ACCLIMATION OF HOLSTEIN CALVES TO TRANSIT STRESS:

THE INTEGRATION OF ENDOCRINE, IMMUNE, AND BEHAVIOR SYSTEMS

A Dissertation

by

AMBER LYNN ADAMS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Animal Science

Acclimation of Holstein Calves to Transit Stress:

The Integration of Endocrine, Immune, and Behavior Systems

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Approved by:

Chair of Committee,	Ted H. Friend
Committee Members,	Luc R. Berghman
	Glenn A. Holub
	Penny K. Riggs
Head of Department,	H. Russell Cross

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ABSTRACT

Acclimation of Holstein Calves to Transit Stress:

The Integration of Endocrine, Immune, and Behavior Systems. (August 2012)

Amber Lynn Adams, B.S., University of Wisconsin-River Falls;

M.S., Oklahoma State University

Chair of Advisory Committee: Dr. Ted Friend

Little is known about the adaptation of livestock to repeated transport. This study determined how repeated transport affected calf feed intake, plasma cortisol (CORT), post-transport behavior, and the expression of immune-related genes. Thirty-six 4-month-old Holstein steer calves were housed in groups of six with each group randomly assigned to either transport (T) or control (C) treatments. The T calves were hauled for 6 h in a 7.3 m x 2.4 m goose-neck trailer, at an average density of 0.87 m^2/calf, every 7 d for five consecutive weeks. Individual daily intake was determined using Calan gate feeders. Blood samples were obtained in the trailer or home pen via jugular venipuncture before loading, and after 2, 4, and 6 h of transport. Samples were analyzed for CORT, serotonin, tryptophan, and the gene expression of interleukin-4 (IL-4), interleukin-6 (IL-6), chemokine (C-X-C motif) receptor 2, interleukin-12, toll-like receptor-4, toll-like receptor-2, and 5-hydroxytryptamine receptor 2A in leukocytes. Behavior was recorded for transported calves at 5-min intervals for 1 h after return to their home pens.

The C calves had a higher feed intake than T calves overall ($P = 0.01$), on the day of transport ($P = 0.007$), and the day after transport ($P = 0.02$). Pre-transport CORT concentrations did not differ by treatment ($P = 0.77$) or trial ($P = 0.32$). However, the T calves had higher response CORT concentrations than C calves during Transport 3 ($P = 0.006$), Transport 4 ($P = 0.001$) and Transport 5 ($P = 0.02$). The T calves had the highest response CORT concentrations after 2 h of transport and the lowest response CORT concentrations after 6 h of transport ($P < 0.0001$). Treatment did not affect gene expression in leukocytes, however, the expression of IL-4 ($P = 0.01$) and IL-6 ($P = 0.05$) was significantly lower after 2 h of transport than any other sampling times. These results suggest conflicting conclusions on whether the calves started to acclimate after being transported five times. However, CORT and gene expression differences occurred in response to the blood sampling regimen, which may provide insight to how calves acclimate during prolonged stress.

DEDICATION

I dedicate this dissertation to my outstanding family and friends for all their support and encouragement. A special thank you goes to my grandparents because their love built the solid foundation for my strong family. I am especially grateful to my mother for teaching me how to work hard, be brave, and live life with integrity. Someday, I hope to have a daughter as sweet as my niece Kaydence. Kaydence, I hope you dream big and always follow your heart in everything you do. Throughout my years in school, a few special friends helped me along the way. Kendra and Michelle, thank you for being my long-time confidantes. Shannon, Cooper, and Mark, thank you for not only helping me with my projects, but also lending an ear and never allowing me to quit. I also have a deep appreciation for my best friend, Kevin, who picked up the pieces when I felt broken. Without all of you, this dissertation would not be possible. Thank you!

ACKNOWLEDGEMENTS

Several people have devoted hours of their time to share advice and help me write this dissertation. Dr. Friend, thank you for serving as my advisor and encouraging me to learn more about the research that has become my passion. Your expertise provided me with opportunities that I would never have experienced, thank you. I also thank Dr. Berghman, Dr. Holub, and Dr. Riggs for contributing to my dissertation as members of my committee. Dr. Berghman taught me how to be a better scientist and inspired me to acquire a stronger appreciation and understanding of immunology. Dr. Holub mentored me and taught me how to be a better teacher. I value how truly dedicated he is to his students. Dr. Riggs opened my eyes to the new, complex world of gene expression. Thank you for taking the time to share your knowledge and walk me through this process. I am also appreciative for Dr. Sawyer's statistical advice and Kelli Kochan's assistance in the laboratory.

I would not be where I am now if I didn't have the support of the people in my hometown community. Thank you Barb, Ms. Berget, Mr. Paul Miller, and the rest of the Plainfield, WI and Hancock, WI communities for having faith in this small-town girl. A special thank you is also extended to Dr. Onan and Dr. Lovern for guiding me through my B.S. and M.S. degrees. Thank you to my fellow graduate students in the Animal Science Graduate Student Association and Graduate Student Council for making me feel like part of the Texas A&M University family. For all of the above-mentioned people in my life, I am truly grateful. Once again, thank you!

NOMENCLATURE

ADG	Average Daily Gain
d	Day
h	Hour
kg	Kilogram
m	Meter
min	Minute
ml	Milliliter
µl	Microliter
ng	Nanogram
pg	Picogram
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RQ	Relative Quantification
THI	Temperature-humidity Index
wk	Week

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INTRODUCTION AND BACKGROUND INFORMATION

Consequences of Stress

Irwin et al. (1979) reported that approximately 1% of cattle die as a consequence of transport stress. Although a rather low percentage of cattle die, transport stress imposes on the well-being of cattle. It is well documented that transport stress is detrimental to not only the health of the cattle, but also causes significant economic losses as well as food safety concerns. For example, previous studies have demonstrated that transport stress leads to increases in heart rate (Stephens and Toner, 1974), respiration rate (as reviewed by Swanson and Morrow-Tesch, 2001), decreases in immune function (as reviewed by Kelley, 1980), and fecal shedding of bacteria (Barham et al., 2002) in cattle.

In order to increase the well-being of animals, researchers attempt to decrease the negative effects of stress that animals experience. Two types of stress are commonly recognized; distress (stress that has a negative impact on the animal) and eustress (stress that has a positive impact on the animal). In humans, an example of distress and eustress would be attempting to avoid a predator and voluntary exercising, respectively (as reviewed in von Borell, 2001). Distress may impose long-term damage to the brain (such as decreased volume of the hippocampus), as seen in post-traumatic stress disorder and

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This dissertation follows the style of the Journal of Animal Science.

childhood abuse patients (Bremner, 1999).

Acute stress activates the hypothalamic-pituitary-adrenal (HPA) axis to stimulate the release of corticotrophin releasing factor (CRF) from CRF neurons, adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, and glucocorticoids from the adrenal cortex (as reviewed by Leonard, 2005). This stress system implements physiological and behavioral changes to help the body return to homeostatic conditions and increase its probability of survival (Tsigos and Chrousos, 2002).

Stress and Cortisol Concentrations

Cortisol is a glucocorticoid released in greater amounts when an animal is under stress (distress or eustress). The amount of stress an animal experiences is commonly quantified by measuring the animal's cortisol concentrations. Handling and transport stress are associated with significant increases in plasma cortisol concentrations in cattle (Fazio et al., 2005) and horses (Clark et al., 1993). While multiple studies have documented the differences between pre-transport and post-transport plasma cortisol concentrations, few studies have measured plasma cortisol concentrations during transportation.

Nwe et al. (1996) noticed goat plasma cortisol concentrations rose immediately after the start of a 6-h transportation to a peak value within 1 h. The plasma cortisol concentrations returned to base values 6 h post-transport. Broom et al. (1996) noted

similar results in sheep plasma cortisol concentrations during a 15-h transport. The most dramatic increases in sheep plasma cortisol concentrations occurred during loading and within the first 3 h of transport. During the remaining 12 h of transport, plasma cortisol concentrations stabilized at higher concentrations than base values. Pig plasma cortisol concentrations rose to peak concentrations within 30 min of transport and then returned to pre-transport concentrations 4 h post-transport (Dalin et al., 1993).

Furthermore, the potential ability of cattle to acclimate to transport stress has not been thoroughly studied. Locatelli et al. (1989) evaluated the effects of repeated 30-min simulated transportation on calf plasma cortisol concentrations and concluded that plasma cortisol increased significantly during transport, but became less noticeable with each successive trial (with 10-d rest periods between trials). Schmidt et al. (2010) measured horse salivary cortisol concentrations during repeated transportation. Transport always caused an increase in cortisol concentrations, but with each successive transport (with 2 to 4 d of rest between trials), a less obvious increase in cortisol was elicited in the horses. While cortisol is a reliable indicator of stress, researchers have not yet been able to differentiate distress from eustress.

Stress and Serotonin Concentrations

Serotonin (5-Hydroxytryptamine or 5-HT) may offer additional insight into the determination of distress versus eustress. Serotonin is a neurotransmitter derived from tryptophan, an amino acid, which is associated with the feeling of well-being. Eighty

percent of serotonin in the body is located within the intestinal tract (enterochromaffin cells), with less than 1 % of serotonin stored in blood platelets (a component of blood plasma), and the remaining serotonin either confined to the brain or free (unbound) in peripheral circulation (Berger et al., 2009). More than 99% of whole blood serotonin is contained in platelets (Anderson et al., 1987).

Serotonin plays a key role in activating the HPA axis to stimulate ACTH and corticosteroid release when the body experiences stress (as reviewed by Firk and Markus, 2007). Once the stress response is initiated, the enterochromaffin cells release serotonin to increase the rate of contractions in the gastrointestinal tract, which may cause diarrhea (Racké and Schwörer, 1991). As the release of serotonin increases, more serotonin is discharged into the blood stream where platelets bind to and store the serotonin. Platelets release serotonin to facilitate blood clotting during bodily injury.

During stress (especially repeated stress), the brain uses serotonin to help the body adapt to the stress by enhancing the negative feedback control of cortisol on the HPA axis (as reviewed by Chaouloff et al., 1999; Robertson et al., 2005). As the brain's reservoirs of serotonin become depleted, the brain recruits tryptophan from the blood to synthesize additional serotonin because tryptophan is able to cross the blood-brain barrier, and serotonin does not have this capability (Dunn, 1988). Decreases in serotonin production in humans are directly correlated with depression and the lack of the feeling of well-being (Svenningsson et al., 2006).

The relationship between blood serotonin concentrations and depression is controversial. For example, Biegon et al. (1990) found that suicidal males had

significantly higher concentrations of blood serotonin than males in the control group. However, Cleare (1997) found that patients diagnosed with major depression had significantly reduced blood serotonin concentrations. Some researchers believe the decreased blood serotonin concentrations measured in depression patients is due to a decrease in tryptophan available for serotonin synthesis, and not due to a direct relationship between serotonin and depression. This concept was also developed to address the effect a loss of appetite (a side-effect of depression) may have on circulating concentrations of tryptophan and serotonin. Researchers cannot resolve this controversy until it is determined how to differentiate between the effects of depression and its side-effects (such as decreased appetite) on serotonin concentrations.

Animal scientists have become increasingly interested in acquiring a better understanding of serotonin and its functions within the body. For example, Bruschetta et al. (2010) measured plasma serotonin concentrations via enzyme-linked immunosorbent assay to examine dairy cattle breed differences and discover other elements that may affect blood serotonin concentrations. The study concluded that plasma serotonin concentrations appeared to be affected by breed, temperature, blood sampling season, and altitude. Furthermore, Kollmann et al. (2008) investigated the effects of tryptophan supplementation on plasma serotonin concentrations by using an enzyme-linked immunosorbent assay. They concluded that tryptophan supplementation did not affect plasma serotonin concentrations. With the limited published data on plasma cortisol, serotonin, and tryptophan concentrations in dairy cattle, a study that examined these blood constituents at set intervals during transportation could provide more insight into

how these three hormones interact during stress. Stress not only affects the endocrine system, but is also greatly impacts the immune system.

Stress and the Immune System

Acute stress may stimulate the immune system by redistributing lymphocytes and macrophages throughout the body (as reviewed by McEwen, 1998), but previous studies have also determined that acute stress (which may include transport stress) may also cause a suppression of the innate immune system. For example, Calcagni and Elenkov (2006) explained that acute stress suppressed innate immune system components, such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ).

Transport stress, in particular, has been shown to also cause decreases in lymphocyte production (Murata et al., 1987). Stull et al. (2004) concluded that long-term transport stress caused a decrease in equine lymphocyte subpopulation counts, but also caused an increase in neutrophil and white blood cell counts. Furthermore, Dixit et al. (2001) noticed no effect of short-term transport on cattle lymphocyte ACTH secretion, but a significant increase in lymphocyte ACTH secretion occurred in animals that were transported long-term. The detrimental influence transport stress may have on the immune capabilities of cattle is especially important for feedlot cattle managers and owners. Feeder and stocker calves undergo various sources of stress (such as weaning

and castration) prior to being transported to feedlots or pastures in which they are exposed to an array of other co-mingled calves that may have contagious illnesses.

In order to optimize calf growth and performance, preventative measures need to be taken to ensure calves maintain healthy immune systems by decreasing the amount of negative stress the calves experience. Before transportation conditions can be improved, though, a better understanding of transportation acclimation and the relationships between acute versus chronic transport stress and immune function must be obtained. Recently, gene expression techniques have been developed which assist with the investigation of the gene expression of specific cytokines and immune system components that influence an individual's susceptibility to disease.

Gene Expression and the Immune System

The development of in vitro polymerase chain reaction (PCR) techniques (Mullis et al, 1987) assisted in increasing the popularity of gene expression research. PCR has become a commonly used procedure to investigate deoxyribonucleic acid (DNA) sequence, phylogeny, gene function, and hereditary diseases. As PCR became more employed in laboratories, researchers began to develop variations of the technique, such as reverse transcriptase PCR. Furthermore, Heid et al. (1996) showed that real-time reverse transcriptase PCR (also known as real-time quantitative PCR) could be used to quantify gene expression. Real-time quantitative PCR can be evaluated using absolute quantification or relative quantification. Absolute quantification compares the quantity

of a detected product to a standard curve, whereas, relative quantification (RQ) compares the quantity of a detected product from a treatment sample to another sample (most likely a control). In order to calculate RQ, the $2^{-\Delta\Delta CT}$ method is one of the most popular calculations (Livak et al., 2001). The analysis of gene expression has provided insight to several human diseases.

For example, genes that may be involved in multiple sclerosis pathogenesis have been identified by evaluating the expression of genes from blood mononuclear cells (Achiron et al., 2004). In another study, Nadler et al. (2000) examined the expression of genes in adipocytes and realized that those genes were expressed at a lower amount in cases of obesity. Also, the expression of the GAP-43 gene has been shown to play a key role in neurodegeneration, such as Alzheimer's disease (de la Monte et al., 1995). Besides its use in human research, gene expression has also been used in animal research, including cattle.

A connection between leptin gene expression and heifer puberty was detected by Garcia et al. (2002) in which a higher expression of leptin was measured during puberty and during increases in body weight. In other examples of gene expression uses in cattle, higher expression of insulin-like growth factor was linked to follicular dominance in cattle (Yuan et al., 1998) and heifers fed a high concentrate diet had a higher expression of approximately 230 genes than heifers fed a forage-based diet (Allen et al., 2012). Research has also been conducted in cattle to take a closer look at how gene expression influences the immune system.

Using a microarray, Coussens et al. (2002) revealed that cows infected with *Mycobacterium paratuberculosis* exhibited a decrease in the expression of 83 genes measured in blood mononuclear cells. Reverse transcriptase real-time PCR techniques (similar to the methods in this study) have been used to examine the expression of cytokine genes in cattle infected with bacteria. For example, Sweeney et al. (1998) noticed that Holstein cows subclinically infected with *Mycobacterium paratuberculosis* had higher expression of the interferon-gamma (IFN-γ) gene than cows clinically infected with *Mycobacterium paratuberculosis*. Thacker et al. (2007) also saw increases in the gene expression of IFN-γ, along with increases in interleukin-4 and decreases in interleukin-10 in cattle infected with *Mycobacterium bovis*. Previous studies that have integrated stress and the immune system using gene expression techniques in cattle are limited. However, Burton et al. (2005) did attempt to explain how gene expression changes as neutrophils are exposed to glucocorticoids during parturition in dairy cows. The conclusions of that study were that glucocorticoids appear to assist with neutrophil development, but also decrease the defense activities of the cells. This finding may help explain why parturient cows (with increased concentrations of glucocorticoids) are more likely to develop inflammatory diseases.

With limited information available about how stress influences the expression of genes related to the innate immune system, and how the expression of these genes changes over time, a study that measured differences in the expression of cytokine genes during stress in an animal model could be useful. Such a study may contribute additional

information this field of research needs in order to better understand human diseases and develop useful forms of treatment for patients.

Objectives and Hypotheses

The objectives of this study were to determine: 1) how dairy calves respond to repeated transport; 2) how endocrine and immune systems respond to the duration of transport; 3) how plasma serotonin, tryptophan, and cortisol concentrations interact as calves experience repeated transport; 4) how repeated transport alters the gene expression of innate immune system components in leukocytes in calves; and 5) how calves react behaviorally to repeated transport.

Cattle transported for long distances experience stress which may ultimately lead to death. Calves with decreased brain serotonin concentrations and increased cortisol concentrations may be under a considerable amount of stress that may cause decreases in immune function. It was hypothesized that transported calves would have more stress (as indicated by cortisol, tryptophan, and serotonin concentrations) and lower immunity (as indicated by reduced gene expression of innate immune system components) than calves that are not transported, and that repeatedly transported calves would show signs of acclimation with subsequent transports.

GENERAL PROCEDURE

Subjects, Housing and Management

This study was approved by the Texas A&M University Animal Care and Use Committee (AUP #2010-202). Thirty-six 4-mo-old Holstein steer calves housed at the Texas A&M University Animal Science Teaching, Research and Extension Center (9.7 km west of College Station, TX, USA) were assigned, according to BW (blocked so each pen had a similar average BW), to 12 m x 6 m pens with six calves in each pen. The average initial calf BW was 146 ± 2.6 kg. The pens were located on the south side of a covered open-sided barn that provided roofing over the feeders and northern half of the pens. The pens were constructed of corral panels on three sides, and were equipped with automatic watering systems, with one water basin shared by two pens. Six Calan gate feeders (American Calan Inc., Northwood, NH) were mounted on the north or covered side of each pen. Two weeks after being regrouped into pens of six, calf feed bunk preferences (number of times each calf attended each feeder) were recorded by direct observation for 4 h during four separate feedings, over the course of 2 d. According to each calf's feeder preferences, each calf was assigned an individual feeder and the corresponding feed bunk key placed on the calf's neck. Pens were scraped clean weekly and the water basins were scrubbed daily.

Transportation of Calves and Sample Collection

Individual feed intake and BW data was collected 7 d prior to the first transport trial and 7 d after the final transport trial. One week after feed bunk assignments were established, transport trials commenced. The calves were fed an ad libitum starter feed (Cornerstone Ampli-calf DX 30®, Purina Mills, St. Louis, MO) mixed with cottonseed hulls and molasses yielding a 19.37% crude protein and 4.12% crude fat diet that was fed twice daily. Excess feed was weighed every evening. Each pen of six calves was randomly assigned to either transport (n = 18 calves) or control (n = 18 calves) treatments. Calves were transported for 6 h in their assigned groups of six in a 7.3 m x 2.4 m goose-neck trailer divided into three equal compartments, at an average density of 0.87 m^2/calf, every 7 d for five consecutive weeks. Each week, all calves were weighed in a squeeze chute prior to loading the transported calves onto the trailer. No calves (including the control calves) had access to feed when transport occurred.

Temperature-humidity index (THI) was calculated using ambient temperature and relative humidity from data loggers (H08-003-02 HOBO devices, Onset Computer Corporation, Pocasset, MA, USA) mounted in the shade in the feed alley next to the feeders of the middle pen and along the front wall of the trailer. Each data logger was attached to Styrofoam (15.24 cm x 10.16 cm) in order to prevent direct contact with the feeder post or trailer wall. The data loggers recorded ambient temperature and relative humidity at hourly intervals from 1 h pre-transport to 1 h after the transported calves returned to their home pens. The THI equation used in this study was THI = ambient

temperature – [0.55 - (0.55 * relative humidity/100)] * (ambient temperature – 58.8), where ambient temperature was recorded in Fahrenheit and relative humidity was recorded as a percentage (NOAA, 1976).

EFFECTS OF REPEATED TRANSPORT ON FEED INTAKE AND BEHAVIOR

Introduction

Stress has been shown to have a negative impact on cattle feed intake, growth and production (Duff and Galyean, 2007). For example, overcrowding has caused dairy cattle to decrease their feeding and rumination activity, along with altering their resting behavior (Grant and Albright, 2001). In comparison, transport stress has affected calf resting behavior during transport. Swanson and Morrow-Tesch (2001) found that calves less than 4 wk of age spent 33 to 36% of their time lying during transport and calves 3 mo of age spent 13 to 42% of their time lying during transport. Several studies have examined the effect of transportation on calf post-transport behavior, but no known studies have compared calf post-transport behavior and feed intake to determine whether calves acclimate to repeated transport. The effects repeated transport may have on calf behavior could affect calf growth and performance.

Calves are exposed to multiple stressors within a relatively short amount of time that may hinder calf growth. In one example, Fisher et al. (1996) demonstrated that the average daily gain (ADG) for calves was reduced post-castration, regardless of the method of castration implemented. Another common stressor calves experience is weaning; early-weaned calves have better gain:feed than normal-weaned calves (Arthington et al., 2005). In addition to castration and weaning, the stress associated with

handling and transport has caused calves to lose 8.1% of their weaning body weight (Phillips et al., 1987). Furthermore, calves exhibit a decreased appetite or willingness to consume feed for up to 3 wk upon arrival at the feedlot (Hutcheson, 1980). The combination of these effects of transport stress on calf growth is detrimental to not only producer profits, but also the well-being of the calves. However, conflicting results from previous studies showed that stress may actually stimulate appetite rather than suppress appetite.

Research focused on the causes of obesity in humans revealed relationships between stress, cortisol concentrations, and eating behavior. For example, women with higher concentrations of cortisol consumed more calories and preferred sweet food on days they were exposed to stress (Epel et al., 2001). Levine and Morley (1981) saw similar results in rats that were exposed to mild tail pinching. Seventy-two percent of the rats displayed ingestive behavior after exposure to the tail-pinching stress. Taking into consideration the current controversy about stress and its effects on eating behavior, the objective of this component of the study was to determine if calves acclimate to weekly transport and how repeated transport affects feed intake, average daily gain, and feed conversion in Holstein calves.

Materials and Methods

Behavioral Observations

After the calves were unloaded and returned to their home pens after each transport, observers recorded behavioral observations for the transported calves for 1 h at 5-min intervals using a scan-sampling method. Each calf was identified by its ear tag number and behavior was classified as standing, walking, trotting/loping, lying, grooming, eating (head inserted inside the feeder), or drinking (nose within 15 cm to water) every 5 min. Prior to the start of the study, all the observers walked the pens together conducting a mock data collection. Because the behavioral observations were discrete categories and informal observations of the observers agreed, measures of inter-observer reliability were not calculated. The recording of the behavior for each pen started immediately after a particular group returned to its pen and the gate to the alley was closed.

Average Daily Gain and Feed Conversion

Mean feed intake, ADG, and feed conversion for each week were calculated using feed intake values for the day of transport and 6 d following transport. For example, Wk 1 represents the feed intake for the day of the first transport (Transport 1) and the next six days. Feed intake (measurements include feed consumed from evening

to evening on a daily basis) and body weight (measured pre-transport each week) data were used to calculate each calf's average daily gain (ADG) and feed conversion (kg feed per kg gain) for the week following each transport trial, and are designated as Wk 1, Wk 2, Wk 3, Wk 4, and Wk 5.

Statistical Analyses

The percent of transported calves eating and lying during each 5-min observation after each transport was analyzed in a mixed model ANOVA in SAS. Final body weight was analyzed using a t-test for two independent samples. Weekly ADG was calculated by dividing the difference in each calf's weight between two consecutive weeks by seven. Because each calf had its individual feeder, each calf served as an experimental unit (total n = 36). Feed intake was analyzed in a mixed model ANOVA using SAS (SAS 9.2, SAS Institute Inc., Cary, NC, USA), with repeated measures for calf(treatment) and an autoregressive covariance. Mean weekly ADG and feed conversion were also analyzed in a mixed model ANOVA (SAS).

Results

All calves used their feeders without any problems before the study began. During transport, all calves were monitored for signs of hyperthermia (including increased respiration rate and shallow breathing), but none were detected. During each

transport trial, most calves were standing in the trailer after 2 h of transport, but were lying after 4 h and 6 h of transport. Also, informal observations indicated that more calves were lying during Transport 5 than Transport 1.

Calf Eating and Lying Behavior

A significant difference ($P = 0.03$) occurred in the mean percent of observations in which calves were eating post Transport 1 (47 ± 4%, Figure 1) and post Transport 5 (35 ± 4%, Figure 2). Also, a significant difference was detected in the mean percent of calves eating during observations post Transport 2 (48 ± 4%) and post Transport 5 (35 ± 4%, $P = 0.01$). After Transport 5, the mean percent of observations in which calves were lying (55 ± 4%, Table 2) was significantly higher than following Transport 1 (41 ± 4%, $P = 0.008$), Transport 2 (37 ± 4%, $P = 0.0007$), and Transport 3 (43 ± 4%, $P = 0.02$). After Transport 1, all calves immediately went to drink upon returning to their pens, the majority of calves were eating during the observation at 5 min, and remained eating until they commenced to lie down during the observation at 35 min. After Transport 5, a majority of calves began drinking upon returning to their pens, were eating during the observation at 5 min, and remained eating until they commenced to lie down during the observation at 25 min.

Across all observation periods, the mean percent of calves eating during each observation peaked 15 min post-transport (94 ± 6%), but then slowly decreased until the lowest mean percent of calves eating was observed 60 min post-transport (0 ± 6%, $P <$

0.0001). Across all observation periods, the mean percent of calves lying down during each observation increased with each consecutive observation during the 1-h post each transport, until all calves (100 ± 6%) were lying down during the observation at 60 min ($P < 0.0001$).

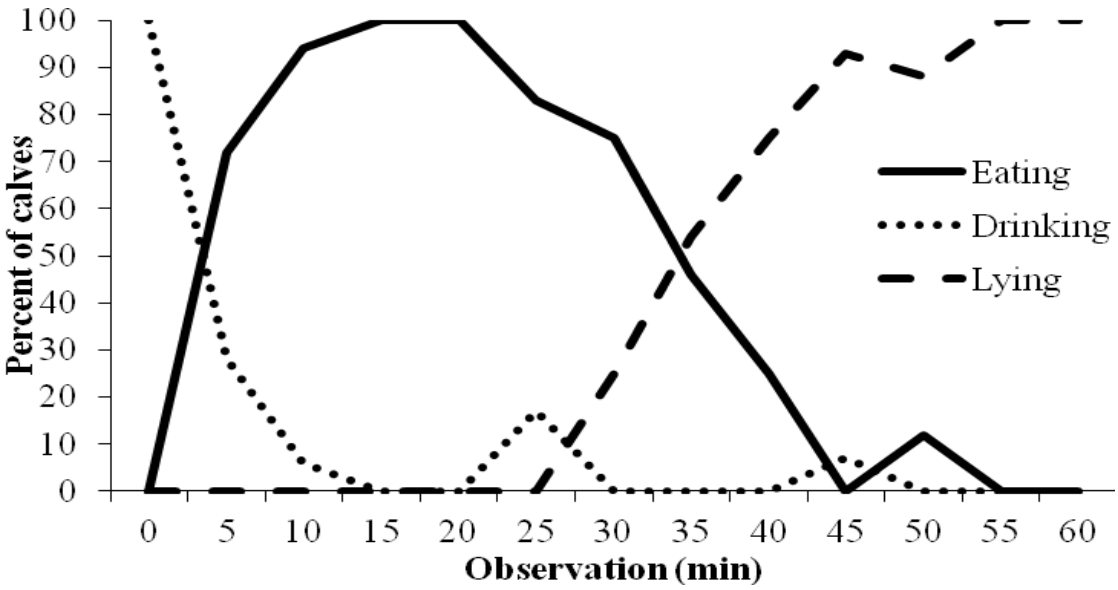

Figure 1. Calf Behavior after Transport 1. The percent of transported calves (n = 18) that were eating, drinking, or lying during each scan at 5-min intervals during the 1-h observation period post Transport 1.

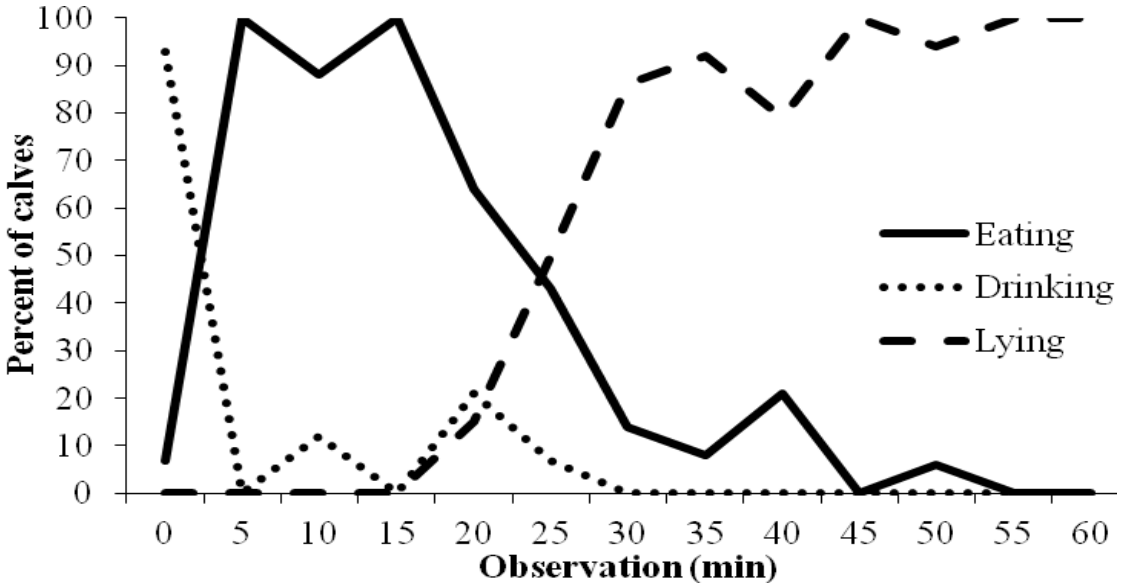

Figure 2. Calf Behavior after Transport 5. The percent of transported calves (n = 18) that were eating, drinking, or lying during each scan at 5-min intervals during the 1-h observation period post Transport 5.

Body Weight and Feed Intake

During the five transport trials, the mean hourly THI within the pens was 66 ± 1.06 and the mean hourly THI within the trailer was 70 ± 1.63. The highest mean pen THI for a trial occurred during Transport 3 (73) and the lowest occurred during Transport 5 (54). Transported and control calves had an average final body weight of 177 ± 2.8 kg and 183 ± 2.4 kg, which did not differ significantly ($P = 0.12$). Calves had significantly higher feed intake during Wk 5 (8.27 ± 0.14 kg) than during Wk 1 (6.44 ± 0.10 kg, $P < 0.0001$, Figure 3).

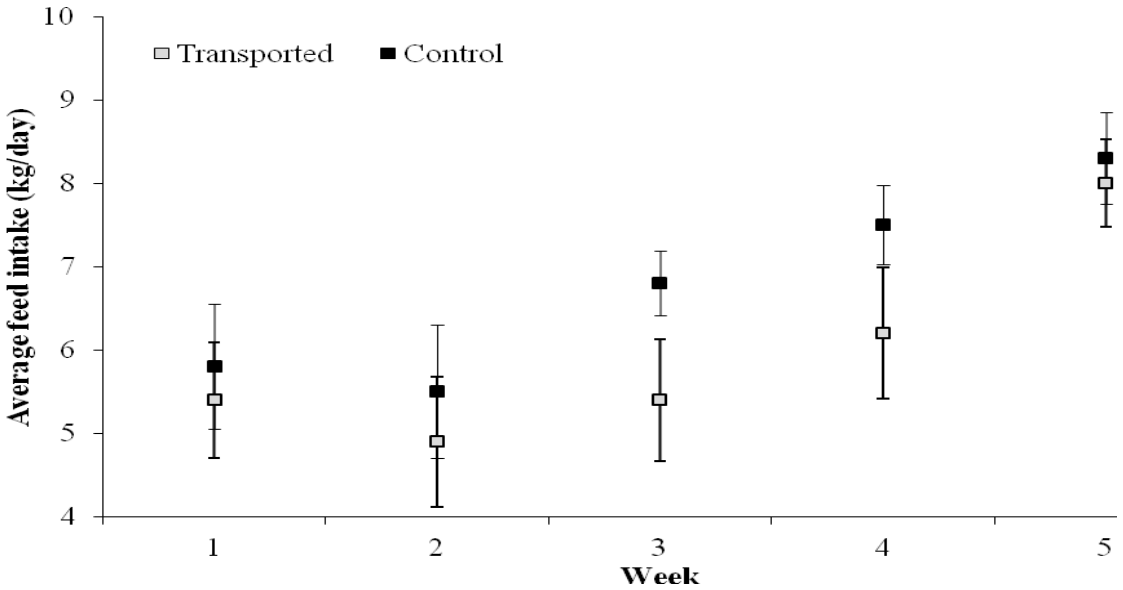

Figure 3. Average Daily Feed Intake per Week. Average (± 1 SD) daily feed intake for the week starting with the day of each transport and the 6 d following each of the weekly transport trials for control (n = 18) and transported (n = 18) calves.

A significant (P = 0.05) treatment by day interaction for feed intake occurred in which all calves decreased their feed intake on the day of transport and increased their feed intake the day after each transport trial, with the exception of Wk 5 (Figure 4). During Transport 5, feed intake increased on the day of transport. Control calves had a higher feed intake than transported calves on the day of each transport (control = 6.78 ± 0.19 kg, transported = 6.01 ± 0.19 kg, P = 0.007, Figure 4) and the day after each transport (control = 7.83 ± 0.20 kg, transported = 7.09 ± 0.20 kg, P = 0.01, Figure 4).

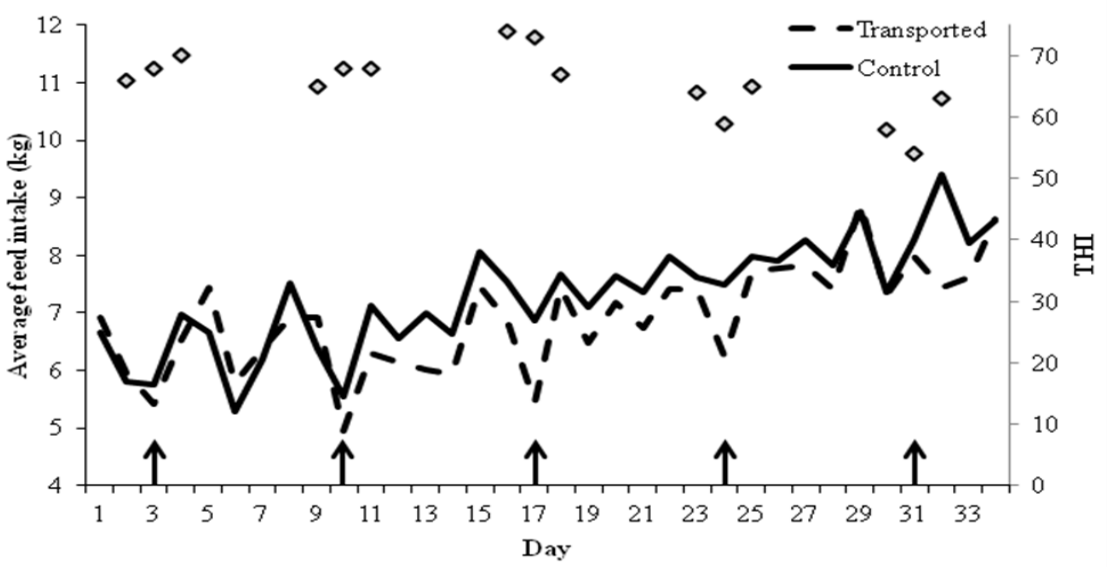

Figure 4. Daily Feed Intake. Daily feed intake for control (n = 18) and transported (n = 18) calves. Arrows indicate day of transport and diamond symbols represent average THI on the day of, 1 d before, and 1 d after each transport.

Average Daily Gain and Feed Conversion

During the 5 wk, control calves (1.53 ± 0.05 kg/d) had a significantly (P = 0.02) higher mean ADG than transported calves (1.36 ± 0.05 kg/d). However, no significant difference in ADG was detected between treatments during Wk 1 (P = 0.72), Wk 2 (P = 0.88), Wk 4 (P = 0.84), or Wk 5 (P = 0.76), but a difference was detected during Wk 3 (control = 1.69 ± 0.19 kg/d, transported = 1.10 ± 0.19 kg/d, P = 0.03, Figure 5). Treatment (P = 0.77) did not significantly affect calf feed conversion, however, the worst (P = 0.07) feed conversions tended to occur during Wk 1 (control = 11.42 ± 0.7 kg feed/kg gain, transported = 8.07 ± 0.7 kg feed/kg gain, Figure 6).

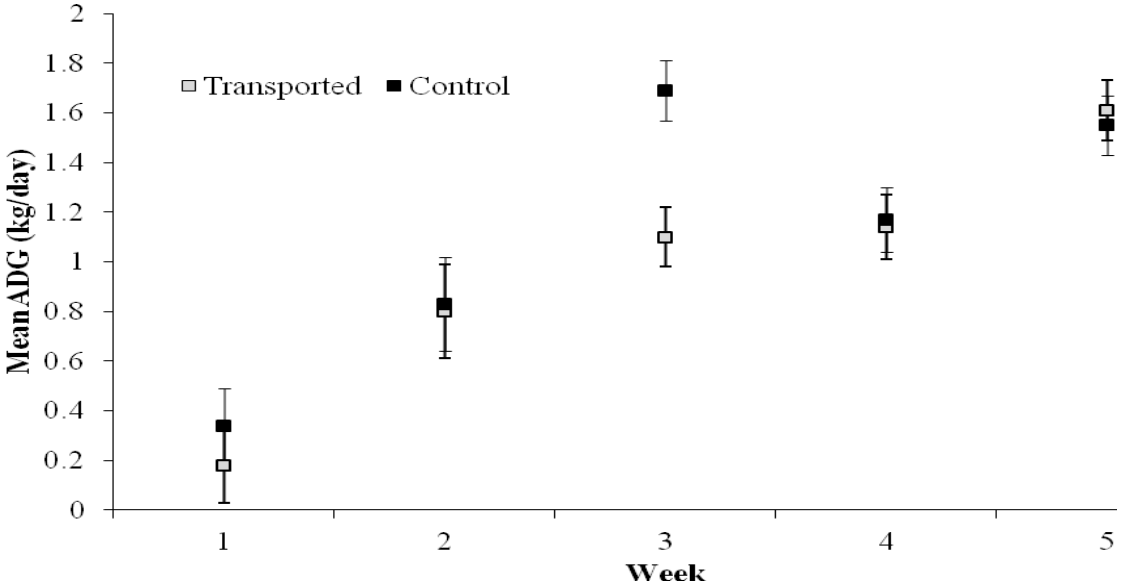

Figure 5. Average Daily Gain per Week. Mean (± SE) average daily gain for the day of each transport and the 6 d following each transport for control (n = 18) and transported (n = 18) calves during the 5-wk-long study. Calf weights were recorded pre-transport on the day of each transport trial, each week of the study.

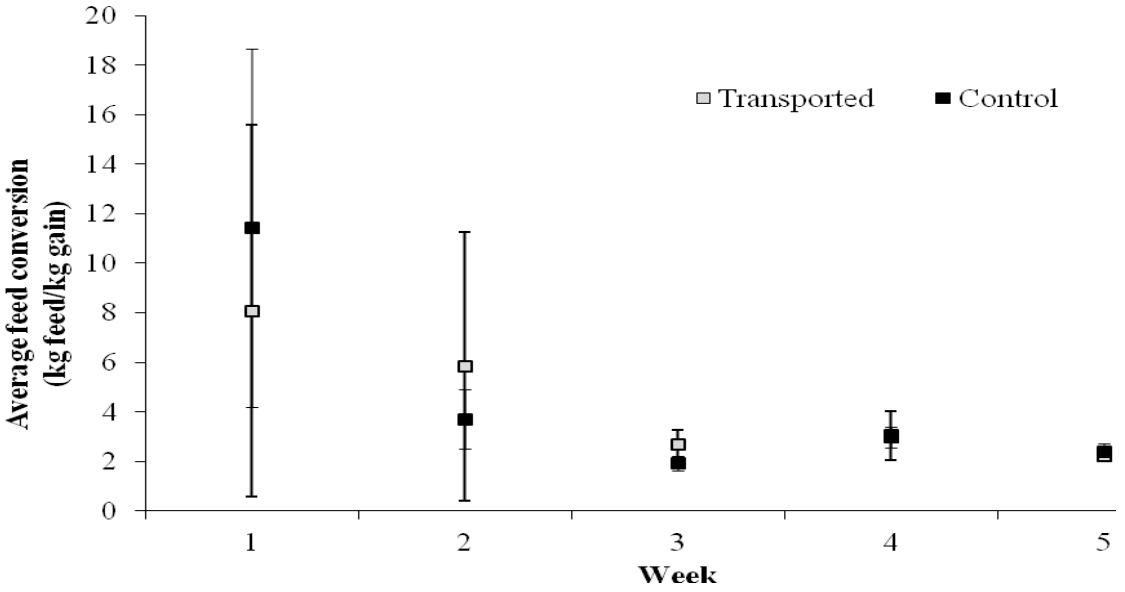

Figure 6. Average Daily Feed Conversion per Week. Average (± 1 SD) daily feed conversion for the day of each transport and the 6 d following each transport for control (n = 18) and transported (n = 18) calves during the 5-wk-long study. Feed intake was measured every 24 h in the evening each day and calf weights were recorded pre-transport each week of the study.

Discussion

Most calves in this study began to eat after 10 min of returning to their home pens and the fewest number of calves ate after 55 to 60 min of returning to their home pens. In general, cattle show signs of fatigue and increased lying behavior after unloading (Swanson and Morrow-Tesch, 2001). However, further research is needed to examine why the calves in this study switched from eating to lying down during the behavioral observation at 35 min post Transport 1 and Transport 2, during the observation at 30 min post Transport 3 and Transport 4, and during the observation at 25 min post Transport 5.

Although not quantified, the percent of calves lying down in the trailer appeared to increase as they spent more time on the trailer and with each successive transport. These informal observations show that most or all of the calves were lying in the trailer after 4 h and 6 h of transport, similar to the results of Kent and Ewbank (1986) in which 1 to 3-wk-old calves transported for 6 h spent more of their time lying (73%) than standing (27%) during transport. In general, the behavior of calves (including the amount of time spent lying) during transport is important because it may be an indicator of the well-being of the calves, but few studies have examined how calf lying behavior during transport is influenced by repeated transport.

The calves used in this study decreased feed intake on the day of transport and then recovered to their normal feed intake the day after each transport trial. The one exception to this occurred during Wk 5, in which feed intake actually increased on the

day of transport, potentially due to the cooler weather during Wk 5. Furthermore, transported calves showed such a significant increase in feed intake during Wk 5 that the overall difference between feed intake in transported calves and control calves became much smaller. The lower feed intake seen on the day of transport in all calves, regardless of treatment, could potentially be attributed to either environmental factors (such as differences in weather), the lack of access to feed bunks during transport hours, or the stress associated with handling during weight data collection. Decreases in feed intake on days of transport for transported calves were expected because previous studies determined that stressed calves decreased their feed intake upon arrival at feedlots (Cole, 1996). However, the calves in this study were returned to their home pen with the same group of calves each week, which may have been a factor in the increases in feed intake observed during Wk 5. Perhaps the increases in feed intake during Wk 5, coupled with more calves observed lying during Transport 5, could be attributed to the calves starting to habituate to the weekly transport.

No known previous studies have monitored changes in feed intake in calves that experience repeated transport, but several studies have determined feed intake in newly-received calves at the feedlot. The results in this study agreed with the results from Arthington et al. (2008) where calf feed intake decreased 1 wk after transportation, and began to increase after 2 wk. In addition, Hutcheson and Cole (1986) noticed that transported calves returned to normal feed intake amounts 2 to 4 wk after arrival at the feedlot. In the Hicks et al. (1998) study, no differences in body weight, feed intake,

ADG, or gain:feed were observed in pigs exposed to 4 h of transport, but this study did observe differences in ADG.

In this study, the transported calves had a lower ADG than control calves overall, with the largest difference occurring during Wk 3. Newly-received calves at the feedlot can experience depressed ADG during the receiving period, or the first 56 d after arrival (Pritchard and Mendez, 1990). Feed conversion, however, did not appear to be affected in this study, possibly because tame calves were used and they were returned to their home pen after each transport trial. Interestingly, a rather large variation in calves was noticed for feed conversion during Wk 1 and Wk 2. Perhaps, the variation in feed conversion decreased after Wk 2 because the calves were older and more adjusted to the Calan gate feeding system.

The calves in this study appeared to have started to habituate to the weekly transport because they were resuming normal amounts of feed intake, and their ADG and feed conversion were comparable to the control calves during Wk 5. Further research on repeated transport over a longer time period (> 5 wk) could help determine whether the calves were truly beginning to habituate to the repeated stress. Also, additional measurements of stress (such as hormone concentrations, etc.) in the calves could provide more insight to whether acclimation occurred.

EFFECTS OF REPEATED TRANSPORT ON PHYSIOLOGY AND GENE EXPRESSION

Introduction

In general, the effect of stress on calf behavior and cortisol concentrations is well documented in previous studies; however, repeated exposure to a stressor is not well-studied. Calves, in particular, experience multiple stressors (including castration, weaning, and branding) during processing that cause changes in cortisol concentrations. For example, Cohen et al. (1990) determined that Holstein calves had higher plasma cortisol concentrations at 3 h and 6 h post-castration than intact calves. Elevated plasma cortisol concentrations have also been detected in calves that undergo abrupt weaning (Hickey et al., 2003) and either hot-iron or freeze branding (Schwartzkopf-Genswein et al., 1997).

Along with the effects of stress on calf hormone concentrations (especially cortisol), changes in immune function can also provide insight into the amount of stress a calf is experiencing. Cattle exposed to stress experience a wide variety of physiological changes, including alterations to the immune system, which play a significant role in the health, well-being, and production of these animals. For example, weaning stress is often associated with increases in circulating neutrophils and decreases in circulating lymphocytes (Blanco et al., 2009 and Hickey et al., 2003), whereas, castration stress typically causes increases in cortisol concentrations and decreases in interferon-gamma

production (Fisher et al., 1997 and Ting et al., 2004). Stress, in general, is well-known for its immunosuppressive effects on cytokines and antibodies (Roth and Kaeberle, 1982), which is sometimes attributed to elevated cortisol concentrations during stress.

Transport stress has been shown to cause increases in cortisol concentrations (Fell and Shutt, 1986 and as reviewed in Tarrant, 1990), increases in IgG concentrations (Mackenzie et al., 1997), and decreases in interferon-gamma production (Earley et al., 2012). Previous studies have determined that high incidences of disease (such as bovine respiratory disease) in newly-received calves at feedlots could be attributed to the amount of stress calves experience prior to arrival at feedlots. According to Snowder et al. (2006), a sharp increase in the number of newly-received calves with bovine respiratory disease in feedlots occurred after 5 d on feed and the number of incidences peaked within 14 d on feed. Furthermore, infectious diseases in cattle (such as bovine respiratory disease) have been associated with higher concentrations of tumor necrosis factor-alpha (TNF-α), which causes weight loss from anorexia and diarrhea (Ohmann et al., 1989). To gain a better understanding about the effects of stress on the immune system, an earlier study (Terrill et al., 2011) investigated whether acute and chronic stress affected the expression of immune-related genes in leukocytes in beef calves. In that study, fifteen cytokine, chemokine, and toll-like receptor genes were identified as being significantly affected by the stress associated with the marketing process (including transport stress). Of the fifteen genes Terrill et al. (2011) identified, six were selected for use in this study, along with one novel gene of interest.

This study focused on the gene expression of interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-12 (IL-12), toll-like receptor-2 (TLR-2), toll-like receptor-4 (TLR-4), chemokine (c-x-c motif) receptor 2 (CXCR2), and 5-hydroxytryptamine receptor 2A (HTR2A) in leukocytes. IL-4, IL-6, and IL-12 are involved in cytokine-cytokine receptor interactions that have been linked to susceptibility to diseases such as asthma, measles, rheumatoid arthritis, influenza A, herpes, cancer, Type I diabetes, malaria, and tuberculosis. IL-4 is known for activating B-cell and T-cell proliferation, stimulating T-cell differentiation, suppressing interferon-gamma (IFN-γ) production, and acting as a regulator of adaptive immunity. IL-6 is involved in the production of neutrophils and plays a key role in mediating fever within the body. IL-12 assists with T-cell differentiation and triggers the production of IFN-γ, along with being linked to autoimmune diseases.

TLR-2 and TLR-4 are both components of the toll-like receptor signaling pathway. TLR-2 is a receptor that is widely known for its recognition of Gram-positive bacteria, in which it activates the production of cytokines (including tumor necrosis factor- alpha and various interleukins) in the innate immune system to destroy the bacteria. In contrast, TLR-4 detects lipopolysaccharide in Gram-negative bacteria and assists with the activation of the innate immune system. CXCR2 is a receptor for interleukin-8, in which it stimulates neutrophil migration to areas of inflammation and has been linked to melanoma growth (Huang et al., 2009). As with most immune system components, the cytokines used in this study are essential to the body's immune response, so moderate amounts of these cytokines are ideal, however, overproduction of

cytokines may lead to hyperactivity of the immune system and potentially the development of autoimmune diseases.

HTR2A is one of the receptors for the neurotransmitter serotonin. Serotonin is considered to be a key component of an individual's well-being as the brain uses it to cope with stressful events. Low concentrations of serotonin have been associated with depression and mental illnesses (Owens and Nemeroff, 1994). Serotonin is derived from the amino acid tryptophan. Serotonin in the brain is replenished by recruiting tryptophan across the blood-brain barrier to synthesize additional serotonin (Dunn, 1988).

The objective of this component of the study was to determine if calves acclimate to repeated weekly transport and the duration of transport, as indicated by pre-transport plasma cortisol concentrations, cortisol concentrations during transport (response cortisol concentrations), and the expression of innate immune system genes in leukocytes. In addition, a pilot study was conducted in junction with this study to determine possible relationships among plasma cortisol, serotonin, and tryptophan concentrations that occur during repeated transport. The hypothesis of this component of the study was that calves exposed to repeated weekly transport would have decreased expression of innate immune system genes in leukocytes (due to the stress and elevated cortisol concentrations) and if the calves acclimated to the stress, then the expression of these genes would return to baseline values.

Materials and Methods

Blood Sample Collection

Blood samples (9 ml) were collected via jugular venipuncture from the calves on the trailer or in their home pens pre-transport, and after 2 h, 4 h, and 6 h of transport. The pre-transport blood samples were collected from calves in their home pens, before any of the calves were handled for weighing, or otherwise disturbed. The calves in this study had been bottle-fed and showed minimal reaction to the researchers entering their pens. Several calves in each group approached and sniffed the researchers, but others in each group appeared to pay no attention. Teams of two people quietly approached a particular calf, and when close to the calf, one person reached for the head and neck area to restrain the calf. The second person knelt in front of the calf and collected the sample. Some calves were easily sampled standing in the middle of the pen, while others were allowed to back up against a wall of the pen. Pre-transport samples from transported calves were collected before control calves were sampled to approximate the time delay that would occur during subsequent sampling.

For the 2 h, 4 h, and 6 h samples, blood samples were first obtained from the transported calves on the trailer, and then from control calves in their home pens. To efficiently collect blood samples from the calves on the trailer, two teams of two people entered from the rear or the front of the trailer and each team focused on collecting samples from all calves within the rear or front compartments before assisting the other

team with the middle compartment of the trailer. Within the trailer, one person from each team held the calves around their necks and restrained them so the second person could collect the samples. Some samples were collected from calves that remained lying during the sampling process.

After collection, blood samples were immediately placed on ice. Within 30 min of collection, blood samples were centrifuged at 3200 rpm for 10 min at room temperature and the plasma from each sample was transferred to individual 5 ml Falcon round-bottom disposable tubes (Falcon tubes, Becton-Dickinson, Franklin Lakes, NJ, USA) and frozen at -20°C for future use.

Selected blood samples from each treatment and sampling time (Transport 1, pre-transport: n = 18 transported and 18 control calves; Transport 1, 6 h: n = 17 transported calves; Transport 5, pre-transport: n = 18 transported and 18 control calves; Transport 5, 2 h: n = 18 transported calves; Transport 5, 4 h: n = 17 transported calves; Transport 5, 6 h: n = 18 transported and 18 control calves) were chosen for gene expression analysis in order to determine whether repeated transport affected the expression of immune-related genes in leukocytes (Transport 1 compared to Transport 5) and whether the expression of these genes changed as calves were exposed to 2, 4, and 6 h of stress. Samples in this subset were filtered to collect leukocytes (LeukoLOCK™ Total RNA Isolation System, Applied Biosystems, Carlsbad, CA, USA) prior to centrifugation. As demonstrated by Terrill et al. (2011), the filtration of samples prior to plasma collection did not affect cortisol concentrations.

Enzyme-linked Immunosorbent Assays

Colorimetric competitive enzyme-linked immunosorbent assays (ELISA) were conducted to detect cortisol concentrations, with an intra-assay coefficient of variation (CV) of 10% or less for each assay. Flat bottom ELISA plates were coated with goat anti-mouse IgG (5 µl/ml, Sigma Aldrich, St. Louis, MO, USA) coating solution and incubated overnight at 37 °C. The following day, the plates were rinsed four times with a phosphate-buffered saline solution containing Tween 20 (PBST, pH = 7.4) and 100 µl of diluted (with PBST) cortisol standards (Assay Designs, Ann Arbor, MI) at 10,000 pg/ml, 5,000 pg/ml, 2,500 pg/ml, 1,250 pg/ml, 625 pg/ml, 313 pg/ml, and 156 pg/ml were added to the plates in triplicate.

Samples were diluted 1:4 with PBST and also run in triplicate (100 µl per well). Blue cortisol conjugate (50 µl, Sigma Aldrich, St. Louis, MO, USA) and yellow cortisol antibody (50 µl, Sigma Aldrich, St. Louis, MO, USA) were added to the wells. The plates were incubated at 37°C for 2 h on a plate shaker at 500 rpm, washed four times with PBST, and the wells were aspirated. A para-Nitrophenyl phosphate (pNpp) substrate solution (200 µl) was added to the plates and incubated at 37° C for 1 h. Stop solution (50 µl, 3 M NaOH) was then added to all wells and the plates were read at an absorbance of 405 nm on a plate reader (Wallac Victor II 1420, Perkin Elmer, Waltham, MA, USA).

Samples with the lowest (0.62 to 1.04 ng/ml) cortisol concentrations (total n = 19; Transport 1, pre-transport: n = 2 control calves; Transport 1, 4 h: n = 4 transported

calves; Transport 1, 6 h: n = 4 transported calves; Transport 2, pre-transport: n = 2 transported and 2 control calves; Transport 2, 2 h: n = 3 control calves; and Transport 2, 4 h: n = 1 control and 1 transported calf) were selected for the determination of serotonin and tryptophan concentrations. These samples were chosen because no known previous studies have attempted to measure plasma tryptophan in relation to plasma cortisol concentrations in livestock. Furthermore, plasma tryptophan concentrations in dairy cows were previously reported at low concentrations (Kollmann et al., 2008). To increase the ability to detect tryptophan in the calves in this study, samples with the lowest cortisol concentrations were used because they were expected to have higher tryptophan concentrations (Dunn, 1988). Plasma serotonin and tryptophan concentrations were determined using pre-established protocols (BA E-8900 Fast Track Serotonin ELISA and BA E-2700 Tryptophan ELISA, Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany). Both protocols were competitive ELISAs in which samples were run in duplicate and the plates were read at an absorbance of 450 nm on the same plate reader described above. Finally, all data from the plate reader was analyzed with StatLIA® computer software (Brendan Technologies, Inc., Carlsbad, CA, USA) to determine plasma cortisol, serotonin, and tryptophan concentrations using the logarithmic curve of the known standards for each assay.

RNA Extraction and Analysis

In order to limit the degradation of sample RNA, samples were processed (filtered to collect leukocytes) and frozen within 1 h post-collection. Samples were filtered to collect leukocytes (LeukoLOCK™ Total RNA Isolation System, Applied Biosystems, Carlsbad, CA, USA), filters were flushed with RNAlater (a preservative), and all filters were stored at -20 °C for future use. Ribonucleic acid (RNA) was extracted and collected from each sample by lysing leukocytes off the filters with 2.5 ml of Lysis/Binding solution, degrading cellular proteins with 25 µl of Proteinase K, capturing the RNA onto binding beads, purifying the RNA using three (750 µl per rinse) rinses of Wash Solution, and eluting the purified RNA from the binding beads with 50 µl of Elution Solution (LeukoLOCK™ Total RNA Isolation System, Applied Biosystems, Carlsbad, CA, USA). All RNA samples were stored at -20 °C until further use.

The quantity of RNA in each sample was measured using UV absorption in a UV spectrophotometer (NanoDrop 2100, Thermo Scientific, Wilmington, DE, USA) and the quality was measured using a combination of microfluidics, capillary electrophoresis, and fluorescence in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with at least a quantity of 80 ng/µl and an RNA Integrity Number of 6.9 (based on a scale of 1 to 10, with 10 representing the best quality RNA) were used to create cDNA and run quantitative reverse transcriptase PCR (qRT-PCR).

Complementary DNA (cDNA) was synthesized for each sample using a reverse transcription kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems,

Foster City, CA, USA) in which 750 ng of RNA was diluted with nuclease-free H_2O for a final volume of 10.2 µl and a cocktail of H_2O, reverse transcriptase buffer, random hexamer primers, and dNTP (deoxyribonucleotide triphosphate) were mixed. For each sample, 15 µl of cocktail (with 1 µl per reaction of reverse transcriptase) and 5 µl of sample RNA were incubated at 25°C for 10 min, 37°C for 2 h, 70°C for 2 min, and 10°C on hold. H_2O was used to dilute cDNA to reach a final ratio of 1:5 and the solutions were then stored at 4°C for 24 h.

Real-time polymerase chain reaction FAST assays were used in this study in which samples were run in duplicate on FAST 96-well plates. Each plate contained assays for eight genes; 18S (reference gene), IL-4 (interleukin-4), IL-6 (interleukin-6), IL-12 (interleukin-12), CXCR2 (chemokine (c-x-c motif) receptor 2), TLR-2 (toll-like receptor-2), TLR4 (toll-like receptor 4), and HTR2A (5-hydoxytryptamine receptor 2A). Ten microliters of Taqman FAST master mix (Applied Biosystems, Foster City, CA), 8 µl of nuclease-free H2O, and 2 µl of the appropriate sample's cDNA were added to each well while the plate was kept on ice. Each plate was covered with an optical cover, centrifuged for 10 s at 700 x g, and run in a 7900HT real-time instrument (Applied Biosystems, Foster City, CA). Amplification data was collected using SDS RQ (relative quantification) Manager software (Sequence Detection System (SDS) Software v2.4.1, Applied Biosystems, Foster City, CA) in which average Ct (threshold cycle) values were used to calculate average Δ Ct (target gene average Ct – 18S average Ct), average ΔΔ Ct (Δ Ct – average Δ Ct for all samples), and average RQ (2^ (-ΔΔ Ct)) values for each sample.

Statistical Analyses

Cortisol concentrations were evaluated on three levels; pooled (all samples), pre-transport (basal, before each transport), and response (after 2 h, 4 h, and 6 h of transport for each transport). Pooled sample analysis was conducted in order to determine how the calves in this study, in general, responded to repeated transport. Pre-transport samples were analyzed separately in order to determine whether pre-transport cortisol concentrations differed from transport trial to transport trial (skewing the results). Response samples were analyzed separately to determine whether any differences could be detected when pre-transport samples were not included in the dataset. Pooled, pre-transport, and response cortisol concentrations were analyzed in a mixed model ANOVA using SAS (SAS 9.2, SAS Institute Inc., Cary, NC, USA), with repeated measures for calf and an unstructured covariance. Transport trial, treatment, and time of sampling (except for the pre-transport data set) served as fixed effects and each calf served as an experimental unit (n = 36). When significant three-way interactions occurred with transport trial, treatment, and time of sampling, analyses were conducted by transport trial. Pairwise comparisons were implemented to further examine significant effects. Regression analysis was used to determine the effect of THI on plasma cortisol concentrations. Pearson correlation tests were implemented to investigate associations among cortisol, serotonin, and tryptophan plasma concentrations. Regression was used to further examine significant associations.

Average RQ values for each sample were used for statistical analyses. Each calf served as an experimental unit (n = 36), however, a sample was not collected from every calf during each sampling time (as discussed above). Average RQ values for each target gene (IL-4, IL-6, IL-12, CXCR2, TLR-4, and HTR2) were analyzed in a mixed model ANOVA using SAS (SAS 9.2, SAS Institute Inc., Cary, NC, USA), with repeated measures for calf and an autoregressive covariance. Three separate models using different subsets of data were implemented to analyze the RQ values. Samples collected from transported and control calves pre-transport during Transport 1 and Transport 5 were analyzed in a model that tested for the effects of treatment group and transport trial to see if any unexpected differences between treatment groups occurred pre-transport. Samples collected from transported calves pre-transport and after 2 h, 4 h, and 6 h of transport during Transport 5 were analyzed in a model that tested for the effects of the time of sampling. Samples collected from transported calves after 6 h of transport during Transport 1 and Transport 5 were analyzed in a model that tested for the effects of transport trial.

Results

Samples were easier to collect from calves in the trailer than calves in the pens, perhaps because the calves in the trailer had less space or were more tired. A few of the calves in the pens appeared to display more avoidance behavior with each successive

sample collection, but the researchers also became more adept at catching and restraining the calves. Regardless, sample collection took < 30 s for each calf.

Calf Plasma Cortisol Concentrations

Pooled Cortisol Concentrations. Over the span of the study, a significant interaction ($P = 0.03$, Figure 7) with transport trial, treatment, and time of sampling occurred in cortisol concentrations. Treatment did not affect pooled cortisol concentrations during Transport 1 ($P = 0.24$), Transport 2 ($P = 0.70$), or Transport 3 ($P = 0.07$). However, transported calves had significantly higher cortisol concentrations than control calves during Transport 4 (transported: 9.51 ± 0.89 ng/ml, control: 5.48 ± 0.92 ng/ml, $P = 0.004$) and Transport 5 (transported: 5.96 ± 0.60 ng/ml, control: 3.96 ± 0.62 ng/ml, $P = 0.03$). Transported calves had significantly lower cortisol concentrations pre-transport (pre-transport, 4.64 ± 0.33 ng/ml) than after 2 h (11.20 ± 0.70 ng/ml, $P < 0.0001$) and 4 h (8.18 ± 1.17 ng/ml, $P = 0.003$) of transport, but no significant difference occurred between pre-transport and the 6 h samples (5.73 ± 0.74 ng/ml, $P = 0.12$).

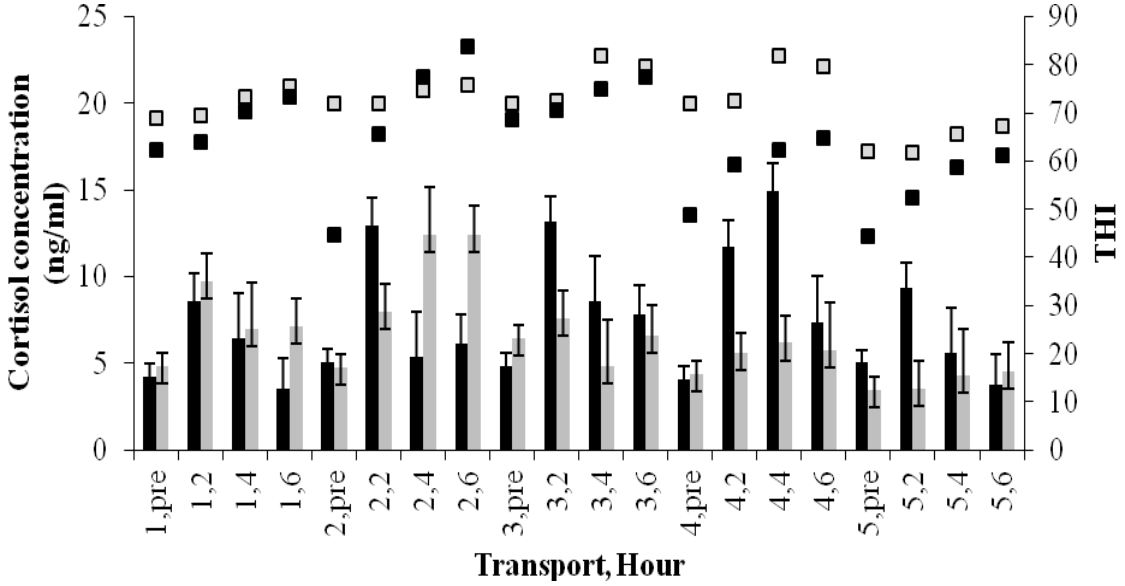

Figure 7. Plasma Cortisol Concentrations on Days of Transport. Mean (± SE) plasma cortisol concentrations for control (n = 18, represented by gray bars) and transported (n = 18, represented by black bars) calves for each of the five transports during the 5-wk-long study. The gray square symbols represent mean THI within the trailer and black square symbols represent mean THI within the pens during each sampling time each transport.

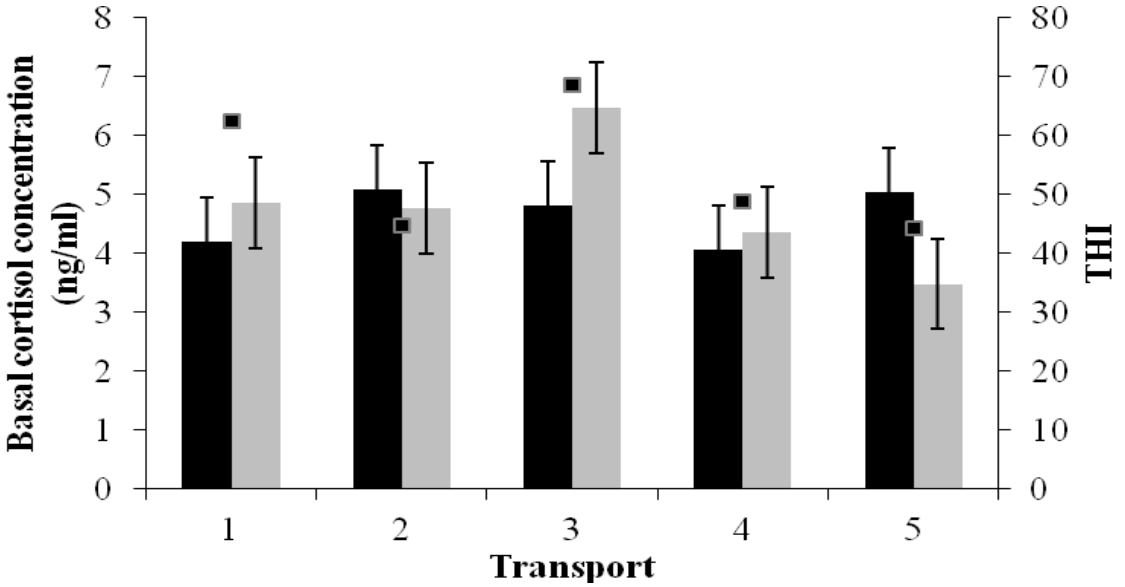

Figure 8. Pre-transport Cortisol Concentrations on Days of Transport. Mean (± SE) pre-transport (basal) cortisol concentrations for control (n = 18, represented by gray bars) and transported (n = 18, represented by black bars) calves for each of the five transports during the 5-wk-long study. The black square symbols represent mean THI within the pens during the pre-transport sampling time each transport.

Pre-transport and Response Cortisol Concentrations. No significant interactions occurred in pre-transport (basal) cortisol concentrations. Also, pre-transport cortisol concentrations did not differ by treatment ($P = 0.77$) or transport trial ($P = 0.32$, Figure 8). A significant ($P = 0.04$) interaction with transport trial, treatment, and time of sampling occurred in response cortisol concentrations. During Transport 1 and Transport 2, treatment did not affect response cortisol concentrations ($P = 0.29$, $P = 0.56$), but all calves had significantly higher cortisol concentrations during the 2 h (9.17 ± 1.27 ng/ml, 11.81 ± 2.86 ng/ml) sampling time than the 6 h (5.33 ± 0.92 ng/ml, $P = 0.02$ and 9.26 ± 2.29 ng/ml, $P = 0.04$) sampling time (Figure 7). During Transport 3 and Transport 4, transported calves had significantly higher cortisol concentrations than control calves after 2 h (Transport 3: transported = 13.16 ± 1.33 ng/ml, control = 7.57 ± 1.37 ng/ml, $P = 0.006$ and Transport 4: transported = 11.72± 1.82 ng/ml, control = 5.62 ± 1.88 ng/ml, $P = 0.03$) and 4 h (Transport 3: transported = 7.83 ± 0.89 ng/ml, control = 6.62 ± 0.92 ng/ml, $P = 0.002$ and Transport 4: transported = 14.92 ± 1.44 ng/ml, control = 6.18 ± 1.49 ng/ml, $P = 0.0002$) of transport (Figure 7). During Transport 5, transported calves had significantly higher cortisol concentrations than control calves after 2 h of transport (transported: 9.32 ± 0.95 ng/ml, control: 3.55 ± 0.98 ng/ml, $P = 0.0002$), but not after 4 h of transport ($P = 0.20$, Figure 7). A trend ($P = 0.06$) was detected in which cortisol concentrations increased by 2.52 ± 0.42 ng/ml as THI increased by one unit.

No relationship was detected between cortisol and serotonin concentrations ($P = 0.54$) or tryptophan and serotonin concentrations ($P = 0.45$), but a significant negative relationship was discovered between cortisol and typtophan concentrations ($P = 0.02$,

Table 1). For every one ng/ml increase in cortisol concentrations, tryptophan concentrations decreased by 0.02 ± 0.01 µg/ml.

Table 1. Mean Concentrations of Plasma Serotonin and Tryptophan of Calves with Lowest Cortisol Concentrations (total n = 19). Samples were from control and transported calves pre-transport and after 2, 4, and 6 h of transport during Transport 1 and Transport 2.

		Concentrations[a]		
Sampling Time	Treatment	Cortisol (ng/ml)	Serotonin (ng/ml)	Tryptophan (µg/ml)
Transport 1				
Pre-transport	Control	6.91	81	10.73
4 h	Transported	7.68	150	9.76
6 h	Transported	7.85	49	10.55
Transport 2				
Pre-transport	Control	6.86	74	10.18
Pre-transport	Transported	9.08	57	3.75
2 h	Control	8.29	68	11.32
4 h	Control	9.09	109	10.90
4 h	Transported	9.16	195	7.4

[a] Standard error is not provided with these means because the sample sizes were too small, but data for these samples is located in Table 3.

Expression of Immune-related Genes in Leukocytes

In this study, TLR-2 expression was not detected. No significant interactions were detected when comparing RQ values for the other genes for transported and control

calves pre-transport during Transport 1 and Transport 5. Expression in the pre-transport samples during Transport 1 and Transport 5 was not affected by treatment (IL-4: P = 0.94, IL-6: P = 0.81, CXCR2: P = 0.62, IL-12: P = 0.85, TLR-4: P = 0.53, and HTR2A: P = 0.49) or time of sampling (IL-4: P = 0.56, IL-6: P = 0.32, CXCR2: P = 0.06, IL-12: P = 0.91, TLR-4: P = 0.54, and HTR2A: P = 0.29). The time of sampling during Transport 5 had no effect on IL-12 (P = 0.20, Figure 9), CXCR2 (P = 0.52, Figure 10), or TLR-4 (P = 0.34, Figure 11) in transported calves. However, transported calves did have lower RQ values for IL-4 and IL-6 during the 2 h (IL-4: 0.66 ± 0.07 RQ and IL-6: 0.64 ± 0.10 RQ) sampling time than during the pre-transport (IL-4: 1.06 ± 0.08 RQ, P = 0.0003 and IL-6: 1.00 ± 0.09 RQ, P = 0.006), 4 h (IL-4: 0.96 ± 0.08 RQ, P = 0.007 and IL-6: 0.94 ± 0.10 RQ, P = 0.03), and 6 h (IL-4: 1.03 ± 0.07 RQ, P = 0.0009, Figure 12 and IL-6: 1.09 ± 0.09 RQ, P = 0.002, Figure 13) sampling times. Also, transported calves had lower HTR2A gene expression after 2 h (0.77 ± 0.16 RQ) of transport than after 4 h (1.19 ± 0.16 RQ, P = 0.005, Figure 14) of transport. No difference (IL-4: P = 0.59, IL-6: P = 0.67, CXCR2: P = 0.81, IL-12: P = 0.40, TLR-4: P = 0.44, and HTR2A: P = 0.66) was detected between samples collected during the 6 h sampling from transported calves during Transport 1 and Transport 5.

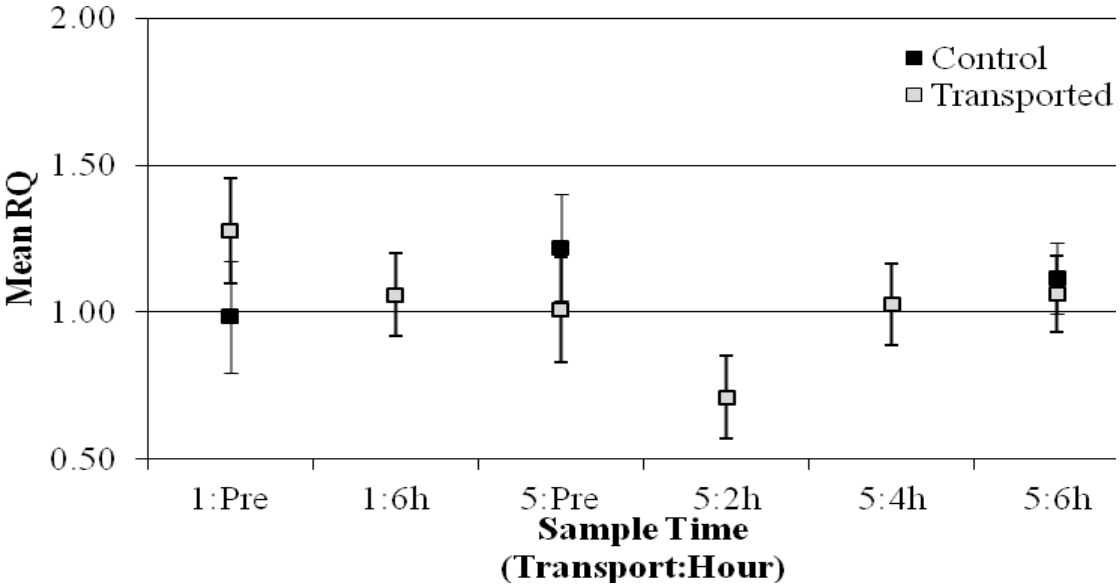

Figure 9. Average RQ Values for IL-12. Mean (± SE) relative quantification (RQ) values for the IL-12 gene in plasma samples from Holstein calves.

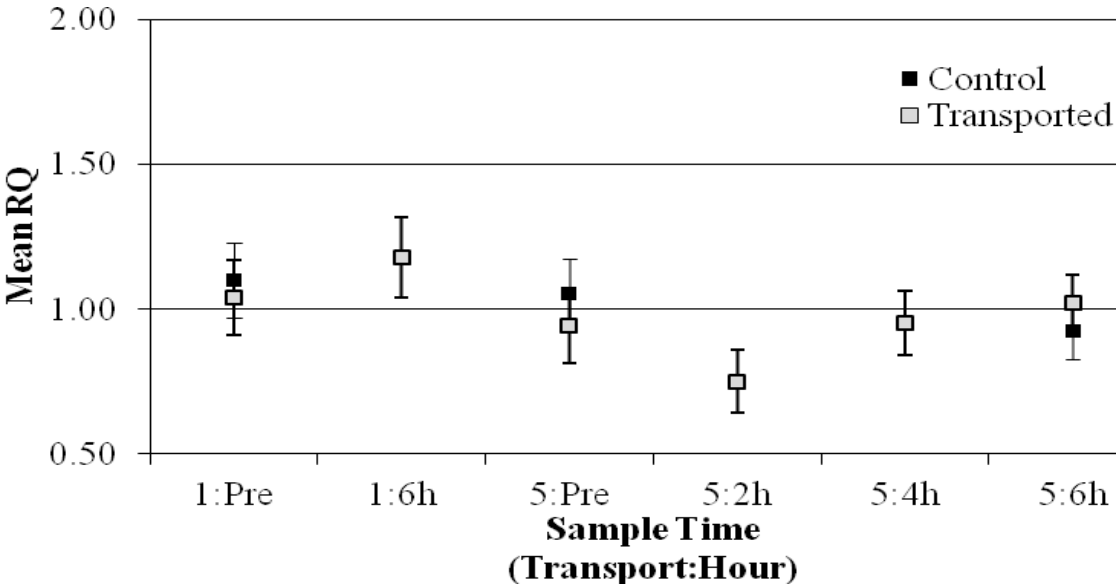

Figure 10. Average RQ Values for CXCR2. Mean (± SE) relative quantification (RQ) values for the CXCR2 gene in plasma samples from Holstein calves.

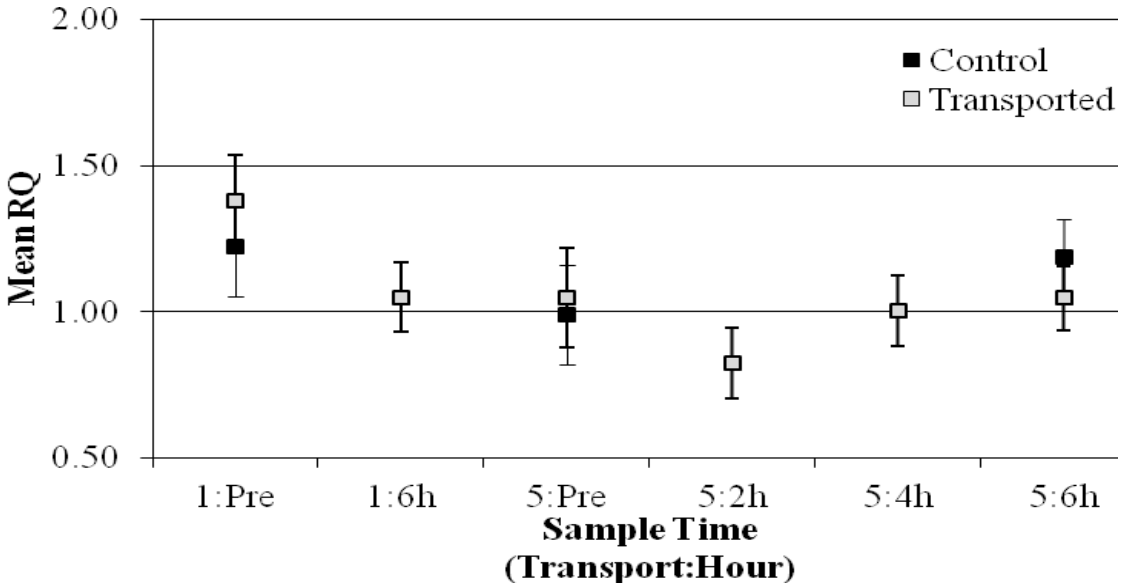

Figure 11. Average RQ Values for TLR-4. Mean (± SE) relative quantification (RQ) values for the TLR-4 gene in plasma samples from Holstein calves.

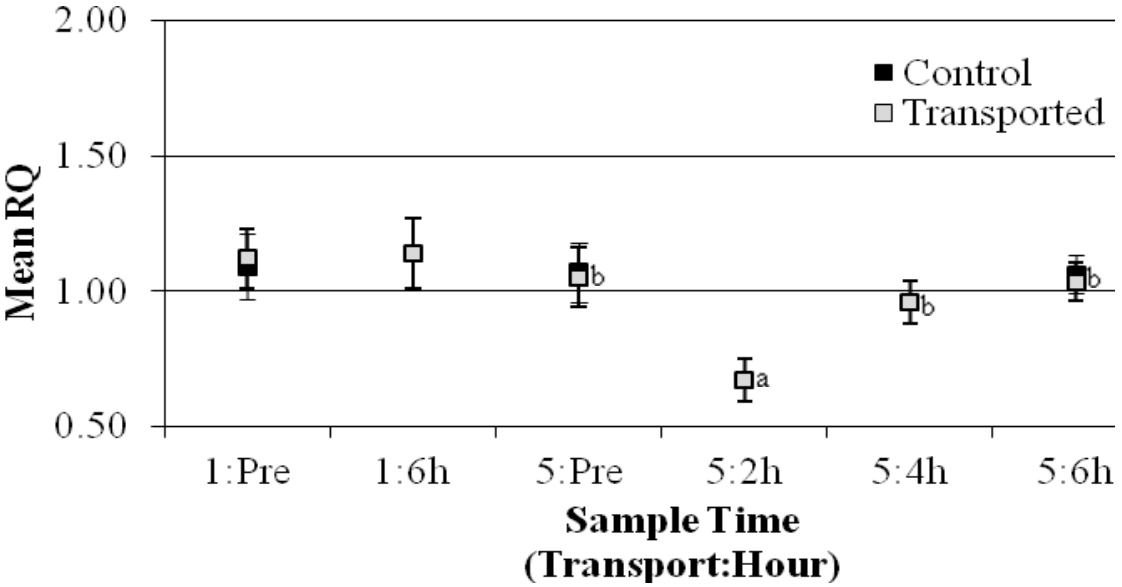

Figure 12. Average RQ Values for IL-4. Mean (± SE) relative quantification (RQ) values for the IL-4 gene in plasma samples from Holstein calves. [a,b] Means from transported calves during Transport 5 without a common superscript differ ($P < 0.05$).

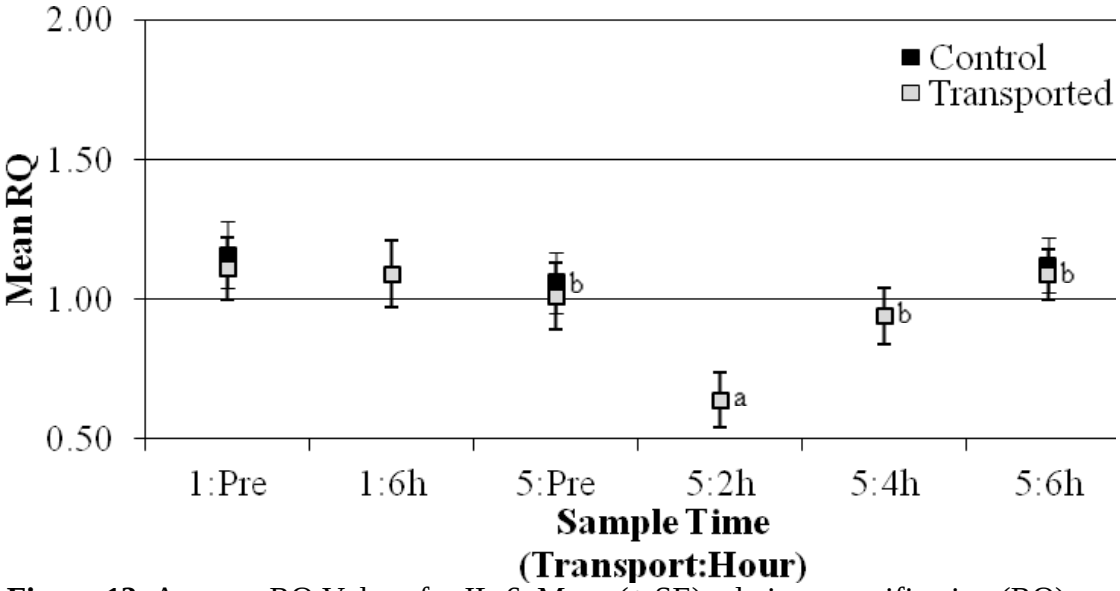

Figure 13. Average RQ Values for IL-6. Mean (± SE) relative quantification (RQ) values for the IL-6 gene in plasma samples from Holstein calves. [a,b] Means from transported calves during Transport 5 without a common superscript differ ($P < 0.05$).

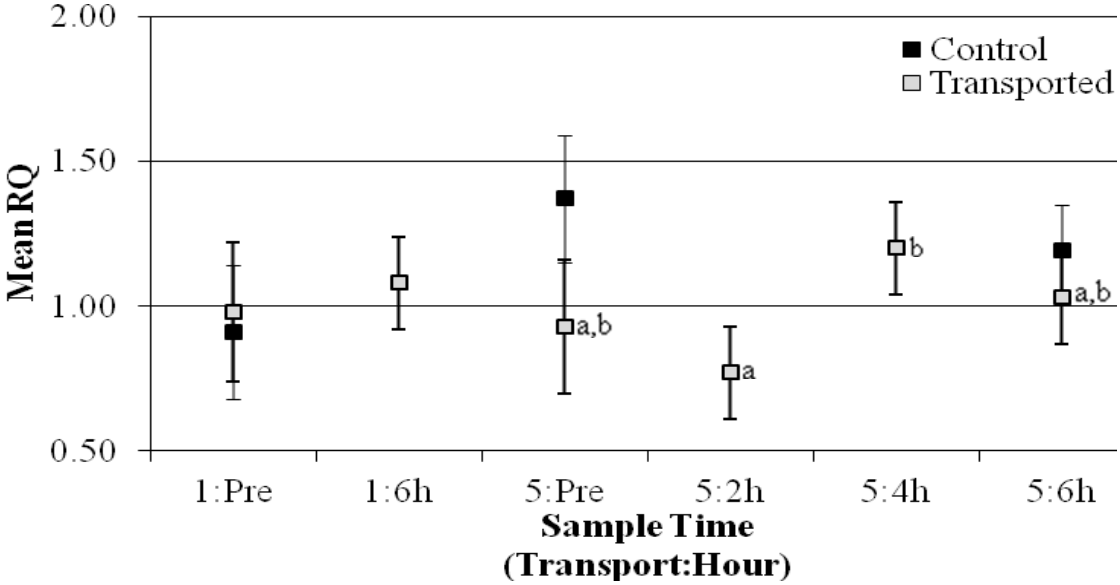

Figure 14. Average RQ Values for HTR2A. Mean (± SE) relative quantification (RQ) values for the HTR2A gene in plasma samples from Holstein calves. [a,b] Means from transported calves during Transport 5 without a common superscript differ ($P < 0.05$).

Discussion

All calves used in this study maintained pre-transport cortisol concentrations that ranged from 3 to 7 ng/ml during each transport trial. Johnston and Buckland (1976) documented a mean resting plasma cortisol concentration of 4.11 ± 0.47 ng/ml for Holstein bull calves. Although the pre-transport cortisol concentrations in this study were similar, the cortisol concentrations in response to transport in this study were much lower than those reported in Johnston and Buckland (1976). After 2 h of transport during Transport 3 in this study, calf cortisol concentrations increased in transported calves to 13.16 ± 1.33 ng/ml. In comparison, after 1 to 2 h of handling and transport in the Johnston and Buckland (1976) study, calf cortisol concentrations peaked at 19.63 ± 2.10 ng/ml. These results may suggest that the calves in this study were not very stressed by the transportation, and perhaps this is an explanation for the lack of an obvious acclimation response in these calves.

In this study, plasma cortisol concentrations for transported calves were affected by repeated transport. Transported and control calves had similar cortisol concentrations during Transport 1, Transport 2, and Transport 3, but then transported calves had higher concentrations than control calves during Transport 4 and Transport 5. On the other hand, transported calves had cortisol concentrations after 4 h and 6 h of transport that seemed to be returning to concentrations similar to the control calves during Transport 5. Locatelli et al. (1989) noticed that calf cortisol concentrations increased with each consecutive 30-min simulated transport (calves were transported three times with 10-d

intervals between transports), but the increase became less significant with repeated transport. In contrast, Fell and Shutt (1986) measured salivary cortisol concentrations from calves exposed to repeated transport and did not have any evidence of acclimation to transportation.

The amount of time spent catching and collecting a blood sample took < 30 s for each calf in this study. After experiencing a stressor, increases in cortisol concentrations are typically seen 1 to 3 min after the stressor is applied (Grandin, 1997). Also, Lay et al. (1992) did not notice a change in cortisol concentrations in dairy cows during hot-iron branding until approximately 5 min after the stressor was applied, so the cortisol concentrations measured in this study should not have been influenced by the stress associated with the sampling process.

A correlation was discovered between cortisol concentrations and THI where cortisol increased as THI increased, but this association was anticipated because both cortisol concentrations and THI are influenced by diurnal effects, and pre-transport (basal) cortisol samples were collected before calves were exposed to a stressor. In addition, transported calves had higher cortisol concentrations than control calves after 2 h of transport during Transport 3, Transport 4, and Transport 5. These results are supported by Nwe et al. (1996) and Broom et al. (1996) in which goats and sheep exhibited the greatest increase in cortisol concentrations during loading and the first 3 h of transport. Dalin et al. (1993) also had similar findings as swine reached peak cortisol concentrations after 30 min of transport.

With the limited number of samples in this study, no significant differences were detected in the gene expression of immune-related genes in leukocytes between transported calves and control calves pre-transport during Transport 1 and Transport 5. While Terrill et al. (2011) documented differences between calves exposed to acute stress and calves exposed to chronic stress (including transport) in six of the genes used in this study, the calves used in that study did not have a recovery period between stressors and were not exposed to repeated transport, unlike the calves in this study. Also, the calves in this study were not exposed to a stressor during the pre-transport sampling. Another explanation for these conflicting results from the two studies may be due to the temperament of the calves used in each study. Besides common differences between dairy and beef breeds, the Holstein calves used in this study were bottle-fed and handled on a daily basis since they were 1 to 2 d old. For example, Hulbert et al. (2011) showed that temperamental bulls had higher cortisol concentrations than calm bulls and calm bulls had elevated neutrophil L-selectin expression, and phagocytic and oxidative burst activity than temperamental bulls, after 4 h of transport. Another factor may be that the amount of handling stress the calves in this study experienced was limited because these calves were selected because they were accustomed to handling and the objective of this study was to focus on the stress associated with transport alone. The expression of TLR-2 in leukocytes was detected in previous studies (Terrill et al., 2011), but not in this study. Given that no expression of TLR-2 was detected in any of the samples in this study, the most likely reason is a failure of the primers used in these assays.

The results in this study indicate that the gene expression of IL-4 and IL-6 in leukocytes were significantly lower after 2 h of transport than after 4 h or 6 h of transport. Majority of the studies in this field claim that stress causes decreases in immune function, but Calcagni and Elenkov (2006) demonstrated that glucocorticoids inhibited IL-12, while up-regulating IL-10 and IL-4. They also commented that stress hormones may induce the production of IL-1, IL-8, and IL-6. Further supporting this, Buckham Sporer et al. (2008) saw the expression of IL-8 genes increase and IFN-γ genes decrease after 4.5 h of transport in beef bulls.

The lack of agreement among these studies makes it apparent that further research is needed in order to understand how innate immune system cytokine genes are regulated during transport stress, along with other types of stressors. Previous studies have determined that pro-inflammatory cytokines (including TNF-α, IFN-γ, and IL-6) impose a metabolic demand on cells (Berg et al., 2003), in which high concentrations of these cytokines are associated with the loss of skeletal muscle mass in humans (Eid et al., 2001). Future research that focused on measuring pro-inflammatory cytokine concentrations and skeletal muscle mass in newly-received calves at feedlots may be able to provide further insight into the connection between stress and weight loss.

Surprisingly, no relationship between plasma cortisol and serotonin concentrations was detected in this study. Plasma serotonin concentrations were expected to increase as plasma cortisol concentrations increased because previous studies have demonstrated that the intestinal tract releases serotonin into circulation as an individual experiences stress (as reviewed by Racké and Schwörer, 1991). However,

a negative relationship between plasma cortisol and tryptophan concentrations was revealed in this study. This relationship was expected because according to Dunn (1988), individuals experiencing stress deplete serotonin sources in the brain and recruit circulating tryptophan to the brain to synthesis additional serotonin. One of the limitations of this study was that only samples with the lowest cortisol concentrations were used to analyze serotonin and tryptophan. Given the relationship between cortisol and tryptophan that was discovered in this study, it would be useful to also analyze serotonin and tryptophan in the samples with the highest cortisol concentrations. This study was the first known study to attempt to make a connection between stress and circulating serotonin and tryptophan concentrations in livestock animals.

In conclusion, the calves in this study had plasma cortisol concentrations that peaked after 2 h of transport and decreased after 4 h and 6 h of transport each transport, but no evidence of acclimation was found with each successive transport. In regards to the expression of immune-related genes in leukocytes, the length of exposure to stress appeared to affect expression, but no differences were detected for repeated transport. An explanation for the lack of acclimation for repeated transport seen in this study could be that the calves were not very stressed from the transport to begin with, given the rather low cortisol concentrations measured in this study. Future research that examines the effects of repeated transport and the duration of transport on the concentrations of proteins (such as cytokines) in cattle blood samples could provide additional information about the effects of transport stress on the innate immune system. The results from this study also indicate that further research about the relationship between plasma cortisol

and tryptophan concentrations, as well as cortisol concentrations and the expression of immune-related genes and proteins would be useful.

CONCLUSIONS

The amount of stress the transported calves in this study encountered had apparent effects on their feed intake, ADG, and plasma cortisol concentrations. Transported calves had higher plasma cortisol concentrations during the 2 h sampling time, lower feed intake, and lower ADG. However, the transport conditions used in this study caused only a mild response, which was expected from bottle-fed and frequently handled Holstein calves. Each week, calves decreased their feed intake on the day of transport and then increased their feed intake the day after transport, except during Wk 5 when the calves actually increased their feed intake on the day of transport. Cooler weather occurred on the day of the 5th transport, which suggests that cooler weather may cause increases in feed intake.

The expression of the IL-4 and IL-6 genes in leukocytes was significantly lower during the 2 h sampling time than any other sampling time, which suggests that the expression of those genes can be altered very quickly after the onset of a stressor. This also suggests a possible negative relationship between cortisol concentrations and the expression of immune-related genes. A significant negative correlation between plasma cortisol and tryptophan concentrations was also detected in this study. These results suggest that when calves experience stress circulating tryptophan is recruited to the brain to synthesize additional serotonin because of physiological coping mechanisms within the brain. These conclusions offer evidence of an interaction among the expression of immune-related genes in leukocytes, hypothalamic-pituitary-adrenal axis activity, and

the physiological activation of coping mechanisms in the brain in calves after 2 h of transport.

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APPENDIX

Table 2. Mean Percent of Calves Eating, Drinking, and Lying after Each Transport. Mean (± SE) percent of calves eating, drinking, and lying for 1 h post-transport. Transported calves (n = 18) were observed in their home pens at 5-min intervals for 1 h after each transport.

	Behavior		
Post-transport	Eating	Drinking	Lying
1	47 ± 12	12 ± 8	41 ± 13
2	48 ± 13	15 ± 7	37 ± 13
3	39 ± 11	18 ± 8	43 ± 13
4	42 ± 12	12 ± 7	46 ± 13
5	35 ± 11	10 ± 7	55 ± 13

Table 3. Mean Concentrations of Plasma Cortisol from Control and Transported Calves. Mean (± SE) concentrations of cortisol (ng/ml) in control (n = 18) and transported calves (n = 18) pre-transport and after 2, 4, and 6 h of transport during the five transports.

	Treatment	
Sampling Time	Control	Transported
Transport 1		
Pre-transport	4.86 ± 0.51	4.21 ± 0.49
2 h	9.75 ± 1.79	8.58 ± 1.79
4 h	6.99 ± 1.91	6.44 ± 1.86
6 h	7.10 ± 1.32	3.56 ± 1.28
Transport 2		
Pre-transport	4.94 ± 0.98	5.08 ± 0.93
2 h	5.14 ± 1.77	13.25 ± 1.72
4 h	12.44 ± 5.38	5.41 ± 3.23
6 h	12.38 ± 3.29	6.15 ± 3.20
Transport 3		
Pre-transport	6.47 ± 0.96	4.82 ± 0.94
2 h	7.57 ± 1.37	13.16 ± 1.33
4 h	4.81 ± 0.81	8.55 ± 0.78
6 h	6.62 ± 0.92	7.83 ± 0.89
Transport 4		
Pre-transport	4.35 ± 0.57	4.07 ± 0.55
2 h	5.62 ± 1.88	11.72 ± 1.82
4 h	6.18 ± 1.49	14.92 ± 1.44
6 h	5.78 ± 0.88	7.34 ± 0.86
Transport 5		
Pre-transport	3.49 ± 0.75	5.04 ± 0.73
2 h	3.55 ± 0.98	9.32 ± 0.95
4 h	4.27 ± 0.76	5.69 ± 0.75
6 h	4.55 ± 0.61	3.79 ± 0.59

Table 4. Serotonin and Tryptophan Concentrations of Calves with Lowest Cortisol Concentrations (total n = 19). Samples were from control and transported calves pre-transport and after 2, 4, and 6 h of transport during the five transports.

			Concentrations		
Calf ID	Treatment	Transport:Hour	Cortisol (ng/ml)	Serotonin (ng/ml)	Tryptophan (µg/ml)
4	Transported	1:6 h	8.66	62	12.7
9	Transported	1:6 h	6.24	39	12.7
9	Transported	2:Pre-transport	9.58	64	3.7
11	Transported	1:4 h	7.05	101	19.9
24	Transported	1:4 h	7.75	185	5.8
33	Transported	1:4 h	8.65	103	6.4
47	Transported	2:4 h	9.16	195	7.4
48	Transported	1:6 h	8.94	60	12.6
48	Transported	2:Pre-transport	8.58	50	3.8
50	Transported	1:6 h	7.55	35	4.3
55	Transported	1:4 h	7.27	209	6.9
28	Control	2:4 h	9.09	109	10.9
38	Control	2:2 h	8.25	68	16.5
39	Control	2:2 h	10.38	94	2.5
52	Control	2:Pre-transport	7.08	60	8.0
58	Control	1:Pre-transport	7.17	74	9.1
59	Control	1:Pre-transport	6.64	90	15.9
59	Control	2:Pre-transport	6.64	88	12.4
59	Control	2:2 h	6.24	42	14.9

Table 5. Mean RQ Values for Immune-related Genes in Control and Transported Calves. Mean (± SE) RQ values (with range of minimum to maximum) of immune-related genes in control (n = 18) and transported calves (n = 18) pre-transport and after 2, 4, and 6 h of transport during the five transports.

Sampling Time	IL-12		CXCR2		IL-4	
	Control	Transported	Control	Transported	Control	Transported
Transport 1						
Pre-transport	0.99 ± 0.19 (0.29 – 2.77)	1.28 ± 0.18 (0.28 – 2.99)	1.22 ± 0.17 (0.31 – 2.75)	1.38 ± 0.16 (0.21 – 3.29)	1.09 ± 0.12 (0.51 – 3.18)	1.12 ± 0.11 (0.36 – 1.94)
6 h	----	1.06 ± 0.14 (0.42 – 2.27)	----	1.05 ± 0.12 (0.30 – 2.37)	----	1.14 ± 0.13 (0.47 – 3.31)
Transport 5						
Pre-transport	1.22 ± 0.18 (0.15 – 3.95)	1.01 ± 0.18 (0.42 – 2.18)	0.99 ± 0.17 (0.49 – 1.62)	1.05 ± 0.17 (0.33 – 1.99)	1.07 ± 0.11 (0.63 – 1.77)	1.05 ± 0.11 (0.60 – 2.27)
2 h	----	0.71 ± 0.14 (0.27 – 1.45)	----	0.82 ± 0.12 (0.28 – 1.88)	----	0.67 ± 0.08 (0.37 – 1.23)
4 h	----	1.03 ± 0.14 (0.49 – 3.35)	----	1.00 ± 0.12 (0.36 – 2.81)	----	0.96 ± 0.08 (0.58 – 1.54)
6 h	1.12 ± 0.12 (0.14 – 3.56)	1.06 ± 0.13 (0.37 – 2.32)	1.19 ± 0.13 (0.42 – 3.08)	1.05 ± 0.11 (0.58 – 1.90)	1.06 ± 0.07 (0.41 – 1.62)	1.03 ± 0.07 (0.65 – 1.94)

Table 5. Continued

Sampling Time	IL-6		TLR-4		HTR2A	
	Control	Transported	Control	Transported	Control	Transported
Transport 1						
Pre-transport	1.16 ± 0.12 (0.53 – 2.52)	1.11 ± 0.11 (0.33 – 2.02)	1.10 ± 0.13 (0.42 – 2.92)	1.04 ± 0.13 (0.29 – 2.07)	0.91 ± 0.23 (0.13 – 1.70)	0.98 ± 0.24 (0.12 – 2.88)
6 h	----	1.09 ± 0.12 (0.46 – 2.72)	----	1.18 ± 0.14 (0.39 – 2.65)	----	1.08 ± 0.16 (0.19 – 2.86)
Transport 5						
Pre-transport	1.06 ± 0.11 (0.49 – 2.00)	1.01 ± 0.12 (0.53 – 2.38)	1.05 ± 0.12 (0.47 – 1.92)	0.94 ± 0.13 (0.44 – 1.69)	1.37 ± 0.22 (0.18 – 5.92)	0.93 ± 0.23 (0.26 – 2.54)
2 h	----	0.64 ± 0.10 (0.27 – 1.58)	----	0.75 ± 0.11 (0.32 – 1.72)	----	0.77 ± 0.16 (0.09 – 2.02)
4 h	----	0.94 ± 0.10 (0.40 – 1.56)	----	0.95 ± 0.11 (0.45 – 1.88)	----	1.20 ± 0.16 (0.38 – 2.81)
6 h	1.12 ± 0.10 (0.39 – 2.31)	1.09 ± 0.09 (0.45 – 2.15)	0.93 ± 0.10 (0.36 – 1.87)	1.02 ± 0.10 (0.44 – 1.89)	1.19 ± 0.16 (0.23 – 2.92)	1.03 ± 0.16 (0.22 – 2.19)

VITA

Name:	Amber Lynn Adams
Address:	2471 TAMU Kleberg Center College Station, TX 77845
Email Address:	amberada7@hotmail.com
Education:	B.S., Animal Science, University of Wisconsin-River Falls, 2007 M.S., Zoology, Oklahoma State University, 2009 Ph.D., Animal Science, Texas A&M University, 2012