ABSTRACT: Acknowledgment that modern livestock production systems impose stress on animals has been accepted by the scientific community and producers. As the economic burden has increased for livestock producers, expectations for animal performance have increased, thus placing more strain on the entire production system. Whether or not periodic exposure to stress within the production system jeopardizes the well-being of animals continues to be an area of debate largely because of the inability to accurately quantify the magnitude and severity of the stress response on other biological systems. Adding to the confusion is the fact that activation of the stress axis can be both beneficial and detrimental to the body depending on the duration of the stress response and the frequency at which an animal is exposed to stressful stimuli. Few would argue that continuous long-term stress inhibits livestock productivity and overall well-being. Less clear is whether or not occasional exposure to acute stress jeopardizes the productivity and well-being of livestock. To fully appreciate the complexity associated with activation of the stress axis and the overall biological impact on the body, one must delve deep into the scientific literature and examine the science in an unbiased manner. It is imperative to appreciate and understand that activation of the stress axis is an essential survival mechanism necessary to maintain homeostasis during biologically challenging times. Acute activation of the stress axis leads to repartitioning of energy to organs and tissues essential for coping with stress, redirection of blood flow from the peripheral to large muscle groups, decreased digestive function, and priming of the immune system to prepare for subsequent infections. Conversely, chronic activation of the stress axis disrupts digestive function, causes catabolism of muscle and adipose tissue, and suppresses overall immune function, thus making an animal more susceptible to disease. But what parameters are needed to distinguish periods of acute stress from those of chronic stress, and what biological markers are the best indicators of “stress” in an animal? Although there are a plethora of physiological responses and endocrine biomarkers that can be quantified, an integrative tool that has been readily embraced by scientists and producers as an effective and efficient indicator of the duration and magnitude of stress that an animal is experiencing has yet to be identified.

Key words: cattle, stress, well-being
INTRODUCTION

The relationship between stress and immune function continues to be an area of considerable scientific interest because of its complex and dynamic characteristics (Fig. 1). The preconceived notions of primarily immuno-suppressive effects of stress hormones have been thwarted by overwhelming evidence of a positive relationship between stress hormones and immunity. Research endeavors are now focusing on the possibility of harnessing the positive attributes of stress hormones related to permissive and/or enhancing effects on immune function.

Perceptions associated with stress axis activation and the subsequent biological responses necessary to return an animal to homeostasis have continued to evolve as evidence delineating stressor-specific responses has mounted. The biological consequences of exposing an animal to periodic acute stress as opposed to chronic stress or exposing a prenatal or neonatal animal vs. an adult animal to the same stressful stimuli are not analogous. The biological system is designed such that it favors survival of the animal through initiation of various coping behaviors and eventual adaptation measures. In fact, survival of the animal depends on the ability to “anticipate” and respond appropriately to these threats.

Elucidation of these basic biological control mechanisms is the first step in gaining a better understanding of how the complex relationship between stress hormones and immune function can be utilized to improve animal health, performance, and overall well-being in modern production settings. We must gain a better understanding of physiological responses and endocrine biomarkers that are indicative of whether an animal is displaying an appropriate stress response or perhaps experiencing an immunological challenge. Additionally, more information is needed to better understand the impact that management decisions have on these tightly associated biological systems, including naturally occurring differences in stress and immune function in beef cattle.

PHYSIOLOGICAL RESPONSES ASSOCIATED WITH STRESS AND IMMUNITY

Because of the intricate relationship between the stress response and immune function, delineating stress-specific vs. immune-specific physiological indicators in beef cattle poses a significant challenge. There are numerous physiological responses that can be associated with both a stress response and an immune response. For example, body temperature, blood flow, digestive capabilities, respiration, and heart rate can all be influenced independently by stress but are also influenced by the immune status of an animal (Apanius, 1998; Moberg, 2000).

With regard to body temperature, our group has previously demonstrated an increase in vaginal temperature in beef heifers exposed to both a combined dose of corticotropin-releasing hormone (CRH) and vasopressin (VP; Carroll et al., 2014) as an initiator of the stress response and a dose of endotoxin (i.e., lipopolysaccharide, LPS; Fig. 2A; Hulbert et al., 2008) as an acute pro-inflammatory immune challenge. Interestingly, the increase in vaginal temperature associated with the CRH-VP challenge was more rapid compared with the increase in vaginal temperature induced by the LPS challenge. However, the magnitude of the increase was greater in the LPS-challenged weaned Brahman heifers compared with the CRH-VP-challenged crossbred finishing heifers. Additionally, whereas the profile of LPS-induced increase in vaginal temperature appeared to be bi-phasic in nature, the CRH-VP-induced increase in vaginal temperature appeared to be multi-phasic, with 5 distinct peaks observed during the 8 h following the challenge. Another interesting observation was that the greatest increase in vaginal temperature observed in the CRH-VP-challenged heifers occurred at approximately 2 h post challenge, followed by an immediate decline. With the LPS-challenged heifers, the greatest increase in vaginal temperature occurred at approximately 2.5 h
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Despite the 2 challenges, a stress challenge and an immune challenge, initiate 2 distinct vaginal temperature profiles, the relevance is associated with the fact that vaginal temperature was increased in both groups of heifers, thus potentially causing difficulty with regard to delineating a stress response vs. immune status in beef cattle on the basis of a single sampling of body temperature. Adding to this complexity are previous reports from our group that demonstrate an increase in body temperature in cattle, associated with either a stress or immune challenge, can vary on the basis of animal temperament, breed, and sex class.

Heart rate, although primarily thought of as a physiological indicator of a stress response to a stimulus (Berntson et al., 1994), be it physical or psychological, may also be indicative of the immunological status of an animal. In previous studies (Hulbert et al., 2008, 2013), we have documented an increase in heart rate associated with independent CRH and LPS challenges in weaned Brahman heifers (Fig. 2B). Although the temporal profiles associated with the increases in heart rate in cattle exposed to CRH and LPS are similar, there are distinct differences associated with the magnitude of these responses. In cattle exposed to an LPS challenge, heart rate increased immediately after exposure to the LPS, reaching an initial peak of approximately 140 beats per min (bpm) at 5 h after the challenge, and remained elevated until at least 12 h after the challenge. However, for the CRH-challenged cattle, an increase in heart rate was not observed until approximately 4 h after the challenge, reaching an initial peak of 108 bpm that was reasonably sustained until at least 12 h post-challenge. At 24 h, heart rate was elevated above prechallenge values in both CRH- and LPS-challenged cattle. Thus, although heart rate measurements are undoubtedly indicative of a stress response, there also appears to be a firm association between heart rate and the pro-inflammatory response induced by LPS in cattle.

ENDOCRINE BIOMARKERS OF STRESS RESPONSES AND IMMUNE FUNCTION

In addition to physiological indicators, our laboratory has explored the possibility of using various endocrine biomarkers such as serum cortisol, various pro-inflammatory cytokines, and various acute-phase proteins as potential biomarkers to help distinguish between stress and immune responses in cattle. Serum cortisol, when collected properly, can be a sensitive and informative indicator of stress within beef cattle. Likewise, the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and IL-6 are considered to be primary indicators of acute inflammation in cattle (Carroll et al., 2009b). During times of inflammation, bacterial infection, endotoxin exposure, neoplasia, or physical injury, acute-phase proteins become important mediators of immunological functions and play an active role in tissue repair and remodeling. Acute-phase proteins have also been suggested to be indicators of animal stress, increasing during periods of transportation and weaning in beef cattle (Arthington et al., 2003). However, as with the physiological responses to stress and immune stimulation, there are similarities that exist in the profiles of these endocrine and acute-phase biomarkers in cattle that are exposed to independent CRH and LPS challenges. For example, in weaned Brahman beef heifers exposed to a CRH challenge (Fig. 3A), we previously reported that peak serum cortisol concentrations occur within 30 min of the challenge and remain elevated above baseline values for approximately 4 h post-challenge (Hulbert et al., 2013). When crossbred beef steers were administered LPS (Fig. 3B), peak serum cortisol concentrations were not observed until 1 h post-challenge and remained elevated above baseline values for approximately 5 h post-challenge (Burdick et al., 2012). Interestingly, although the
overall response profiles appear to be very similar between CRH- and LPS-challenged cattle, the magnitude of the responses were 3-fold greater for LPS.

Serum concentrations of the pro-inflammatory cytokines, TNF-α and IL-6, increased in both CRH- and LPS-challenged calves; however, there were some striking differences in the overall profiles. For CRH-challenged heifers, serum concentrations of TNF-α and IL-6 did not increase above baseline concentrations until 3 h post-challenge (Fig. 3A). Peak concentrations were observed at 3.5 h post-challenge for TNF-α and at 3 to 3.5 h for IL-6. Following these peak responses, an immediate decline was observed in serum concentrations of TNF-α and IL-6, which returned to baseline concentrations within 30 min of post-peak concentrations. However, for LPS-challenged steers, serum concentrations of TNF-α and IL-6 peaked at 1 and 2.5 h post-challenge, respectively (Fig. 3B). As with the CRH-induced profiles, following peak concentrations, both TNF-α and IL-6 immediately began to decline within 30 min; however, the decline was more gradual as baseline concentrations were not reached until 4 and 6 h post-LPS challenge, respectively.

In cattle, numerous proteins have been classified as acute-phase proteins, including haptoglobin, serum amyloid A, α1-acid glycoprotein, ceruloplasmin, α1-antitrypsin, α1-antichymotrypsin, α2-macroglobulin, and fetuin (Godson et al., 1995). It was suggested previously that haptoglobin is the most widely studied acute-phase protein in cattle (Horadagoda et al., 1999). Several studies have implicated haptoglobin as a primary indicator of stress in cattle exposed to various managerial stimuli, including transportation, commingling, and weaning (Conner et al., 1988; Arthington et al., 2003). In addition to its role during a stress response, haptoglobin is the major hemoglobin-binding protein that prevents oxidative damage to organs and tissues by binding to free hemoglobin. Specifically, the amount of ferrous Fe available for use by bacteria is limited by the formation of haptoglobin-hemoglobin complexes, resulting in the bacteriostatic effect of haptoglobin. An increase in secretion of haptoglobin from hepatocytes is tightly coupled with an increase in the secretion of pro-inflammatory mediators such as IL-6. It is this strong relationship that has prompted researchers to investigate a regulatory role for haptoglobin with respect to immune function (Huntoon et al., 2008) in addition to its relationship to the stress response.

In an effort to further distinguish the haptoglobin response in cattle, we previously demonstrated that both CRH and LPS induce significant, albeit different, increases in serum concentrations of haptoglobin (Carroll et al., 2009a). In a study with yearling Brahman bull calves that were independently challenged with either CRH or LPS, serum concentrations of haptoglobin were significantly increased at 4 h post-challenge. In the CRH-challenged bull calves, serum concentrations of haptoglobin returned to baseline concentrations by 6 h post-challenge and remained relatively stable through 24 h post-challenge. However, in the LPS-challenged bull calves, serum concentrations of haptoglobin remained elevated above baseline concentrations, reaching peak concentrations at 24 h post-challenge. Given that both CRH and LPS induce an acute increase in circulating concentrations of cortisol and pro-inflammatory cytokines, it is tempting to assume that these stress- and immune-related biomarkers are responsible for the subsequent increases observed in circulating concentrations of haptoglobin. Interestingly, one could make the case that this is, indeed, the scenario in the CRH-challenged bull calves given that circulating concentrations of haptoglobin are more closely associated with the response profiles of cortisol and the pro-inflammatory cytokines. However, this is not the case with regard to LPS-induced haptoglobin secretion as the sustained increase in circulating concentrations of haptoglobin is extended well beyond the duration observed for cortisol and pro-inflammatory cytokines. However, given gradual increases in circulating concentrations of haptoglobin and the extended duration of this response, the haptoglobin profiles may be reflecting a delayed and more stable response.
than that of other stress- and immune-related biomarkers. Regardless, circulating concentrations of haptoglobin in the bovine appear to be reflective of a stress response and are an indicator of acute inflammation initiated by a bolus endotoxin challenge. The magnitude and duration of the haptoglobin response appears to be more specific to the stimuli, thus indicating that haptoglobin profiles may be more informative with regard to animal health and well-being than absolute concentrations obtained via single time-point blood samples.

**METABOLIC MARKERS OF STRESS RESPONSES AND IMMUNE FUNCTION**

Increases in metabolites (e.g., glucose and NEFA) and metabolic hormones (e.g., insulin) are observed in response to normal feeding behaviors. However, metabolism and metabolic parameters are also key participants in stress and immune interactions. Although the relationship between the stress response and immune function has been established for some time, the link between immune function and metabolism, despite being theoretically understood for many years, has only recently received appropriate attention. Furthermore, the link between stress and metabolism, specifically glucose kinetics, has been established since the original discovery of potential actions of “glucocorticoids” on circulating glucose concentrations in adrenalectomized rodents (Long et al., 1940). Energy availability and redistribution greatly influence the outcome of stress and immune responses and therefore directly influence animal performance, health, and well-being following such challenges. Consequently, it is imperative to gain an understanding of the differences and similarities observed between responses induced by acute stress and those induced by an acute immune challenge. Herein we share recent data from our laboratory profiling metabolic differences following a dual CRH-VP challenge to mimic an acute stressor and a LPS challenge to acutely stimulate a pro-inflammatory immune response.

A dual CRH-VP challenge in finishing beef heifers induced a rapid increase in glucose concentrations (Fig. 4A) that peaked within 30 min post-challenge and then declined back to basal concentrations by 2 h post-challenge (J. A. Carroll, unpublished data). The temporal glucose response to the CRH-VP challenge is in contrast to the delayed and decreased response observed following LPS challenge in crossbred beef steers (Burdick Sanchez et al., 2014b). Glucose concentrations in steers were elevated above basal concentrations at 2 h and then again from 3 to 3.5 h following administration of a bolus intravenous injection of LPS. Subsequently, glucose concentrations decreased below basal concentrations and remained low for the duration of the 8-h challenge period.

**Figure 4.** Serum concentrations of (A) glucose, (B) insulin, and (C) NEFA were measured at 30-min intervals in beef calves that were exposed to either a combined dose of corticotropin-releasing hormone (CRH; 0.3 µg/kg BW) and vasopressin (VP; 1.0 µg/kg BW) or an endotoxin challenge (lipopolysaccharide, LPS; 0.5 µg/kg BW) administered at time 0. Values represent the change in circulating concentrations from baseline values from samples collected at 30-min intervals from -2 to 0 h.

Similar observations were made for concentrations of insulin, the major metabolic regulating hormone in the body. A dual challenge with CRH and VP in finishing heifers induced a rapid increase in insulin within 30 min post-challenge (Fig. 4B). This was followed by a gradual decline to basal concentrations by 2 h post-challenge. Challenge with LPS also induced an increase in insulin,
yet the increase and peak concentration were not observed until 2 h after LPS administration and had a magnitude that was 2-fold lower than what was observed in response to the CRH-VP challenge.

In addition to glucose, fatty acids can be used as a source of energy by many body systems, and under some induced or naturally occurring conditions may be the major source of energy used for homeostatic processes. In a recent study (J. A. Carroll, unpublished data) we have observed that concentrations of NEFA gradually increase in response to a CRH-VP challenge beginning at approximately 2.5 h and continue to steadily increase for 8 h post-challenge (Fig. 4C). In contrast, concentrations of NEFA induced by a LPS challenge display a more rapid and multi-phasic response profile. Specifically, 3 distinct peaks in NEFA concentrations were observed at 0.5, 4, and 7.5 h post-LPS challenge. Similar to NEFA concentrations induced by the CRH-VP challenge, NEFA concentrations remained elevated relative to baseline values for 8 h after challenge with LPS but to a far lesser extent than what was observed in response to the CRH-VP challenge.

Concentrations of NEFA can be increased by several endocrine factors, including but not limited to ACTH, cortisol, epinephrine, norepinephrine, and glucagon. These factors stimulate the release of lipases from the cytosol, subsequently resulting in the hydrolysis of triglycerides within adipose tissue and the release of fatty acids. Because these factors can be increased in response to exposure to a stressor as well as activation of the immune system, this clearly demonstrates the existence of a complex regulatory system between the stress and immune axes and metabolism.

The differential metabolic responses to a CRH-VP vs. a LPS challenge are easily explained when one considers the biological needs of the animal during times of stress vs. during an immune challenge. The fight or flight response triggered in reaction to stress requires a rapid and immediate response to the increasing energetic needs of an animal. In support of our observations, challenge with ACTH increases concentrations of glucose and insulin in plasma (Bazhan and Zelená, 2013). The energetic demands of the immune system in response to an antigen are far greater and longer in duration than those elicited by an acute stressor. For example, Kluger and Rothenberg (1979) stated that to increase body temperature 1°C, a 10% to 13% increase in metabolizable energy was needed. This increased energy demand does not account for the energy required for antibody and acute-phase protein synthesis or other immune processes that require additional energy resources outside of what would be required under normal homeostatic conditions. The multiphasic NEFA response observed following exposure of steers to LPS supports this increased demand for energy. The further increase in NEFA after the LPS-induced insulin and glucose responses indicates that the energy demand was greater than what could be provided by glucose alone.

Various factors can influence the strength of an immune response and thus the energetic demand of such a response. For example, the magnitude and duration of an immune response can be influenced by the virulence or immunogenicity of a pathogen, as well as the exposure rate of a specific antigen. Additionally, the development of a state of immunotolerance, as observed in persistently infected cattle, for example, may diminish or abolish an immune response and thus may require less energy mobilization and redistribution.

These data demonstrate that metabolic variables are yet another set of endocrine biomarkers that can be used to distinguish between stress and immune system activation in beef cattle. Although the temporal patterns and magnitude are challenge dependent, single-sample measurements are not likely to differentiate whether an elevation in these variables is due to acute stress or to acute immune activation. Further, these data demonstrate the multidisciplinary approach that must be taken during research, as manipulations to one body system can clearly influence several other systems.
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Interestingly, most people would consider leukocytosis to be indicative of disease, infection, or even cancer and leukopenia to be indicative of reduced hematopoiesis or a side effect associated with chemotherapy. In livestock production, leukocytosis is typically associated with an ongoing infection, and the animals are typically treated in such a manner. However, recent data from our laboratory provide some interesting insight into the concentrations of total white blood cell and differential white blood cell population shifts that occur in the bovine when it is exposed to an acute simulated stress (i.e., a combined dose of CRH and VP) or a bolus of endotoxin (i.e., LPS) as an initiator of the acute pro-inflammatory response.

In finishing crossbred beef heifers that were given a combined dose of CRH and VP (Fig. 5A), leukocytosis was observed by approximately 1.5-fold at 2 h following the challenge (Carroll et al., 2014). Likewise, lymphocytosis was observed by approximately 1.7-fold 2 h post-challenge. The leukocytosis and lymphocytosis persisted throughout 8 h post-challenge. Circulating concentrations of neutrophils were initially similar to the responses observed in total white blood cells and lymphocyte concentrations, peaking at 2 h post-challenge. In contrast, following the peak in neutrophil concentrations, a rapid neutropenia was observed throughout the 8-h period. However, when crossbred beef steers were challenged with an intravenous bolus dose of LPS (Fig. 5B), significant leukopenia, lymphopenia, and neutropenia were observed 1 h post-challenge (Burdick Sanchez et al., 2014a). As with the CRH-challenged heifers, circulating concentrations of neutrophils in LPS-challenged steers remained below baseline concentrations for 8 h post-challenge. Starting at 6 h post-LPS challenge, total white blood cells and lymphocyte concentrations gradually began to replete by 8 h post-challenge.

The peripheral neutropenia in both CRH- and LPS-challenged calves is not surprising and most likely reflects migration of these cells out of the circulation into various tissues. However, it may also be influenced by a decrease in hematopoiesis or increased apoptosis of neutrophils. The CRH-induced migration of neutrophils may be indicative of an immune-priming event in a preparatory manner associated with anticipated microbial invasion. For the LPS-induced neutropenia, the body would most likely interpret the circulating endotoxin as an indicator that microorganism (gram-negative bacteria) invasion has already occurred, and the neutrophils are being redirected to tissues or lymph nodes in search of the invader. However, this is where the similarities end with regard to the observed immune cell profiles. Given that the total white blood cell population is primarily composed of lymphocytes (i.e., ~70% of white blood cells are lymphocytes), it is not surprising that the observed changes in total white blood cell concentrations are similar to the changes observed in lymphocyte concentrations. It is interesting that whereas the LPS challenge caused a predictable acute decrease in lymphocytes, the CRH challenge elicited an opposing response of an acute increase in circulating concentrations of lymphocytes. Again, given that these two challenge models stimulate both a stress response and a pro-inflammatory cytokine response, one would speculate that the controlling factor(s) that mediates circulating concentrations of white blood cell populations is not a conserved mediator that is shared between these two distinct, but overlapping, endocrine responses.

SUMMARY AND CONCLUSIONS

These results clearly demonstrate that the different stress responses in beef calves, simulated by CRH, CRH and VP, or induced by endotoxin, are composed of specific immunological and stress hormone profiles that may be as unique as the stressors themselves. Although these results demonstrate stressor-specific responses in indices typically associated with animal health, further research is needed to fully elucidate the profiles of indicators of immunological and stress responsiveness to various managerial-, environment-, and/or pathogen-induced stressors. The results also imply that assessing the immunological status of an animal via a single parameter measured at a single time point may not be an accurate depiction of the animal’s overall health and could potentially be more reflective of a stress response associated with animal processing or handling. Developing methods to effectively attenuate potentially detrimental effects of various stressors encountered by livestock depends on understanding these unique stressor- and immunological-specific responses. However, as scientists continue to explore the intricate relationship between the stress and immune axes and their overlapping and biologically unique aspects, it is imperative that assiduousness is used with regard to potential variables (i.e., age, breed, environment, prior exposure, gender, health status, animal temperament, etc.) that are known to alter this convoluted relationship.

LITERATURE CITED


