EFFECTS OF ACUTE AND CHRONIC STRESS ON IMMUNE- AND INFLAMMATORY-RESPONSE GENE EXPRESSION IN BEEF CALVES

A Thesis

by

COOPER LEE TERRILL

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

MASTER OF SCIENCE

December 2011

Major Subject: Animal Science

Effects of Acute and Chronic Stress on Immune- and Inflammatory-response Gene Expression in Beef Calves

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Approved by:

Chair of Committee,	Ted Friend
Committee Members,	Jason Sawyer
	Penny Riggs
	Luc Berghman
Head of Department,	H. Russell Cross

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ABSTRACT

Effects of Acute and Chronic Stress on Immune- and Inflammatory-response Gene Expression in Beef Calves. (December 2011)

Cooper Lee Terrill, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Ted Friend

Transport stress research has shown correlations among stress, morbidity, and mortality in calves subjected to the traditional U.S. market system, indicating the possibility of compromised immune function. The objective of this study was to determine if expression of specific immune and inflammatory response genes differed between calves that were subjected to either an acute stress (AS, handled and weaned for 1.5 h) or a chronic stress (CS, weaned, handled and transported for 3 to 4 d). Two groups of forty calves, *Bos taurus* (n = 20) and crossbred calves (n = 20), weighing 181 kg to 250 kg were used in each of two trials. Jugular veni-puncture blood samples (9 ml) were collected from AS calves 1.5 h after the start of handling and separation from their dam. Samples were collected from CS calves during processing after arrival at a north Texas feed lot. RNA for gene expression analysis was extracted from peripheral blood leukocytes obtained from blood samples by a filtration method. During the second trial, the filtrate was centrifuged for measurement of plasma cortisol. A diagonal covariance mixed model ANOVA was used to determine effects of treatment, breed, and breed by treatment interaction on cortisol concentrations. Expression values for each gene were analyzed using linear models that considered the

effects of treatment (AS and CS) and breed (*Bos taurus* and crossbred calves) comparing each trial separately. Mean plasma cortisol concentrations did not differ between AS (16.40 ± 1.08 ng/ml) and CS calves (18.06 ± 1.14 ng/ml) ($P > 0.296$). The interaction of effects was detected for 2 genes in Trial 1, and 3 genes in Trial 2 ($P < 0.029$). Breed was influential for 5 genes in both Trial 1 and 2 ($P < 0.046$). Significant differences were found in relative quantification for 30 genes in Trial 1 and 36 genes in Trial 2, in which CS calves had greater expression than AS calves ($P < 0.047$). Fifteen of those genes were common between the two trials with mean treatment differences of RQ values from the 15 genes ranging from 0.309 to 913.19, excluding outliers. Similar elevated cortisol concentrations in both the AS and CS calves indicated that both groups experienced significant stress. However, changes in gene expression differences were greater in the calves subjected to CS, indicating that gene expression may be more useful than cortisol for identifying detrimental long-term stress.

DEDICATION

To my supportive, patient and loving family that has stood beside me through all these years, my gratitude can't be expressed. The values you instilled in me, along with the constant support you provided, allowed me to develop into the man I am today with the ability to take on this challenge. Without my family standing by my side, I would not have been able to achieve my goals. Thank you for listening to me and keeping me sane long enough to complete this degree! I love y'all!

To my advisor, friend and colleague Dr. Glenn Holub, who offered me my first opportunities in both research and teaching. Your advice and guidance were instrumental in both my curricular and professional development, often pointing out that the ability to achieve is there; you just need to grasp it, while reminding me that there is more to life than just school. Your values and lead by example style encouraged me to keep moving forward, and advice and guidance through my college career made my time here so much smoother.

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To Dr. Joe Townsend, without whom this would not have been possible. I thank you for taking a chance on a fellow Aggie and giving me the opportunity to be here today.

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NOMENCLATURE

BRD	Bovine Respiratory Disease
BVD	Bovine Viral Diarrhea
h	Hour
min	Minute
d	Day
kg	Kilogram
m	Meter
km	Kilometer
SE	Standard Error
L	Liter
mL	Milliliter
ng	Nanogram
µl	Microliter
RQ	Relative Quantification values
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction

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INTRODUCTION

History

During the Civil War era in America, beef cattle were maintained over large areas in the South. In order to get these cattle to packing plants in the North, the cattle had to be rounded up and driven to the rail lines where they spent days in rail cars being transported North. The industry of that time differed from our modern one in many ways. Cattle were finished on grass and modern medicines weren't available. Steers weren't ready for slaughter until they reached four to five years of age and were shipped by rail in the South to packers in the North. After slaughter the meat was shipped east to feed the rapidly growing populations in New England. More supply was needed as populations increased and technology developed allowing for the ability to store and transport beef more efficiently. By the 1970's large scale feed operations were possible due to hybrid grains and new irrigation techniques. These large operations rapidly developed across the South and Midwest United States, and flourished. Once again the industry shifted as packing plants moved closer to the large feed lot operations allowing for faster processing with less profit loss from shrinkage in shipping. Today feeder cattle are transported directly from ranches to auctions by truck and trailer where they are sold. Order buyers buy cattle at auctions to fill orders for feedlots and haul these cattle to their own holding facilities to sort and complete the orders. These calves are then hauled to feed lots in tractor trailers. After arrival, the beef calves remain in feedlots till they are

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This thesis follows the style of Journal of Animal Science.

ready for slaughter at 10 to 12 months of age. These large feed lots are located in the central United States and currently hold the majority of the current 11 million cattle on feed.

Modern Practice

In our modern world there is an increasing demand to provide beef to an ever growing number of people. With a rapidly increasing demand, supply can sometimes struggle, causing prices to increase. Current prices have been influenced by the recent economic hardships. This accompanied by lack of rainfall and a shift from corn being produced for cattle feed to the production of ethanol. Profits have also fallen due to increased costs of input, thus creating a shortage of cattle. Worldwide events have created an even greater demand as droughts, floods, and other natural disasters have decimated food supplies for millions. In response to these problems, producers face the possibility of sending younger, smaller cattle through the market to start them on feed. These cattle have a risk of contracting Bovine Respiratory Disease (BRD), which is related to such factors as age class (weanling vs. yearling), body weight (proxy for age), procurement method (ranch direct or sale barn), and amount of comingling before and after arrival (Wildman et al., 2008). Older, larger animals like those shipped in the past may have had a much greater immune advantage than the animals we ship today. Until recent years, little attention has been paid to transport stress in market cattle. However, it is clear that there are significant physiological changes occurring in cattle as a result of transport and handling. These physiological changes lead to reduced immune system

function, increased disease incidence, and decreased carcass quality (Fike & Spire 2006). With compromised immune systems, pathogens are able to spread rapidly causing concern for the health of the animal as well as possible secondary effects such as food poisoning in humans.

Pathogen Shedding

Animals that are under chronic stress, which leads to immune-suppression, have an increased pathogen shedding incidence (Hussein et al., 2001, Isaacson et al., 1999). Market calves are being penned and transported in close quarters with animals that could potentially infect each other. These close quarters and multi-deck design of cattle trailers used to haul calves to feed lots increases the exposure to pathogens shed by infected calves. Barham et. al, (2002) showed prevalence levels of Salmonella on hides and in feces in the feed lots increased 1200% and 250%, respectively, upon final arrival at the packing plant. The results of this study demonstrate that transportation is a stressor for cattle, as evidenced by the increased shedding of *Salmonella* spp. during transit.

Bovine Respiratory Disease

Very few animals are exposed to the high stress environment that sometimes occurs in our cattle marketing system. Weaning and arrival at a feed lot can be one of the most stressful periods in the life of a calf (Boyles et al., 2007). These cattle are subjected to multiple stressors as they travel through our traditional market system. Calves are often weaned, hauled to auction where they are sold, and penned with animals with

which they may not be familiar. They may be reloaded when an order buyer's transport vehicle arrives at the auction, shipped to a sorting facility where the animals will be once again unloaded, sorted and re-penned with animals of similar size and breed to fill an order for a feed lot. Generally, they are sold within a 24 hour period after arriving at the order buyer and then loaded onto a truck and transported to feedlots in the panhandle of Texas, Oklahoma, or the Midwestern United States. These animals arrive at the feedlots displaying symptoms of stress, including weight loss and dehydration. They will also be penned one more time before processing at the feedlot. Calves that are mixed and confined with other calves before final arrival at the feedlot have a high probability of prolonged exposure to stressed cattle that may be shedding pathogens. This exposure, coupled with immune suppression from prolonged stress, may increase the morbidity in these feedlot cattle to as high as 30%, and the industry uses the term "at risk" to describe such calves. According to Buhman et al. (2000) 91 percent of calves diagnosed with respiratory disease are diagnosed within 27 days after arrival at the destination feed lot.

An increasing economic and health problem at feedlots is the incidence of BRD. Even so, an estimated 60 percent of calves in cow-calf operations are not vaccinated against such BRD pathogens as *Pasteurella haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus* before weaning (Step et al., 2007). A high percentage of these calves are not retained for back-grounding, a practice that will aid in stress reduction and adaptation as they are moved from a controlled environment to unfamiliar stocker operations or feedlots (Step et al., 2007). BRD is reported to be the most prevalent disease responsible for morbidity and mortality in feedlots (Kilgore et al., 2005) with the

majority of deaths occurring within 45 days of arrival at their destination feed lot (Edwards, 1996; Loneragan et al., 2001). Studies have shown that BRD causes substantial economic losses resulting from decreased feed efficiency, increased veterinary costs, lower final body weights, lower average daily gain, and lower final carcass weights (Edwards, 1996, Gardner et al., 1999).

Stress Indicators

Stress can be defined as the body's way of responding to any kind of demand, whether physical or psychological. When the brain interprets the presence of a stressor it acts on the hypothalamus causing the secretion of corticotrophin releasing hormone or CRH. CRH acts on the anterior pituitary stimulating the release of adrenocorticotrophic hormone (ACTH). ACTH is carried by the blood to the adrenal gland where it acts on the cortex to trigger the release of glucocorticoids including cortisol. The medulla of the adrenal gland responds to ACTH by secreting neurotransmitters that are important in the fight or flight response. The body's response to an initial stressor includes increased heart rate, blood pressure and respiratory rate, supplying the body with increased blood flow and oxygen.

Hans Selye defined stress as "mutual actions of forces that take place across any section of the body, physical or psychological, which threaten homeostasis (Chrousos, 1998)." He subjected rats to stressors for long periods of time observing enlarged adrenal glands, ulcer development, and the atrophy of the rat's immune system. He coined the term General Adaptation Syndrome to describe these responses to chronic

stressors. He saw that the natural processes used to defend the body could actually be harmful if chronic stress was present. He described three stages of stress. The first, alarm stage, was initiated when a stressor was first encountered. Following stimulation from CRH the pituitary gland began producing ACTH which in turn caused the adrenal cortex to release mineralocorticoids and glucocorticoids, one of which is cortisol, which target organs throughout the body (Selye, 1951). During this stage the sympathetic nervous system is engaged supplying the body with increased blood flow and oxygen. The second stage, adaptation, includes a reduction in the initial hormone response while the body continued to fight the stressor and the immune system became more suppressed. In the final stage, exhaustion, the body has depleted all resources and fails to resist the stressor. In the most extreme cases of exhaustion, death can result.

Several physiological indicators of stress have been used to identify and study transport stress in horses, with cortisol concentrations having been most commonly used in recent studies (Stull 1999, Stull & Riedick 2002). Garey et al. (2010) showed that horses subjected to short transport times of approximately 6 hours had increased cortisol concentrations compared to those that were not transported. Cortisol is widely used as an indicator for stress in livestock as well. For example several prior studies have determined that circulating cortisol concentrations are a useful indicator of stress in animals such as goats (Kannan et al., 2000). Transport and handling of cattle, based on a comparative response in circulating corticoid levels, is considered to be one of the most potent stressors for cattle (Johnson and Buchland, 1976).

Good versus Bad Stress

The immune system's response to stressors has been studied for years, due to its sensitivity to stressful situations. The general concern with stress is that it can be destructive and can lead to immune suppression. However, in recent years it has been demonstrated that stress may be more than just a detriment to the immune system. Milla´n et al. (1996) showed that serum antibody response to sheep red blood cells in rats exposed to short term restraint stress was enhanced. Rhesus monkeys subjected to social reorganization stress resulted in significantly higher lymphocyte proliferation of higher ranking animals (Clark et. al 1996). High ranking animals within small stable groups had greater lymphocyte proliferation in response to mitogens than those animals that were subordinate. However, these results disappeared when the animals were placed in larger groups. These data support the idea of short term stress enhancing the immune system. While research shows that immune suppressive effects of acute stress may be doubtful, there is evidence that chronic stress is immune suppressive (Wrona et al., 2001; Dhabhar and McEwen 1996; Dhabhar and McEwen 1997).

Bovine Genome and Gene Expression

The recent mapping of the bovine genome has made it possible to analyze and study gene expression that is critical in the immune defense response in cattle. This may soon enable genome-assisted selective breeding, as well as marker assisted vaccinations and the development of innate immunologicals used as anti-infective agents (Seabury et al. 2010).

The ability to map genetic pathways and identify genetic expression should allow for the differentiation between acutely and chronically stressed animals. Gene expression data also offer the ability to identify genes that are critical in immune function pathways and pathogen recognition. Multiple genetic pathways are of great interest, including the chemokine and cytokine receptor pathway, and toll-like receptor signaling pathway or TLRP.

The cytokine – cytokine receptor interactions are involved with innate and adaptive inflammatory responses, as well as cell growth, differentiation, apoptosis, and repair processes to restore homeostasis. Cytokines are extracellular proteins which are important regulators and mobilizers of cells in the immune response. Various cells throughout the body release cytokines in response to an invasive stimulus (Kanehisa et al., 2000, 2010). Cytokines induce responses by binding to receptors on the cell wall of target cells.

The chemokine signaling pathway is a diverse pathway activated by chemokine receptors on immune cells when a pathogen is detected. Some chemokines are involved with the inflammatory immune response function as chemo attractants for leukocytes, recruiting monocytes, and neutrophils from the blood at the point of inflammation from a foreign body. They are released mainly in response to pro-inflammatory cytokines and aid in guiding cells of both the innate and adaptive immune system. Chemokines are small peptides that enable cell trafficking through directional cues making them vital to the immune response (Kanehisa et al., 2000, 2010). Chemokines also regulate certain

biological processes of hematopoietic cells leading to cellular activation, differentiation and survival.

One pathway of great interest is the TLRP, which contains toll like receptors that are localized on the cell surface and detect bacterial and viral infections. Toll-like receptor activation is instrumental in guiding activation of immune responses through macrophage and dendritic cell activation (Iwasaki and Medzhitov 2004, Seabury et al., 2010). The ability to identify at what point TLR receptors such as TLR-2, TLR-4, and TLR-9 are activated would allow for a much better understanding of the immune response recognition system. Better understanding of how the immune system responds to threats will allow for an improved ability in the production of prophylactic medicines to treat and protect our animals and livelihood.

Knowledge of these genes, coupled with the recent understanding of innate immunity and its contribution to initiating the adaptive immune response, should give a greater insight into the effectiveness of our current vaccines and will aid in improving our next generation vaccines by allowing a more direct application to disease resistance. This is important as the estimated economic impact of diseases such as shipping fever is 800 to 900 million dollars in losses each year (Chirase and Greene, 2001).

Objectives and Hypothesis

The objective of this study was to determine the response of immune related inflammatory genes in cattle when the cattle were subjected to a period of transport and arousal, approximately 4 d, when compared to cattle exposed to a stressor lasting 1.5 h.

Gene expression data from pro-inflammatory genes such as chemokines, cytokines, and interleukins in cattle may be useful in determining the underlying mechanisms involved in the impaired immune function and susceptibility to respiratory disease observed in cattle after the transportation and handling process. If we can better understand the inflammatory response in cattle and how it develops over time, we can better understand the underlying causes of diseases like BRD and BVD, and potentially reduce morbidity and mortality while increasing profitability. A second objective was to determine the response of cortisol concentrations in these same calves.

It may be hypothesized that, cattle subjected to longer periods of stress in the form of the traditional market system, may display differing gene expression of certain pro-inflammatory genes, as well as differing cortisol concentrations than cattle exposed to an acutely stressful event such as handling and processing.

MATERIALS AND METHODS

Animals and Treatments

Two trials were conducted one year apart, in which similar groups of acute stressed (AS) and “at risk” or chronic stressed (CS) calves were observed. The calves were selected based on weight, 204 to 250 kg, and similar breeding to typical Texas market calves. Therefore, Angus calves (*Bos Taurus)* and calves with Brahman or Nellore influence (crossbred) were used. The two treatments in each trial were conducted within a 14 day period of each other. The treatments could not be conducted simultaneously because of the availability of herds and variation in the actual shipments of the cattle to the feed lot.

The AS calves consisted of 20 *Bos taurus* and 20 crossbred calves that were randomly selected based on weight and breed from separate herds owned and maintained by the Texas Agrilife Research Station at McGregor, Texas. These calves were maintained with their dams on warm season perennial forages, predominantly bermuda and kleingrass, until the morning of sample collection when they were rounded up and brought into onsite holding pens for routine health processing. Creep feed was not provided. The AS calves were rounded up in their pasture the morning of collection and driven to onsite holding pens at the research station prior to blood sampling. These calves were then separated from their dams and moved through the working chute system where they were weighed, vaccinated, ear tagged, bled for sample collection, and castrated if bull calves. While they were separated physically from their dams, it remained possible for them to maintain partial visual and vocal contact.

The “at risk” or CS calves consisted of 20 *Bos taurus* and 20 crossbred calves were selected from multiple herds across Texas and Louisiana and purchased for a north Texas Feedlot near Dalhart, Texas by a central Texas cattle buyer. This cattle buyer gathers weaned calves from livestock auctions in Texas, Oklahoma, Arkansas, and Louisiana and then resells and transports the calves to feed lots in Texas, New Mexico and the midwestern United States. The cattle buyer was instrumental in helping us identify suitable groups of cattle in advance of the calves arriving at the north Texas feed lot. The history of the calves prior to being purchased by the central Texas cattle buyer was not known, but they were likely typical of other cattle that are weaned immediately prior to shipping. The CS calves were weaned from their dams and hauled to an auction where they were purchased by our cattle buyer. The calves were then loaded onto a tractor trailer and hauled to the central Texas cattle buyer’s livestock yard, where they were unloaded, re-penned, and matched with cattle of like weight and breed to fill an order for our cooperating feed lot. After completing the order, the cattle were loaded onto a tractor trailer for transport to the destination feed lot where they were unloaded and held in pens for approximately 12h prior to processing, sample collection, and placement in the lot. The calves were located throughout the trailer with no control over the compartment in which they were transported. The calves had access to water while in the holding pens at the livestock buyer’s yard and the feed lot. The cattle were transported in a 15.24m long x 2.6m wide x 3.71m high, fixed axle, cattle pot tractor trailer (Wilson Trailers, Sioux City, Iowa) pulled by a tractor (Figure 1).

Figure 1. Example of the type of **t**ractor and trailer used by a central Texas cattle buyer for transport of calves to a north Texas feed lot.

Sample Collection

AS calves were held in pens after being separated from their dams until processing began that morning. All CS calves were held in pens at the cooperating feed lot until processing began the morning after arrival. The calves were then moved through the chute system and weighed before being caught in the hydraulic squeeze chute. The head was mechanically restrained for the period of sample collection, approximately 1 min (Figure 2). Blood samples (9mL) were collected via jugular veni-puncture using a 20 gauge x 1 inch needle with holder (Vacutainer® Becton, Dickinson and Company, Franklin Lakes, NJ) and 12ml plastic evacuated collection tubes containing sodium heparin (Vacuette, Greiner Bio-One, New York, NY). After each tube was full, the tube was immediately inverted several times over approximately thirty seconds to allow uniform mixing of blood with the anticoagulant. The tube was then placed in ice until

sample collection was complete. Once a sample was collected the calf was released into a holding pen.

The AS calves were sampled on two separate days because the *Bos taurus* and crossbred herds were processed at separate times. Processing for AS calves began at approximately 8:30am on collection day. The first twenty calves matching the weight range (204 to 250kg) for this study were sampled within 1.5 h of the first disruption of the calves. The CS calves were sampled approximately 12 h after unloading at a north Texas feed lot near Dalhart, TX. Processing began at approximately 6:30am at the feed lot's facilities. The first twenty calves matching the weight range and breed for this study were sampled after spending 3 to 4 d being transported.

Figure 2. Photograph of sample collection via jugular veni-puncture while calf was restrained in squeeze chute for routine health processing.

The blood in each tube in Trial 1 and 2 was processed through a leukocyte capture and preservation filter (LeukoLOCK™ Total RNA Isolation System, Applied Biosystems, Carlsbad, CA). The filters were processed further by adding a chemical preservative

(RNAlater, Applied Biosystems, Carlsbad, CA) and stored at -20ºC for future RNA extraction.

In only Trial 2 the filtered blood was captured in a glass evacuated collection tube with no chemical additives (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). These tubes were then centrifuged at 3300 rpm for 10 min and the plasma was collected and placed in 5mL Falcon tubes (BD, Bedford, Massachusets) and stored at -20ºC for hormone analysis.

Cortisol Assays

Ambion, the manufacturer of the leukocyte filtration system, instructed us that there should be no effect from filtering out leukocytes on hormone concentrations due to the large size of pores in the filter. The filter is designed to capture the larger leukocytes while letting other material through.

Cortisol concentrations were analyzed by colorimetric ELISA from the plasma samples taken in Trial 2. No plasma samples were collected during Trial 1. A competitive ELISA protocol was developed using a goat anti-mouse IgG (Sigma Aldrich, St. Louis, MO) coating buffer at 5 µg/mL. Collected samples were added in duplicate wells, allowing for binding of the primary antibody. A commercially produced conjugated cortisol and an alkaline-phosphatase conjugated secondary antibody (Assay Designs, Ann Arbor, MI) were then added to each well of the plate to form an immune complex. A para-Nitrophenyl phosphate (pNpp) substrate solution was added to all of the wells, binding the enzyme attached to the secondary antibody (Figure 3). This

allowed for colorimetric detection on a photometric multi-label plate reader at an optical density of 405 nm (Wallac Victor II 1420, Perkin Elmer, Waltham, MA). Known concentration standards were used on every plate which allowed comparisons between plates to be optimized. Data obtained from the plate reader were then inputted into a curve-fitting software program (StatLIA®, Brendan Technologies, Inc., Carlsbad, CA) that calculated total concentrations of each hormone assay based on averages of the duplicate samples in comparison to the generated logarithmic curve of the known standards.

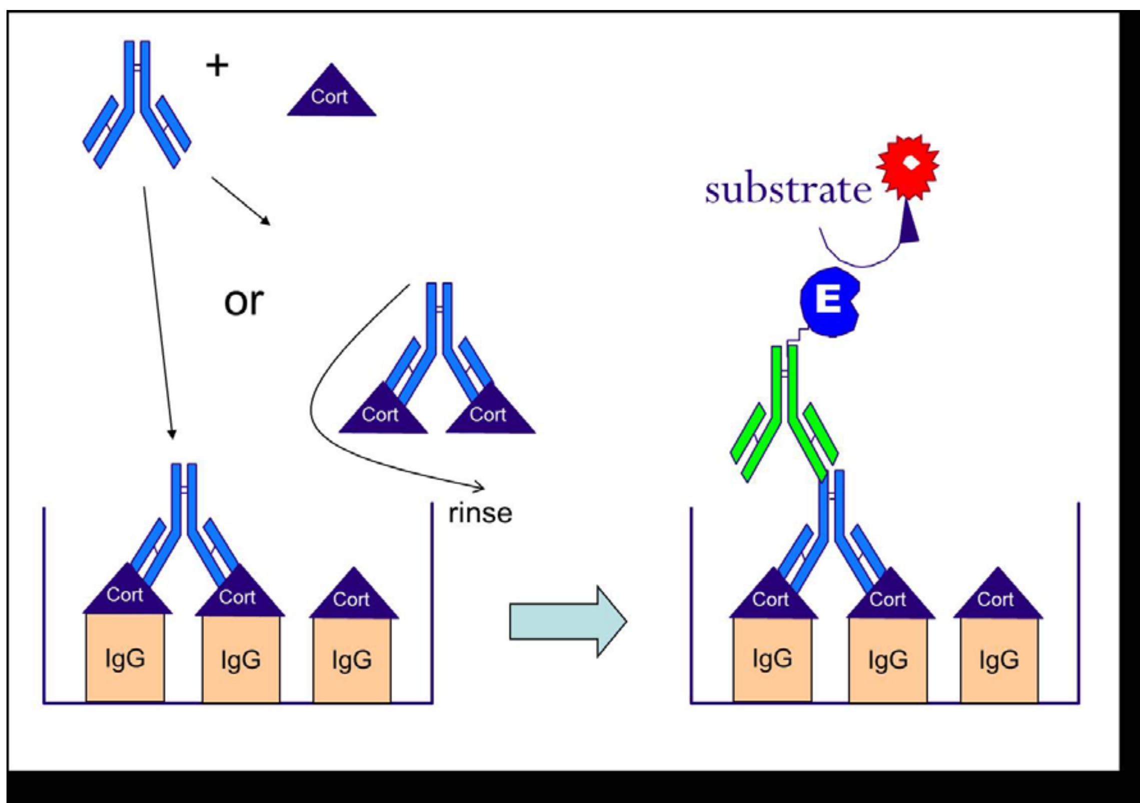

Figure 3. Diagram of competitive cortisol ELISA performed to detect plasma cortisol concentrations in AS and CS beef calves.

RNA Extraction

The protocol developed by the manufacturer of the leukocyte capture filtration kit (LeukoLOCK^{TM} Total RNA Isolation System, Applied Biosystems, Foster City, CA) was followed, except for three amended steps. These were amended to increase yield while decreasing degradation of RNA during the extraction process. The step to remove the supernatant from the 15mL tube after centrifuging was changed. In this step the protocol calls for pipetting out the waste. At this point in the extraction process the beads have been firmly pelleted in the bottom of the 15mL tube and the liquid can simply be poured off in order to save time. This step is very time consuming if pipettes are used. Also, two, double wash steps were modified, as the steps call for the dispersion of binding beads. This step requires two washes to attempt to collect all RNA bound binding beads that may remain in the tube. It was modified to transfer the beads while still pelleted, allowing for transfer of all beads at once to help prevent the possibility of lost sample. All edits to the protocol resulted in higher quality and yield than in previous extractions.

The cells were first rinsed with a phosphate buffered solution to remove any remaining preservative from the filters. Each filter was then flushed with a cell lysing solution and the cell lysate was transferred to a corresponding 15 mL centrifuge tube. The RNA was then isolated using RNA binding beads and repeatedly washed using isopropanol. After further washing, a DNase treatment was added to degrade any contaminating DNA that remained in the product. The final RNA product was eluted from the RNA binding beads back into solution using 50 µl of an elution solution. To

reduce possible degradation of RNA from multiple freeze/thaw events during testing and analysis the RNA-containing supernatant from each sample was then transferred to two new processing tubes for analysis. A 5 µl aliquot was used for quality and quantity testing while the remaining supernatant was used for PCR analysis. The aliquots were stored at -80ºC for future analysis.

RNA Analysis

Gene expression was analyzed from samples with acceptable quantity and quality as determined from the 5 µl aliquot. Samples were considered to have sufficient quantity of RNA when containing 80 ng/ µl or more. Quantity of RNA was measured using a NanoDrop 2100 (Thermo Scientific, Wilmington, DE). The remaining portion from this aliquot was used to determine quality of RNA verified by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples determined to contain 350 ng/µl RNA or more were diluted with DNase free water to prevent clogging of the pins in the Bioanalyzer. Samples were individually evaluated for degradation and were selected based on results evaluating the RNA ratio between the 18s and 28s peaks produced on an electropherogram (Figure 4). The area between the peaks was observed for lack of "noise" which could represent degradation or excess RNA. Due to the dilution of samples with greater than 350ng/µL of RNA, samples with noise in this area were considered to have significant degradation as opposed to excessive RNA, and were not used. A RNA Integrity Number (RIN) from 1 to 10, with 10 being the highest quality was produced by the software representing the quality of

RNA present in the sample. Samples were acceptable for quality when they had a RIN above 6.9 with minimal degradation observed. However, in cases where a RIN was not provided by the system due to system error, quality was based on the peak ratio evaluation. Applying the RIN while also examining the electropherogram output between the 18s and 28s peak allowed us to select the highest quality samples with the lowest degradation.

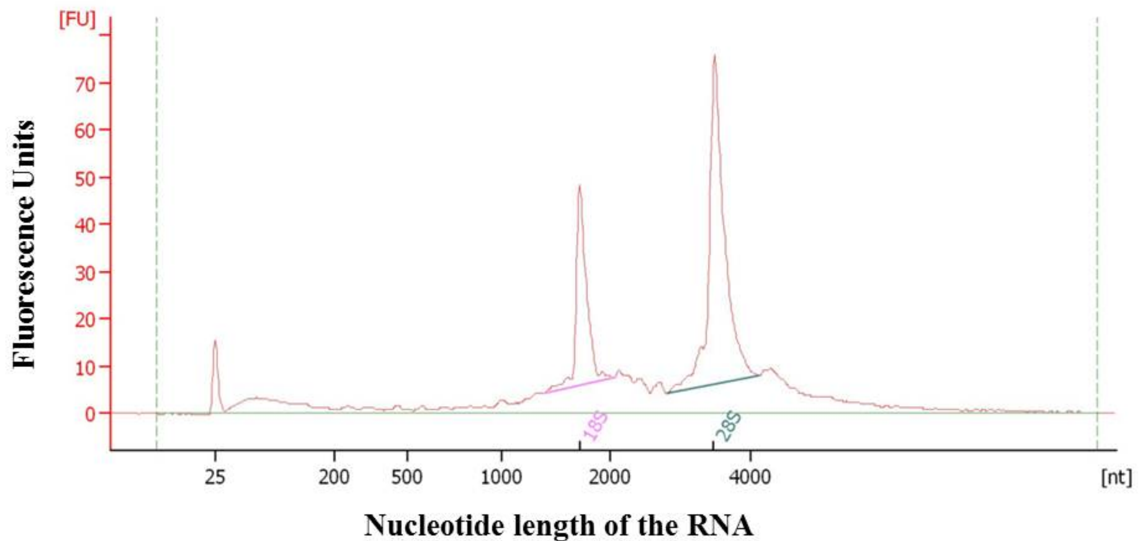

Figure 4. Electropherogram of RNA sample from Bioanalyzer. Quality of RNA was determined by examining area between 18s and 28s peak with the goal of selecting samples with the least disturbance.

The quantity of mRNA for genes of interest was measured in all samples from Trial 1 and 2 by quantitative reverse transcriptase PCR (qRT-PCR). Low density array cards (LDA) containing 96 bovine genes that are involved in the inflammatory response were purchased (Applied Biosystems, Foster City, CA). Primer/probe assays in the card

included chemokines, cytokines, interleukins and members of the tumor necrosis factor family (Appendix Table 1). For qRT-PCR, 750 ng of RNA was reverse transcribed into cDNA according to the manufacturer's instructions in the included protocol (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Samples from Trial 1 (26) and Trial 2 (37) were then applied, four samples per LDA card and the cards were centrifuged two consecutive times for 1 min at 12000 rpm using a Sorvall Legend™ centrifuge (Kendro Scientific, Asheville, USA). The LDA were then sealed using TaqMan low density array sealer (Applied Biosystems, Foster City, CA) and the cDNA amplified in a fast real-time PCR system (ABI 7900HT, Applied Biosystems Inc., Foster City, CA).

Data was normalized for the amount of RNA in the reaction, to an endogenous control included on the card. Eighteen S was used as our control gene for these assays. The selected calibrator was an untreated control. Raw C_t values may falsely represent variation between samples and should not be used. Data were analyzed to arrive at Relative Quantification values (RQ) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Relative quantification describes the changes in expression of the target gene relative to a reference group.

Statistical Analysis

To determine plasma cortisol concentration effects on breed, treatment, and breed by treatment during Trial 2, data were analyzed using a diagonal covariance mixed model ANOVA (SAS 9.1, SAS Institute, Inc., Cary, NC).

Gene expression data were in terms of relative quantification (RQ values) for significant differences in amplified genes in each of the two trials with mean treatment differences of RQ values from the amplified genes recorded. Year one included 86 genes in 26 animals while year two included the same 86 genes in 37 animals. RQ expression values for each gene were analyzed using general linear models for each of the two trials separately (SAS 9.1, SAS Institute, Inc., Cary, NC) and then compared. All RQ expression values equaling zero were removed from the data to prevent errors in SAS during analysis. Fixed effects were treatment (AS and CS), breed (*Bos taurus* calves and crossbred calves), and the interaction of breed by treatment. Least squares means were presented only from models with significant effects ($P < .05$).

RESULTS

Cortisol

Weather conditions were similar each day of collection. Samples were collected in early morning to avoid diurnal effects on cortisol concentrations for both AS and CS calves. Forty (20 B*os taurus;* 20 crossbred calves) samples were collected in each treatment, providing a total of eighty samples.

Mean plasma cortisol concentrations (Table 1) did not differ significantly (*P* > 0.296) between AS (16.40 ± 1.08ng/ml) and CS calves (18.06 ± 1.14ng/ml). Plasma concentrations were not significantly influenced by breed (*P* > 0.529, Table 1) or breed by treatment (*P* > 0.909, Table 2).

Table 1. Mean concentrations of plasma cortisol (ng/ml) in acutely and chronically stressed calves.

Variable	Treatment		P value
	Acute	Chronic	
Treatment	16.40 ± 1.08	18.05 ± 1.13	*P* > 0.296
Breed	16.59 ± 1.21	18.64 ± 1.13	*P* > 0.529

Table 2. Mean concentrations of plasma cortisol (ng/ml) in acutely and chronically stressed calves for the interaction between breed and treatment (*P* > 0.9097)

Breed	Treatment	
	Acute	Chronic
Bos taurus (Brd 1)	16.81± 1.52	18.64± 1.61
Crossbred calves (Brd 2)	15.99± 1.52	17.47± 1.61

RNA Analysis

Three Taqman expression assays, in addition to the 93 bovine assays, were included on the LDA array as housekeeping genes. These primers were selected for their minimal differences in RQ values for all samples indicating minimal variation in inflammatory response for the target species. The lack of variation makes these primers useful for correction of data. However, it should be noted that the primer for LSP-1 (Figure 21) would make a great reference gene in future studies.

Viable RNA for genetic expression evaluation through PCR was produced from only 26 animals from Trial 1. Quantity and quality problems were discovered for the other calves during testing on the Nanodrop and Bioanalyzer. A problem was discovered in a certain lot of binding beads used to bind the RNA during the extraction process. The beads continually dispersed instead of remaining as a pellet that was necessary for capture and binding of RNA. This caused a poor yield of RNA in the final supernatant. Two animals were very high responders displaying RQ values in some cases as high as 10 standard deviations from the mean. These animals were considered outliers and removed from the statistical model as they had an effect on the significance of gene expression values across most samples. While these two animals were removed for statistical reasons, their biological importance should be noted. Both of these animals were retreated for respiratory illness while one of the high responders ultimately died in the feed lot. A total of five out of the 26 animals that were used for RNA evaluation in Trial 1 were retreated by the feed lot for respiratory disease after initial processing and placement in the lot, and two of those ultimately died.

Trial 2 samples yielded a higher number of quality RNA samples allowing us to run 37 samples using qRT-PCR. These samples displayed more uniform gene expression values, while yielding three high responders that were removed from the statistical analysis. A total of 4 of the 37 animals with useable RNA in Trial 2 required further medical treatment for respiratory sickness after processing and placement in the feed lot. Of the animals removed as statistical outliers, two of the three were animals that were retreated for illness in the yard. No animals in Trial 2 died after processing and placement in the feedlot. The two animals which were ill were only retreated once according to medical records.

RNA quality between the two Trials remained consistent. Samples were selected using the same criteria for each year providing similar RNA quality over the whole study.

Gene expression analysis in Trial 1 and 2 produced quality amplifications of 30 and 36, respectively, out of 93 target genes (Appendix Table 3). There were 15 common genes between the two trials that displayed significant expression (Appendix Table 4) as measured by the $2^{-\Delta\Delta Ct}$ method after PCR produced C_t values (cycle at which the amplification curve crossed the threshold) (Appendix Figures 9 - 23). These genes displayed significantly different amplification ($P < .047$) in calves subjected to CS than those calves subjected to AS, indicating that chronic stress exposure alters amplification of these genes in peripheral blood leukocytes. The genes that had significant treatment effects in both trials are shown in Figures 5 and 6. While differences in gene expression of the crossbred calves appeared more exaggerated than *Bos taurus* calves, no breed

differences (P > .05) nor interaction of effects (P >.05) were seen in these genes. Additionally, three genes in Trial 2 displayed highly significant different amplification (P < .0001, Figure 7) in calves subjected to AS than those subjected to CS, indicating that acute stress exposure alters amplification of these genes in peripheral blood leukocytes.

The genes were mapped revealing significant gene overlap (Huang et al., 2009a; Huang et al., 2009b, Appendix Figures 26 - 27) through biological pathways which affect immunity (Schnare et al., 2001; Rot & Andrian, 2004; Aderem & Ulevitch, 2004).

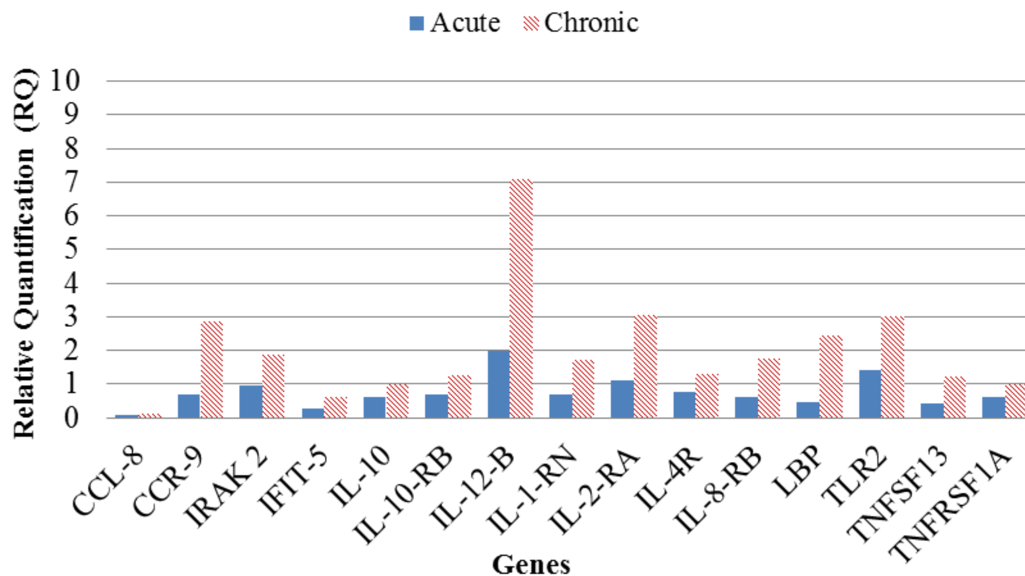

Figure 5. Mean *Bos taurus* gene expression (RQ) values in which CS calves had a greater response than AS calves (P < .047) in Trial 1 and 2, excluding outliers.

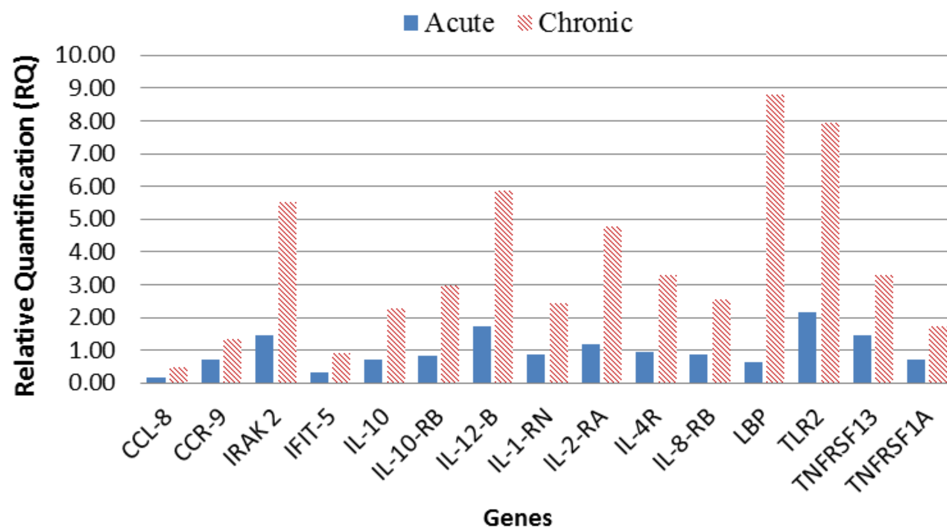

Figure 6. Mean crossbred calves gene expression (RQ) values in which CS calves had a greater response than AS calves (*P* < .043) in Trial 1 and 2, excluding outliers.

Two out of the five animals sampled in Trial 1 that were retreated for illness were considered extreme outliers. Both of these animals were retreated for illness by the feed lot with one animal ultimately dying. In Trial 2, four animals were retreated for illness. Two of the four animals that were sampled were considered extreme outliers showing extreme expression of most genes tested in the study (Figure 7). These extreme animals were biologically significant due to their poor health, infection and death. The RQ values in these animals were well out of range with the other animals recorded. While they were not the only animals treated for illness it is interesting to note that 45% of the 9 animals which were retreated or died were statistical outliers due to extremely high RQ values.

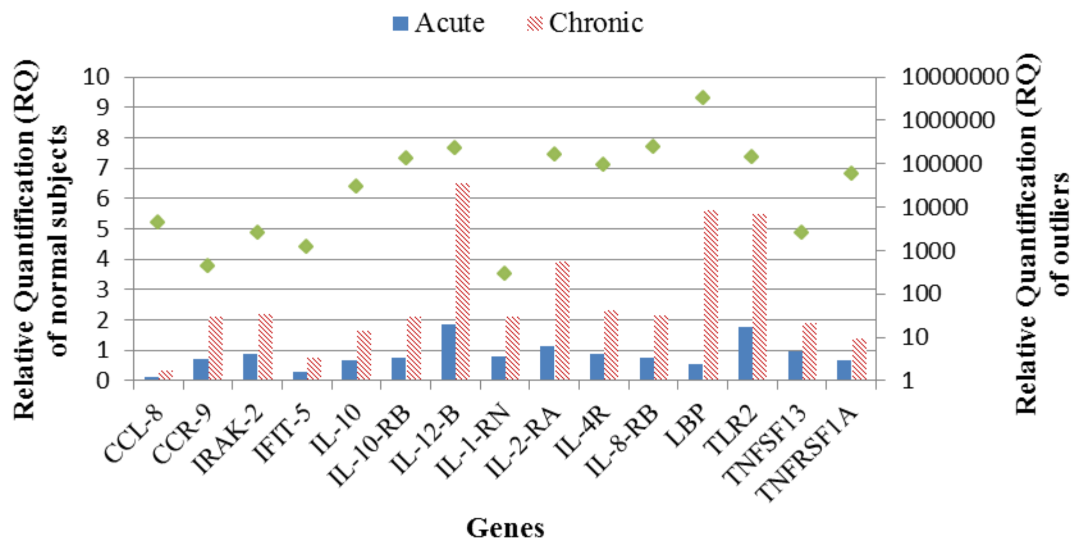

Figure 7. Mean *Bos taurus* and crossbred calves gene expression (RQ) values in which CS calves had a greater response than AS calves ($P < .047$) in Trial 1 and 2, including outliers. Normal subjects are presented on the left axis and are represented by the bar graph. The mean gene expression values of all statistical outliers is represented using a log base 10 axis on the right, and are represented by the separate data points.

In contrast to the above data, three genes in Trial 2 showed differences ($P < .0001$, Figure 8) in up regulation of gene expression in AS when compared to CS animals. These genes however only approached significance in Trial 1 ($P < .054$). In both Trials the AS had more expression than the CS.

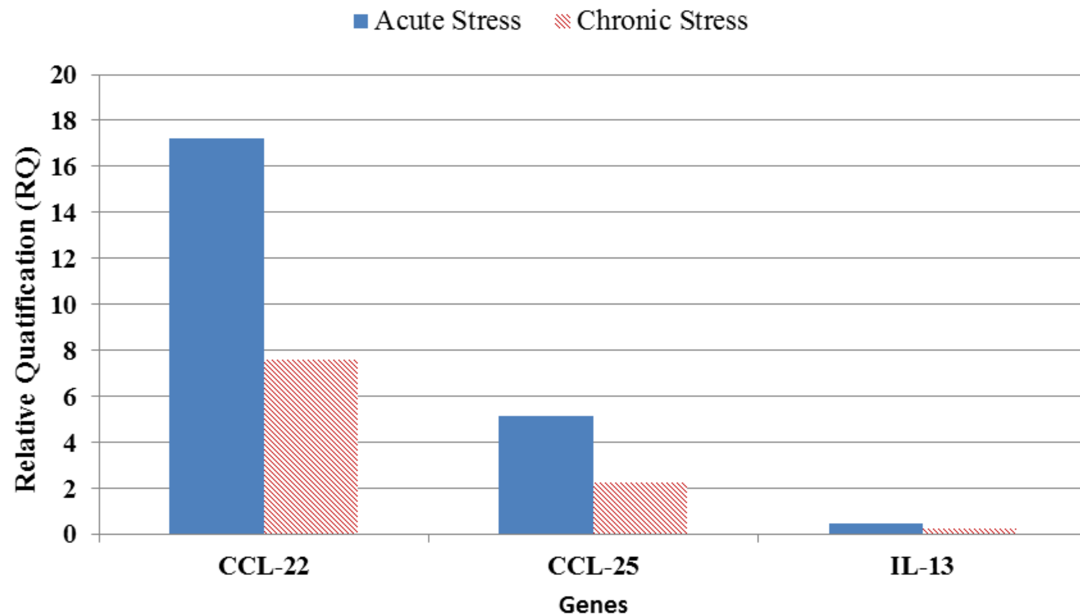

Figure 8. Mean *Bos taurus/indicus* cross gene expression (RQ) values in which AS calves had a greater response than CS calves ($P < .0001$) in Trial 2 excluding outliers. Gene expression data presents up regulation of these genes during acute stress when compared to chronic stress. These same genes approached significance in Trial 1 ($P <$ .054).

DISCUSSION

Results from this study indicate that gene expression data may be a more useful tool in determining chronic stress than cortisol hormone assays. When a stressor is applied to the body, the hypothalamus produces CRH which acts on the anterior pituitary. ACTH is released stimulating cortisol production by the adrenal cortex. Immediate effects of cortisol release include increased blood sugar through gluconeogenesis, among other things. When released in response to stress, cortisol can have positive effects such as a quick burst of energy, lower pain sensitivity, heightened cognitive abilities and an enhanced immune system. However, when exposed to stress over a long period of time, prolonged increases in cortisol concentrations can begin to produce negative effects. These negative effects such as impaired cognition, high blood pressure, and suppressed immune and inflammatory responses are detrimental to proper homeostasis in the body and can lead to disease and ultimately death. Cortisol is rapidly released with the initial spike beginning within three to five minutes of the stressor being applied and peak within 10 to 20 min (Grandin 1997). This rapid response prevented obtaining resting cortisol concentrations on the calves in this study as calves were either brought up from holding pens at the feed lot or rounded up and separated from their dams prior to processing and blood collection.

Blood samples were taken in the morning from both AS and CS calves in order to avoid diurnal effects. Cortisol concentrations showed no significant difference between the cattle exposed to an acute stressor and those exposed to chronic stressors in

this study. However, in both treatments plasma cortisol concentrations were elevated when compared to basal concentrations in a study conducted in our lab by Adams et. al. (2011). The mean initial plasma cortisol concentrations in that study were in the range of 4 to 6 ng/mL. In that study, 36 Holstein calves were used to study acclimation of cattle to transport. Eighteen calves were designated as controls with the remaining 18 being subjected to periods of transport lasting 6 h at weekly intervals for 5 wk. Blood samples were collected from the calves in their home pens within a 2 min window per calf, providing accurate baseline cortisol concentrations on these calves. Samples were also collected at hours 2, 4, and 6 during transport showing an increase in cortisol concentrations from the baseline. It is possible that Holstein calves have lower baseline concentrations do to a calmer demeanor. However a study by Henricks et. al (1984) studied six bull and six heifer angus cross beef calves that were halter broke in order to easily gain access to blood samples. In that study the calves were fitted with a catheter from which blood was collected every 15 min for 6 h in order to determine sex differences in cortisol concentrations. Results from that study supported the baseline concentrations found by Adams et. al. (2011) with bull calves baseline cortisol at 3 ng/mL while heifers were at 6 ng/mL.

The unavailability of multiple sampling points in this study made it difficult to determine the actual dynamics of the cortisol concentration. Without a sample taken immediately prior to gathering the cattle we were not able to establish a baseline of plasma cortisol concentrations. What we can see from the collected data is that there is no significant difference in concentrations of plasma cortisol in calves that are exposed

to either an acute stressor or as those exposed to long term chronic stress. These data contradict the Warriss et. al, (1995) study, which showed cortisol concentrations were increased during loading and unloading, but decreased during long periods of travel. In that study 24 market aged steers were transported for three different periods of time (5, 10, and 15 h). They found little evidence of the 15 h transport being more stressful than the 10 h transport, instead seeing the highest response in the 5 hour transport. Cortisol increases were due to the stress of loading and the initial transport but decreased as the transport continued. When compared to our study, it suggests that data collected at the feed lot showing similar concentrations as those observed from the AS calves, may be influenced more by the unloading and handling of the cattle before sampling, than by transport. In our study calves exposed to chronic stress were subjected to at least three loading and unloading bouts between ranch, auction, buyer yard, and feed lot, which may have caused repeated spikes of cortisol as the animal endured these stressors.

The management of cattle prior to shipping to market can have an effect on the health and growth of calves. Animals which have been preconditioned including castration and or vaccination prior to being transported to feed lots have shown to be highly effective in the ability to fight off infection (Duff & Gaylean 2006). We had full control over the handling of the AS calves and know their history. Both the *Bos taurus* and crossbred herds of AS cattle were maintained on essentially the same forage and pasture and were not weaned or handled prior to the commencement of this study. The "at risk" CS calves were purchased at several auctions across Texas and Louisiana. While these calves were selected by our buyer as traditional market calves, it is unclear

what type of health management each calf had been exposed to. We can assume that some calves were weaned as they were being shipped to auction. However, others may have been weaned for a few days before being sold. Also, some calves may have been castrated and vaccinated prior to being sold and shipped to the feedlot. Calves that were picked up first at auctions were exposed to more trailer travel time than those picked up last on the way back to the cattle buyer's livestock yard. No health records prior to purchase by our cattle buyer are available on these calves. Other stressors these cattle may have been subjected to could be crowding on trucks and the possibility of falling while in transport. With these calves coming from multiple locations, and our not having access to them until arrival at the destination feed lot, our data collection pertaining to potential stressors other than transport was quite limited.

To determine effects on the immune system we analyzed gene expression of 93 bovine genes important in the inflammatory response. The results of the two trials presented 30 and 36 genes respectively that displayed significantly increased gene expression in the CS over the AS calves when amplified on LDA plates. There were 15 significant genes that were common between both Trials 1 and 2. These 15 genes are of particular interest because of their individual involvement in three immune pathways. The genetic pathways include the chemokine and cytokine receptor pathway, and Toll-like receptor signaling pathway or TLRP. The pathway of greatest interest, TLRP, contains toll-like receptors which are localized on the cell surface as well as intracellular and detect extracellular or endosomal pathogen-associated molecular patterns. Toll-like receptor activation is instrumental in guiding activation of immune responses through

dendritic cell and macrophage activation. The ability to identify at what point TLR receptors are activated would allow for a much better understanding of the immune system recognition system. Unfortunately, it is not apparent at what point during the 3 to 4 d of transport and handling these genes begin to be up-regulated. However, throughout the period of chronic stress the "at risk" calves were subjected to, these genes were up regulated affecting immune function in the process.

Trial 2 identified three genes with significantly increased gene expression in AS calves when compared to CS calves. These genes can be found in some of the same pathways as the 15 genes of interest in CS. CCL-25 is believed to play a role in developed in T-cell development. It is chemotactic for macrophages, and dendritic cells an CCL-25 stimulates its effects by binding chemokine receptors such as CCR9. IL-13is a cytokine secreted by T helper cells and is a mediator of allergic inflammation and disease in many tissues. More study is needed to determine if these genes could be useful in determining acute stress.

The gene of most interest from this study is the Toll-like receptor 2 or TLR-2, which is expressed on the surface of innate immune cells such as macrophages and dendritic cells and expressed most abundantly in peripheral blood leukocytes. TLR-2 can detect bacterial and viral infections by recognizing peptidoglycans, in addition to the lipoproteins and lipopeptides on membranes of Gram-positive or negative bacteria and mycoplasma (Takeuchi et al., 1999). TLR-2 encoded proteins are members of TLR family which are fundamental in the activation of the innate immune system as well as pathogen recognition. They are essential in recognizing pathogen-associated molecular

patterns or PAMPs which are expressed on bacterial or viral agents, and provoke the production of cytokines which are essential in an effective immune response. Some of these cytokines are Tumor necrosis factor, Interleukin 8 and Interleukin 12. When TLR-2 is activated by bacterial lipoproteins such as those on the cell wall of BRD pathogens; the production of pro-inflammatory cytokines is up-regulated leading to a host of responses including innate and adaptive inflammatory defense, cell growth and differentiation, and repair processes aimed at re-attaining homeostasis. It should be noted that while there are data available on what each gene does, we are only looking at up-regulated genes in blood leukocytes. Each tissue containing these same genes can react in a positive or negative way. Without further study of these genes, and developing a time line for their up-regulation, as well as determining concentrations of their corresponding proteins in the blood, it is difficult to accurately identify their true effect on the immune system.

Biological relevance of these data is clearly indicated from the health data received from the cooperating feed-yard in which Trial 1 had 19.2 % morbidity with a 7.6 % death loss among animals we sampled with viable RNA. Trial 2 calves sustained no death loss but had 10.8 % morbidity among sampled calves used in the study. While it is noted that animals exhibiting extremely high RQ values were among those that were retreated and or deceased, it is also important to note that “at risk” animals that did not display extreme values also became ill. Whether these animals were in the preliminary stages of infection or their immune system was better equipped to handle the pathogen is unknown.

Leukocyte RNA extraction was selected for this project due to the practicality of sample collection relative to stage of growth and maturity of the subject animals. The cattle used in this study were owned by independent outside sources and were not at the endpoint of the market process. While RNA may be extracted from a variety of tissues and organs, this limited our sample collection such that blood samples or skin samples (i.e. ear notches) were the most practical choices. In light of BVD testing using ear notches, we were unable to execute the latter option on these calves. While other tissues may display a more time sensitive alteration of expression values due to differences in cell turnover rate, leukocyte cell extraction was more practical for the use in live animals.

CONCLUSIONS

Comparison of plasma cortisol concentrations between both treatments indicated both groups of calves experienced a stressful event. Cortisol concentrations were elevated in both AS and CS calves due to the amount of elapsed time from initial disruption of cattle to sample collection on the morning of collection. However, while cattle exposed to the acute stressor had increased plasma cortisol concentrations they did not display the increased expression of immune inflammatory response genes that we saw from the calves subjected to chronic stress. While the AS calves were experiencing a stressful event, it appears their immune function was not being affected as it related to peripheral lymphocytic gene expression.

While cortisol concentrations were increased and statistically similar in both the case of AS and CS calves, it is apparent that differences in genetic expression is more useful in studying immune suppression in beef cattle subjected to traditional marketing systems. Gene expression data collected in these two trials identified 15 genes of interest using qRT-PCR that are intrinsically involved in innate and adaptive immune function of the bovine. Mean treatment differences of RQ values from the 15 genes ranged from 0.309 to 913.19 excluding extreme statistical outliers. These genes show promise in evaluating stress related physiological reactions in beef cattle when exposed to chronic stress, especially when supported by morbidities and mortalities reported by the feed lot.

The results of this study suggest the expression data of the genes presented in this study can be used to identify chronically stressed animals due to the effect these same

genes have on the immune system of beef calves. However, further analysis is needed to determine at what point these genes begin to up-regulate to help determine a more accurate effect of chronic stress on the immune response. Further analysis is also needed on the genes that were up regulated during AS when compared to CS to determine if these effects were true. This information may help determine at what critical points "at risk" calves are most susceptible to infection. The ability to develop a timeline of lymphocytic gene expression on calves which have no preconditioning will provide insight into the immune defense response in beef calves ability to prevent infection.

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APPENDIX

Table 3. List of 93 genes present on bovine LDA with significance listed.

Result	Gene ID	Gene Description
*	BOLA-DMA	ATP-binding cassette, sub-family F (GCN20
-	BOLA-DQB	B-cell CLL/lymphoma 6 (zinc finger protein 51)
-	BOLA-DRA	Complement component 3
-	CCBP2	Complement component 4A (Rodgers blood group)
**	CCL11	Chemokine (C-C motif) ligand 11
-	CCL19	Chemokine (C-C motif) ligand 19
-	CCL20	Chemokine (C-C motif) ligand 20
**	CCL22	Chemokine (C-C motif) ligand 22
**	CCL25	Chemokine (C-C motif) ligand 25
*	CCL28	Chemokine (C-C motif) ligand 28
X	CCL8	Chemokine (C-C motif) ligand 8
*	CCR1	Chemokine (C-C motif) receptor 1
**	CCR4	Chemokine (C-C motif) receptor 4
-	CCR5	Chemokine (C-C motif) receptor 5
X	CCR9	Chemokine (C-C motif) receptor 9
*	CCRL1	Chemokine (C-C motif) receptor-like 1(Protein Coding)
-	CD40	CD40 (TNF receptor superfamily mbr 5) (Protein Coding)
-	CD40LG	CD40 ligand (TNF superfamily, mbr 5, hyper-IgM syndrome)
*	CRH	Corticotrophin Releasing Hormone
**	CSF1	Colony Stimulating Factor 1(Macrophage)
*	CSF1R	Colony Stimulating Factor 1 Receptor
**	CXCL2	Chemokine (C-X-C motif) ligand 2
**	CXCL5	Chemokine (C-X-C motif) ligand 5
-	CXCL9	Chemokine (C-X-C motif) ligand 9
**	CXCR3	Chemokine (C-X-C motif) receptor 3
*	CXCR5	Chemokine (C-X-C motif) receptor 5
*	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
-	HSF1	Heat Shock Factor 1 (Protein Coding)
**	HSF2	Heat Shock Factor 2 (Protein Coding)
-	HSP90AB1	Heat Shock Protein 90 kDa (Cytosolic Class B mbr)
-	HSPA14	Heat Shock Protein 70 kDa (Protein 14)
-	HSPA1A	Heat Shock Protein 70 kDa (Protein 1A)
-	HSPA8	Heat Shock Protein 70 kDa (Protein 8)

* Genes that amplified and showed differences in expression (P<.05) between treatments in Trial 1
** Genes that amplified and showed differences in expression (P<.05) between treatments in Trial 2
X Common genes between Trial 1&2 that showed differences in expression (P<.05) between treatments
\- Genes showing no significant differences between treatments in Trial 1 or 2

Table 3. Continued

Result	Gene ID	Gene Description
**	HSPA9	Heat Shock Protein 70 kDa (Protein 9)
-	HSPB1	Heat Shock Protein 27 kDa (Protein 1)
-	HSPB6	Heat Shock Protein 20 kDa (Alpha Crystalin beta-6)
-	HSPB8	Heat Shock Protein 22 kDa (Protein 8)
**	HSPCA	Heat Shock Protein 90 kDa Alpha (cytosolic) Class A mbr
*	IFI47	Interferon Gamma (Inducible Protein 47)
X	IFIT5	Interferon-induced protein with tetratricopeptide repeats
-	IFNG	Interferon Gamma,
*	IK	IK Cytokine (down regulator of HLAII)
X	IL10	Cytokine (Interleukin 10)
X	IL10RB	Cytokine (Interleukin 10) alpha Receptor
-	IL11RA	Cytokine (Interleukin 11) alpha Receptor
X	IL12B	Cytokine (Interleukin 12 Natural killer cell stim. Factor 2)
-	ILRB12	Cytokine (Interleukin 12)
**	IL13	Cytokine (Interleukin 13)
*	IL16	Cytokine (Interleukin 16)
-	IL17A	Cytokine (Interleukin 17)
**	IL18	Cytokine (Interleukin 18)
*	IL1A	Cytokine (Interleukin 1) alpha
-	IL1B	Cytokine (Interleukin 1) beta
*	IL1F5	Cytokine (Interleukin 1 family, mbr 5) delta
X	IL1RN	Cytokine (Interleukin 1 receptor antagonist)
-	IL2	Cytokine (Interleukin 2)
-	IL23R	Cytokine (Interleukin 23) Receptor
X	IL2RA	Cytokine (Interleukin 2) alpha Receptor
*	IL4	Cytokine (Interleukin 4)
X	IL4R	Cytokine (Interleukin 4) Receptor
-	IL5	Cytokine (Interleukin 5)
-	IL6	Cytokine (Interleukin 6)
-	IL6R	Cytokine (Interleukin 6) Receptor
X	IL8RB	Cytokine (Interleukin 8) beta Receptor
X	IRAK2	Interleukin receptor associated kinase
-	IRF6	Interferon regulatory factor 6
-	IRGC	Immunity related GTOase, cinema
-	KLRA1	Killer cell lectin-like receptor (subfamily A, mbr 1)
**	KLRD1	Killer cell lectin-like receptor (subfamily D, mbr 1)
-	KLRK1	Killer cell lectin-like receptor (subfamily K, mbr 1)
X	LBP	Lipopolysaccharide binding protein

* Genes that amplified and showed differences in expression ($P<.05$) between treatments in Trial 1
** Genes that amplified and showed differences in expression ($P<.05$) between treatments in Trial 2
X Common genes between Trial 1&2 that showed differences in expression ($P<.05$) between treatments
- Genes showing no significant differences between treatments in Trial 1 or 2

Table 3. Continued

Result	Gene ID	Gene Description
**	LOC529196	Chemokine (C-C motif) Receptor type 1-like
**	LSP1	Lymphocyte-specific protein 1
**	NKG7	Natural killer cell group 7 sequence
**	PTGDR	Prostaglandin D2 Receptor
-	PTGER4	Prostaglandin E Receptor (subtype EP4)
**	PTGFR	Prostaglandin F Receptor
**	SERP1	Stress-associated endoplasmic reticulum protein 1
-	SFTPA1B	Surfactant protein (A1B)
-	SFTPD	Surfactant protein (D)
-	TLR10	Toll-like Receptor 10
X	TLR2	Toll-like Receptor 2
-	TLR6	Toll-like Receptor 6
**	TLR9	Toll-like Receptor 9
-	TNF	Tumor Necrosis Factor
X	TNFRSF1A	Tumor Necrosis Factor Receptor (superfamily, mbr 1A)
**	TNFRSF25	Tumor Necrosis Factor Receptor (superfamily, mbr 25)
-	TNFRSF4	Tumor Necrosis Factor Receptor (superfamily, mbr 4)
-	TNFRSF9	Tumor Necrosis Factor Receptor (superfamily, mbr 9)
X	TNFSF13	Tumor Necrosis Factor Receptor (superfamily, mbr 13)
-	TNFSF13B	Tumor Necrosis Factor ligand (superfamily, mbr 13)
*	TNFSF8	Tumor Necrosis Factor ligand (superfamily, mbr 8)

* Genes that amplified and showed differences in expression ($P<.05$) between treatments in Trial 1
** Genes that amplified and showed differences in expression ($P<.05$) between treatments in Trial 2
X Common genes between Trial 1&2 that showed differences in expression ($P<.05$) between treatments
\- Genes showing no significant differences between treatments in Trial 1 or 2

Table 4. List of 15 inflammatory response genes common between Trial 1 and 2 which showed increased expression in the CS calves compared to AS calves.

Gene ID	Gene Description
TNFRSF1A	Tumor Necrosis Factor Receptor (superfamily, mbr 1A)
TLR2	Toll-like Receptor 2
IRAK1	Interleukin-1 receptor-associated kinase 1
LBP	Lipopolysaccharide binding protein
IL8RB	Cytokine (Interleukin 8) beta Receptor
IL4R	Cytokine (Interleukin 4) Receptor
IL2RA	Cytokine (Interleukin 2) alpha Receptor
IL1RN	Cytokine (Interleukin 1 receptor antagonist)
TNFSF13	Tumor Necrosis Factor (superfamily, mbr 13)
IFIT5	Interferon-induced protein with tetratricopeptide repeats
CCL8	Chemokine (C-C motif) ligand 8
IL10	Cytokine (Interleukin 10)
IL10RB	Cytokine (Interleukin 10) alpha Receptor
IL12B	Cytokine (Interleukin 12 Natural killer cell stimulus, Factor 2)
CCR9	Chemokine (C-C motif) receptor 9

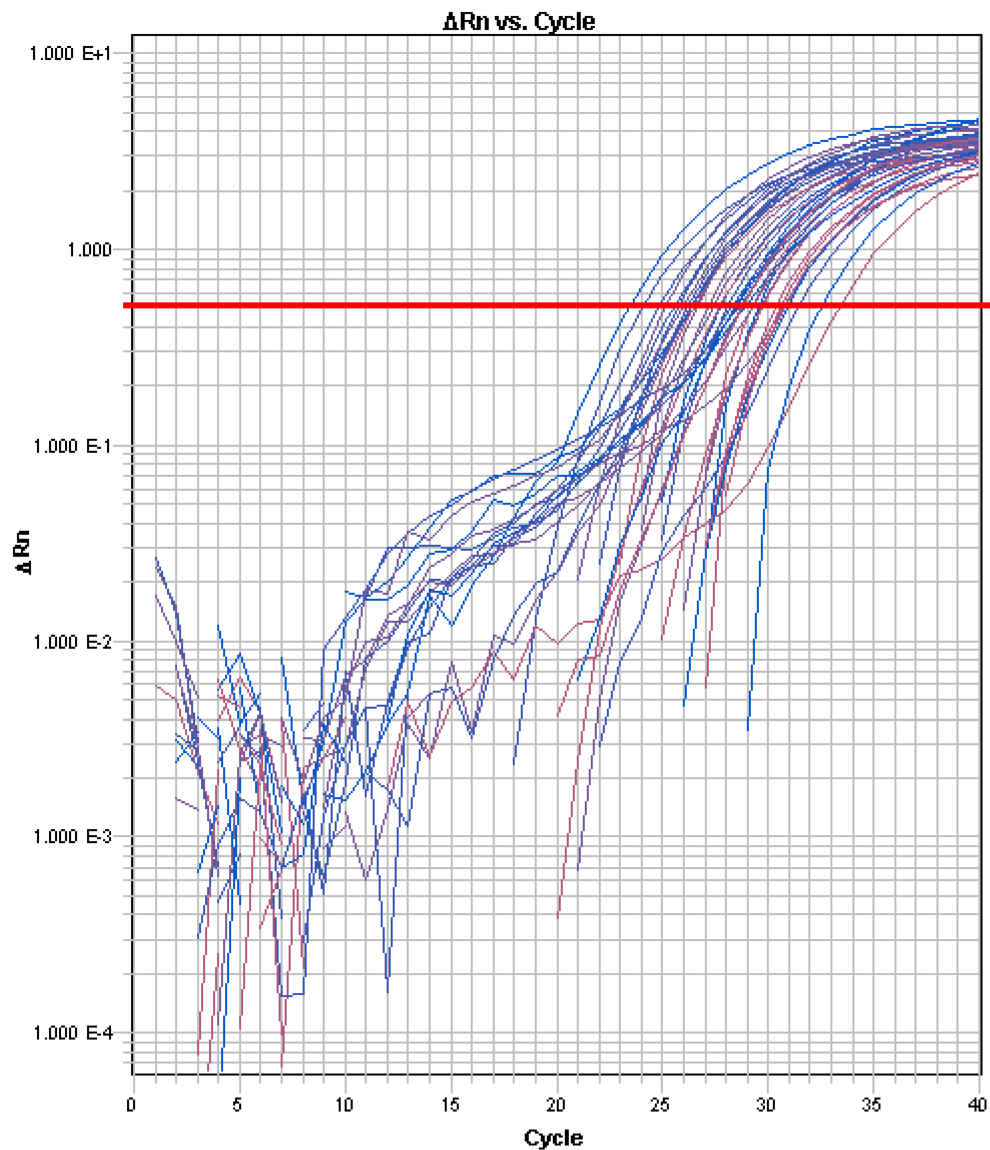

Figure 9. Amplification plot of gene expression for primer CCL-8. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

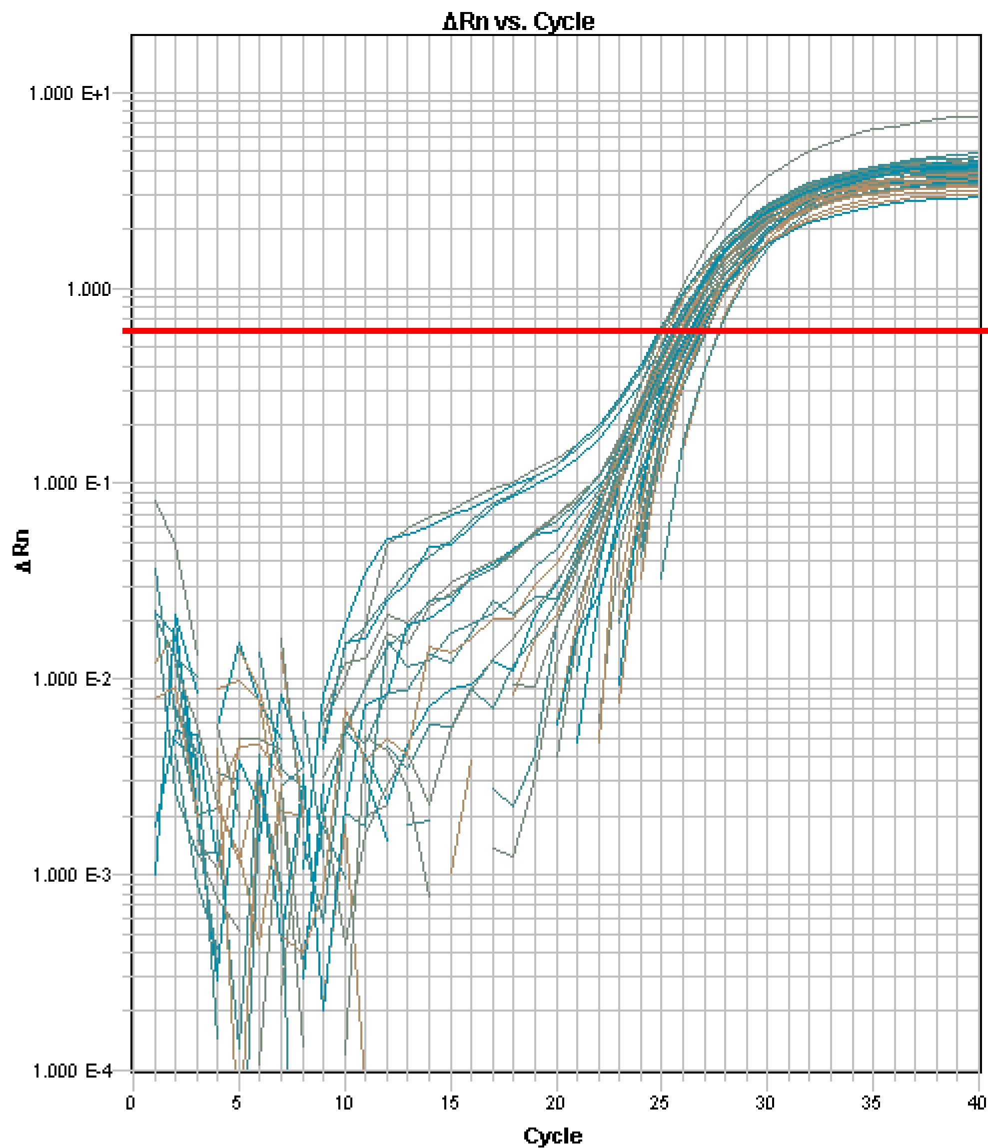

Figure 10. Amplification plot of gene expression for primer CCR-9. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

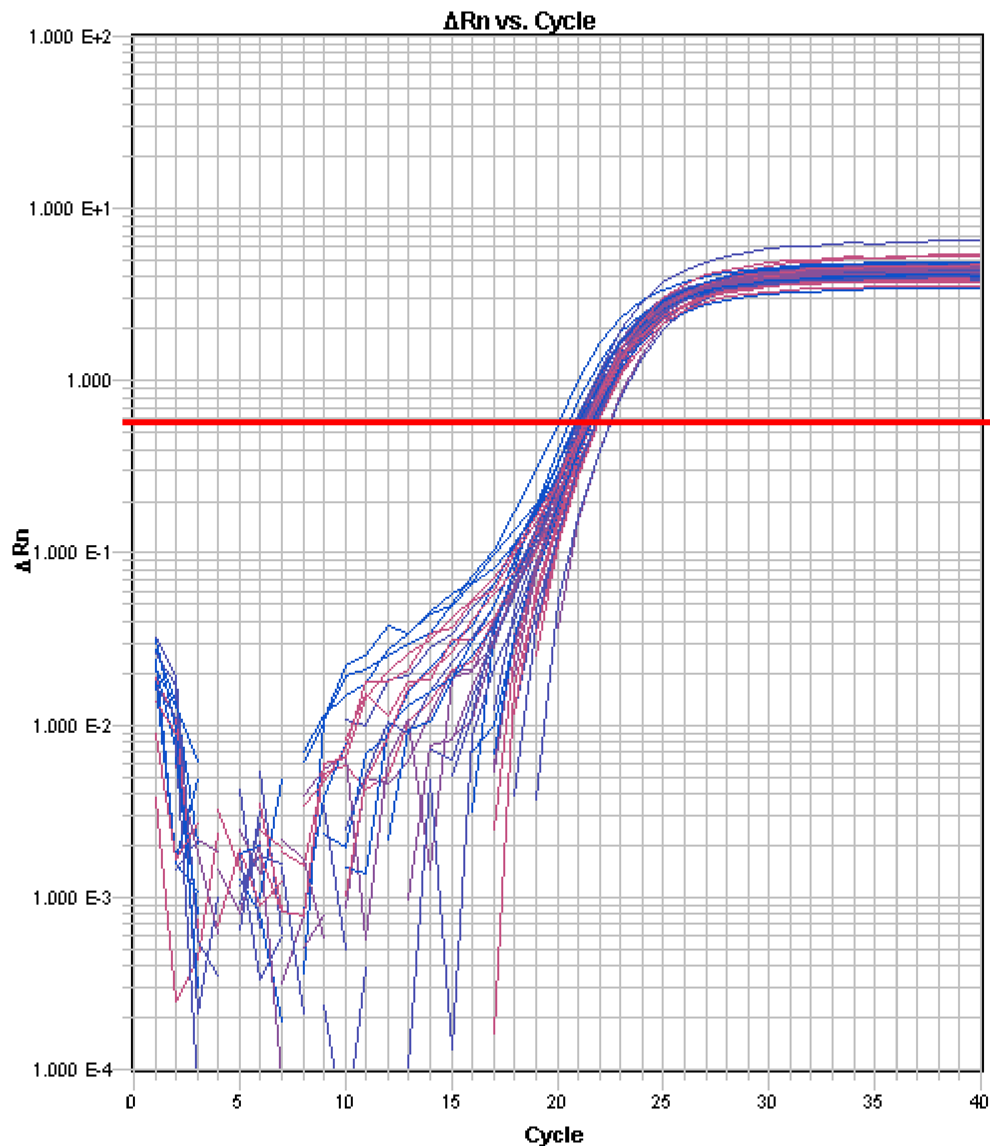

Figure 11. Amplification plot of gene expression for primer TNFSF13. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

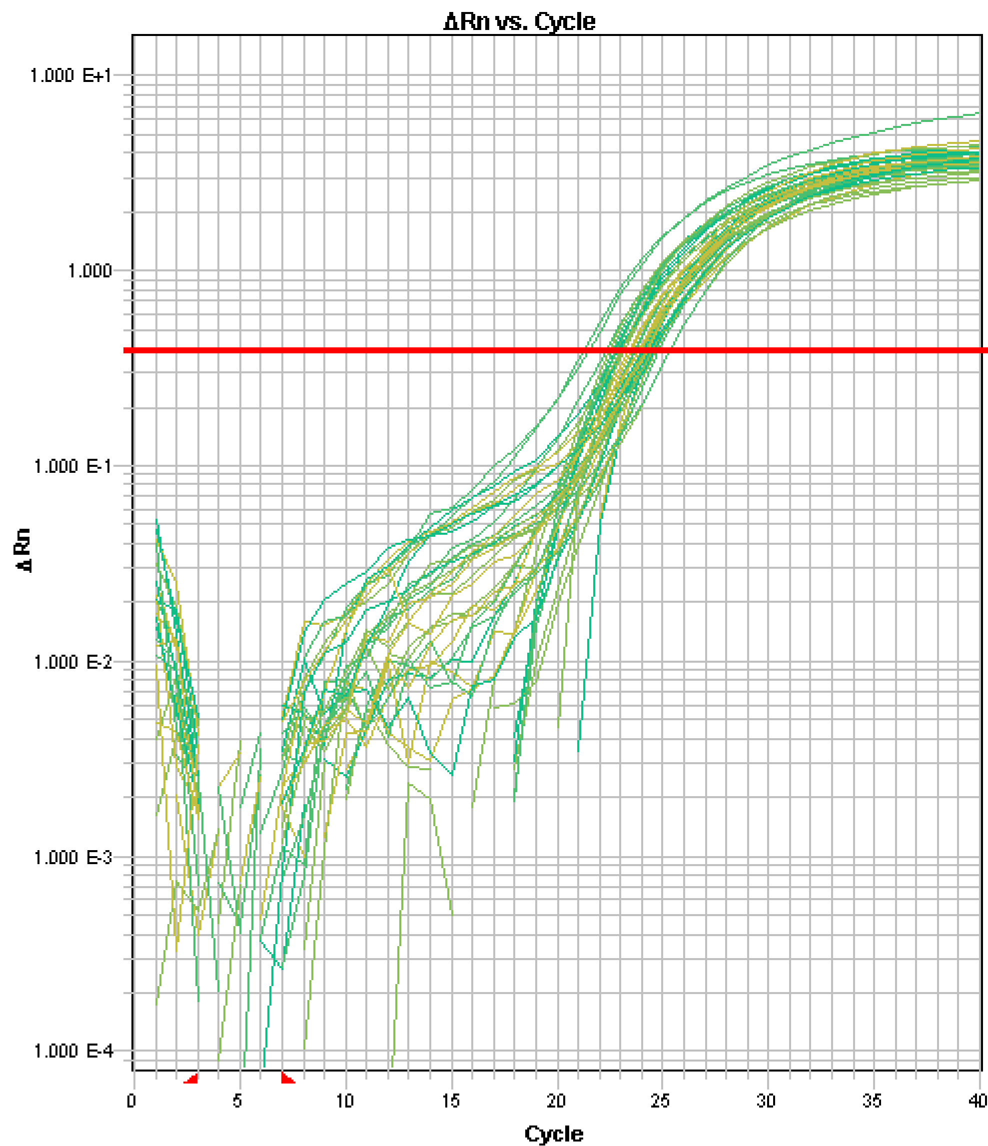

Figure 12. Amplification plot of gene expression for primer IFIT-5. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

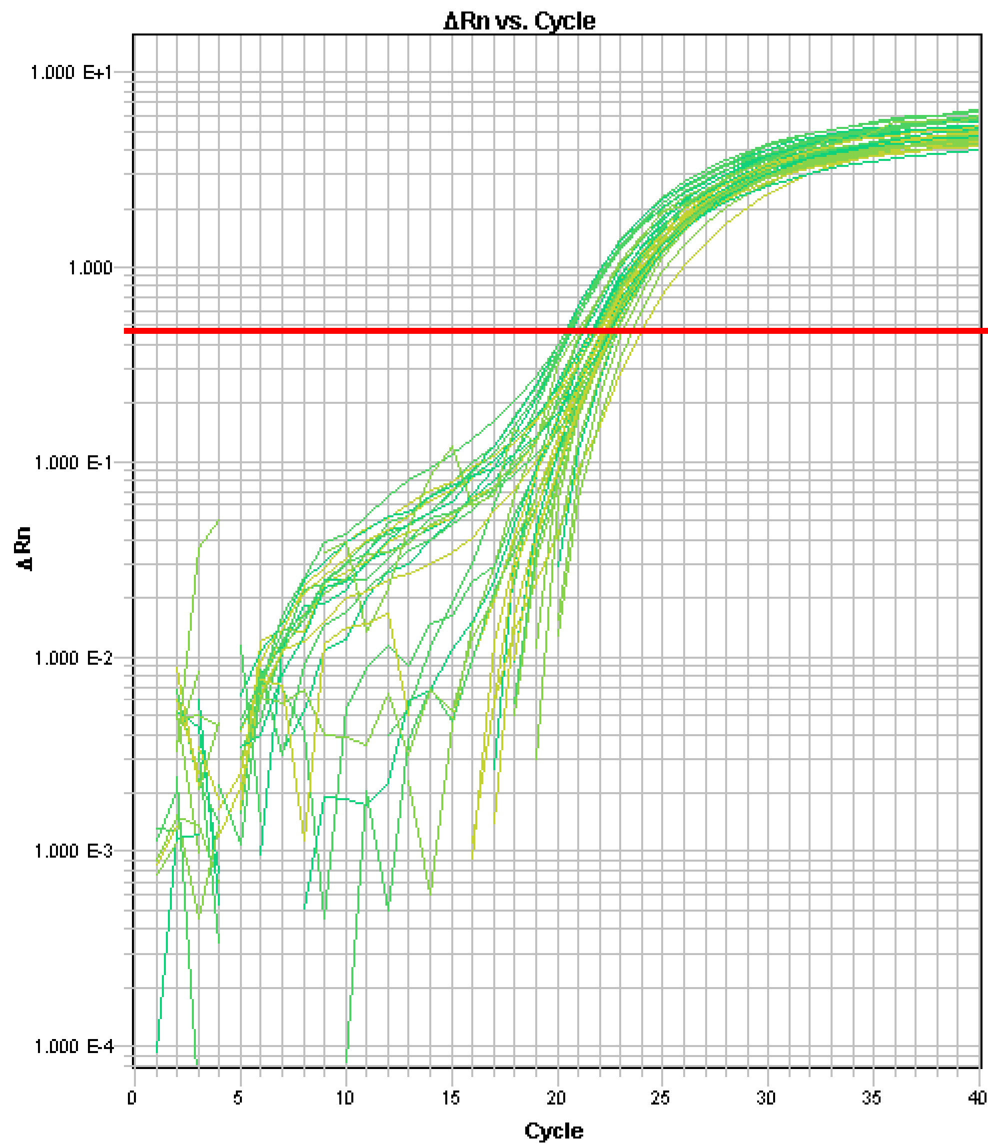

Figure 13. Amplification plot of gene expression for primer IL-1RN. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

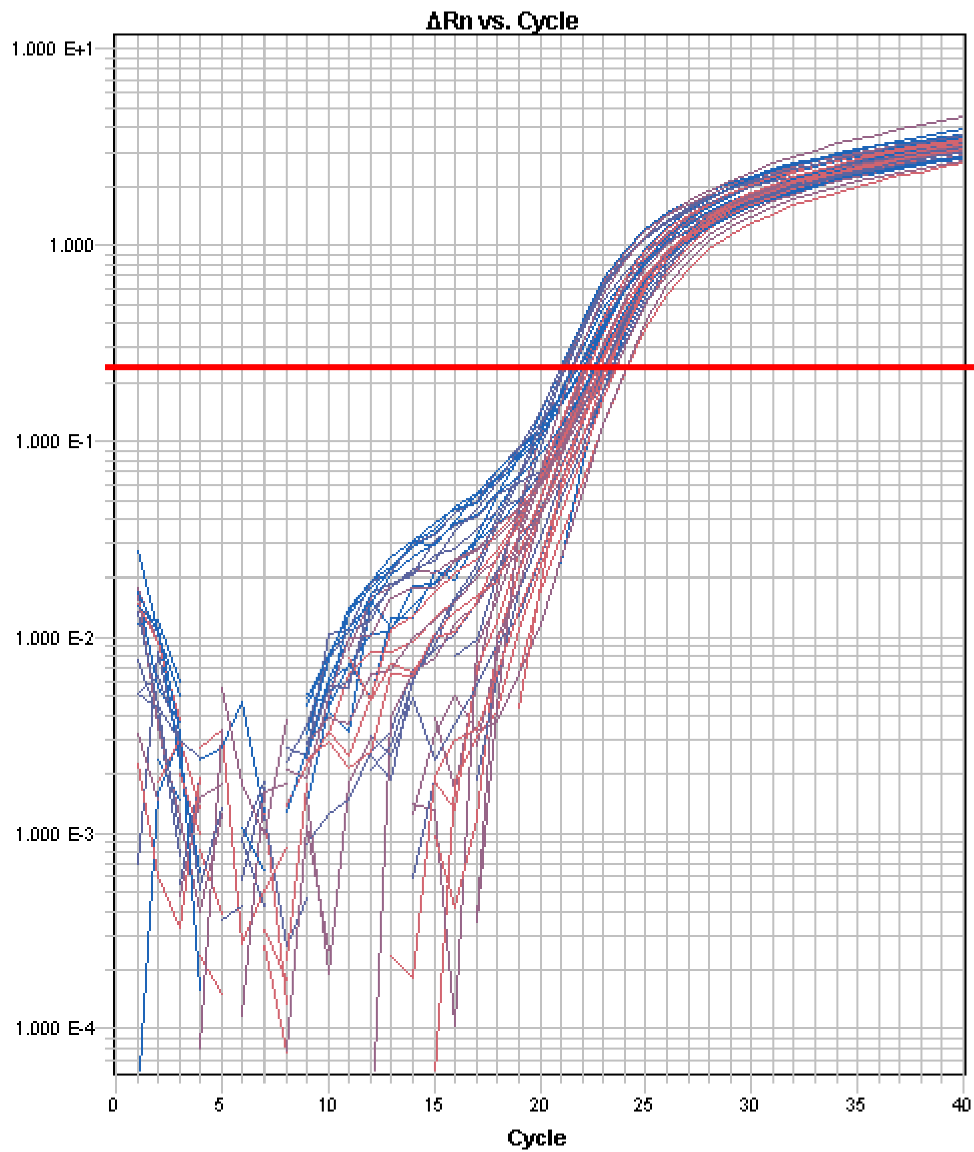

Figure 14. Amplification plot of gene expression for primer IL-2RA. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

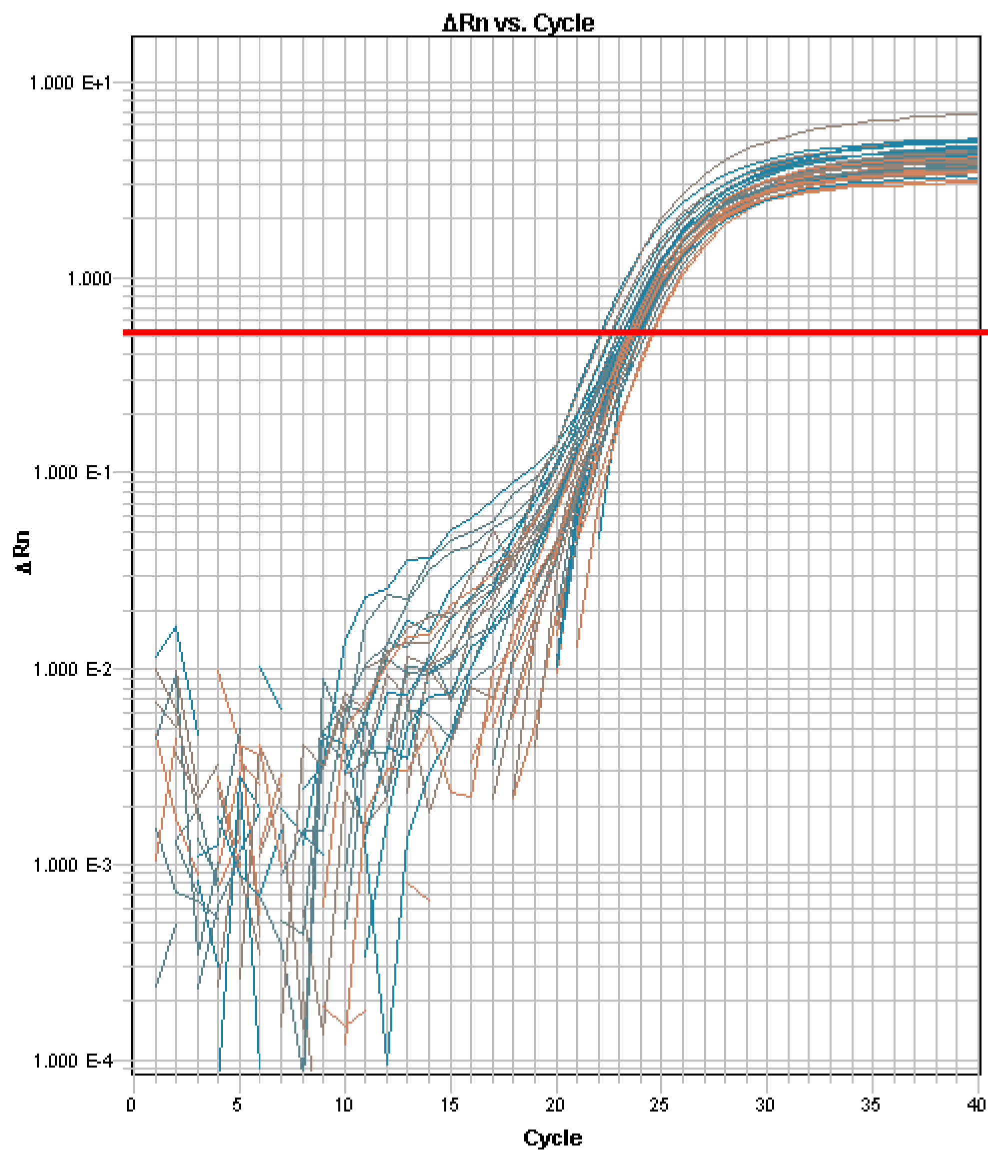

Figure 15. Amplification plot of gene expression for primer IL-4R. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

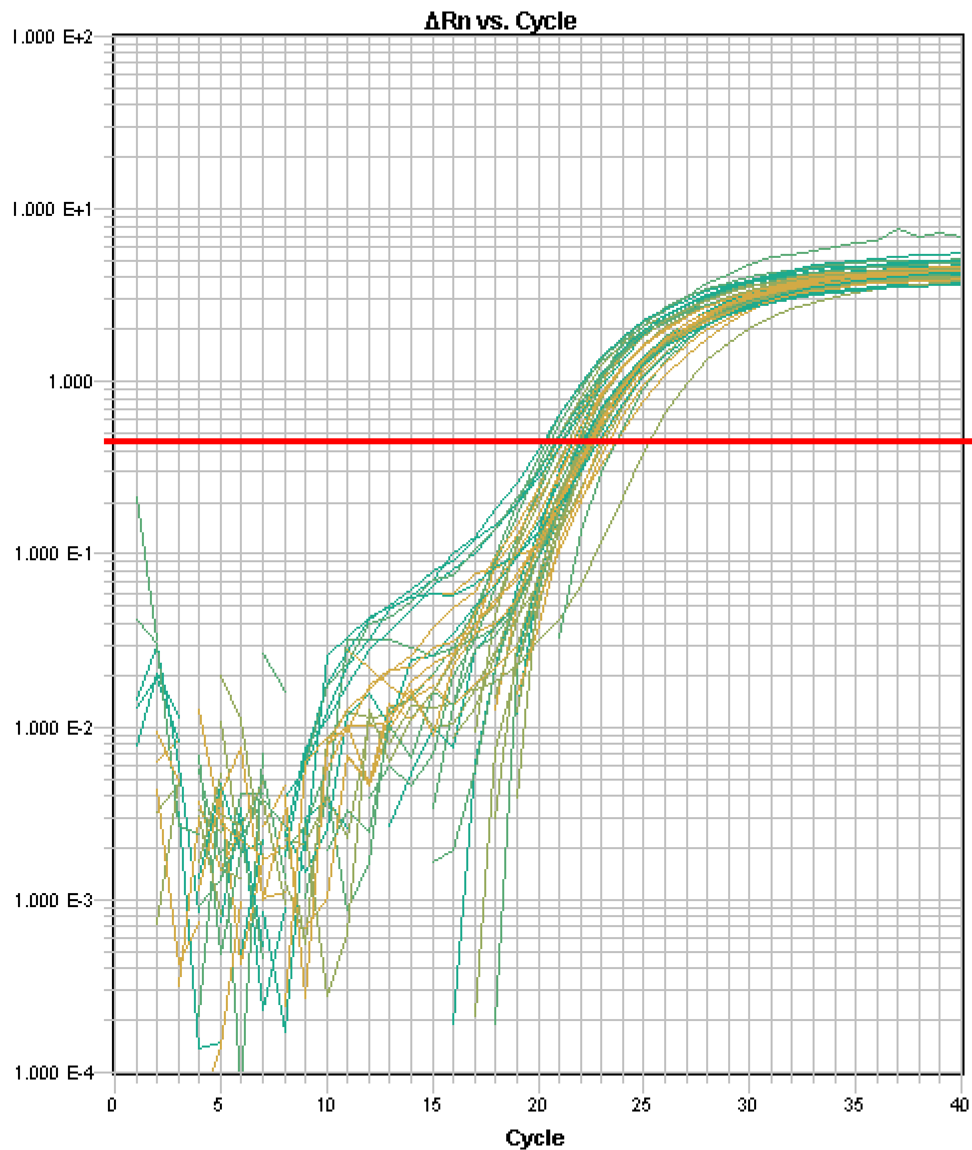

Figure 16. Amplification plot of gene expression for primer IL-8RB. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

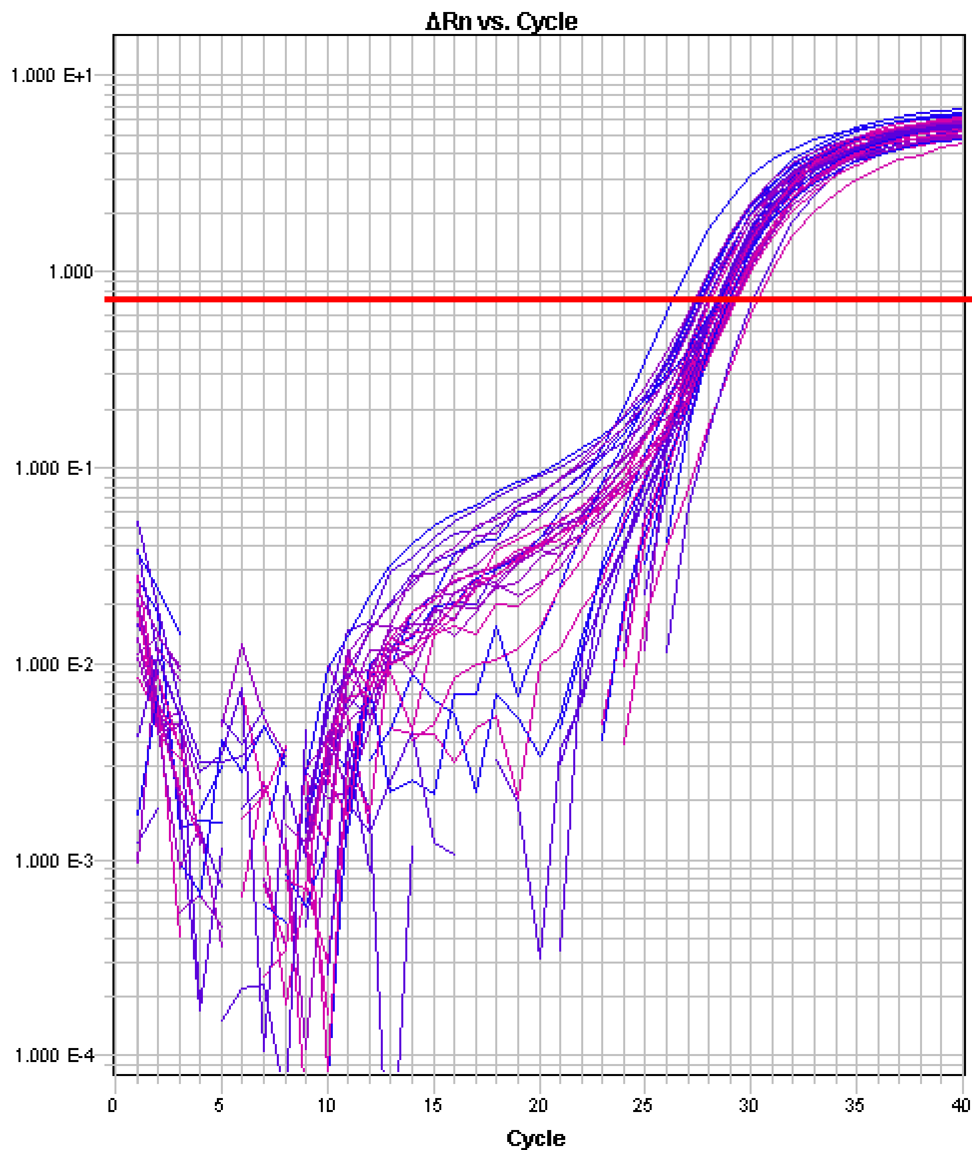

Figure 17. Amplification plot of gene expression for primer IL-10. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

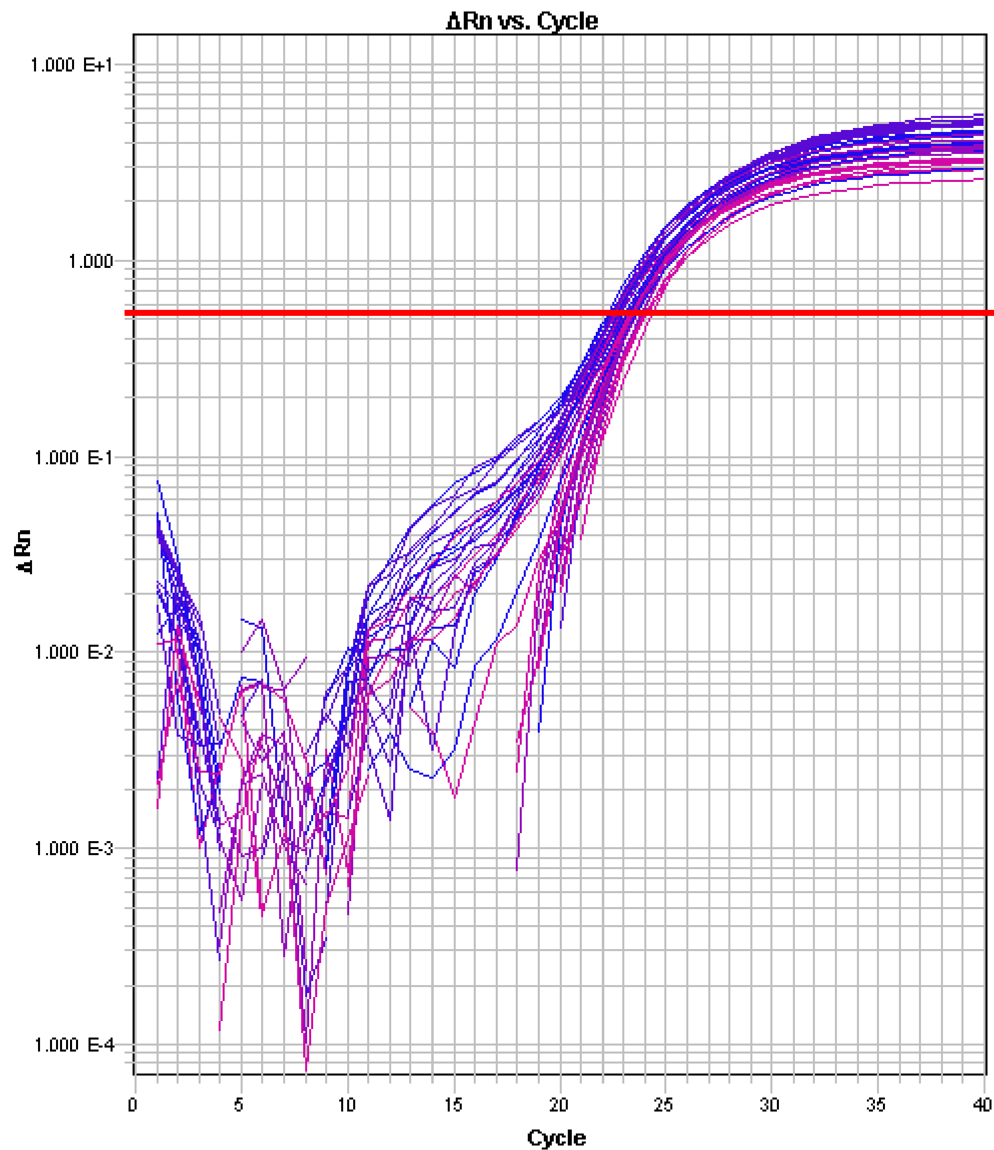

Figure 18. Amplification plot of gene expression for primer IL-10RB. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

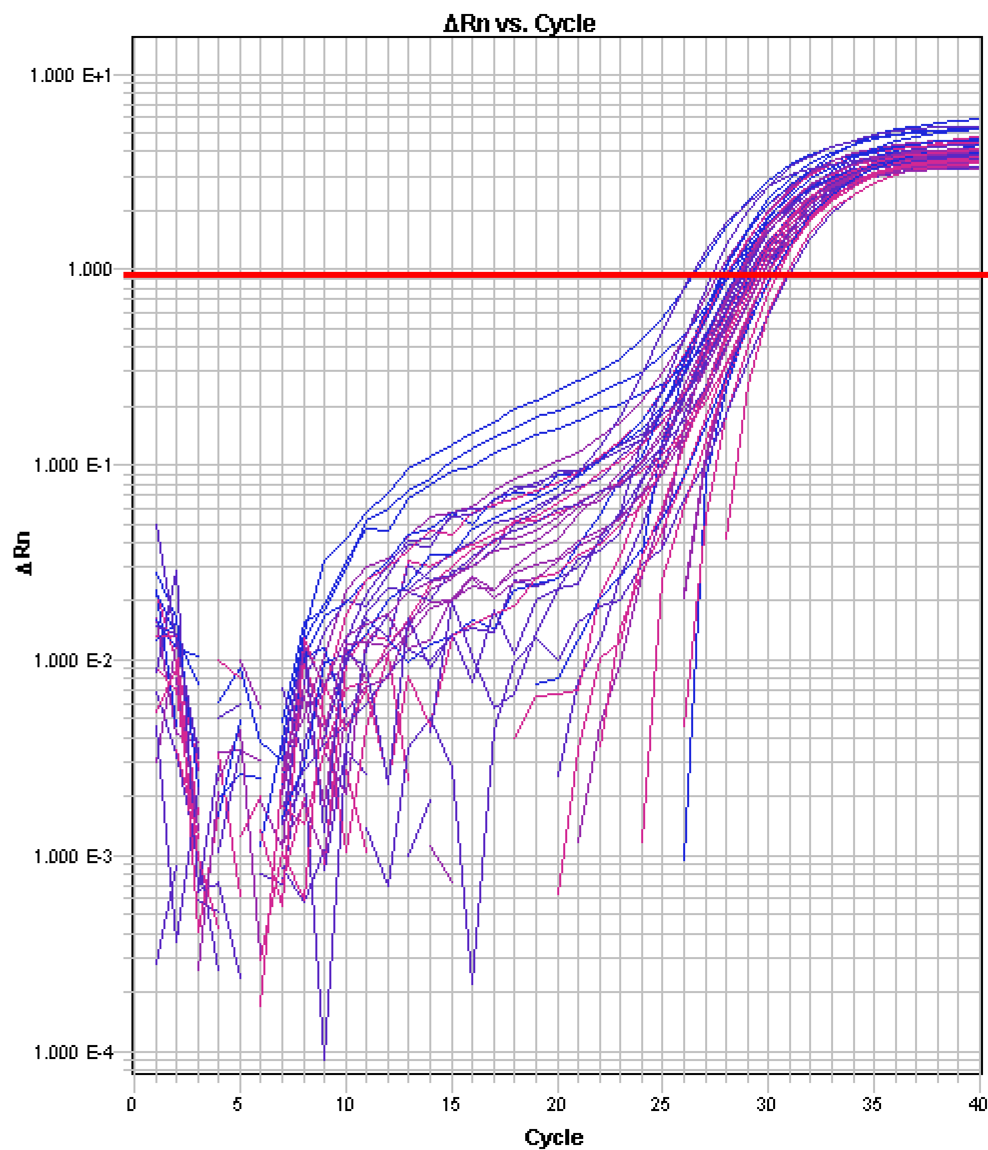

Figure 19. Amplification plot of gene expression for primer IL-12B. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

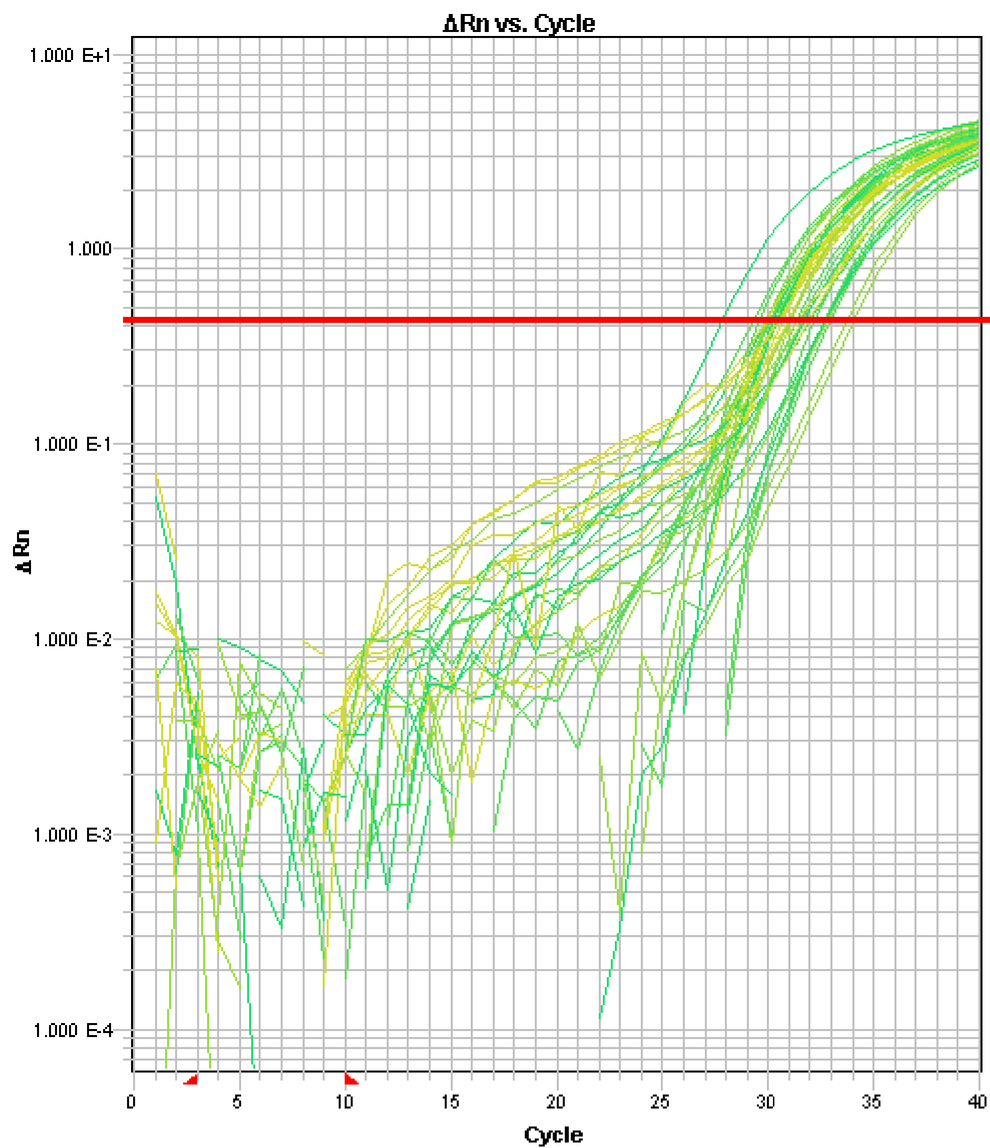

Figure 20. Amplification plot of gene expression for primer LBP. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

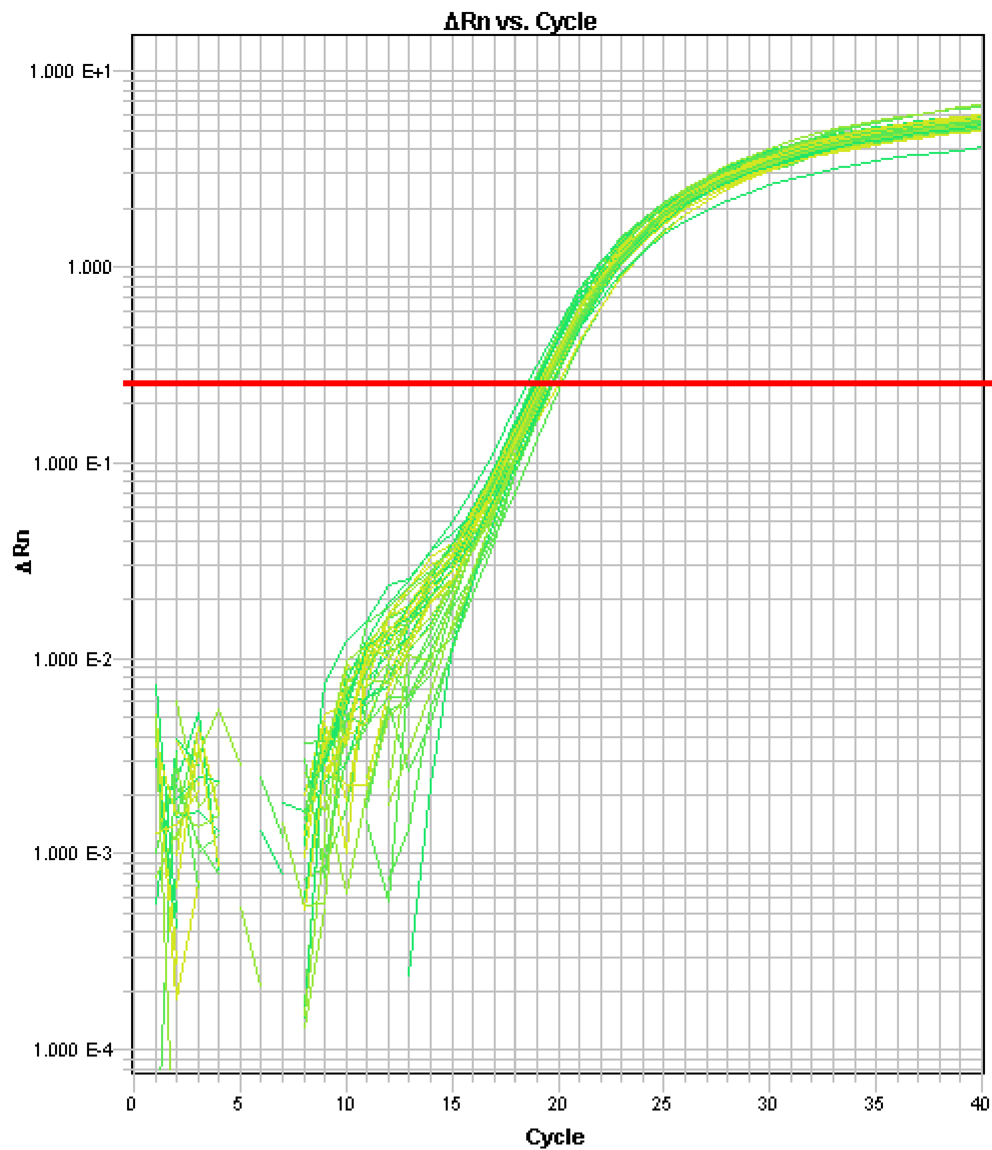

Figure 21. Amplification plot of gene expression for primer IRAK-1. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

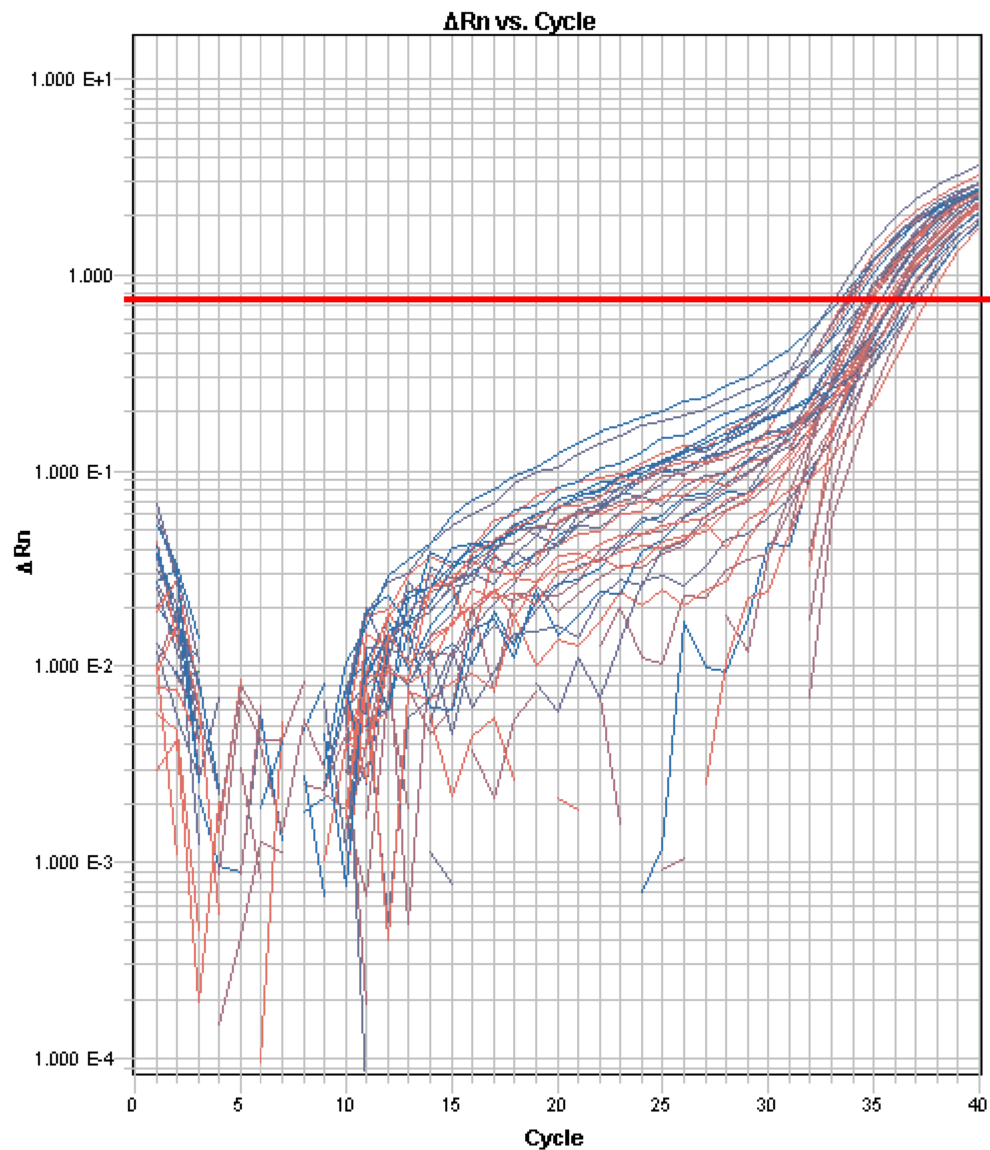

Figure 22. Amplification plot of gene expression for primer TLR-2. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

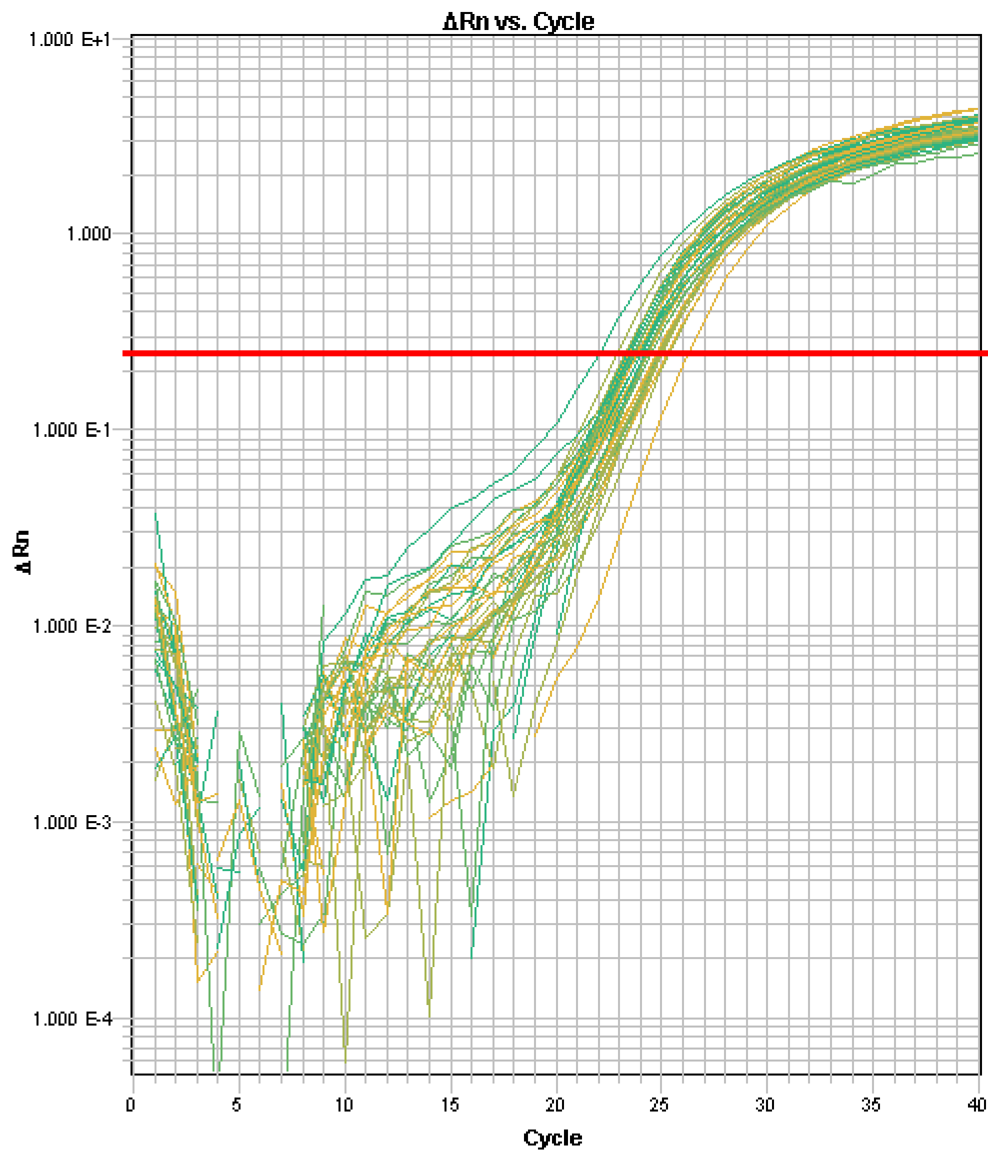

Figure 23. Amplification plot of gene expression for primer TNFRSF-1A. Each line represents an individual sample. Space between lines indicate differences in gene expression. All samples that failed to amplify are absent for clarity. The red line indicates threshold.

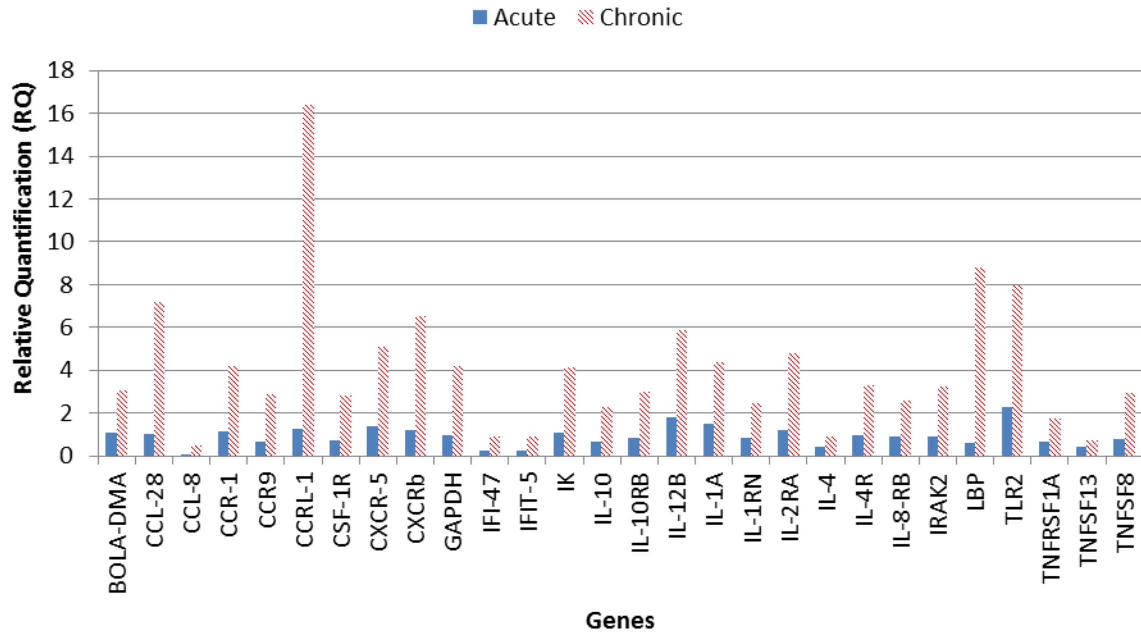

Figure 24. All mean *Bos taurus* and crossbred calves gene expression (RQ) values in which CS calves had a greater response ($P < .047$) than AS calves in Trial 1. CRH and IL-1F5 were removed.

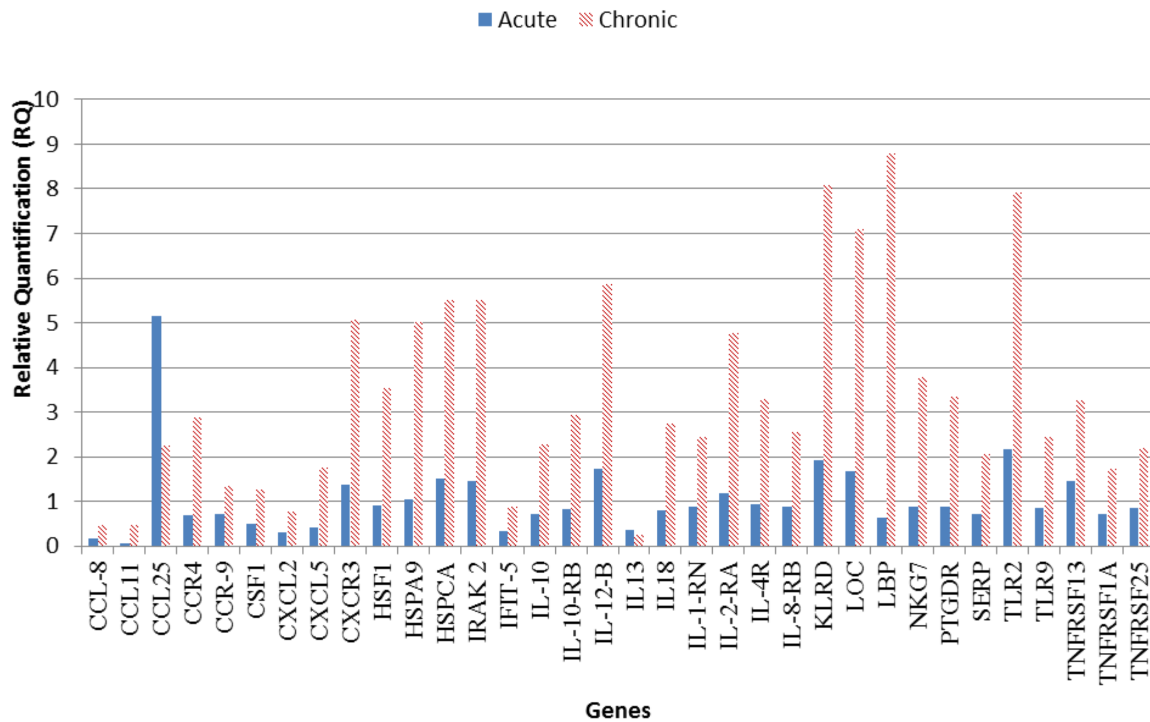

Figure 25. All mean *Bos taurus* and crossbred calves gene expression (RQ) values in which CS calves had a greater response ($P < .047$) than AS calves in Trial 2. CCL-22, LSP-1 and PTGFR removed due to high RQ values.

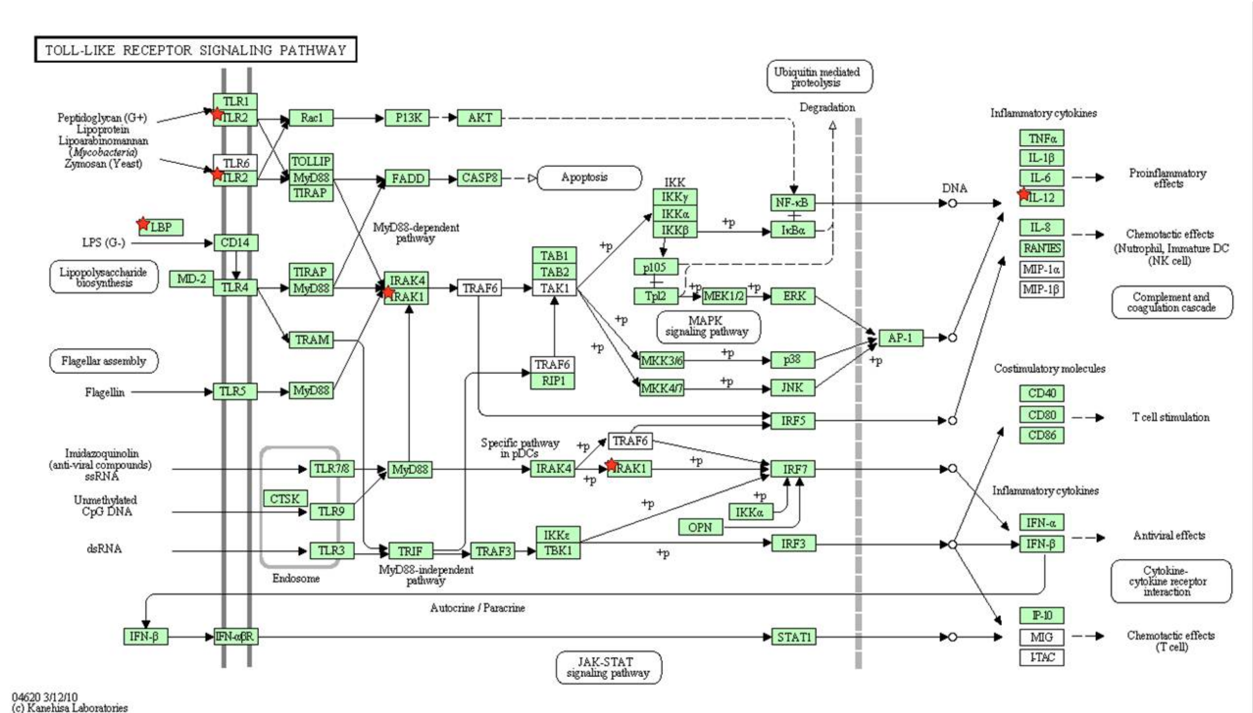

Figure 26. Toll-like receptor pathway with relevant individual genes identified in Trial 1 and 2 highlighted using a red star.

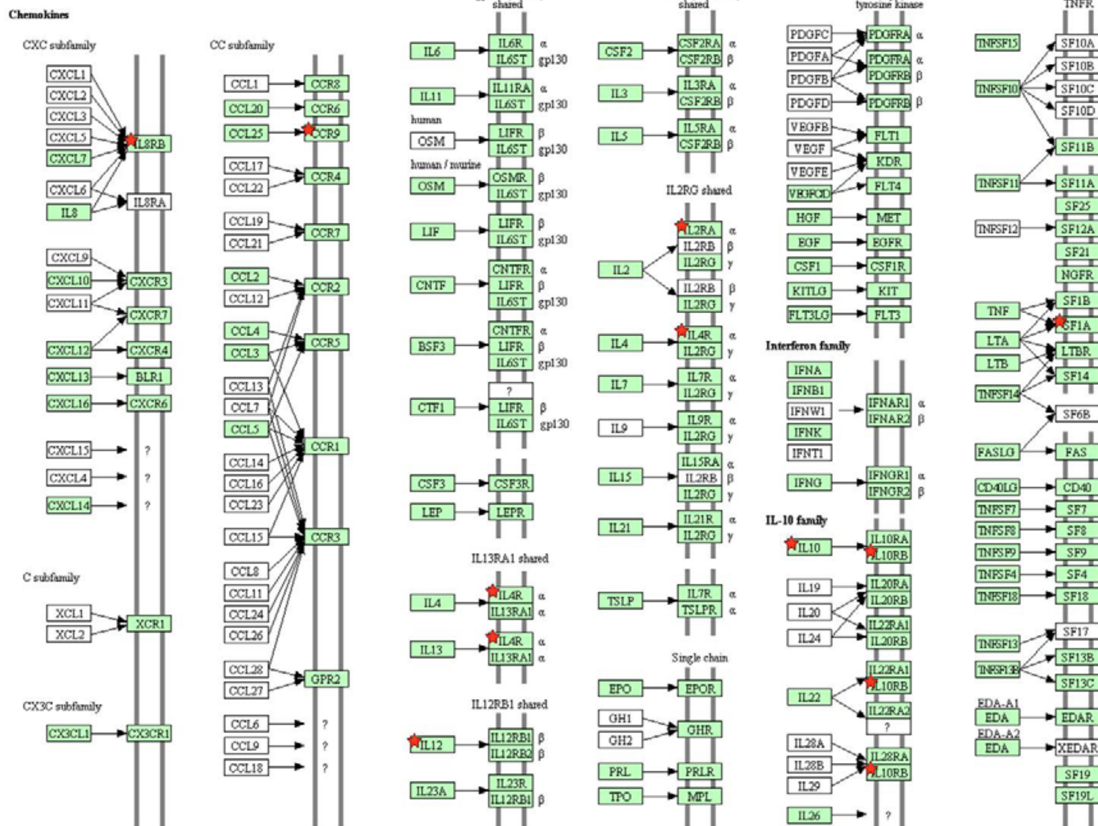

Figure 27. Cytokine – Cytokine Receptor Interaction with relevant individual genes identified in Trial 1 and 2 highlighted using a red star.

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