sodium fluoride (NaF) (Sigma Aldrich, St. Louis, MO, USA), 1 mM β-Glycerophosphate (Sigma Aldrich, St. Louis, MO, USA), 1 µg/mL Leupeptin (Sigma Aldrich, St. Louis, MO, USA), 1 µg/mL Aprotinin (Sigma Aldrich, St. Louis, MO, USA), 1 mM Phenylmethylsulphonyl fluoride (PMSF) (Sigma Aldrich, St. Louis, MO, USA) and distilled deionized water was added to each frozen pellet of 10 X 10^6 PBMC. Cells were then placed on ice for 10 minutes and centrifuged at 1000 X g for 10 minutes at 4 °C. A 70 µL aliquot of the supernatant was then mixed with 10 µL of the activation mix, containing 50 % glycerol (Bio Basic Canada Inc., Ontario, Canada), 60 mM $MgCl_2$ (Bio Basic Canada Inc., Ontario, Canada), 0.05% v/v Polyoxyethylene lauryl ether (Brij-35) (MP Biomedicals, CA, USA), 0.25 mg/mL BSA, and 500 µM ATP, before layering on the peptide array and incubating at 37 °C for 2 hours. Arrays were washed 10 times with 50 mL 0.1 M PBS containing 0.1 % Triton-X-100. A final wash was performed with with 50 mL distilled deionized water. Pro-Q Diamond phosphoprotein stain (Invitrogen, NY, USA) was used to stain phosphorylated peptide on the arrays for 1 hour at room temperature. Pro-Q destain containing 20% acetonitrile (EMD Chemicals, Inc., Gibbstown, NJ) and 50 mM sodium acetate (Sigma Aldrich, St. Louis, MO, USA) was used to wash the arrays twice for 10 minutes at room temperature. Distilled water was then used to wash arrays for 10 minutes at room temperature

to complete the destaining procedure. Slides were then dried by placing them in a 50 mL polypropylene tube with a kimwipe (Uline, Canada, Ontario, Canada) placed at the bottom. The arrays were then centrifuged at 300 X g for 5 minutes at room temperature. Arrays were read using a microarray scanner Gene Pix Profession 4200A with fluorescence detected at wavelength 532 nm (Molecular Devices, LLC, CA, USA). GenePix 6.0 Software (Molecular Devices, LLC, CA, USA) was used to collect intensities of individual peptides. Background intensities were normalized for spots on the array (mean intensities = background intensities - foreground intensities). Peptide arrays were used to analyze PBMCs collected from the high and low serum cortisol responder sub-groups following the first, second, and third restraint episode.

2.11 Analysis of Peptide Array Data

Kinome profiles were determined by uploading an input file in a tab-delimited text format for a program called Platform for Integrated, Intelligent Kinome Analysis 2 (PIIKA 2) (http://saphire.usask.ca/saphire/piika/index.html) (Trost *et al*., 2013). 1-Pearson correlation for distance metric and McQuitty linkage method were used to perform hierarchical clustering. Peptides with inconsistent phosphorylation patterns among the technical replicates on the array were excluded by performing a Chi-square test. Peptides differentiating between high and low responders in each of the three restraints were identified by performing t-test comparisons of individual peptides. Further validation of kinome data was based on the significant differentiated peptides.

2.12 RNA Extraction

PBMC pellets stored in 1mL Trizol were thawed and 20 µL chloroform was added before vortexing. Samples were then placed on ice for 10 minutes before centrifuging at 14,000 rpm for 15 minutes at 4 °C. The top layer was transferred into 550 µL isopropanol (Sigma Aldrich, St. Louis, MO, USA) with 2.5 µL of 20 mg/mL glycogen, incubated at -70 °C for 15 minutes, then centrifuged at 14,000 rpm for 15 minutes at 4 °C. The RNA pellet was washed twice with 1 mL 70% ethanol and a third wash of 1 mL 100% ethanol (Sigma Aldrich, St. Louis, MO, USA) before air-drying the pellet. RNA pellets were re-suspended in 20 µL DNase RNase free water (Life Technologies, Burlington, Ontario, Canada). RNA was quantified with

a Nanodrop (Nanodrop 1000, Thermo Scientific, Waltham, MA, USA) before placing adding RNA to a 50 µL reaction, which contained 5 µL 10 X DNase Buffer (Life Technologies, Burlington, Ontario, Canada), 0.5 µL DNase I (Life Technologies, Burlington, Ontario, Canada), 17 µL of RNA sample, and 27.5 µL DNase RNase free water. The reaction mixture was incubated at 37 °C for 5 minutes (Life Technologies, Burlington, Ontario, Canada). Then the treated samples were transferred into 500 µL Trizol in 1.5 mL tubes. 110 µL chloroform was added to the tube, then vortexed, placed on ice for 10 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The top layer was transferred into a mixture of 275 µL isopropanol and 1.25 µL of 20 mg/mL glycogen, incubated at -80 °C for 15 minutes, then centrifuged at 14,000 rpm for 15 minutes at 4 °C. Supernatants were removed and the pellet was washed twice with 70 % ethanol and a third time with 100% ethanol. Pellets were dried on ice in the fume hood, then re-suspended in 20 µL DNase RNase free water. RNA concentration and quality were then determined with a bio-analyzer (Agilent Technologies, Mississauga, Ontario, Canada).

2.13 cDNA Synthesis

Each reaction consisted of 10 µL 2X RT Reaction mix, 2 µL RT Enzyme mix, 500 ng RNA, and DEPC-treated water to a final 20 µL volume in a PCR tube. RNA was reverse transcribed in the thermocycler (BioRad, Mississauga, Ontario, Canada): 25 °C for 5 minutes, 60 °C for 60 minutes, and 70 °C for 15 minutes. DNase RNase free water was used to dilute synthesized cDNA to a final volume of 60 µL and then stored at -20 °C.

2.14 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Primers including p21 (Fw 5'TCCAAGGACTTTTTCCATTTGC3'; Rv 5'TCTGACTCCTTCAGCTGTTATTCAA3') and β-actin (Fw 5' AGGCATCCTGACCCTCAAGTA 3'; Rv 5' GCTCGTTGTAGAAGGTGTGGT 3') were used for qRT-PCR. Samples were amplified at 50 °C for 2 minutes. 95 °C for 30 seconds, then 40 cycles of 95 °C for 15 seconds; 60 °C for 30 seconds; 72 °C for 30 seconds, and 30 cycles of dwell time for 10 seconds were performed. The generation of a single PCR product for each PCR reaction was evaluated by agrose gel (p21: amplication size of 75 bp; β-actin: amplicon

size of 116 bp). PCR standard curves were completed to determine an amplification efficiency of 1.98 for p21 and 2.0 for β-actin. Sequence analysis of the amplicons confirmed the appropriate gene identity for each PCR product.

2.15 Statistical Analysis

Data were analyzed for normal distribution using SPSS version 22 (SPSS Inc., Chicago, Illinois, USA) before subsequent analysis. Serum cortisol levels, serum glucoses levels, entry orders, N/L ratios, chute behaviors, exit frames were given individual rankings based on mean values for each restraint episode. A positive ranking represented values greater than the group mean while a negative ranking indicated values below the group mean. Values closer to the mean were assigned smaller ranking values. Rankings were summated across three restraints. Rankings for exit velocity were similarly calculated. Exit frame numbers exceeding the mean numbers of frames were given a positive ranking but indicated a slower exit speed when compared to negative rankings. The association between summated rankings of serum cortisol levels, serum glucose levels, N/L ratios, entry order rankings, chute behaviors, and exit rankings were assessed using Spearman ranking correlation coefficients. Unpaired t tests were performed to compare serum cortisol levels, entry orders, exit frames, chute behaviors, serum glucose levels, glycogen contents, gene expression values of p21 for low versus high responder sub-groups. Habituation or sensitization patterns for physiological and behavioral responses were analyzed using a one-way ANOVA to analyze data from all animals and restraints as well as for high and low responder subgroups. GraphPad Prism Version 6.10 software (GraphPad Software, Inc., San Diego, CA, USA) was used for all correlation coefficient analyses, unpaired t tests, and one-way ANOVA.

3. Results

3.1 Serum Cortisol

Table 3.1. Serum cortisol levels in response to restraint stress.

	First Restraint		Second Restraint		Third Restraint		Sum Ranking[3]
Animal	Cortisol[1]	Ranking[2]	Cortisol	Ranking	Cortisol	Ranking	
208	**110.0**	**5**	**114.0**	**5**	**108.0**	**1**	**11**
209	75.0	-5	100.0	1	122.0	3	-1
210	**105.0**	**3**	**148.0**	**10**	**165.0**	**8**	**21**
211	**140.0**	**9**	**153.0**	**11**	**151.0**	**6**	**26**
212	**116.0**	**6**	**128.0**	**7**	**154.0**	**7**	**20**
213	99.0	1	78.4	-3	82.2	-10	-12
214	**78.9**	**-4**	**73.9**	**-6**	**62.4**	**-12**	**-22**
215	84.4	-2	79.7	-2	107.0	-1	-5
216	108.0	4	88.8	-1	103.0	-2	1
217	**64.8**	**-7**	**59.3**	**-7**	**94.1**	**-7**	**-21**
218	126.0	8	76.4	-4	111.0	2	6
219	84.1	-3	104.0	2	103.0	-2	-3
220	**59.3**	**-8**	**46.4**	**-9**	**70.4**	**-11**	**-28**
221	**55.5**	**-9**	**59.0**	**-8**	**84.1**	**-8**	**-25**
222	102.0	2	106.0	3	125.0	4	9
223	119.0	7	114.0	5	103.0	-2	10
224	73.7	-6	111.0	4	83.0	-9	-11
225	93.0	-10	134.0	9	99.6	-5	-6
226	85.8	-1	131.0	8	133.0	5	12
227	93.0	-10	75.6	-5	98.8	-6	-21

1. Serum cortisol expressed as nmol/L.
2. Rankings were based on the mean serum cortisol level for the group mean on each restraint day. Animals with serum cortisol levels higher than mean were given a positive ranking whereas animals with serum cortisol levels below the mean were given a negative ranking.
3. Summated rankings for the three restraint periods.

Serum cortisol levels for individual calves following each of the three restraint periods are represented [**Table 3.1**]. The magnitude and range of serum cortisol levels (46.4 nmol/L to 165.0 nmol/L) in the current investigation are consistent with previous studies of stress responses in cattle (Mormede *et al*., 2007). Relative rankings for each restraint and summated rankings for the three restraints were calculated to reveal the temporal consistency of the serum cortisol responses of individual animals to restraint stress [**Table 3.1, Figure 3.1 A**].

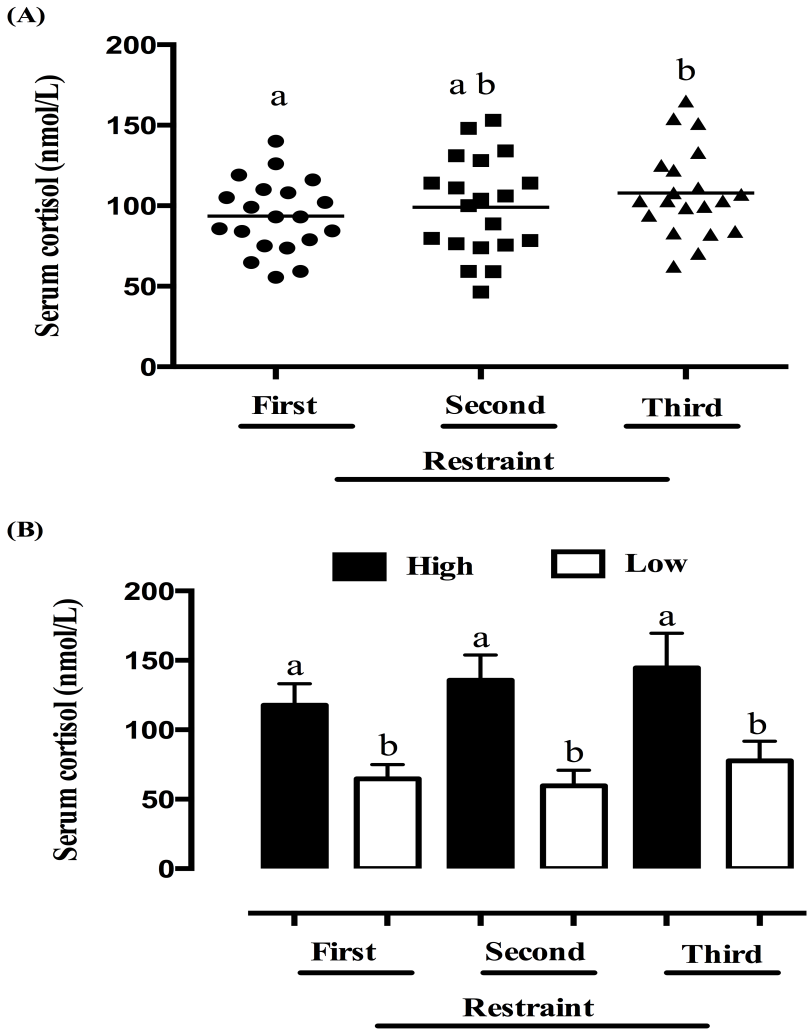

Figure 3.1. Serum cortisol response. (A) Serum cortisol levels (nmol/L) in calves subjected to repeated three restraints for 5 minutes (n = 20); (B) Serum cortisol levels in calves subjected to three 5-min restraints (n=4/group) at weekly intervals. Differences between serum cortisol levels (nmol/L) in high responders and low responders were compared using an unpaired t-test. Data presented are mean values with 1 SD. Different letters (a, b) denote significant ($p < 0.05$) difference between high and low responders.

Animals 208, 210, 211, 212 were selected to represent high cortisol responders while animals 214, 217, 220, 221 were selected to represent low cortisol responders based on these rankings. Significant differences in the serum cortisol levels between high responders and low responders were found across each of the three restraint episodes ($p < 0.01$ for the first and third restraints and $p < 0.001$ for the second restraint) [**Figure 3.1B**]. This demonstrates the existence of naturally occurring and temporally stable differences in how individual animals respond to restraint stress with respect to serum cortisol. Whether these differences in cortisol responses correlated with other behavioral, physiological and biochemical responses were then investigated.

3.2 Behavioral Scores (Entry Order, Chute behavior& Exit Velocity)

Three aspects of behavioral responses to restraint stress were quantified and ranked. Data related to entry order [**Table 3.2**], chute behavior [**Table 3.3**], and exit frames [**Table 3.4**] for each animal across the three restraint episodes are represented. Correlations analyses between serum cortisol levels and these behavioral scores across all animals and restraints were subsequently completed. In addition, these correlations were also subsequently investigated for the sub-groups of animals classified as high and low serum cortisol responders.

3.3 Leukocyte Counts

The physiological response to restraint stress was investigated by analyzing relative changes in blood neutrophils and lymphocytes. The differential cell counts were performed for each animal following each restraint episode and N/L ratios were determined and ranked [**Table 3.5**]. Similar to the analysis performed for the behavioral read-outs, efforts were made to correlate these responses to serum cortisol scores. There were no significant correlations between serum cortisol levels and N/L ratios [**Table 3.6**].

Table 3.2. Entry Order Rankings.

Animal	First Restraint	Second Restraint	Third Restraint	Sum of Entry Order[2]	Rank of Entry Order[3]
208	12	15	16	43	3
209	11	4	5	20	-8
210	1	14	11	26	-5
211	18	13	10	41	2
212	19	19	19	57	8
213	16	7	2	43	3
214	6	8	6	20	-8
215	3	5	13	21	-6
216	9	9	9	27	-3
217	5	6	1	12	-11
218	20	16	17	53	7
219	14	10	7	31	-1
220	17	2	2	21	-6
221	10	3	4	17	-9
222	4	11	12	27	-3
223	15	20	15	50	6
224	8	12	8	28	-2
225	13	17	18	48	5
226	7	18	14	39	1
227	2	1	3	6	-12

1. Animals were ranked from 1 to 20 (first to last) to indicate order of chute entry on each restraint day.
2. Order of entry for each calf was summated for the three restraints period.
3. A positive ranking was assigned when the summated value exceeded the group mean (later entry) and a negative ranking (earlier entry) when summated value was less than the group mean.

Table 3.3. Chute behavioral scores relative to restraint stress.

Animal	First Restraint[1]	Second Restraint	Third Restraint	Sum of Chute Behavioral Scores[2]	Rank of Chute Scores[3]
208	3	1	1	5	-8
209	5	5	3	13	8
210	3	4	4	11	5
211	3	1	3	7	-1
212	5	1	3	9	2
213	5	2	4	11	5
214	1	1	1	3	-9
215	2	3	2	7	-1
216	2	2	2	6	-5
217	1	1	1	3	-9
218	2	2	2	6	-5
219	3	3	2	8	1
220	3	2	4	9	2
221	1	1	1	3	-9
222	2	3	2	7	-1
223	5	5	4	14	9
224	1	3	3	7	-1
225	4	3	5	12	7
226	3	3	4	10	4
227	1	2	3	6	-5

1. Chute behavioral scores per animal were assigned using a 5-point system. Lower chute scores reflect calmer behavior while higher scores reflect more agitation.
2. Sum of chute behavioral scores from all three restraints periods.
3. Ranking of summated chute behavioral scores was given a positive ranking when the summated value exceeded the mean (agitation) and a negative ranking when summated value was less than the mean (calmer).

Table 3.4. Exit velocity and rankings subjected to restraint stress.

Animal	First Restraint[1]	Second Restraint	Third Restraint	Sum of Exit Velocity[2]	Rank of Exit Velocity[3]
208	4	4	10	18	7
209	3	4	5	12	1
210	5	2	2	9	-5
211	2	2	2	6	-12
212	3	3	2	8	-7
213	3	5	5	13	4
214	3	5	7	15	6
215	3	4	3	10	-1
216	3	3	4	10	-1
217	5	3	4	12	1
218	3	3	3	9	-5
219	2	2	6	10	-1
220	4	4	5	13	4
221	2	3	2	7	-10
222	3	4	3	10	-1
223	2	3	3	8	-7
224	3	3	2	8	-7
225	6	3	3	12	1
226	2	3	2	7	-10
227	5	8	6	19	8

1. Number of video frames (0.03 s) recorded while animal travelled a distance of 1.63 m after exiting the head-gate.
2. Summation of frames recorded from all three restraints periods.
3. Ranking of summated frames was given a positive ranking when the summated value exceeded the group mean (slower exit velocity) and a negative ranking when summated value was less than the group mean (more rapid exit velocity).

Table 3.5. Neutrophil to lymphocyte ratios relative to restraint stress.

	First Restraint		Second Restraint		Third Restraint		Sum Ranking[3]
Animal	N/ L[1]	Ranking[2]	N/ L	Ranking	N/ L	Ranking	
208	0.43	-3	0.33	-5	0.20	-9	-17
209	0.83	6	1.00	10	0.55	4	20
210	0.38	-6	0.90	9	0.80	8	11
211	0.40	-4	0.62	4	0.31	-3	-3
212	0.63	3	0.35	-4	0.26	-7	-8
213	0.49	-1	0.22	-10	0.31	-3	-14
214	0.29	-9	0.25	-8	0.09	-11	-28
215	0.25	-11	0.43	-2	0.51	1	-12
216	0.88	7	0.64	6	0.71	7	20
217	0.35	-8	0.64	6	0.23	-8	-10
218	0.08	-12	0.32	-6	0.53	2	-16
219	0.52	1	0.49	-1	0.33	-1	-1
220	0.53	2	0.52	1	0.30	-5	-2
221	0.38	-6	0.55	2	0.19	-10	-14
222	0.11	8	0.73	8	0.59	5	21
223	0.76	5	0.60	5	0.53	2	12
224	0.49	-1	0.23	-9	0.33	-1	-11
225	0.71	4	0.39	-3	0.81	9	10
226	0.40	-4	0.26	-7	0.66	6	-5
227	0.26	-10	0.57	3	0.28	-6	-13

1. N/ L = neutrophil to lymphocyte ratio in blood smears.
2. Rankings were based on the mean N/L ratios for each restraint day. Animals with N/L higher than the group mean were given a positive ranking whereas animals with N/L below the group mean were given a negative ranking.
3. Summed rankings for each animal across the three restraint periods.

3.4 Serum Cortisol Level and Entry Order

A strong association between summated rankings of serum cortisol and rankings of entry orders (r_s= 0.593, p=0.006) for the entire group (n=20) suggests that animals with a higher summated ranking of serum cortisol were more likely to have a later entry into the chute [**Table 3.6**].

This association was also observed when comparing the selected sub-groups of high and low serum cortisol responders. Again, high serum cortisol responders had significantly higher entry orders (p <0.01) than the low serum cortisol responders for the last two of the three restraint episodes [**Figure 3.2**].

Table 3.6. Correlation coefficients and significance level of rankings

	Serum Cortisol	Serum Glucose	Chute Behaviors	Entry Orders	Exit Frames	Neutrophil/ Lymphocyte ratios
Serum Cortisol	/	r_s =0.73 p= 0.0002***	r_s=0.344 p=0.137	r_s=0.593 p=0.006*	r_s=-0.504 p=0.023*	r_s= 0.373 p=0.105
Serum Glucose	/	/	r_s=0.429 p=0.059	r_s=0.712 p=0.0004***	r_s=-0.4 p=0.081	r_s=0.068 p=0.776
Chute Behaviors	/	/	/	r_s= 0.423 p=0.063	r_s= -0.165 p=0.488	r_s= 0.581 p= 0.007**
Entry Orders	/	/	/	/	r_s= -0.337 p=0.146	r_s= 0.043 p=0.857
Exit Frames	/	/	/	/	/	r_s= -0.241 p=0.306

* indicates p value is less than 0.05; ** indicates 0.01 <p <0.05; *** indicates p < 0.001.

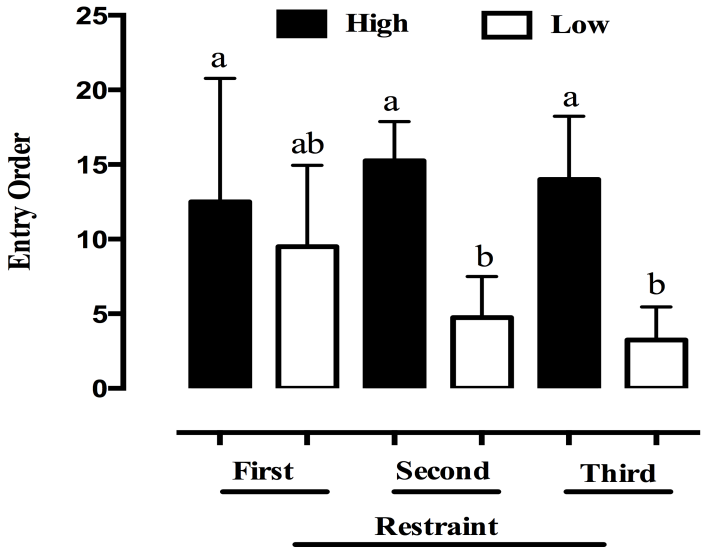

Figure 3.2. Chute Entry Orders (n = 4/group). Order that calves entered into the squeeze chute. Differences between entry orders (1-20) for high responders and low responders in each restraint were compared using an unpaired t-test. Data presented are mean values with 1 SD. [ab]Different letters (a, b) denote statistically significance when comparing between groups ($p < 0.05$).

3.5 Serum Cortisol and Chute Behavior

In contrast, summated ranking of serum cortisol levels and the ranking of behavioral responses in the chute were weakly positively correlated for the entire group, although this association was not statistically significant ($r_s = 0.344$, $p = 0.137$) [**Table 3.6**]. This was also reflected in the comparison of chute behaviors between sub-groups of animals representing high and low serum cortisol response. There was only a significant difference for one of the three restraint episodes (first restraint $p = 0.03$; second restraint $p = 0.55$; third restraint $p = 0.35$) [**Figure 3.3**].

3.6 Serum Cortisol and Exit Frames

The summated rankings of exit frames were negatively correlated with summated rankings of serum cortisol levels when analyzing data from all of the animals and all restraint episodes ($r_s = -0.504$, $p = 0.023$), suggesting that animals with lower exit frame scores (greater exit velocities) were more likely to have a higher summated ranking for serum cortisol levels [**Table 3.6**]. However, there was no significant difference when comparing exit velocities between the high and low responder groups for the three restraint episodes [**Figure 3.4**].

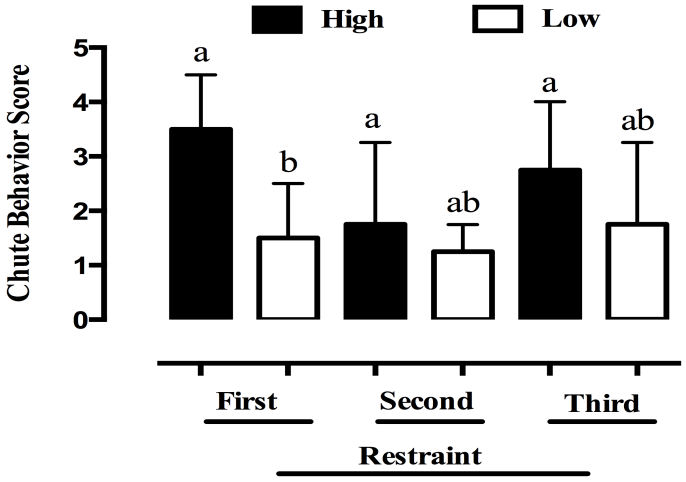

Figure 3.3. Chute behavior scores for calves subjected to three 5-min restraints. Differences between high serum cortisol responding group and low serum cortisol responding group in chute behavioral scores (1-5) in each restraint was compared using an unpaired t-test. Data represented are mean values with 1 SD. [ab]Different letters indicate statistically significant differences between treatment groups ($p < 0.05$).

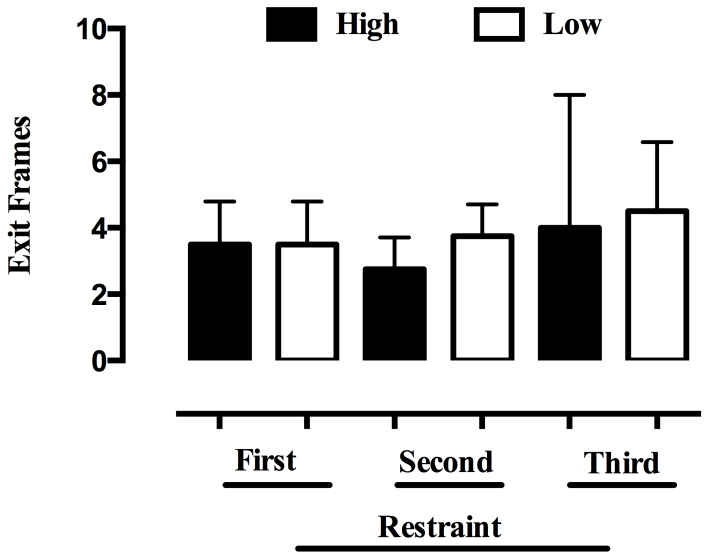

Figure 3.4. Video frames for exit velocity of animals subjected to three 5-min restraint episodes. Differences between exit frames for high responders and low responders in each restraint were compared using an unpaired t-test. Data presented are mean values with 1 SD. No differences between comparisons.

3.7 Serum Cortisol and Leukocyte Count

Across all animals and restraint episodes the summated rankings of the N/L ratios were positively correlated with summated rankings of serum cortisol levels ($r_s = 0.373$, $p = 0.105$) [**Table 3.6**]. While this relationship was not statistically significant, the trend suggests that animals with higher summated rankings of serum cortisol levels have higher N/L ratios. There was no significant correlation between serum cortisol and N/L ratio when comparing the high and low serum cortisol groups [**Figure 3.5**].

3.8 Biochemical Analysis (Kinome Analysis)

Kinome analysis was performed to assess potential differences in cellular signaling between the high and low serum cortisol responding animals. Hierarchical clustering of 24 kinome datasets (4 animals from each of the high and low cortisol responding groups at three time points) showed a tendency, but not absolute difference, for clustering on the basis of the serum cortisol sub-grouping [**Figure 3.6**] ($p = 0.15$). This trend suggests the potential existence of stable differences in signaling activity within the PBMCs of high and low serum cortisol responders. Importantly, this analysis included data relating to all the peptides on the array to reflect global signaling patterns. Differences in signaling relating to the acute stress responses may be limited to a small subset of these peptides.

Distinct clustering of the kinome datasets typically reflects the differential phosphorylation of a subset of the peptides represented on the arrays. T-test comparisons were performed to investigate peptides whose phosphorylation was consistently different between the high and low cortisol responding groups, This analysis revealed 45 whose patterns of phosphorylation showed consistent and significant difference between the high and low cortisol responding groups [**Table 3.7**]. Each of the phosphorylation events represented by these peptides may reflect signaling responses that are either differentially activated by the acute stress response or that contribute to the differential stress responses. Of these 45 phosphorylation events we selected a subset for detailed validation analysis. In particular, five peptides related to glycogen metabolism, apoptosis, and lymphocyte migration that demonstrated differential patterns of phosphorylation between the high and low serum cortisol groups across all restraint episodes [**Table 3.8**]. This suggests that these phosphorylation events represent conserved signaling differences between the phenotypic groupings.

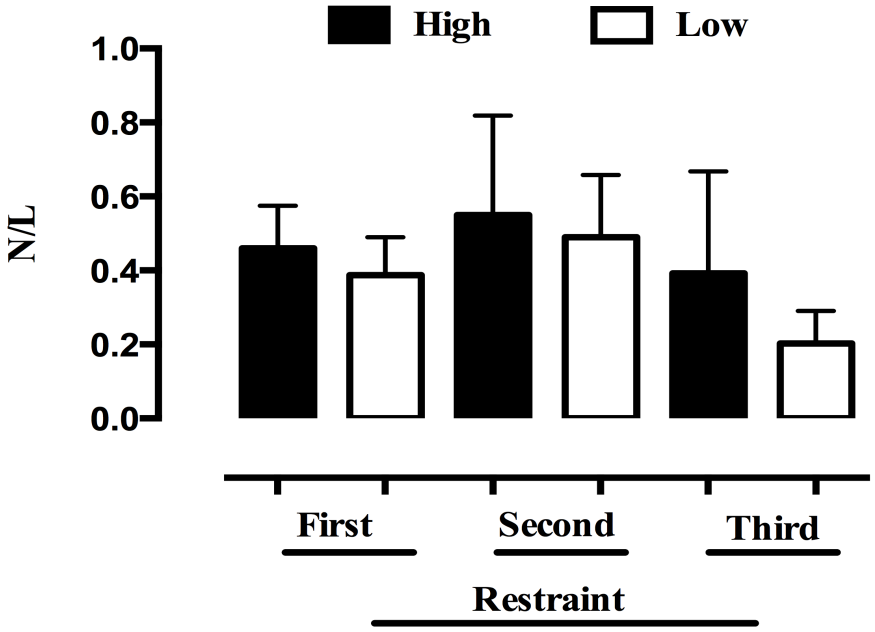

Figure 3.5. Neutrophil to lymphocyte (N/L) ratios for calves subjected to three repeated restraint for 5 minutes (n = 4/group). Differences between N/L in high responders and low responders were compared using an unpaired t-test. Data are represented mean values with 1 SD.

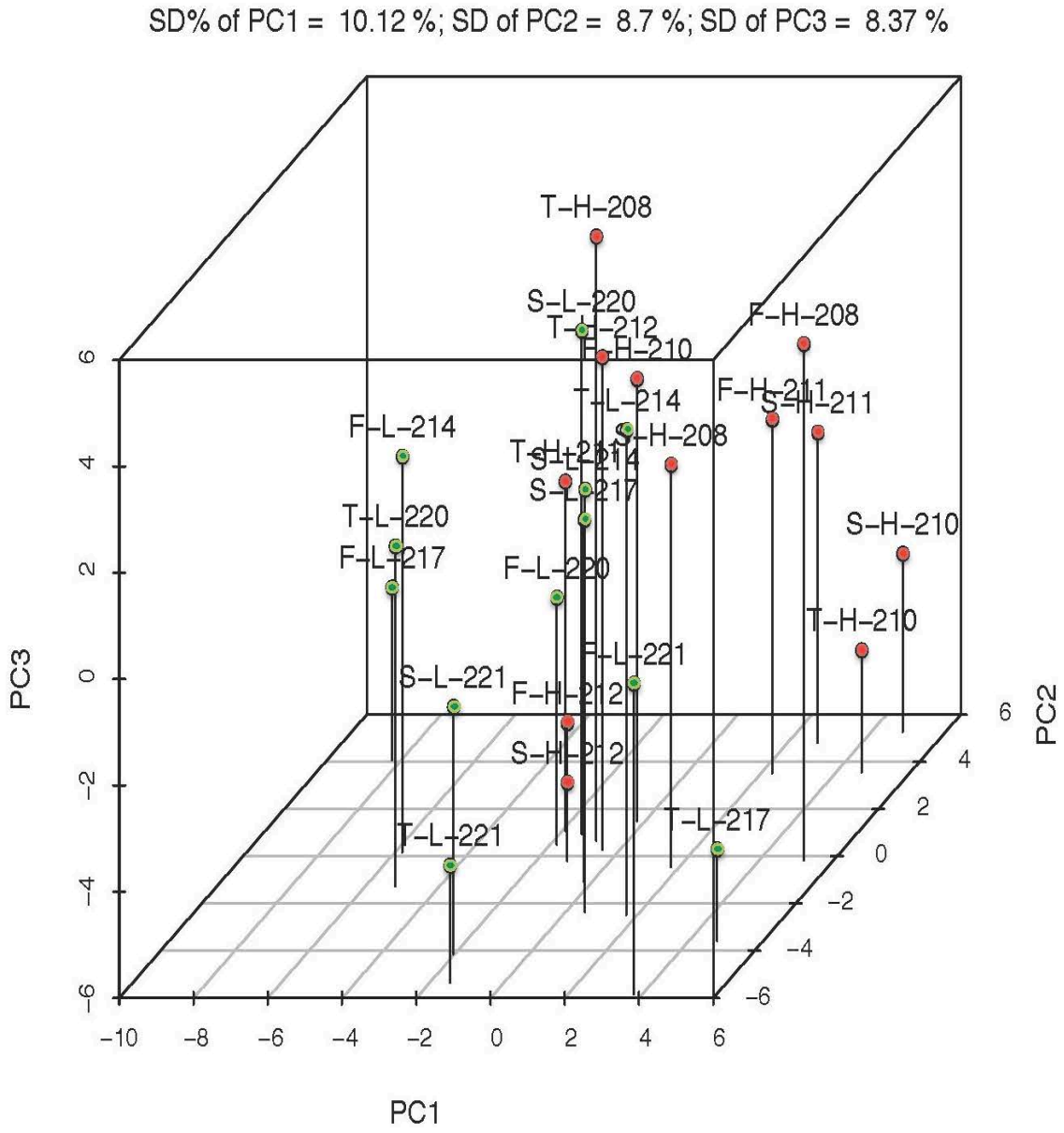

Figure 3.6. Cluster analysis of kinome data of high (n = 4) and low (n = 4) responders of calves following three replicate 5-min restraint periods. Principle component analysis of high responders and low responders subjected to restraints (n = 8/restraint). The first letter indicates first (F), second (S), or third (T) restraint. The second letter represents either high (H; red) or low (L; green) responders. Animal numbers occur at the end of each label.

Table 3.7. Peptides consistently discriminating between high and low serum cortisol responders for all three restraint episodes.

Peptide Name	Site[1]	Biological Function[2]
Eukaryotic translation initiation factor 3 subunit J-A (elF3)	S11	Protein synthesis
Signal transducing adapter molecule 1 (STAM-1)	S156	Upon IL-2 and GM-CSL stimulation, it plays a role in signaling leading to DNA synthesis
Mitogen-activated protein kinase 1(MAPK1)	T141	Involves in cell growth, adhesion, survival and differentiation
Serine/threonine-protein kinase 10	S437	Lymphocyte migration
[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1	Y136	Glucose and fatty acid metabolism regulation
TNF receptor-associated factor 2 (TRAF2)	S11 (human)/S20 (cow)	Cell survival and apoptosis
Macrophage colony-stimulating factor 1 receptor (CSF1R)	Y809 (human)/Y807 (cow)	Regulation of survival, proliferation and differentiation of hematopoietic precursor cells;promotes the release of proinflammatory chemokines.
NF-kappa-B inhibitor alpha (NFKBIA)	T291	Nuclear factor.
SHC-transforming protein 1 (SHC-1)	Y349	Isoform p46Shc and isoform p52Shc promotes the cytoplasmic propagation of mitogenic signals; Isoform p66Shc promotes apoptosis.
Acetyl-CoA carboxylase 2 (ACACB)	Y1489 (human)/ Y1363 (cow)	Catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonly-CoA
Adiponectin receptor protein 1 (ADIPOR1)	Y85	Regulates glucose and lipid metabolism. ADIPOQ-binding activates a signaling cascade that leads to increased AMPK activity, and ultimately to increased fatty acid oxidation, increased glucose uptake and decreased gluconeogenesis.
Hormone-sensitive lipase (LIPE)	S865 (human)/S554 (cow)	Converts cholesteryl esters to free cholesterol for steroid hormone production
Toll-like receptor 3 (TLR3)	Y759; T818	Involved in cytokine secretion and inflammatory responses
Serine/threonine-protein kinase 10	S13	Regulation of lymphocyte migration
RAF proto-oncogene serine/threonine-protein kinase	Y341	Regulates Rho signaling and migration, and is required for wound

(RAF1)		healing; restricts caspase activation
Cyclin-dependent kinase 2 (CDK2)	T160	Involved in the control of cell cycle
Galactokinase (GALK1)	Y236	Major enzyme for galactose metabolism
1-phosphatidylinositol 3-phosphate 5-kinase (PIKFYVE)	Y1772 (human)/Y1776 (cow)	Required for endocytic-vacuolar pathway and nuclear migration
Nuclear factor of activated T-cells, cytoplasmic 2 (NFATC2)	T116 (human)/T115 (cow)	Plays a role in the inducible expression of cytokine expression genes in T-cells
Dual specificity mitogen-activated protein kinase kinase 4 (MAP2K4)	T261 (human)/T274 (cow)	Essential component of the stress-activated protein kinase/ c-Jun N-terminal kinase (SAP/JNK) signaling pathway. MAP2K4 is required for maintaining peripheral lymphoid homeostasis
Polycystic kidney disease and receptor for egg jelly-related protein (PKDREJ)	Y2235 (human)/Y2242 (cow)	May have a central role in fertilization
NT-3 growth factor receptor (NTRK3)	Y516 (human)/Y533 (cow)	Upon activation activates MAPK signaling, which controls cell survival and differentiation
Protein kinase C epsilon type (PRKCE)	S729	Involves in cytoskeletal proteins, such as cell adhesion, motility, migration, and cell cycle
Glycogen synthase kinase-3 alpha (GSK3A)	Y279 (human)/Y191(cow) (cow)	Inactivate glycogen synthase
Nuclear receptor coactivator 1 (NCOA1)	S569 (human)/S572 (cow)	Nuclear receptor coactivator that stimulates the transcriptional activities in a hormone-dependent fashion. Involved in the coactivation of glucocorticoid receptor
Cyclin-dependent kinases regulatory subunit 2 (CKS2)	S51	Binds to the catalytic subunit of the cyclin dependent kinases and is essential for their biological function
cAMP-dependent protein kinase type I-beta regulatory subunit (PRKAR1B)	Y312 (human)/ Y166 (cow)	Regulatory subunit of the cAMP-dependent protein kinases involved in cAMP signaling in cells
5'-AMP-activated protein kinase subunit beta-2 (PRKAB2)	S184	Non-catalytic subunit of AMP-activated protein kinase, an energy sensor protein kinase that plays a key role in regulating cellular energy metabolism
Protein kinase C alpha type	Y658	Involves in cytoskeletal proteins,

	(human)/Y562 (cow)	such as cell adhesion, motility, migration, and cell cycle
Alpha-enolase (ENO1)	Y44	Plays a part in various processes such as growth control, hypoxia tolerance and allergic responses
Aconitate hydratase, mitochondrial (ACO2)	S559	Catalyzes the isomerization of citrate to isocitrate
Calcium/calmodulin-dependent proteinkinase type II subunit gamma (CAMK2G)	T287 (human)/T266 (cow)	Ca^{2+} transport
Phosphatidylinositol 3-kinase regulatory subunit beta (PIK3R2)	Y464 (human)/ Y460 (cow)	A kinase that phosphorylates Phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-triphosphate
Eukaryotic elongation factor 2 kinase (EEF2K)	S78 (human)/ S76 (cow)	Regulates protein synthesis by controlling the rate of peptide chain elongation
Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	Y265	Major phosphatase for microtubule-associated proteins
Utrophin	S1258 (human)/S1217 (cow)	May play a role in anchoring the cytoskeleton to the plasma membrane
Mitogen-activated protein kinase 8	Y185	Involved in various processes such as cell proliferation, differentiation, migration, transformation and programmed cell death
MAP kinase-activated protein kinase 3 (MAPK3)	T201 (human)/ T203 (cow)	An essential component of the MAP kinase signal transduction pathway
SH3 domain-containing kinase-binding protein 1	S587 (human)/S551 (cow)	Involved in the regulation of endocytosis and lysosomal degradation of ligand-induced receptor tyrosine kinases
TGF-beta receptor type-1 (TGFBR1)	T204 (human)/T200 (cow)	Regulates cell cycle arrest in epithelial and hematopoietic cells, control of mesenchymal cell proliferation and differentiation, wound healing, extracellular matrix production, immunosuppression and carcinogenesis
Dual specificity mitogen-activated protein kinase kinase 1(MKK1/MAP2K1)	S218 (human)/S222	An essential component of MAP kinase signaling, involves in diverse biological functions such as cell growth, adhesion, survival, differentiation, and apoptosis

Ras guanyl-releasing protein 3 (RASGRP3)	T133	Guanine nucleotide exchange factor for Ras and Rap1
Protein-tyrosine kinase 2-beta (PTK2B)	Y580	Regulates reorganization of the actin cytoskeleton, cell polarization, cell migration, adhesion, spreading and bone remodeling
E3 ubiquitin-protein ligase CHIP (STUB1)	Y49	Promotes proteasomal degradation

1. Orthologous phosphorylation sites for human and bovine proteins from which peptides were derived for the array;
2. Biological functions that correspond with phosphorylation of sites listed for each protein.

Table 3.8 Staining intensities of peptides that discriminated between high (n= 4/group) and low (n =4/group) serum cortisol responders for the three restraint episodes.

Peptide[1]	First Restraint			Second Restraint			Third Restraint		
	High[2]	Low[3]	p-value[4]	High	Low	p-value	High	Low	p-value
Glycogen phosphorylase	7.82	7.88	0.33	7.74	7.95	0.09	8.23	8.53	0.04
GSK3A	11.26	11.08	0.09	11.27	10.96	0.01	11.34	10.98	0.004
STK-10	7.20	7.48	0.04	7.21	7.89	0.00005	7.83	8.05	0.04
TRAF2	7.16	6.84	0.0004	7.09	6.72	0.006	7.70	7.33	0.001
MKK1	7.48	7.38	0.02	6.53	5.92	0.0002	7.09	6.85	0.03

1. GSK3A: Glycogen Synthase Kinase 3 alpha; STK-10: Serine/threonine-protein kinase 10; TRAF2: TNF receptor-associated factor 2; MKK1: Mitogen-activated protein kinase kinase 1.
2. Average intensities of high responders (Animal # 208, 210, 211, 212)
3. Average intensities of low responders (Animal # 214, 217, 220, 221)

3.9 Validation of Kinome Results

3.9.1 Glycogen Metabolism

Differential phosphorylation of peptides corresponding to the enzymes glycogen phosphorylase and glycogen synthase kinase anticipate differential metabolism of glycogen in the high and low cortisol responding groups [**Table 3.7** and **Table 3.8**]. Consistent higher intensities of the Y279 phosphorylation site of glycogen synthase kinase in high responding group across all restraint episodes predict a reduction in glycogen stores. Specifically, the Y279 phosphorylation site of glycogen synthase kinase has been associated with activation of this enzyme for subsequent phosphorylation and inhibition of glycogen synthase (Doble and Woodgett, 2003). Inhibition of glycogen synthase, the main anabolic enzyme for the generation of glycogen, favors the shift from glycogen to free glucose. Further, as the anabolic

and catabolic metabolism of glycogen are known to be reciprocally regulated (Tymoczko *et al*., 2013) this would logically predict an activation of the enzymes involved in the breakdown of glycogen. Consistent with this hypothesis, array data revealed a consistent and significant difference in the phosphorylation of Y727 of glycogen phosphorylase between the high and low responding groups. While the biological function of this phosphorylation event has not been clearly defined, the results of the array, and subsequent results of the functional validation of changes in serum glucose levels as well as levels of glycogen stores within the PBMCs, support the conclusion that these phosphorylation events result in decreased glycogen stores.

The implications of kinomic data analysis were validated by quantifying glycogen stores present in the PBMCs isolated from the high and low serum cortisol responder subgroups at the end of each restraint episode. A significant ($p < 0.05$) decrease in glycogen stores was detected in PBMCs of animals in the high serum cortisol responding groups for two of the three restraint episodes [**Figure 3.7 A**].

To further validate the implication from kinome data for differential metabolic activity in the high and low serum cortisol responders, the level of serum glucose following each of the three 5-min restraint episodes was also measured [**Table 3.9 and Figure 3.7 B]**. Serum glucose levels were significantly greater in the high serum cortisol responding group when compared to the low responding groups for all restraint episodes (first restraint $p = 0.029$; second restraint $p = 0.029$; third restraint $p = 0.033$) [**Figure 3.7 C**].

For all 20 restrained animals, there was a strong correlation between serum glucose levels and serum cortisol levels ($r_s = 0.73$, $p = 0.0002$) [**Table 3.6**]. Further, serum glucose displayed a similar trend when analyzing correlations with behavioral measures. This included a significant correlation with entry order ($r_s = 0.712$, $p = 0.0004$), chute behavior ($r_s = 0.429$, $p = 0.059$) [**Table 3.6**]. This is also reflected in the high serum cortisol responding animals having significantly and consistently higher serum glucose levels than the low cortisol responders for all restraint episodes [**Figure 3.7 C**]**.**

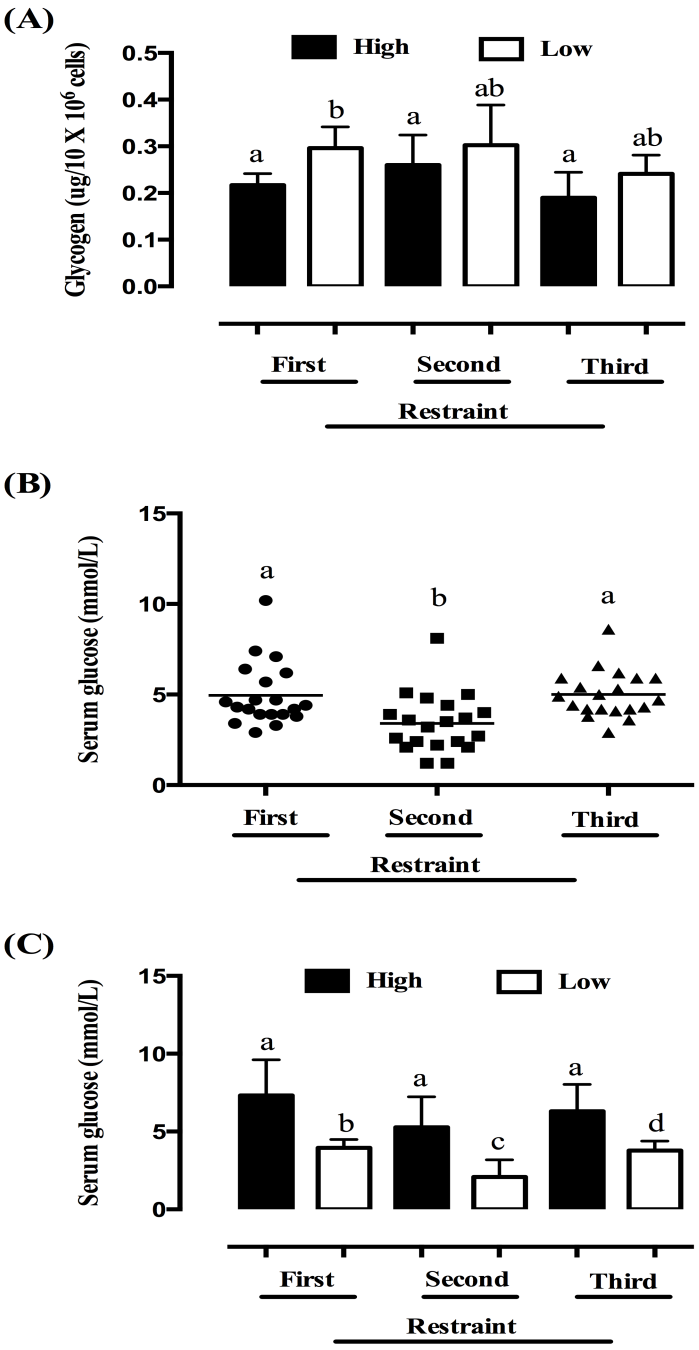

Figure 3.7. Carbohydrate responses for high and low serum cortisol responders following **three replicate 5-min restraint episodes.** (A) Glycogen levels (μg/10 X 10^6 cells) in high and low serum cortisol responder calves following restraint for 5 min (n = 4/group). Differences in glycogen levels were analyzed using an unpaired t-test. (B) Serum glucose levels in calves subjected to three replicate restraint episodes (n=20). (C) Serum glucose in high and low responder calves following restraint for 5 minutes (n=4/group). Differences between glucose levels (mmol/L) for high responders and low responders in each restraint were compared using an unpaired t-test. Data represented are mean values with 1 SD. ^{abcd}Differences between letters show statistical significance ($p < 0.05$).

Table 3.9. Serum glucose levels in response to restraint stress.

Animal	First Restraint		Second Restraint		Third Restraint		Sum Ranking[3]
	Glucose[1]	Ranking[2]	Glucose	Ranking	Glucose	Ranking	
208	4.6	-3	3.9	4	4.7	-2	-1
209	4.2	-6	2.7	-2	4.2	-5	-13
210	10.2	6	5.1	9	6.6	8	23
211	7.1	4	4.0	5	5.3	2	11
212	7.4	5	8.1	10	8.6	9	24
213	4.4	-4	2.4	-4	5.4	3	-5
214	4.7	-1	3.5	1	4.3	-4	-4
215	4.7	-1	3.2	-1	5.0	1	-1
216	4.3	-5	2.2	-6	4.2	-5	-16
217	3.4	-12	1.2	-9	2.9	-11	-32
218	6.4	3	3.7	3	5.9	4	10
219	3.9	-8	2.1	-7	4.2	-5	-20
220	3.9	-8	1.2	-9	4.1	-8	-25
221	3.8	-11	2.4	-4	3.8	-9	-24
222	3.3	-13	3.6	2	5.9	4	-7
223	6.2	2	4.4	6	4.9	-1	7
224	3.9	-8	2.6	-3	4.4	-3	-14
225	5.7	1	4.8	7	5.9	4	12
226	4.2	-6	5.0	8	6.2	7	9
227	2.9	-14	2.1	-7	3.6	-10	-31

1. Serum glucose expressed as mmol/L.
2. Rankings were based on the mean serum glucose level for the group on each restraint day. Animals with serum glucose levels higher than mean were given a positive ranking whereas animals with serum glucose below the mean were given a negative ranking.
3. Summated rankings for the three restraint periods.

3.9.2 Apoptosis

Data from the kinome analysis also revealed consistent differential phosphorylation of TNF receptor-associated factor 2 (TRAF2) and Mitogen-activated protein kinase kinase 1 (MKK1). These two proteins are known to be involved in differential regulation of apoptosis [**Table 3.7 and Table 3.8**]. Higher intensity labelling of peptides corresponding to site S11 of TRAF2 and S218 of MKK1 in the high responding group suggests increased phosphorylation activation, which has been reported to correspond inhibition of apoptosis (Yeh *et al*., 1998; Karl *et al*., 2014). Expression of the p21 gene has been used as a biomarker of apoptosis (Sporer *et al*., 2008). To verify whether there was differential activation of apoptosis between the high and low cortisol responding groups, expression of p21 in PBMCs immediately after each restraint episode was quantified through qRT-PCR. Expression of p21 was significantly greater ($p < 0.05$) in the group responding to restraint with elevated serum cortisol for two of the three episodes with 3.9 and 3.1 fold changes relative the low responders group [**Figure 3.8**].

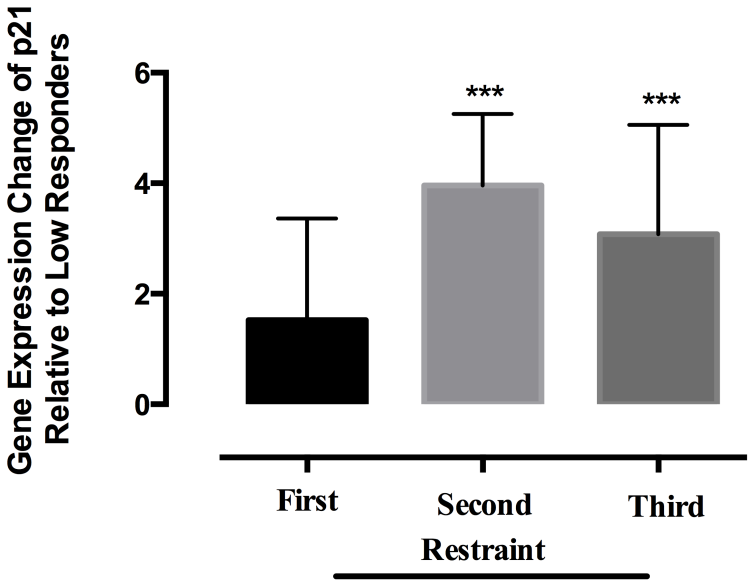

Figure 3.8. p21 gene expression in high responders relative to low responders (n=4) following three replicate restraint episodes. P21 gene expression in high versus low serum cortisol responders were quantified using qRT-PCR. *** ($p < 0.001$).

3.10 Sensitization/Habituation to Repeated Restraint Stress

Repeated exposure to a stressor may result in either sensitization or habituation of biological responses to the stressor (Mormede *et al*., 2007). Sensitization results in enhanced stress responses, while habituation diminishes responses to stress. ANOVA analysis was performed to determine there were significant changes in the magnitude of any of the measured physiological and behavioral parameters when comparing among the three restraints episodes. When comparing data from all 20 animals, there was a significant ($p = 0.02$) increase in serum cortisol levels during the third versus first restraint episode [**Figure 3.1A**]. This suggests that calves have become sensitized to the 5-min restraint period when there was a 7-day interval between episodes. No significant difference among restraint episodes was observed, however, when comparing high ($p = 0.14$) versus low ($p = 0.22$) serum cortisol responders [**Figure 3.1B**]. Additionally, serum glucose levels varied significantly ($p < 0.0001$) when comparing data from all animals ($n = 20$) for the second versus first and third restraint episodes, but there was not a temporal pattern consistent with either habituation or sensitization [**Figure 3.7B**]. Interestingly, a significant difference was also observed when comparing the first restraint versus second restraint ($p = 0.013$) as well as the second restraint versus third restraint ($p = 0.03$) for the low serum cortisol responder sub-group but no significant temporal differences were observed among restraints for the high responders [**Figure 3.7C**]. There was no temporal pattern consistent with either habituation or sensitization when comparing N/L ratios [**Figure 3.5**]. In contrast to physiological responses, no significant temporal change in responses was observed when comparing behavioral responses among restraint episodes for either all 20 animals or the high versus low serum cortisol responder sub-groups [**Figure 3.2, 3.3, 3.4**]. These results suggest that physiological and behavioral responses may be subject to distinct regulatory mechanisms that influence sensitization or habituation of responses.

4. Discussion

The priority of this investigation was to monitor physiological and behavioral responses of cattle to a brief restraint stress to observe individual animal variance in these responses and determine if some animals consistently displayed stress tolerant versus stress sensitive responses. The young animals selected for the study had limited prior contact with humans and had not previously been restrained in a chute. Responses were evaluated by measuring both behavioral (chute entry order, chute behavior and chute exit velocity) as well as biochemical and physiological variables, including serum cortisol levels, cell differential counts and the analysis of phosphorylation-mediated signal transduction activity in blood leukocytes. The parameters measured were then used to determine how well behavioral and physiological responses correlated and to determine if specific responses segregated within two sub-groups of animals identified as stress tolerant versus sensitive, on the basis of serum cortisol levels immediately following each restraint episode.

Cortisol levels in body fluids and tissue is often employed as a biomarker of stress responses. While this biomarker reflects hypothalamus-pituitary-adrenal axis activity it does not reflect the activity of the sympathetic-adrenal medullary axis and may not correlate with the diverse behavioral and physiological responses observed following a variety of stressors (reviewed in Chen *et al*., 2015). Therefore, analyzing correlations between serum cortisol and behavioral, physiological and biochemical responses to a specific stressor may assist in understanding the mechanisms of stress as well as identifying better biomarkers of stress tolerant versus stress sensitive animals. These correlations were investigated from two perspectives. Firstly, correlations among the parameters measured were analyzed for the full cohort of animals (n = 20) through summation ranking analysis. Secondly, sub-groups of animals that consistently represented the two extremes of the serum cortisol responses were identified and differences in the responses of these two phenotypically distinct sub-groups were considered. Furthermore, these two sub-groups were then used for a kinomic profiling of PBMCs to determine if physiological responses could be identified that consistently differentiated between these sub-groups of animals.

When studying stress responses it is important to be aware that experimental manipulations may in themselves impact responses, especially when animals are repeatedly exposed to the same stressor. Therefore, data was analyzed for potential patterns that might reflect either habituation or sensitization to the restraint procedure that was repeated at weekly

intervals. While a sensitization pattern was observed for serum cortisol levels when analyzing the full cohort of animals, this pattern was not apparent for the two sub-groups with low and high serum cortisol levels. The lack of apparent sensitization within the two sub-groups may reflect that the over-riding effect of other factors that determine these extreme phenotypes or may simply reflect the lack of statistical power inherent in with a reduced group size. No significant habituation or sensitization patterns were observed for either serum glucose levels, N/L ratios, or behavioral responses. Therefore, we conclude that the experimental design and procedures used in the current study were adequate for the identification of animals that were either consistently tolerant or sensitive to restraint stress.

4.1 Induction of Serum Cortisol in Response to Restraint Stress in Cattle

Serum/plasma cortisol levels are frequently used to quantify the magnitude and duration of stress responses in animals (Andrade *et al*., 2001; Herskin *et al*., 2007; Szenci *et al*., 2011; Carroll *et al*., 2009). There were, however, apparently contradictory results when cortisol levels were used to monitor the HPA response to restraint stress (Andrade *et al*., 2001; Herskin *et al*., 2007; Szenci *et al*., 2011). For example, Brahman cows adapted to handling prior to an experiment, displayed declining levels of serum cortisol when subject to daily 10-min restraint episodes over a 19-day interval (Andrade *et al*., 2001). In contrast, elevated plasma cortisol concentrations were observed following a single 15-min restraint of Holstein-Friesian cows (Herskin *et al*., 2004). Moreover, plasma cortisol levels were significantly increased when pregnant Holstein-Friesian heifers were subjected to a single 2-hour restraint period (Szenci *et al*., 2011). These variances in cortisol responses highlight the potential impact of the duration of restraint stress, prior human-animal interaction, and adaptation to frequent exposure to the same stressor. In the present investigation, the range of serum cortisol levels detected following a 5-minute restraint period was consistent with previous studies that reported serum cortisol levels of 60 to 200 nmol/L following a stressful event and a basal serum cortisol level of 15 nmol/L (Lay *et al*., 1992; Boissy *et al*., 1997). Further, we observed animal-specific differences in the magnitude of HPA response to restraint stress that were consistent among multiple restraint episodes [**Table 3.1**]. This observation supports the conclusion that some animals may be resistant or more sensitive in responding to restraint stress. According to Mormede and colleagues, genetic factors, environmental influences and the other factors, such

as handling history, might contribute to individual animal variation in HPA axis activity (Mormede *et al*., 2007). In the present study, animals were selected that had little prior contact with humans and no history of restraint in a chute. Serum cortisol levels may also be influenced by the method used to collect blood samples. However, to minimize this potential source of variability, all the animals were handled by the same individual throughout all replicate experiments and blood collection was performed immediately after the restraint period. .

4.2 Cortisol and Entry Order

The strong correlation between serum cortisol levels and chute entry order is consistent with a previous investigation, which reported that higher serum cortisol levels in beef cattle correlated with later entry order into a squeeze chute ($p = 0.10$) (Bristow and Holmes, 2007). A stronger correlation between serum cortisol and entry order in the current study versus the Bristow and Holmes investigation may be a consequence of a shorter restraint interval (5 min vs 90 min) that more closely connect HPA activation during chute entry with the time of serum collection. Moreover, the stronger correlation between serum cortisol levels and chute entry order when analyzing data from all 20 animals, rather than the sub-groups based on of low and high serum cortisol levels, may simply be a consequence of the greater statistical power associated with larger animal numbers.

4.3 Cortisol and Chute Behavior

Previous study of restraint stress in cattle failed to find a significant correlation between serum cortisol responses and the chute behavioral responses of animals when restrained in a chute (Curley *et al*., 2006). This is consistent with the current study, where we observed no significant correlation between serum cortisol levels and behavior when analyzing all animals ($n=20$) for multiple restraint episodes ($r_s = 0.344$, $p = 0.137$). Further, differences in chute behavior were only significant ($p < 0.05$) during the first restraint episode when comparing the sub-groups of high versus low serum cortisol level animals [**Figure 3.3**]. This observation suggests that the greatest behavioral response may be induced when animals with no prior restraint experience are first subjected to restraint. There may then be a rapid behavioral

adaptation to restraint that results in no significant behavioral difference among animals following the second and the third restraint. Studies in other species have also demonstrated a disassociation between behavioral and HPA responses induced by restraint stress. For example, habituation of struggling behavior in male Sprague-Dawley rats was induced by a 30-min restraint but adrenal gland removal failed to alter the struggling behavior. It was concluded that struggling behavior was not determined by HPA activity (Grissom *et al*., 2008). Moreover, Gagliano and colleagues demonstrated that a 2-hour restraint stress repeated every other day did not alter adrenocorticotropic hormone responses, but reduced the degree of anxiety behavior in rats (Gagliano *et al*., 2008). The agreement between this investigation and studies with rats is consistent with a disassociation between behavioral responses to restraint and HPA activity. In future studies, it may be informative to measure SAM activity to determine if it has a greater effect on behavioral responses.

4.4 Cortisol and Exit Velocity

A significant relationship between serum cortisol levels and the number of video frames measuring exit velocity was observed when analyzing data from all 20 calves for all three restraint episodes ($p = 0.023$). This observation is consistent with data from a previous study with Brahman bulls (Curley et al., 2006). However, no significant differences in exit velocity were observed when comparing the sub-groups of high and low serum cortisol responders. This may simply be a consequence of the relatively small number of animals analyzed and the minor variation in recorded frame number for the short distance monitored after animals were released from the chute.

4.5 Cortisol and Differential Counts

No significant correlation was observed between serum cortisol levels and the N/L ratio calculated for all 20 animals from multiple restraint episodes ($p = 0.11$). There is limited information available regarding the impact of restraint stress on blood leukocyte populations in cattle. In contrast, Earley and colleagues reported that transportation significantly elevated neutrophils (neutrophilia), decreased lymphocytes (lymphopenia), which significantly increased the N/L ratio in comparison to non-transported bulls (Earley *et al*., 2010). The same effects

have also been detected in calves that were subjected to abrupt weaning (Lynch *et al*., 2010). This contradiction with our current study may reflect the relatively short duration of the restraint stress which may not allowed adequate time to alter leukocyte trafficking in blood. The neutrophilia and lymphopenia reported in previous stress studies has been attributed, in part, to a gradual down-regulation of the surface adhesion molecule, CD62L, on neutrophils (Weber *et al*., 2001). The 5 min restraint period used in the present study may not allow sufficient time for this change to occur.

4.6 Sensitization/Habituation

The specific type, time frame, frequency, or strength of a stressor may contribute to the habituation or sensitization of a stress response. In the current investigation, significant sensitization of serum cortisol responses was observed during the third consecutive restraint. In contrast, habituation of plasma cortisol levels was observed on the second test day when Brahman cattle were subjected to daily 10-minute restraint periods on 19 consecutive days (Andrade et al., 2001). Moreover, cattle that were handled every 10 days had lower serum cortisol concentrations than those handled in the squeeze chute every 20 days (Goonewardene et al., 2000). These observed difference may arise for a number of reasons. Firstly, the one-week interval between restraint episodes in our study was selected to minimize possible habituation but this interval resulted in a sensitization of the HPA response. Secondly, calves in our study were reared in an extensive grazing situation within a herd of over 150 cows. The only human contact and restraint prior to our study was at the time of branding and vaccination (occurring at 4 to 8 weeks of age) as well as during transportation to the VIDO research facility (6-8 months of age). These interactions did not involve restraint in a chute. In contrast, the Brahman cattle used in the study by Andrade and colleagues (Andrade *et al*., 2001) were 5 to 8 years old and had been handled regularly for two years prior to the experiment. Furthermore, restraints in these studies were performed more frequently and were of longer duration. This might provide more time for cattle to adapt to each restraint. Interestingly, there was no evidence for either habituation or sensitization when analyzing serum glucose levels for either all animals or the two sub-groups of low and high serum cortisol. There was, however, significant variation in serum glucose levels when comparing among replicate restraint

experiments. This may suggest that regulation of serum glucose may relate more to other factors than the HPA axis.

4.7 Stress and Kinome Data

4.7.1 Stress and Carbohydrate Metabolism

Phosphorylation of glycogen phosphorylase is involved in the breakdown of glycogen (Roach *et al*., 2012). The differential phosphorylation of glycogen phosphorylase observed for high and low serum cortisol groups was revealed by kionme analysis. However, the functional consequences of Y727 phosphorylation, the phosphorylation site identified in glycogen phosphorylase, has not been determined. In contrast, increased phosphorylation of the Y279 site in glycogen synthase kinase, is known to be associated with an inhibition of glycogen synthase activity (Doble and Woodgett, 2003). Thus, kinome results are consistent with the differential regulation of glycogen stores observed for high and low serum cortisol groups [**Figure 3.7A**].

The range of serum glucose levels (1.2 mmol/L to 10.2 mmol/L) was greater than previously reported following the one-minute restraint of 2-year-old non-pregnant fighting breed cows (6.7 ± 1.4 mmol/L) (Sanchez *et al*., 1996). This may indicate that the duration of a restraint episode may contribute to the magnitude of change in this metabolic response. Comparisons between the high and low serum cortisol groups were consistent with both the PBMC glycogen stores and kinome data. As glucose is one of the final products of glycogen breakdown (Roach *et al*., 2012), greater serum glucose levels in the high serum cortisol group is consistent with a reduction in glycogen stores relative to the low serum cortisol group. Serum glucose was strongly correlated with serum cortisol and followed the same trend when analyzing correlations with behavioral measures. These results suggest that serum glucose has the potential, similar to serum cortisol, to be used as a biomarker for detecting responses to a brief stress, such as restraint, in healthy animals.

4.7.2 Stress and Apoptosis

Kinome data analysis for high and low serum cortisol responders suggested differential phosphorylation of two proteins related to the regulation of apoptosis. Expression of the p21 gene has been used as a biomarker of apoptosis (Sporer *et al*., 2008). Increased expression of the p21 gene in high responders after two of the three restraint episodes indicated that high

apoptosis may be increased in the high responders relative to the low responders. This observation is consistent with a previous study demonstrating that transportation induced higher relative expression of p21 compared with non-transported control animals (Sporer *et al*., 2008). However, this data is not consistent with the differential phosphorylation intensities observed for TRAF2 and MKK1, which suggests decreased apoptosis in high responders versus low responders. This inconsistency may reflect a possible inhibitory effect by p21 on apoptosis (Cartel *et al*., 2002).

4.7.3 Stress and Lymphocyte Migration

Serine/theronin-protein kinase 10 (STK-10) is associated with the regulation of lymphocyte migration (Belklna *et al*., 2009) and this protein may be involved in the reduction in circulating lymphocytes frequently observed following cortisolemia (Earley *et al*., 2010). Consistent changes in the phosphorylation intensity of STK-10 were observed for the three restraint episodes. There was, however, no significant effect on the neutrophil to lymphocyte ratio observed at the end of each restraint episode [**Figure 3.5**]. It is possible that the kinomic responses we observed represent early cell signaling events that precede changes in circulating blood leukocyte populations. The 5-minute time frame of the current study may not be sufficient to manifest significant changes in the lymphocyte trafficking.

4.8 Glucose as Potential Biomarker of Brief Stress

Changes in carbohydrate metabolism that increase the availability of free glucose through catabolism of glycogen is a well-documented response to acute stress (Rhoads *et al*., 2013). Within the present study, a significant correlation was observed between serum cortisol and serum glucose levels suggesting that serum glucose levels may serve as a potential biomarker of an acute stress response in healthy cattle. There are a number of practical advantages to measuring serum glucose as opposed to serum cortisol levels when monitoring an acute stress response. Firstly, in terms of the costs, it is possible to use relatively inexpensive, commercially available blood glucose meters and strips. The cost of a blood glucose test is approximately $1.7/sample which is $8.3/sample less than the cost of a serum cortisol assay. Secondly, blood glucose levels can be determined within seconds beside the chute and requires little technical expertise. In contrast, measuring blood cortisol assays requires a larger volume

of blood, requires a much longer time to process samples, and must be performed within the context of a well equipped research lab.

Collectively, these characteristics suggest that quantification of serum glucose might be an effective tool for monitoring livestock responses to a variety of stressors. It is important to appreciate, however, the complex nature of serum glucose regulation. Hormones such as insulin, epinephrine, and cortisol are all involved in regulating glucose metabolism (Mehla *et al*., 2014; Kolli *et al*., 2014; Rose *et al*., 2013; Lowenberg *et al*., 2006; Tirone and Brunicardi, 2001). While insulin is responsible for glucose clearance from the blood, epinephrine and cortisol increase blood glucose through promoting glycogenolysis or gluconeogenesis, respectively (Tirone and Brunicardi, 2001). In addition to this hormonal regulation, blood glucose levels can be generated from hepatic glycogen and be regulated by the central nervous system. Further, the ingestion of carbohydrates, proteins and fats can all serve as sources for producing glucose (Tirone and Brunicardi, 2001). The complexity of the mechanisms regulating blood glucose may impose limitations, or require special consideration, when using glucose as a biomarker of stress responses. For example, conflicting results have emerged over whether shipping stress of cattle increases (Earley *et al*., 2010), decreases (Bernardini *et al*., 2012) or fails to alter (Fisher *et al*., 2014) serum glucose levels. These contradictory results most likely reflect variables such as feed availability as well as the duration and nature of the transportation.

A few studies highlight the potential to use glucose in chronic stress studies and stress related diseases. For example, glucose levels were reported to be lower in stressed rats than control rats when rats were subjected to chronic unpredictable stress for 35 days (Karagiannides *et al*., 2014). Although this change in serum glucose is opposite to that observed during our acute stress study, it may be possible to use glucose as an indicator for chronic stress. It has also been observed that both male and female patients with stress-related exhaustion had lower plasma glucose levels than that of controls (Sjors *et al*., 2013). However, the use of glucose as an indicator of human diseases might be further complicated by the incidence of hyperglycermia and hypoglycermia (Hermayer *et al*., 2015).

4.9 Translation to Human Health

Stress has been implicated as a contributing factor in a large variety of human diseases, such as anxiety/depression (Bao *et al*., 2007), post-traumatic stress disorder (PTSD) (Jones *et al*., 2011), cardiovascular diseases (Steptoe *et al*., 2012), and cancer (Moreno-Smith *et al*., 2010). These are diseases that affect a large percentage of the population (Dupont *et al*., 1996; Lepine, 2002), resulting in substantial economic costs. The current investigation may benefit research related to stress-related human diseases in terms of two aspects. Firstly, the large animal model used in this study may better represent similar stress responses in human than mice models. Mice have been extensively used in research to mimic human diseases because they can easily be handled and genetically manipulated, and well-defined tools and tests are available to measure responses. It has recently been reported, however, that the immune system in mice differs substantially from that of humans (Seok *et al*., 2013). Moreover, the genetically manipulated mouse models can not represent the genetic diversity among human populations, which raises doubts regarding the general applicability of results generated in mouse models. This suggests that stress-related studies might be less relevant when using rodent models since stress has an effect on immune responses. Further, using outbred animal models provides the biological diversity that may reveal the diversity of responses possible when a stressful event is encountered. To better understand the mechanisms regulating these diverse responses in a mammalian species, it may be useful to select individuals displaying extreme responses. Secondly, saliva cortisol is commonly used as a measurement of anxiety (Hardeveld *et al*., 2014), which only provides crude assessments to reflect the complexity biological responses in patients. Thus, to develop a more accurate parameter to measure stress responses in patients would be of great benefit. According to this current study, serum glucose may have the potential to serve as an indicator for human diseases that are not compounded by other diseases such as hyperglycermia and hypoglycermia.

4.10 Stress and Antibiotics

The wide use of antibiotics in animal production is beginning to raise considerable concern regarding the emergence of antibiotic resistant bacteria, which is compromising the control of infectious disease (Anderson and Hughes, 2014). For example, Penicillin was used to treat human infections caused by a pathogen *Stapbylococcus aureus*. However, penicillinase

was detected in *Staphylococcus aureus* strain, resulting the use of another antibiotic Methicillin in 1950s. Methicillin-resistant *staphylococci aureus* (MRSA) were detected in 1961. MRSAs are resistant to all currently available antibiotics, and have been detected in dairy cows, horses, and chickens, indicating the spread between humans and animals (Doyle *et al.,* 2012). Improving stress management may be one way to reduce the risk of infectious disease and decrease our reliance on the use the antibiotics to treat animal diseases and promoting animal production. A direct correlation has been established between stress and increased mortality (Hodgson *et al*., 2012) and decreased animal production (O'Brien *et al*., 2010). Therefore, minimizing stress responses through changes in management procedures and animal handling may be of great benefit in reducing our reliance on antibiotics. To achieve these objectives, however, it will be necessary to have quantitative measures of stress responses so we can monitor the efficacy of new animal production systems.

5. Conclusion

Significant correlations were observed between serum cortisol, serum glucose and specific behavioral responses of cattle to restraint stress. Serum cortisol and glucose levels were closely correlated but serum glucose was more of two of the three behavioral responses, including entry order (r_s = 0.712, p = 0.0004), chute behavior (r_s = 0.429, p = 0.059). This observation supports the conclusion that serum glucose has a similar potential as serum cortisol to be used as a rapid test to measure an acute stress response in healthy cattle. Habituation/sensitization patterns were evaluated across all physiological and behavioral parameters for all 20 animals and for the subsets of high and low HPA responders. Serum cortisol responses increased significantly with consecutive restraint episodes while serum glucose and behavioral responses did not showed any habituation or sensitization pattern.

A priority of this investigation was the identification of quantifiable responses (behavioral or physiological) that could be used to identify animals consistently displaying either a weak or strong response to restraint. Such biomarkers could potentially be used to evaluate whether changes in management practices reduce stress and may be used to facilitate the selection of animals with a calmer disposition and a greater tolerance for routine handling procedures. Kinome data provided insight into the extremes of stress tolerant or sensitive

animals by suggesting ten conserved phosphorylation events. The importance of an animal being able to tolerate a brief period of restraint is highlighted by a possible association between toe-tip necrosis syndrome in cattle with excessive struggling while restrained in a chute (Gyan *et al*., 2015). An objective, rapid, inexpensive, and quantitative biomarker that measures stress during restraint would provide a valuable tool to further explore possible associations between stress responses and an increased susceptibility to specific disease. This investigation indicates that measuring serum glucose levels may provide an effective tool for this type of study.

6. References

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7. Appendices

Hair Samples Collection

Hair samples were collected using electric clippers to remove hair from an area on the neck measuring two inches by two inches. Hair was collected 16 days prior to the first restraint and one week after the last restraint. Hair that regrew in the clipped area throughout the stress experiment was collected one week after the last restraint. All hair samples were labeled and stored in individual dry paper envelopes at room temperature.

Hair cortisol Extraction

Extraction methodology was modified from Macbeth and colleagues (Macbeth *et al*., 2010). A 100 mg aliquot of each hair sample was placed into a 5.0 mL 12 X 75 mm disposable culture tube (VWR International LLC, Radnor, PA) and then washed 3 times with 4 mL 98% Methanol (HPLC-grade) (EMD Chemicals, NJ, USA) for 3 minutes. Each wash was completed on the Fisher-Scientific 346 Hematology and Chemistry Mixer (Fisher Scientific Company, Ontario, Canada). Methanol was discarded, then all samples were examined to ensure removal of any dirt, debris, or other contamination. Washed and cleaned hair samples were put into a covered petri dish on a filter paper and allowed to dry for 24 hours. Washed and dried hair samples were ground in 10 mL stainless grinding jars with a single 12 mm stainless steel grinding ball at 30 Hz with a Retsch MM301 Mixer Mill for 2 minutes (Retsch Inc., Pennsylvania, USA). Specific grinding time was determined by the quantity of hair. Powdered hair was transferred into unstatic 1.5 mL eppendorf tubes and a 25 mg aliquot was fransferred into unstatic 0.6 mL eppendorf tubes. 500 µL 98% methanol was added into each sample, gently vortexed for 10 seconds and placed on a slowly spinning rotator for 24 hours to extract cortisol. All samples were then vortexed for 40 seconds and centrifuged for 15 minutes at 1800 X g at 20 °C. The entire supernatant was transferred into 12 mm glass tubes and dried at 38 °C for 15 minutes under a gentle stream of nitrogen gas (6.5 LPM). To ensure all extracted cortisol was collected, another 500 µL 98% methanol was added to the 0.6 mL tubes and vortexed for 40 seconds and centrifuged at 1800 X g for 15 minutes at 20 °C. The second supernatant was collected into the glass tubes with the first dried supernatant and dried with a similar procedure as the first supernatant. This procedure was then repeated a third time. Cortisol was concentrated at the bottom of glass tubes after using 400 µL, 200 µL, and 150 µL

98% methanol to rinse the sides of the tubes. Glass tubes were dried using nitrogen gas (6.0 LPM) at 38°C after each rinse. 200 µL 1 X extraction buffer was then added to the dried glass tubes which were covered with parafilm and gently vortexed for 10 seconds. All glass tubes were stored overnight in the dark at 4 °C. Reconstituted samples were vortexed on the lowest setting for 40 seconds and scrapped down with a pipet tip. All glass tubes were centrifuged at 300 X g at 20 °C for 5 minutes to bring all fluid to the bottom. Supernatant was then transferred into 0.6 mL eppendorf tubes and centrifuged at 1800 X g at 20°C for 5 minutes to remove any remaining powder. Extracts were stored at -80°C until ready to assay with a cortisol ELISA kit (Oxford Biomedical Research, Oxford, MI, USA).

Hair Cortisol Measurement

Hair cortisol levels were measured by an enzyme-based immunoassay ELISA kit (Oxford Biomedical Research, Oxford, MI, USA). A series of standards 0, 0.04 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.4 ng/mL, 1.0 ng/mL, 2.0 ng/mL, and 10.0 ng/mL were made using 1µg/mL cortisol standard solution. Samples were vortexed, centrifuged for one second, and mixed well before adding to the ELISA plate. 50 µL of standards and samples were added in duplicate to the corresponding wells of the microplate. 50 µL diluted cortisol horseradish peroxidase concentrated conjugate (Cortisol-HRP conjugate) was added to each well. The plate was incubated at room temperature for an hour. The plate was washed three times with 300 µL diluted wash buffer per well. 150 µL 3,3',5,5'- Tetramethylbenzidine (TMB) substrate was added to each well and incubated for 30 minutes in the dark at room temperature. Plates were read at wavelength 650 nm. According to the manufacturer, cross reactivity of the Cortisol-HRP Conjugate with the following hormones: Prednisolone (47.42%), Cortisone (15.77%), 11-Deoxycortisol (15%), Prednisone (7.83%), Corticosterone (4.81%), 6-β-Hydroxycortisol (1.37%), 17-Hydroxyprogesterone (1.36%). Cross reactivity with other endogenous steroids (deoxycorticosterone, progesterone, betamethasone, dehydroepiandrosterone, dexamethasone, beclomethazone, d-aldosterone, testosterone, 17α-hydroxypregnenolone, androstendione, cholesterol, and estradiol) is less than 1%.

Table A1. Hair cortisol levels in response to restraint stress.

Animal	Whole Hair Prior to Restraint[2]		Whole Hair After Restraint[3]		Regrowth Hair After Restraint[4]	
	Original[5]	Transformed[6]	Original	Transformed	Original	Transformed
208	1.59[1]	0.20	2.08	0.32	2.50	0.40
209	3.58	0.55	2.27	0.36	1.63	0.21
210	11.39	1.06	3.14	0.50	3.35	0.53
211	1.62	0.21	1.58	0.20	3.46	0.54
212	1.26	0.10	1.67	0.22	3.10	0.49
213	0.84	-0.08	1.03	0.01	2.23	0.35
214	2.14	0.33	1.97	0.29	2.42	0.38
215	1.78	0.25	1.22	0.09	2.78	0.44
216	2.15	0.33	1.97	0.29	2.42	0.38
217	2.00	0.30	3.78	0.58	3.60	0.56
218	0.76	-0.12	2.04	0.31	1.97	0.29
219	1.12	0.05	2.29	0.36	2.03	0.31
220	1.02	0.01	2.08	0.32	2.33	0.37
221	2.74	0.44	2.72	0.43	3.52	0.55
222	2.74	0.44	2.35	0.37	2.53	0.40
223	1.63	0.21	3.31	0.52	2.68	0.43
224	2.14	0.33	2.63	0.42	2.78	0.44
225	3.23	0.51	4.75	0.68	3.35	0.53
226	2.79	0.45	2.59	0.41	3.37	0.53
227	2.71	0.43	2.16	0.33	2.14	0.33
Mean		0.30		0.34		0.42
1SD		0.26		0.16		0.10

1. Hair cortisol expressed as pg/mg.
2. Cortisol levels in whole hair for each animal prior to the first restraint.
3. Cortisol levels in whole hair for each animal after all three restraints.
4. Cortisol levels in regrowth hair for each animal after all three restraints.
5. Hair cortisol concentrations directly assessed by enzyme immunoassays.
6. Log-transformed hair cortisol levels corresponding to the original levels.

Restraint effects on whole hair cortisol levels, regrowth hair cortisol levels, as well as correlation between cortisol levels in whole hair and regrowth hair after restraints were investigated. Further comparisons were made between whole hair cortisol levels following the arrival of cattle at the VIDO research facility, whole hair cortisol levels seven days after all restraints were performed, and regrowth hair cortisol levels seven days after the third restraint were performed. The dataset was log-transformed since whole hair cortisol levels on the arrival date failed to satisfy the normality test. Similarly, both whole hair cortisol levels and regrowth hair cortisol levels seven days after the third restraint were also log-transformed [**Table A1**].

There was a significant difference between log-transformed whole hair cortisol levels on the arrival date (prior to restraint) and log-transformed regrowth hair cortisol levels ($p = 0.037$) but no significant difference between the log-transformed whole hair cortisol levels prior to or after repeated restraint episodes. This indicated that cortisol levels in whole or was not significantly altered by the three restraint episodes.