2019

RELATIONSHIPS BETWEEN ANIMAL TEMPERAMENT AND SYSTEMIC IMMUNE RESPONSES IN BEEF CATTLE EXPOSED TO CONDITIONS ASSOCIATED WITH CONVENTIONAL MANAGEMENT

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Digital Object Identifier: https://doi.org/10.13023/etd.2019.033

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky

Director: Dr. Eric S. Vanzant, Associate Professor of Animal and Food Sciences

Lexington, Kentucky

2018

Abstract of Dissertation

RELATIONSHIPS BETWEEN ANIMAL TEMPERAMENT AND SYSTEMIC IMMUNE RESPONSES IN BEEF CATTLE EXPOSED TO CONDITIONS ASSOCIATED WITH CONVENTIONAL MANAGEMENT

Measures of temperament have been shown to influence physiological responses. Exit velocity (EV) has been identified as an objective, robust measure of temperament that can be used to predict subsequent performance of cattle. Additionally, previous studies from our lab indicate this measure of temperament may be related to production of interferon-γ (IFN-γ), a cytokine associated with cell-mediated immunity (CMI). Whereas research has investigated effects of EV upon immune responses, the overall goal of these studies was to examine this relationship under a variety of scenarios including human handling, transportation, and exposure to endophyte-infected tall fescue (E+) for determination of its ability to influence CMI in cattle.

In each of 5 experiments, calves were classified as either high or low EV animals, based upon measurements obtained prior to initiation of experimental periods. The hypothesis for these studies was that calves with high exit velocities would have lower systemic immune responses to applied treatments. Two experiments were designed to examine the relationship between exit velocity and lymphocyte IFN-γ production during and following a period of exposure to E+ seed and increased temperature humidity index conditions. Preliminary measures of this cytokine indicated a positive relationship with EV. During application of heat and E+ treatment application, no differences in IFN-γ production were detected between EV or endophyte treatment groups. However, in both experiments, after temperatures were returned to thermoneutral and E+ heifers were placed on the endophyte-free treatment, the positive relationship between exit velocity and total lymphocyte production of IFN-γ observed in baseline samples was reestablished. Similarly, during an experiment examining IFN-γ production by lymphocytes in steers during the 4 weeks following a 10h, 805 km transport study, average lymphocyte production of IFN-γ was higher and lymphocyte proportions producing IFN-γ lower in low EV steers, but total lymphocyte production of this cytokine did not differ between exit velocity treatments. In a grazing and finishing study, cattle were placed on E+ or novel endophyte pastures, with balanced representation of low and high EV treatments within each pasture. During the subsequent finishing period, blood samples for lymphocyte IFN-γ production were collected from a single high EV calf from each pasture group. Neither endophyte nor exit velocity was detected to be related with lymphocyte production of IFN-γ. In an experiment examining changes in cytokine gene expression changes during acclimation to human handling, IFN-γ, Il-6, IL-10, and IL-12 were observed to increase linearly over the experimental period in all calves, irrespective

of exit velocity designation. In the same experiment, whole period pro-inflammatory tumor necrosis factor-α expression was higher for high EV calves, but interferon-γ (IFN-γ) was lower in this same treatment group. These studies, cumulatively, indicate EV may be related to systemic production of IFN-γ, but abrupt changes to an animal's environment may serve to mask this relationship.

Keywords: lymphocyte, cattle, fescue, IFN-γ, temperament

Alexander Williams Altman

March 18, 2019
Date

RELATIONSHIPS BETWEEN ANIMAL TEMPERAMENT AND SYSTEMIC IMMUNE RESPONSES IN BEEF CATTLE EXPOSED TO CONDITIONS ASSOCIATED WITH CONVENTIONAL MANAGEMENT

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To God, for His holy purpose

Acknowledgements

I would first like to thank my Father in Heaven for all His blessings in my life, and all the opportunities which He has afforded me. In his letter to the Romans, Paul eloquently described God as one who continuously "works for the good of those who love him, who have been called according to his purpose." (Romans 8:28) He is the firm foundation on which my life is built, and it is that firm foundation that allows me to continue through this world with the confidence that I will always be provided with all I need to accomplish His purpose for my life. I owe all to Him, and my life is devoted to serving Him.

I would next like to thank my beautiful wife, Melissa, for her unfailing love and unwavering devotion. Her support is something I have come to depend on over the last few years, and I can't begin to fully express how blessed I feel to have her in my life. Wives have been described as lifesavers of their husbands. Melissa truly has been a lifesaver from the first day I met her, and my hope is that I will continue to be the man she deserves in her life.

I would also like to thank my parents and siblings for helping me to grow into the man I have become and continuously encouraging me to be the best I can. My parents have been excellent examples to me of how to approach each task life has to offer and given me the love that every person needs from their parents. My siblings have always been there to keep me humble and prop me up when my confidence began to fail. I wish everyone could be as blessed with family as I have been.

Finally, I want to thank my committee for their guidance through this process and assistance in completing this dissertation. Their patience with me through this process is a

testament to the dedication and professionalism with which they approach their jobs each day. I wish them all the best in their future endeavors. Specifically, I want to thank Dr. Vanzant for taking a chance on me as first a Masters, and then a Doctoral, student, and providing me with the opportunity to obtain both degrees.

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Chapter 1: Literature Review

Introduction

Regardless of the production scenario, calves will experience some form of stress, or a disruption to homeostasis following perceived endangerment that induces an adaptive response (Holsboer and Ising, 2010), within their lifetime. Common stressors experienced by beef calves may involve exposure to novel situations such as weaning, transport, and introduction to new herd mates (Chen et al., 2015), but may also include environmental factors, such as extreme ambient conditions (Hahn, 1999). Physiological responses to these stimuli may depend on a variety of factors including the duration of the stressor (Chappell et al., 1986; Dhabhar and Mcewen, 1997), previous exposure to the stimuli (Cordero et al., 2003), and current immune status of the animal (Von Borell et al., 2007). Additionally, these physiological responses may have further implications on growth performance and immune system responses (Duff and Galyean, 2007).

Human presence may be perceived by the calf as a threat, and as such may be considered a stressor. An animal's ability to identify and properly respond to a threat is vital to ensure its survival (Taylor et al., 2000). One visible mechanism through which this response proceeds is the fight-or-flight response, which is dependent upon acute changes in the adrenal gland (Cannon, 1929; Taylor et al., 2000; Goldstein and Kopin, 2007). Controlled by the sympathetic-adrenal-medullary axis (SAM), fight-or-flight responses occur very rapidly after perception of the stimulus by the animal, resulting in a cascade of neurological signals, from the cerebral cortex to the hypothalamus, which activate the autonomic nervous system (Lynch, 2010). This activation results in the production of catecholamines through 2 related, yet very distinct, pathways: 1) a direct

response is the production of norepinephrine by peripheral sympathetic nerve endings and 2) indirectly, epinephrine and a small amount of norepinephrine are produced by the innervated adrenal medulla (Griffin, 1989; Chen et al., 2015). These are the compounds directly responsible for the behavioral modifications associated with fight-or-flight responses (Chen et al., 2015).

In addition to activation of the SAM axis, exposure to a stressor also activates the hypothalamic-pituitary-adrenal (HPA) axis. When stimuli are perceived by the calf, the neuropeptides vasopressin and corticotropin-releasing hormone are released by the paraventricular nucleus of the hypothalamus, stimulating the anterior pituitary to synthesize and release adrenal corticotropin hormone (ACTH; Maier and Watkins, 1998; Burdick et al., 2011). This hormone then interacts with the adrenal cortex to induce production and release of glucocorticoids (Maier and Watkins, 1998). However, it is important to note that these two axes are intimately related. Glucocorticoid production by the HPA axis can be used to further enhance fight-or-flight responses by enhancing SAM axis catecholamine production, and catecholamines regulate hormone release from the hypothalamus, pituitary, and adrenal cortex (Carroll and Forsberg, 2007).

Although early models of stress suggested commonality in the neural and endocrine responses to various stressors (Selye, 1950), recent models have evolved to account for evidence that different stressors have unique mechanistic 'fingerprints' (Goldstein and Kopin, 2007). That evidence includes work which has shown that both the type and duration of stress can result in not only different endocrinological responses, but also different immunological responses (Dhabhar and McEwen, 1997; Bowers et al., 2008). Generally, acute stress responses are considered adaptive, whereas chronic

exposure to stress is typically associated with maladaptive, or pathological responses (McEwen, 2004).

Temperament

There is considerable variation among individual animals in how potent responses are following exposure to stressors. 'Temperament' is a descriptive term commonly used for characterizing differing animal responses to particular, common stressors (Friedrich et al., 2015). However, temperament is an aggregate of responses encompassing multiple traits that are both expressed and unobserved, making it exceedingly difficult to achieve a true measure of this behavioral complex (Kilgour et al., 2006; Réale et al., 2007; Friedrich et al., 2015). The concept of temperament generally implies an element of stability over time and the presence of intra-animal variation within a given species (Réale et al., 2007). Because temperament is considered to represent consistent differences in individual sensitivity to stressors over the long-term, it would seem that temperament-related influences on the SMA and HPA axes and their sequalae would align with those associated with chronic, as opposed to acute stress.

'Measures of temperament' do not directly assess differences in temperament among a group of animals, per se, but instead measure differences in various behavioral traits associated with temperament. With domesticated animals like cattle, many of the temperament-related measures of interest are those that involve amenability to interactions with humans. For example, measures such as chute scores, exit velocities, and pen scores are often considered to be measures of variation in animal response to interaction with humans (Adamczyk et al., 2013).

There are several reasons for measuring temperament-related behaviors, such as explaining differences in mating success and social status within a group of animals (Réale et al., 2007), or in gaining an understanding of how a particular response variable might be related to production characteristics of importance, e.g., growth and immunological function. Such relationships between outward, behavioral signs of differences (i.e. temperament-related measures) and physiological outcomes have been described. For example, withcattle, the HPA-derived hormone cortisol has been reported to be positively correlated with exit velocity (Fell et al., 1999; Curley Jr et al., 2006). So, in some ways, measures of temperament can be considered relatively easily monitored, behaviorally exhibited proxies of the tolerance of animals to stress-inducing conditions. However, because the common measures of cattle temperament are designed to assess animal reactions to human presence and/or activity, it remains unclear how such measures might relate to other commonly encountered stress-inducing scenarios which may or may not directly involve human interaction. Furthermore, given that different stressors result in different stress response patterns in a variety of animal models (Goldstein and Kopin, 2007; Bowers et al., 2008), and that the relationship of specific temperament-related measures to specific stressors is ill-defined and likely complex, the challenges associated with attempting to relate physiological outcomes to specific temperament-related measures should be apparent. With the current state of knowledge, it would be premature to attempt to develop comprehensive models to predict physiological outcomes associated with specific temperament-related measures for domestic livestock. What is currently needed is empirical data demonstrating such relationships. To be useful, such determinations need to be conducted using specific

temperament-related measures under specific management scenarios, with care taken not to confound interpretations by attempting to relate responses to broadly-defined concepts such as 'stress' and 'temperament', per se. With beef cattle production, one potentially useful temperament-related measure is chute exit velocity.

Exit Velocity

As a commonly used measure of animal behavior, exit velocity serves as a reliable, objective measure of temperament-related responses that is relatively inexpensive and does not interfere with the duration or efficiency of typical cattle processing practices. Exit velocity is calculated as the amount of time it takes an animal to traverse a set distance after release from the head gate, and can be easily applied to any production system (Burrow et al., 1988), as it only requires two set points and a timer. This moderately heritable (Burrow and Corbet, 2000) measure has been reported to be highly repeatable over time, (Petherick et al., 2002; Curley Jr et al., 2006; Bruno et al., 2016), with the first measure of exit velocity on cattle reported as a good predictor of subsequent measures, indicating only a few measurements may be needed to obtain a reliable assessment of a given animal's temperament using this methodology (Petherick et al., 2003).

Despite its popularity as a measure of temperament, there is some debate regarding what aspect of behavior exit velocity measures in cattle. Although it is often assumed that measures of temperament are indications of the variability between individual animals in fear responses (Petherick et al., 2002; Adamczyk et al., 2013), it has been suggested exit velocity and other measures of temperament may be measuring

multiple behavioral traits (Réale et al., 2007; Friedrich et al., 2015). Kilgour et al. (2006) used principle component analyses to examine which of their applied behavioral tests (i.e. fear of human, flight time after restraint, flight distance, etc.) explained the greatest amount of variation between animals in terms of behavior and ability to cope with various situations. The behavioral tests were initially assessed for variation within each test, repeatability of a given measure among animals, relationships between each of the tests, and the frequency of responses for each test. The behavioral tests that best accounted for the variation observed during the initial tests included flight time (similar to exit velocity, but without dividing time by distance), open-field test (i.e. visual isolation from herd mates), restraint test (time to catch the animal in the head gate), and a "following" test (how much area the animal covers when a human approaches from behind). Collectively, the authors described these behaviors as a measure of general agitation, as it is unknown what the combination of these tests describes. In particular, the tests which made up this first principle component did not involve the same level of human interaction, with two of them including no human presence at all. As these tests were determined to be related, this may indicate that exit velocity is measuring something in addition to fear of humans (Kilgour et al., 2006; Petherick et al., 2009b), but these factors remain unknown at this time. Petherick et al. (2009a) distinguished between the temperament-related facets of "general agitation" and "fear of people". In their work, by establishing a testing scenario in which exit velocity (or "flight speed") was assessed with animals blinded to the presence of humans, they concluded that exit velocity was associated with general agitation, whereas tests determining how closely cattle approached humans were more definitively associated with fear of people. Thus, the

complexity of exit velocity may provide an element of stability, making it resistant to the influences of variation in human handling and other environmental stimuli (Petherick et al., 2002; Petherick et al., 2003; Petherick et al., 2009a).

Due to this relative stability and repeatability across serial measurements, exit velocity may serve as a useful sorting factor for finishing cattle, as several studies have noted this measure of temperament is related to calf feedlot growth performance. Cattle with high exit velocities upon entry to the feedlot have been found to have decreased feed intake (Nkrumah et al., 2007; Cafe et al., 2011; Bruno et al., 2016) and average daily gain (Burrow and Dillon, 1997; Falkenberg et al., 2005; Bruno et al., 2016). However, there are mixed accounts of the relationship between exit velocity and gain efficiency, with reports of both no relationship (Cafe et al., 2011, Brahman Cattle; Bruno et al., 2016; Bruno et al., 2017) and both increased (Café et al., 2011, Angus cattle) or decreased efficiency in high exit velocity calves (Petherick et al., 2002). Cafe et al. (2011) asserted any relationship between this measure of temperament and growth performance is largely due to behavioral, rather than metabolic, influences on intake. This interpretation appears to assume that decreased intake is a consequence of decreased feeding time rather than the converse. It would seem that a more parsimonious explanation is that DMI is reduced in high exit velocity animals as a consequence of direct appetite suppressive effects of the endocrine changes associated with stress responses.

Handling

Increased handling of young calves has been demonstrated to decrease measured exit velocities (Burrow, 1991), possibly as a consequence of instilling a sense of trust,

rather than fear, of humans in calves at an early age. This effect was deemed more effective if the intensified handling period lasted longer than 3 months (Burrow, 1991). Interestingly, Petherick et al. (2009a) reported that cattle exposed to poor handling practices (i.e. increased noise, confined for extended periods in the working chute, slapping with open hands, handling of heads, etc) during backgrounding had a more rapid decrease in exit velocity over time than did calves minimally exposed to human contact, indicating that this measure of temperament is an innate trait that is not necessarily reflective of previous handling experiences. Others (Petherick et al., 2002; Curley Jr et al., 2006; Bruno et al., 2016) have also noted decreasing exit velocities in cattle following repeated exposure to human handling.

Transportation

Transport and handling are interlinked in the beef industry and may pose as predominant stressors that lead to immunosuppression as animals are introduced to new environments and social structures while interacting over a short period of time with new human handlers (Trunkfield and Broom, 1990; Chen et al., 2015; Brown and Vosloo, 2017). Transportation, in particular, has been described as one of the most stressful events in a calf's life (Mormede et al., 1982; Riondato et al., 2008). Periods of transport are associated with increases in cortisol and epinephrine concentrations noted in cattle following this activity (Burdick et al., 2010; Hulbert et al., 2011), with a positive relationship occurring between these hormones and exit velocity (Burdick et al., 2010). Effects of exit velocity on other variables in response to transportation have been noted in

cattle but are primarily immunology related and will be explored further in the next section.

Immunology

The immune system is a highly developed system whose purpose is seek out and eradicate any foreign material it encounters (Farmer et al., 1986). These responses can be broadly classified as either local or systemic based on extent the of the immune response. Local immune responses are contained within a certain area or tissue, whereas systemic immune responses affect multiple areas or tissues (Merriam-Webster, 2001). In either case, an effective immune response is the one that utilizes effector cells in an efficient manner to eradicate the insulting material from the body.

To facilitate this purpose, diverse signaling molecules known as cytokines (Janssen et al., 2010) are synthesized and secreted by several cells within the body including macrophages, monocytes, dendritic cells, and T cells (Fiorentino et al., 1991; Fadok et al., 1998; Geissmann et al., 2010) to regulate both innate and adaptive immune systems (Vilček and Feldmann, 2004). These molecules, as will be evidenced below, are involved in all aspects of immunity, and are key in directing the entire system toward an appropriate response.

The immune system is separated into two major branches: innate and adaptive (Murphy et al., 2012). The innate system is present at birth and is the generalized response to any foreign body (Lynch, 2010), and can be used to direct the adaptive immune response (Iwasaki and Medzhitov, 2010). This system is comprised of multiple first responders that serve as sentries for foreign material, recruiters for other immune

system components needed to remove the insulting material from the body, and antigen presenting cells (Medzhitov and Janeway, 1997).

Despite the distinction between innate and adaptive immunity, it is important to note that many of the members of the innate branch required for initiating the immune response are also critical for sustaining the adaptive response. For example, to combat rapidly replicating pathogens, components of innate immunity, such as macrophages and dendritic cells, may be utilized at sites of infection to activate and support B and T cell responses (LeGrand and Alcock, 2012; LeGrand and Day, 2016). Macrophages and dendritic cells are immune system mediators which serve as antigen presenting cells, or cells which phagocytize foreign material and present it to B and T cells to activate the adaptive immune system (Hamilos, 1989), and are primarily resident (macrophages) or circulatory (dendritic cells) in nature (Geissmann et al., 2010). However, these two cell types are also responsible for production of many of the effector molecules, or cytokines and chemokines, produced in the body. This synthesis is required during the innate response to assist in recruiting the correct cells to the site of inflammation, and in the adaptive response for differentiation, recruitment, and suppression of B and T cells (Geissmann et al., 2010).

Adaptive Immunity

The primary goal of an immune response is to eradicate the pathogen with minimal damage to host, which is accomplished through development of increased specificity to both recognize and eliminate the pathogen (LeGrand and Day, 2016). The adaptive branch of the immune system is developed throughout the course of an animal's

life, is heavily influenced by the pathogens encountered, and represents the level of specificity to which the immune system combats a given pathogen (Schenten and Medzhitov, 2011). Although this system is generally discussed separately from the innate system, and its effects are slower to appear, it is important to recognize that these two systems are intertwined and dependent upon one another for effectively combating a pathogen. Cell surface receptor expression of B and T cells, as determined by random chain pairing and rearrangement of somatic DNA, dictates the efficacy and efficiency of any adaptive immune response (Germain, 2002). An example of establishing adaptive immunity is through vaccination, which typically utilizes attenuated-live or killed pathogens administered to a subject to facilitate host development of antibodies and T cells specific to the pathogen(s) present in the vaccine (Murphy et al., 2012). This establishment of primary immunity is desirable to impede replication and promote eradication of the pathogen once it has been encountered, thereby reducing and/or preventing bodily harm (LeGrand and Day, 2016).

Adaptive immunity can be further broken down into humoral and cell-mediated responses. The simplest description to differentiate between these two responses is how the encountered antigens are processed: humoral responses address extracellular bacterial pathogens, whereas cell-mediated responses are used in eradication of intracellular bacterial and viral pathogens (Murphy et al., 2012).

T cells

T cells are developed in the thymus and divided into $CD4^+$ and $CD8^+$ lineages based upon the antigen recognition proteins on their surfaces (MHC class-II and MHC

class-I, respectively; Germain, 2002). $CD4^+$ T cells, commonly referred to as T helper cells, are involved in B cell activation, macrophage activation, neutrophil recruitment, and immune suppressive activity, in addition to other crucial roles within the immune system including production of a wide array of cytokines (Kumar et al., 2014; Zhao et al., 2018). This class of T cells is further divided into 5 subsets (T_H1, T_H2, T_{Reg}, T_H17, and T_{FH}) based upon effector functionality (Murphy et al., 2012). However, this review will focus on T_H1 and T_H2 subsets. These two subsets are equally important to host health, but act in support of different responses. The T_H1 subset assists with the detection and removal of intracellular pathogens (i.e. cell-mediated responses), whereas the T_H2 subset serves as a mediator of allergic, extracellular pathogen, and antibody production responses (i.e. humoral immunity; Scott, 1993; Romagnani, 1999; Dimitrov et al., 2004).

The balance between these two subsets is crucial to initiating and maintaining the appropriate immune response for a given invading pathogen and is dependent upon the cytokines to which undifferentiated $CD4^+$ T cells are exposed (Vukmanovic-Stejic et al., 2000). T_H2 T cells are activated by monocyte-derived interleukin-10 (Murphy et al., 2012). Upon activation, T_H2 T cells produce interleukin-4, which functions as the cell survival and growth factor for this $CD4^+$ subset (Minshall et al., 1997). Both interleukin-4 and interleukin-10, individually and in synergism, have been reported to suppress dendritic cell production of interleukin-12 and, by extension, cell-mediated responses (Koch et al., 1996).

In contrast to T_H2 cells, T_H1 T cells require interleukin-12 and interleukin-2 for activation and survival. Interleukin-12, which is secreted by activated dendritic cells and macrophages, is the cytokine responsible for T_H1 differentiation (Hsieh et al., 1993) and

inhibition of interleukin-4 production by T_H2 T cells (Manetti et al., 1993). Binding of this cytokine to T_H1 T cells and natural killer cells stimulates production of IFN-γ by this subset and $CD8^+$ T cells (Kobayashi et al., 1989; Hsieh et al., 1993). In addition to IFN-γ, T_H1 T cells also produce interleukin-2, which is used to stimulate proliferation and survival of these T cells (Duke and Cohen, 1986), sustaining and potentially prolonging the cell-mediated response. However, T cells activated in the presence of this cytokine also have enhanced expression of pro-apoptotic FasL and suppressed expression of the anti-apoptotic FLIP, indicating IL-2 may also assist in activating a feedback control mechanism to control T cell proliferation (Refaeli et al., 1998).

Interferon-γ

Upon recognition of an antigen or mitogen, activated $CD8^+$ and $CD4^+$ T_H1 T cells and natural killer cells produce IFN-γ (Emery et al., 1988; Fisher et al., 1997). Once released, this potent cytokine is used to activate macrophages (Nathan et al., 1983) and natural killer cells (Glaser et al., 1986). Activation of macrophages induces production of IL-12, which, as mentioned above, is used to differentiate $CD4^+$ T cells to the T_H1 subset, thereby potentially increasing the cell-mediated response. Increased production of IFN-γ has also been associated with increased Ig class switching in B cells, suppressed T_H2 differentiation, and increased expression of MHC class I on T cells (Murphy et al., 2012). Collectively, this indicates IFN-γ production assays are useful measurements of cell-mediated immune response capabilities in cattle (Fisher et al., 1997).

However, the immune system, much like other systems in the body, relies on balance to maintain homestasis. For example, increased concentrations of IFN-γ have

been associated with increased mortality in cattle after exposure to a viral pathogen alone (Van Wyk et al., 2016), and a secondary bacterial infection following primary infection with a virus (Hodgson et al., 2012). These experiments were designed to mimic bovine respiratory disease, a debilitating complex that plagues the beef industry with lost revenue in the form of high morbidity and mortality rates. It is currently unknown what constitutes the 'ideal' range of IFN-γ in cattle, making it necessary to investigate production of this cytokine as it relates to other measurable parameters. A possible explanation for the variation in concentrations of this cytokine in cattle may relate to measures of temperament.

Temperament and the immune system

Literature examining the relationship between temperament and the immune system in humans indicates a strong relationship between the HPA axis and cytokine production, such as pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α (Beishuizen and Thijs, 2003). Interleukin-1β, classically identified only as IL-1, has been of primary interest of researchers examining the behavior-immunology relationship, as this cytokine has been shown in multiple species to increase production of glucocorticoids by the HPA axis (Besedovsky et al., 1986; Dunn, 2000), with lesser responses also observed for IL-6 and TNF-α (Dunn, 2000). Collectively, these studies indicate an interlinking of temperament and immunity. Evaluation of these relationships may further assist in explaining some of the variation associated with vaccination efficacy, morbidity incidence, and mortality rates in cattle.

Interleukin-1β, interleukin-6, and tumor necrosis factor-α

In addition to being sensitive to cytokines, hormone production by the HPA and SAM axes (glucocorticoids, norepinephrine, and epinephrine) can influence the T_H1/T_H2 ratio thereby influencing cytokine production. For example, binding of glucocorticoids to receptors expressed on immune cells suppresses T_H1 cytokine production and stimulates production of T_H2 cytokines, such as IL-10 and IL-6, through β_2 adrenergic receptors (Elenkov et al., 1999; Komori, 2017). Conversely, binding of the hormone serotonin increases production of T_H1 cytokines IL-1β, IFN-γ, and TNF-α (Komori, 2017). Cortisol and norepinephrine have been demonstrated, *in vitro*, to suppress peripheral blood mononuclear cell (PBMC) production of lymphocyte growth hormone, which is necessary for lymphocyte proliferation, leading to a subsequent decrease of IFN-γ and a lower T_H1/T_H2 ratio (Malarkey et al., 2002). Maintenance of this balance is crucial to initiating the appropriate immune response since, as mentioned above, the two subtypes are suppressive toward one another.

Reports of the effects of HPA axis products on TNF-α production are inconsistent. Administration of glucocorticoids has been reported to suppress TNF-α production, with alleviation of this suppression occurring following administration of glucocorticoid receptor blockade (Barnes, 1998; Roggero et al., 2006). In *Bos indicus* cattle, injection of corticotropin releasing hormone stimulated an increase of IL-6 plasma concentrations relative to baseline, but similar increases were not observed in IL-1β, IFN-γ, or TNF-α concentrations (Cooke and Bohnert, 2011). However, in a follow-up study examining the influence of increasing concentrations of this hormone (i.e. 0, 0.1, and 0.5 μg CRH/kgBW) on these 4 cytokines, only TNF-α concentrations were influenced by treatment, with increased concentrations of this cytokine detected for the 0.1 μg

CRH/kgBW treatment compared with the other two levels (Cooke et al., 2012). Collectively, these studies indicate a mechanism other than the HPA axis may influence synthesis of TNF-α.

One such mechanism may be temperament, as measures of this behavioral trait have been associated with cytokine production. Rats with higher locomotion scores, which was used as an indication of temperament, produced lower TNF-α levels following a tail nicking stressor, with increased corticosterone production observed in the same group (Cavigelli et al., 2008). Attenuation of TNF-α production was observed in patients infused with epinephrine, as compared with non-infused patients, following administration of an LPS injection, indicating that catecholamines also serve as a suppressing agent to this pro-inflammatory cytokine (van der Poll et al., 1996). Deficiencies in IL-6 production have been associated with increased fearfulness in mice subjected to a maze with various obstacles (Armario et al., 1998). These mice were less exploratory and curious, but more cautious, as they moved about the maze.

Contrasting with elevated levels of IL-6, increased genetic expression of IFN-γ (as determined through SNP- genotyping using Allele Specific-Polymerase Chain Reaction) has been associated with inhibitive behaviors such as harm avoidance, introversion, decreased exploratory excitability, anticipatory worry, fear of uncertainty, and fatigability and asthenia in humans (MacMurray et al., 2014). Similarly, aggressive and dominant behavior in mice following suppression of IFN-γ has been reported (Hardy et al., 1990; de Groot et al., 1999; Bartolomucci et al., 2001) and may be related to an overall shift in responses to favor T_H2 over T_H1 (Bartolomucci et al., 2001). In addition, IFN-γ may also play a critical role in enhancing HPA axis suppression of pro-

inflammatory cytokine production. Treatment of murine macrophages with this cytokine induced an increased expression of glucocorticoid receptors by the macrophages, thereby increasing the sensitivity of these cells to the inhibitory actions of HPA products on proinflammatory cytokine production (i.e. TNF-α, IL-6, IL-12, etc.; Salkowski and Vogel, 1992). Collectively, these studies demonstrate the integrated nature of temperament with the immune system and indicate that future research investigating these relationships may better facilitate understanding of morbidity differences among cattle under similar conditions.

Transportation, temperament, and immune function

Following a period of transport, cattle may have altered cell-mediated immune responses, potentially making them more susceptible to disease (Simensen et al., 1980; Earley et al., 2017). Riondato et al. (2008) examined changes in leukocyte, neutrophil, and lymphocyte numbers in 24 male Blonde d'Aquitaine calves using a transportation model. Immediately following transportation, leukocyte numbers increased without any change in the other two cell types. However, neutrophil and leukocyte numbers were decreased 24 hours and 7 days after transport relative to pre-transport values. Interestingly, despite an overall increase in the number of lymphocytes present, the proportion of T cells were decreased immediately after transport. Similarly, Stanger et al. (2005) reported decreased leukocyte and eosinophil numbers and decreased proliferation of PHA-stimulated lymphocytes in *Bos* indicus steers, relative to pre-transport levels, subsequent to a 72-hour transport period. However, samples obtained from Brahman steers prior to and following a 16-hour transport period and stimulated with bovine viral

diarrhea virus had higher IL-4 and IFN-γ in $CD8^+$, $CD4^+$ T_H1, and $\gamma\delta^+$ T cells in transported, as compared with non-transported, steers (Van Engen et al., 2016). The discrepancy between these two studies may be attributable to differences in temperament, which were not accounted for in either study.

Temperament, as determined by averaging exit velocity and pen score, has also been reported to have a positive relationship with neutrophil counts and the neutrophil:mononuclear cell ratio, but a negative relationship was observed with adhesion molecules and neutrophil activity following a 24-hour transport period, indicating higher neutrophil numbers may be needed in temperamental cattle to overcome the deficiency in neutrophil function (Hulbert et al., 2011). However, other studies have reported no change in rectal temperatures following transport (Tarrant et al., 1992; Behrends et al., 2009; Burdick et al., 2010), although temperamental bulls have been reported to maintain higher rectal temperatures during transport in comparison to calmer counterparts (Burdick et al., 2010). Thus, temperament may assist in explaining some of the variation in immunity observed among beef calves following exposure to sudden changes in their environment. Another contributing factor to the observed variability in immune responses among animals may be due to nutritional differences, such as the debilitating effects associated with consumption of endophyte-infected tall fescue.

Fescue

Established by settlers and pioneers after its arrival from western Europe, tall fescue (*Festuca arundinacea*) has remained a popular choice due to its adaptability, ease of establishment, resilience, productivity, and appearance (Stuedemann and Hoveland,

1988). This cool season perennial is prevalent in the southeast United States, accounting for greater than 20 million hectares of pasture and hayfields (Bacetty et al., 2009). This area, known as "the fescue belt" (Campbell, 2012), is home to approximately 20% of the beef cows in the United States (West et al., 2007; Waller, 2009).

Many tall fescue varieties maintain a symbiotic relationship with the endophytic fungus *Epichloë coenophiala*, which provides the plant with many desirable traits to increase persistence against insects, drought, grazing tolerance, and poor soil conditions (Stuedemann and Hoveland, 1988). However, cattle grazing tall fescue pastures can experience a syndrome known as "fescue toxicosis", which has been associated with adverse growth, reproductive, and immunological issues (Hoveland, 1993; Allen and Segarra, 2001).

Currently, the mechanism(s) underlying the observed symptoms, including potential synergistic effects of the compounds produced by the endophytic fungi, how these compounds are metabolized in the rumen, and how metabolites of these compounds may further instigate these observed signs and symptoms of endophyte-infected (E+) fescue ingestion, have yet to be fully elucidated. This paucity of knowledge stems partly from the lack of a reproductive stage in the endophytic fungus (Latch, 1997), leading to a discrepancy in infection rate of this fungus in tall fescue progeny, causing variability in toxin production across pastures and years (Bacon et al., 1977; Welty et al., 1994). In addition, exposure of E+ seeds to ammoniation (Simeone et al., 1998a; Simeone et al., 1998b), heat, fungicides, the combination of increased temperatures with low moisture during storage (Siegel et al., 1987), and light and air (Garner et al., 1993) can adversely affect the viability and potency of this endophytic fungus.

Tall Fescue Alkaloids

One major unknown factor regarding animal consumption of E+ tall fescue is how each of the alkaloids synthesized by the endophytic fungus affect physiological responses in livestock. The main three compound groups produced are loline alkaloids, pyrrolopyrazine alkaloids, and ergot alkaloids, with the ergot alkaloids noted to be the most prevalent group of the three (Yates et al., 1985; Bush and Burrus, 1988; Jones et al., 2003). There have been several studies in cattle examining effects of these compounds on various responses including changes in vasculature (Oliver et al., 1993; Klotz et al., 2008), daily gains (Hoveland et al., 1983; Camp, 1986; Schmidt and Osborn, 1993), respiration (Jackson et al., 1984), and immunity (Filipov et al., 1999; Saker et al., 2001), but the differing concentrations of both fungi and toxins between tall fescue plants make it difficult to investigate these physiological responses effectively, particularly since some of these compounds are controlled substances.

Ergovaline, either individually or in synergism with other compounds, is believed to be at least one of the causative agents for many of the observed negative effects of fescue toxicosis, accounting for up to 80% of the observed decrease in average daily gain (Lyons et al., 1986; Peters et al., 1992; Porter, 1995; Liebe and White, 2018), with average seed concentrations of this alkaloid ranging from 0.1 and 6.0 μg/g (Belesky et al., 1988; Porter, 1995). To reduce the potentially high levels of ergovaline associated with E+ fescue, it is recommended these pastures be managed through mowing or continuous, heavy grazing to prevent seed head formation, as animals will selectively graze seed heads (Schmidt and Osborn, 1993). Similarly, dilution of E+ pastures with a non-infected forage, such as clover, has been demonstrated to mitigate several of the observed signs of E+ ingestion (Roberts and Andrae, 2004).

The negative effects associated with consumption of E+ tall fescue are commonly divided into four main categories: fescue foot, fat necrosis, reproductive problems, and summer slump.

Fescue Foot

Fescue foot was first reported in New Zealand (Cunningham, 1949). This disorder is attributable to a constriction of peripheral blood vessels in response to consumption of E+ tall fescue (Garner and Cornell, 1978). Appearing as quickly as 3 to 7 days after initial exposure to infected fescue, early clinical signs include an arched back, weight loss or reduced weight gain, and hyperemia at the coronary band of the rear leg (Bush et al., 1979) The onset of fescue foot is more commonly observed in cooler environments, as the colder weather causes vasoconstriction, which when induced in already constricted blood vessels due to alkaloid consumption (Ball, 1997), results in severe blood flow restriction to, and occurrence of gangrene in, the feet, ears, and tail tip, and, in severe cases, may lead to sloughing of the hoof (Garner and Cornell, 1978).

Fat Necrosis

Characterized by hard fat nodules, fat necrosis is another condition observed in cattle consuming E+ tall fescue (Thompson and Stuedemann, 1993). These nodules are typically found in the mesenteric adipose tissue along the entire intestinal tract, deeper yellow streaked with white and orange compared with the pale yellow observed in normal fat (Bush et al., 1979), and irregularly shaped small to large sized masses embedded within normal fat deposits (Townsend et al., 1991). Higher levels of nitrogen

fertilizer use in E+ pastures are believed to exacerbate development of fat necrosis (Stuedemann et al., 1985). Formation of the necrotic hard-fat in luminal spaces within the abdominal cavity has been reported to cause constriction of the intestines and subsequent restriction of digesta flow in the intestines (Williams et al., 1969; Wilkinson et al., 1983; Schmidt and Osborn, 1993; Thompson and Stuedemann, 1993).

Reproductive Performance

Consumption of E+ fescue by beef cattle has also been attributed to reproductive problems in females (Campbell, 2012), including a reduction in pregnancy (Schmidt and Osborn, 1993) and calving rates (Porter and Thompson Jr, 1992). Conception may also be delayed with exposure to E+, with an estimated conception rate decrease of 3.5% for every 10% increase in fungal infection of tall fescue (Schmidt et al., 1986; Schmidt and Osborn, 1993). A study by Burke et al. (2001) examined the difference between environmental conditions (heat stress vs thermoneutral) and endophyte treatment (E+ vs E-) on follicular and luteal dynamics and serum concentrations of estradiol and progesterone. Corpus luteum size was not affected by endophyte treatment, but consumption of E+ reduced serum estradiol concentrations at thermoneutral temperatures and serum progesterone during heat stress. Additionally, shorter luteal phases of the estrous cycle have been reported in E+ heifers (Jones et al., 2003). Estrogen, which has been reported to influence population proportions (Paavonen et al., 1981; Sthoeger et al., 1988; Jenkins et al., 2001), has been shown to influence noted to vary throughout the estrous cycle (Lyimo et al., 2000). Collectively, this may indicate that consumption of E+

feeds influence endocrine responses associated with normal reproductive function in female cattle.

Summer Slump

The most extensively studied of the four categories, summer slump, more commonly called fescue toxicosis, refers to the decreased growth performance, increased body temperatures and endocrine imbalances of cattle grazing E+ tall fescue during hot summer months (Schmidt and Osborn, 1993). Across the spring and summer grazing season, average daily gain for cattle on E+ pasture is estimated to decrease approximately 45 g/d for every 10% increase in the number of toxic endophyte-infected tillers present in the pasture (Williams et al., 1984; Crawford et al., 1989). It's possible these poor gains are a result of depressed intakes. The observed decreased intake may be related to an inability to dissipate heat properly (Aldrich et al., 1993), as consumption of fescue is associated with increased vasoconstriction (Solomons et al., 1989; Rhodes et al., 1991), possibly related to decreased concentrations of nitric oxide, a biochemical used by the body to dilate blood vessels (Al-Tamimi, 2002). Compared with cattle grazing E- pastures, those on E+ pastures during the summer spend more time grazing at night than the heat of the day (Bond et al., 1984b). Cattle exposed to a cyclic heat stress model (22°C to 33°C) while consuming E+ seed were found to have lower dry matter intakes than E- cattle in the same environment (Aldrich et al., 1993).

Residual influences of E+ consumption during grazing on subsequent feedlot growth performance has also been postulated in cattle. However, these growth performances have been reported to be, relative to E- cattle, both increased (Cole et al.,

2001; Duckett et al., 2001) and not different (Parish et al., 2013) during finishing. The apparent discrepancy in these studies may be attributable to differences in severity of nutrient restriction during grazing. Compensation for slowed growth due to restricted nutrition may be observed in ruminants once the restriction has been alleviated, with the degree to which these compensatory responses are manifested relating to the severity of the restriction experienced by the animal (Ryan et al., 1993). Interestingly, these compensatory responses are initially observed as increased efficiencies in gain and later apparent as increased dry matter intakes (Ryan et al., 1993). These differences in efficiency may relate to the observed reduced liver and gastrointestinal weights in animals on lower planes of nutrition (Murray et al., 1977; Johnson et al., 1987; Carstens et al., 1991). Following removal of the nutrient restriction, these relatively smaller organs may be responsible for increased protein deposition, making the animal more growth efficient (Carstens et al., 1991).

The decreased performance of cattle during hot summer months may also influence the immune system. Summer slump has been suggested to negatively impact beef cattle immunity (Saker et al., 1998; Allen and Segarra, 2001), with decreased leukocyte counts, MHC class II cell surface expression, and monocyte phagocytic activity reported in cattle following ingestion of E+ feeds (Saker et al., 1998). However, increases in humoral immunity have been consistently observed in cattle grazing E+, as compared with E-, pastures (Dawe et al., 1997; Rice et al., 1997), indicating a potential increase in responsiveness to vaccination in these cattle. But, rather than resulting as a direct effect of alkaloid consumption, this mechanism may also relate to the severity of nutrient restriction experienced by the animal rather than a direct effect of toxic

endophyte consumption. Dew (1989) reported that steers feed an E+ seed diet had similar titers to sheep red blood cell as those observed for steers on an E- seed diet, indicating that humoral responses are not influenced by endophyte treatment when cattle are on the same plane of nutrition.

Prolactin

Originally investigated for its involvement with mammary growth and lactation (Trott et al., 2008), prolactin has become the benchmark serum metabolite for studying fescue toxicosis in cattle, as consumption of toxic endophyte results in decreased prolactin synthesis in the anterior pituitary (Schillo et al., 1988). Synthesis of this hormone is positively correlated with daylength, resulting in a seasonal change in prolactin levels throughout the year (Bourne and Allen Tucker, 1975; Lincoln et al., 1978). Circulating serum prolactin concentrations are also controlled through the D_2 dopamine receptor, as stimulation of this receptor can suppress secretion of this hormone (Ben-Jonathan, 1985; Lamberts and Macleod, 1990). The decrease in prolactin during E+ exposure is through this receptor pathway, as ergovaline is a known dopamine agonist (Strickland et al., 1992; Strickland et al., 1994).

Prolactin is synthesized in the anterior pituitary (primary secretion site), hypothalamus, cerebral cortex, hippocampus, amygdala, septum, caudate putamen, brain stem, cerebellum, spinal cord, choroid plexi, circumventricular organs, placenta, amnion, decidua, uterus, mammary gland, and several lymphocytes (Freeman et al., 2000). This hormone is noted to be an active component of the immune system, as it can be secreted by immune tissues and has been reported as stimulatory, inhibitory, or ineffectual upon

immune cells (Yu-Lee, 1997; Marketon and Glaser, 2008). This apparent discrepancy among studies may reflect a dose-dependent nature of this hormone on immune cells, with a stimulatory effect observed at low concentrations and inhibitory effect at concentrations outside the physiological range (Yu-Lee, 1997). However, several studies using prolactin receptor knock-out mice have demonstrated that under normal conditions, healthy mice do not need prolactin for T and B cell and macrophage function, despite the appearance of this receptor on many of these cells (Dorshkind and Horseman, 2000).

Whereas prolactin is ineffectual on immune cell function under normal conditions, the application of stress may provide insight into the role of this hormone within the immune system. Mice under stressful conditions exhibit increased lymph node cellularity and antigen-specific proliferative responses, which may be due to prolactin counteracting the apoptotic effects of glucocorticoids (Dorshkind and Horseman, 2000). *In vivo* and *in vitro* apoptosis of T cells in rodents has been observed following administration of dexamethasone (Wyllie, 1980; Compton and Cidlowski, 1986; Krishnan et al., 2003), but is reversed when prolactin is administered (Krishnan et al., 2003). Thus, the decreased prolactin commonly associated with cattle grazing tall fescue may compromise the ability of these animals to mount a proper immune response, with high exit velocity cattle potentially further inhibited due to the increased glucocorticoids commonly associated with that subset of animals. Disease progression may also impact how much prolactin influences immune responses. A systemic lupus erythematosus (SLE) study in humans corroborates this theory, as incubation of PBMC cell cultures with prolactin has been observed to induce spontaneous production of IgG (Jacobi et al., 2001).

Hypothesis and Specific Aims

Collectively, the literature discussed in this review indicate the immune system maintains a strong relationship with animal temperament. Additionally, these studies indicated that exposure to stimuli deemed negative for cattle performance, such as tall fescue and transportation, are generally associated with negative effects on immune function. Therefore, the overall hypothesis for this dissertation was that animal temperament, as measured by exit velocity, would be related to variation in systemic immune responses of cattle, with exposure to negative stimuli (i.e. transportation, endophyte-infected tall fescue, and human handling) suppressing immune function to a greater extent in calves with high exit velocities.

Specific aims were as follows:

1. To determine if there is relationship between animal temperament, as measured by exit velocity, and cell-mediated immunity, as measured by production of interferon-γ, in the absence of other treatments.
2. To determine if consumption of endophyte-infected tall fescue exacerbates the effects of animal temperament on cell-mediated immune responses.
3. To determine if previous exposure to endophyte-infected tall fescue pastures influences subsequent feedlot growth performance, cell-mediated immune responses, titer response to vaccination, and carcass quality.
4. To determine if human handling induces different cytokine gene expression profiles in high and low exit velocity calves.

5. To determine if cell-mediated immunity is impacted differentially in low and high exit velocity animals during periods of transportation.

Chapter 2: Interactions between animal temperament and exposure to endophytic tall fescue: effects on cells of the innate and humoral immune systems in beef heifers

Abstract

Two experiments (n=12 Angus heifers/experiment) were performed to investigate the influence of exit velocity (evaluated at weaning) and consumption of endophyte-infected tall fescue seed on peripheral lymphocyte production of interferon-γ (IFN-γ). Heifers in both studies were selected from calves born on the University of Kentucky's Little Research Center, with selection in both studies based on weaning exit velocity measurement. In experiment 1, calves were randomly selected from, and representative of, all measured exit velocities within calf crop for that year. In experiment 2, calves were selected from those with the 9 fastest and 9 slowest exit velocities in the following year's calf crop. In both experiments, heifers were assigned to either high or low exit velocity treatments based on relative ranking, and endophyte treatments (endophyte-infected or endophyte-free fescue seed in ration; E+/E-), and treatment combinations were balanced by body weight. Rations (restricted to 1.8 x NEm) consisted of cottonseed hulls, cracked corn, soybean meal, and molasses, were balanced to meet vitamin and mineral requirements of growing heifers, and top-dressed with fescue seed each morning. Estrous was controlled using melangestrol acetate. Experiment 1 was divided into four phases (baseline, increased THI conditions, increased THI/endophyte treatment, and thermoneutral phases) and experiment 2 into two phases (increased THI/endophyte treatment, thermoneutral phases). During endophyte treatment phases, heifers were fed their respective E+/E- seed as part of their diet. During all other phases, all heifers were fed E- seed. In the first experiment, the proportion of lymphocytes producing IFN-γ was

decreased during the heat/endophyte phase in E+ heifers (P=0.03) and increased in E+ high exit velocity animals during the thermoneutral period (P=0.07). Average lymphocyte production of IFN-γ was higher in E+, and total lymphocyte production of IFN-γ was increased in high exit velocity heifers (P=0.10). In experiment 2, average lymphocyte production of IFN-γ was greater in E+ (P<0.01) and high exit velocity (P=0.05) heifers, and total IFN-γ lymphocyte production was lower in E- low exit velocity heifers (P=0.08) during the thermoneutral period. No differences in proportions of lymphocytes producing IFN-γ were detected during any period. These results indicate peripheral lymphocyte production of IFN-γ is influenced by both exit velocity and endophyte treatments following periods of increased THI.
Keywords: endophyte, lymphocyte, cattle, IFN-γ, heat

Introduction

Tall fescue (*Festuca arundinacea*) is the most prevalent forage produced in the Southeastern United States (Pendlum et al., 1980). Thus, many of the beef cattle raised in this region are exposed to tall fescue and its endophytic alkaloids from an early age. This grass has been found to contain an endophytic fungus (*Epichloë coenophiala*), which provides the plant with characteristics that allow for increased hardiness and drought resistance (Hill et al., 1991). The same fungus providing benefits to the plant is also detrimental to grazing animals, resulting in decreased gains and overall poor performance by the animal (Stuedemann and Hoveland, 1988). The negative effects of the alkaloids produced by this fungus upon the animal are compounded during times of heat stress (Spiers et al., 2012). Relatively little is known about the immunological implications of

endophyte exposure in cattle. Filipov et al. (1999) reported modulation of acute phase responses to ergovaline exposure. Similarly, steers grazing endophyte-infected tall fescue pastures and exhibiting characteristic signs of fescue toxicosis maintained lower major histocompatibility class II expression and phagocytic monocyte activity compared with steers grazing endophyte-free pastures (Saker et al., 1998; Saker et al., 2001). However, in all three of these studies, the authors were unable to separate direct effects due to ergovaline from potential effects of alkaloid consumption on DM intake. Thus, whether alkaloids per se directly influence immunological responses in cattle remains unknown.

One important immunological effector protein, or cytokine, that is crucial for combating viruses and intracellular bacteria is interferon-γ (IFN-γ). This cytokine is produced by natural killer cells, natural killer T cells, $CD8^+$ T cells, and $CD4^+$ T_H1 T cells. Interferon-γ is utilized by the immune system to upregulate differentiation of $CD4^+$ T cells to the T_H1 subset, which further enhances defenses against viruses and intracellular bacteria (Schoenborn and Wilson, 2007). Additionally, IFN-γ stimulates production of other pro-inflammatory cytokines through activation of macrophages (Boehm et al., 1997). Furthermore, recent studies indicated IFN-γ may differ among animals with different temperament rankings. Work from our laboratory has demonstrated that heifers with higher exit velocities had depressed systemic IFN-γ concentrations (Altman, 2015) and increased rectal temperatures (Altman et al., 2016) during an acute phase response to LPS injection.

A wide range of studies have demonstrated that temperament is correlated with growth rates in growing and finishing cattle, and that chute exit velocity has been the temperament-related measure most consistently related to growth. In addition to

relationships with growth, researchers have generally found that immunological function is compromised in so-called 'temperamental' cattle (Oliphint, 2006; Burdick et al., 2011). Oliphint (2006) reported that cattle with poor temperament scores had lower *in vitro* lymphocyte proliferative responses. Higher cortisol release, which has been correlated with poor temperament scores (Fell et al., 1999), may be associated with a decrease in lymphocyte proliferation in cattle (Blecha and Baker, 1986), and has been shown to reduce IL-12 and IFN-γ production in humans (de Jong et al., 1999). Combined, these studies suggest that temperament effects may be particularly important during the feedlot receiving phase, especially when considering the implications of suppressed immunological function coinciding with a period of high stress and exposure to novel pathogens. Periods of adjustment, such as during weaning and feedlot receiving, are stressful events for the calf which may compromise the immune system (Blecha et al., 1984; Duff and Galyean, 2007). An animal's instinctive response to stimuli, or temperament, may further compound any negative effects of a new environment. Potential interactions between temperament and endophyte exposure on immunological function might help explain phenotypic variation among individual animals in health responses during the feedlot receiving phase subsequent to fescue grazing.

The hypothesis for these experiments was that calves with higher exit velocities would maintain lower lymphocyte IFN-γ production and consumption of endophytic alkaloids would further decrease this response in an additive fashion (i.e. that exit velocity and alkaloid consumption do not interact). Experiment 2 (EXP 2) was developed to refine the model used in EXP 1, specifically by focusing on calves selected from the two extremes of the measured exit velocity range to enhance the ability to detect potential

effects of this factor upon lymphocyte IFN-γ production Furthermore, we hypothesized that these responses would be particularly evident while animals were consuming alkaloids and that they would return to baseline levels after alkaloids were removed from the ration. Thus, our objective was to determine whether animal temperament (as measured by exit velocity) and endophytic alkaloid consumption interact in their effects on the humoral and cell-mediated immune responses of healthy cattle, and to describe the general nature of the effects of both factors.

Materials and Methods

All methods were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Each experiment used a completely randomized design with repeated measures. There were two dietary treatments (endophyte-infected (E+) and endophyte-free (E-) fescue seed) and heifers were categorized within one of two flight response designations (high/low), as determined by exit velocity (see below). Endophyte treatment assignments were balanced for weight and exit velocities. Heifers were placed in individual pens (3.0 x 3.7 m) inside an environmentally controlled research building for the duration of the experiments.

Experiment 1

Cattle and Processing

Twelve Angus heifers (277 ± 29.3 kg BW; 248 ± 21 d of age) out of 8 sires, born on the University of Kentucky Oran C. Little Research farm, were utilized in this

experiment. At weaning, heifers were vaccinated against bacterial and viral pathogens (Bovi-Shield Gold 5, Zoetis, Florham Park, NJ; Once PMH, Merck Animal Health, Summit, NJ; Somubac, Zoetis; Ultrachoice 7, Zoetis; Autogenous Pinkeye, Central KY Vet Center), and given an injection of anthelmintic (Dectomax, Zoetis). Exit velocity, a measure of temperament, was also recorded at this time, and was calculated as the time it took for a heifer to transverse 1.68m upon release from the headgate, as measured using an infrared trip-wire system (Bruno et al., 2016). These measures were used to designate calves as either high or low exit velocity, based upon relative position in the exit velocity spectrum for these 12 heifers (1.20 to 3.06 m/s, mean = median = 1.96 m/s). Although exit velocity measures vary over time, and it may seem natural that averages of serial measures might produce estimates with lower variance, studies from our lab and others indicate that initial measures of exit velocity appear to be more valuable predictors of future performance and carcass characteristics due to the naivety of the animal (Behrends et al., 2009; Bruno et al., 2016; Bruno et al., 2017). Following the weaning period, heifers were halter broken for adaptation to human handling.

The experiment consisted of 4 periods: thermoneutral and all calves on E- diet (d1-21; P1), increased temperature-humidity index conditions (THI) and all calves on E- diet (d22-28; P2), increased THI and calves on respective E+ and E- diets (d29-50; P3), thermoneutral and all calves on E- diet (d51-78; P4). Body weights were recorded at the start of the experiment (d1), during weekly blood collections (d8, 15, 22, 29, 36, 43, 50, 57, 64, 71), and at the end of the experiment (d78). All calves were naïve to endophyte infected fescue, thereby preventing any potential effects of prior exposure from

influencing results. Daily feed and water consumption were recorded throughout the experimental period.

The basal diet (Tables 2.1 and 2.2) was prepared prior to initiation of the experiment, without inclusion of fescue (E+ and E-) seed. This was to ensure only the presence of ergovaline (10µg/kg BW) differed between treatment diets. Rations were restricted to 1.8 x NEm, and initially provided based upon starting weights, then increased to match current body weight at the start of the fescue exposure period to ensure heifers received the targeted ergovaline dose. Pens were cleaned prior to feeding and heifers were fed once daily, at 0700 a.m., Heifers were randomly assigned to treatment diets, with groups balanced for weight and weaning exit velocity. All feed offered to heifers was consumed throughout the duration of the study.

Melangesterol acetate (MGA; 0.5 mg/hd/d) was provided to control for the effects of estrous and offered prior to the basal diet each day to ensure all heifers consumed the daily dosage. Whereas this medicated feed additive has been reported to negatively influence immunological responses (Corrigan et al., 2007), the noted differences in that study were to agents of the innate immune system involved with oxidative burst, with no differences observed in lymphocyte and monocyte concentrations due to MGA inclusion. Conversely, several studies have demonstrated an influence of estrogens, whose synthesis is inhibited by MGA, on lymphocyte populations (Paavonen et al., 1981; Sthoeger et al., 1988; Jenkins et al., 2001). Estrogen concentrations will vary over time as a result of estrous cycling (Lyimo et al., 2000). Thus, as both experiments within the current study were specifically interested in production of interferon-γ by lymphocytes, and experiment

2 additionally examined humoral responses to an extracellular bacterial challenge, inclusion of MGA to control for these potential fluctuations was deemed necessary.

Fescue seed (E+/E-) was top-dressed on the basal diet and mixed by hand each morning to ensure appropriate seed inclusion for each heifer. Endophyte-infected seed was only provided to E+ cattle during P3 of the study, with E- seed included in place of E+ seed during P1, P2, and P4. All heifers, regardless of diet and phase, consumed all offered rations each day.

Environmental temperatures were monitored using a Hobo environmental temperature logger set to record ambient temperature and humidity every 5 minutes (Fig. 1.1). On d1, heifers were randomly assigned to individual pens (3.0 x 3.7 m) inside an environmentally controlled barn, where they remained for the duration of the study. Ambient temperature was set at 22.2°C and remained at this level during P1of the experiment. Calves were fed E- fescue seed with the basal diet during this time to establish baseline levels of lymphocyte IFN-γ production. At the start of P2, ambient temperatures were set to cycle between 33.3°C during the day and 22.2°C at night. This was to simulate average temperature fluctuations experienced by cattle in Kentucky during July and August, when effects of fescue toxicosis are at their most severe (Hemken et al., 1981) and has been previously shown to be sufficient for inducing signs of fescue toxicosis in cattle during periods of tall fescue consumption (Spiers et al., 2012). Temperature humidity index (THI) was calculated as described by Hahn (1999):

$$THI = \text{Dry Bulb Temperature} + (0.36 * \text{Dew Point Temperature}) + 41.2$$

Increased THI conditions continued through P3, when heifers were started on their respective endophyte seed treatments and remained on these diets until the end of this period (d50). At the start of P4, ambient temperatures returned to continuous thermoneutral conditions, and all heifers returned to the E- diet until the conclusion of the study on d78.

Blood Sampling

Heifers were placed in a headgate once a week, pre-prandial, for collection of blood samples (d1, d8, 15, 22, 29, 36, 43, 50, 57, 64, 71, and 78). A total of 45 mL of blood was collected from every heifer via jugular venipuncture into 3-15 mL Na-heparin tubes (i.e. "red top tubes" with 1 drop of 1,000 usp·mL^{-1} heparin added) to isolate peripheral blood mononuclear cells (PBMCs). These isolated PBMCs were then utilized for *in vitro* analysis of IFN-γ production by lymphocytes.

PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples following a modified protocol of Breathnach et al. (2006) and . Briefly, samples were centrifuged at 800 x g for 30 minutes with a slow brake. The buffy coat was removed, added to 10 mL phosphate buffer solution (PBS), layered over 10 mL Ficoll-Paque Plus™ solution (Amersham Biosciences, Piscataway, NJ), and spun at 500 x *g* for 30 minutes with a slow brake. Cells were harvested, added to 20 mL PBS, and centrifuged at 500 x *g* for 10 minutes with a fast brake. All subsequent centrifugation was performed at 300 x *g* for 10 minutes with a fast brake. Supernatant was removed and cell

pellets resuspended in 5 mL PBS. To this suspension, 10 mL D_2O and 10 mL RPMI were added, with suspension diluted to a final volume of 45 mL with PBS, after which the suspension was centrifuged. Cells were then washed in PBS again, recentrifuged, and resuspended in 10 mL of PBS. From this 10 mL suspension, 100 µL were mixed with 900 µL of PBS to be counted using a Vicell Counter-XR (Beckman Coulter, Miami, FL). Resulting data was used to calculate volume needed from 10 mL suspension to plate at 4 x 10^6 cells/mL. Desired volume was transferred to a 15 mL tube and centrifuged. Supernatant was removed and cells resuspended in 4 mL cRPMI (consisting of 10% fetal bovine serum, 1% penicillin-streptomycin-glutamine, 0.1% 2-mercaptoethanol, and 88.9% RPMI, and plated in 4 wells of a 24 well plate at 1 x 10^6 cells/mL.

Stimulation of samples

PBMC samples were arranged in duplicate in a 24-well plate with each sample type having a control well and stimulated well. Two µL of brefeldin A (BFA) were added to each well to prevent protein secretion (i.e. IFN-γ; Fujiwara et al., 1988) and allow for measurement of IFN-γ production in control and stimulated cells. The control sample wells additionally received 10 µL of phorbol 12-myristate 13-acetate (PMA)/ionomycin (Breathnach et al., 2006) to preferentially activate and maximize IFN-γ production by T_H1 lymphocytes (Baran et al., 2001). Following inoculation, well-plates were incubated in 5% CO_2 for 4 hours at 37°C (Breathnach et al., 2006). At the completion of the 4-hour incubation period, 200 µL were transferred from each sample well to a 96 well plate and centrifuged at 500 x *g* for 5 minutes. Supernatant was removed from the wells, and cells

were fixed in 100 µL of 2% paraformaldehyde, and the plates were placed in a 4°C refrigerator overnight (Breathnach et al., 2006).

Intracellular staining and flow cytometry

Following overnight incubation in the refrigerator, cells were centrifuged at 500 x g and resuspended in 150 µL saponin buffer (1% FBS, 0.1% saponin, and 0.1% sodium azide). Prior to flow cytometry analysis, cells were stained with mouse IgG1 anti-bovine IFN-γ FITC conjugated antibody (Thermo-Fisher, Waltham, MA) at a concentration of 10 µg antibody/mL saponin buffer. Suspended cells were incubated on ice for 30 minutes, then centrifuged at 500 x *g* for 5 minutes twice, with intermediary resuspension in saponin buffer. After the second centrifugation, cells were resuspended in FACS buffer in preparation for analysis. Using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), PBMC samples were gated around lymphocyte populations using forward and side scatter parameters. Thirty thousand gated events per sample were acquired for this analysis. Control samples were gated at 1%, with stimulated samples compared to the control sample to determine treatment responses. Samples were analyzed for percent of gated samples expressing IFN-γ and the mean expression of IFN-γ per cell within the gated population (MFI). Post-analysis, these two parameters were multiplied together to obtain an approximate measure of total IFN-γ production by the gated lymphocyte population (Darrah et al., 2007).

Experiment 2

The following year, twelve Angus heifers out of 8 sires (247 ± 28.2 kg BW; 219 ± 15.2 days of age), born on the University of Kentucky Oran C. Little Research farm, were split into high (n = 6; weaning exit velocity = 3.15 ± 0.496 m/s) and low (n = 6; weaning exit velocity = 1.42 ± 0.338 m/s) exit velocity treatments and placed on either a endophyte-infected (E+) or endophyte-free (E-) diet, so that a 2 x 2 factorial treatment structure was created. Calves were weaned, weighed, and evaluated for weaning exit velocity (EV) 50 days prior to the start of the experiment. Exit velocity, obtained at weaning, was used as a measure of temperament, and calculated as the time it took calves to transverse 1.68m upon release from the headgate. From these measures, calves with the highest (n = 6) and lowest (n = 6) exit velocities measured for heifers in that calf crop (n = 47) were selected, as we believed differences in IFN-γ production by lymphocytes would be more evident between the extremes of the flight response spectrum. Subsequent to the weaning period, heifers were halter broken for adaptation to human handling. During this time, heifers were vaccinated against bacterial and viral pathogens (Bovi-Shield Gold 5, Zoetis, Florham Park, NJ; Once PMH, Merck Animal Health, Summit, NJ; Somubac, Zoetis; Ultrachoice 7, Zoetis).

The basal diet was similar to and prepared as described for EXP 1 (Tables 2.3 and 2.4). Feed was again offered at 1.8 x NE_m, based on initial BW, and remained constant throughout the experimental period, with melangesterol acetate provided at 0.5 $mg \cdot hd^{-1} \cdot d^{-1}$ to prevent estrous cycling during the study. Endophyte treatments were applied during P1 (d1 to 28) of the experiment, with the appropriate seed (E+/E-) provided for each heifer. Calves on the E+ treatment received endophyte-free seed in place of endophyte-infected seed during P2 (d29 to 57) to observe residual effects of endophyte

exposure upon immune responses. Heifers consumed all offered feed each day, ensuring all calves consumed targeted amounts of ergovaline. Water intake for each heifer was also recorded each morning. Pens were sprayed clean daily to remove any fecal material and urine present in the pen to maintain a clean area for the calves

Body Weights

Calf body weights were recorded at the start (d1) and conclusion of the experiment (d57). To reduce the risk of exposing heifers to outside pathogens, blood samples were collected in the animal pen rather than the head chute as in EXP. 1. Therefore, we were did not collect interim body weight data in EXP 2. Thus, average daily gain (ADG) and gain to feed (G:F) were determined using only experiment start and ending weights.

Room and Rumen Temperature

At the start of the experiment, pen room temperatures were programmed to follow the cyclical temperature pattern to establish an increased THI environment during P1, as described for EXP 1. Room THI was also calculated as described in EXP 1. Environmental conditions in the pen room were monitored using a Hobo environmental temperature logger set to record ambient temperature and relative humidity every 5 minutes. Temperatures were lowered to continuous thermoneutral (22°C) following the E+ consumption period (d29) and remained there for the duration of the study.

On day 15 of the study, a temperature-monitoring rumen bolus (SSL001-CT, SmartStock, Pawnee, OK) was placed in each heifer. Boluses were set to record

temperature every 15 minutes, and wirelessly transmitted this data to a computer in the next room. Boluses continued recording data through d 48.

Blood Sampling

Methods for collection of blood samples for *in vitro* lymphocyte analysis were as described for EXP 1, with the exception that samples were collected in the pen rather than in a head gate. This change in methodology was an attempt to minimize stress associated with collections and the chance of exposure to any pathogens existing outside the pen room. Additionally, as the experiment was shorter in duration, samples were only collected on d8, 15, 29, 36, 43, and 57. Samples were collected on d1, but due to a malfunction of the flow cytometer, those results were excluded from analysis. Following this incident, sample analysis was performed using an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA). An additional 10 mL of blood was collected via jugular venipuncture into 10 mL additive-free Vacutainer (Becton Dickinson, Franklin Lakes, NJ) tubes for serum analysis of humoral immunity on d57.

Humoral Immunity

Treatment effects on humoral immunity were assessed by serum antibody titer responses following a vaccination protocol timed to produce a peak response on the last day of the experiment. To accomplish this, animals received a primary vaccination against *Leptospirosis pomona* (L5 SQ, Merck) on d29, and a booster vaccination on d43. Serum blood samples were collected on d57 and subjected to a microscopic agglutination test for detection of *Leptospirosis pomona* antibodies as described by Bruno et al. (2018).

Statistics

Experiment 1

Measures of total lymphocyte population and average lymphocyte production of IFN-γ, in addition to proportions of lymphocytes producing this cytokine, from d1, 8, 15, and d22 (i.e. pre-heat and pre-endophyte treatment measurements) were averaged separately to obtain a representative value for each animal's baseline IFN-γ production. As part of our assessment of the potential validity of using these values to assess exit velocity as a predictor of a calf's vulnerability to intracellular pathogens, these baseline values were regressed against weaning exit velocity to ascertain if a relationship between baseline cell-mediated immunity and exit velocity existed. In addition, the responses from d1 of EXP 2 (obtained using the same flow cytometer as EXP 1) were included in this regression analysis. One heifer in EXP 2 was excluded in this regression due to low cell count numbers. Proc Reg of SAS (9.4; Cary, N.C.) was utilized to perform this analysis, with weaning exit velocity (as a continuous variable) and experiment (EXP 1/EXP 2) included in the model statement.

Weekly peripheral blood mononuclear cell data from d8 through d78 were analyzed using the mixed procedure of SAS (9.4; Cary, N.C.), with endophyte treatment (E+ and E-), weaning exit velocity designation (high and low), and sampling date as the main effects, and Kenward-Roger selected as the denominator degrees of freedom method. Average lymphocyte production of IFN-γ production and the proportion of lymphocytes producing this cytokine obtained from the flow cytometer analysis were analyzed independently and multiplied together to approximate the total lymphocyte

population production of IFN-γ (Darrah et al., 2007), which was subsequently natural log transformed to obtain a normalized data set for the assumptions of ANOVA. Data was analyzed separately within the 4 periods, as described above: d8, 15, 22 (P1; no endophyte or heat); d 29 (P2; heat but no endophyte); d36, 43, 50 (P3; heat and endophyte); d57, 64, 71, 78 (P4; no heat or endophyte). Data from P1, 3, and 4 were analyzed using the repeated measures option with a first-order autoregressive covariance structure, with sampling date included as the repeated effect in the model. An average of the three sampling points from P1 was included as a covariate in the model statement for each of the three IFN-γ measurement parameters to account for variability among samples, as the covariate was parallel among treatments across time.

Water consumption was averaged by week and divided by the animal's body weight to standardize values as a percentage of body weight, and natural log transformed to meet the assumptions of ANOVA. Water consumption data from before day 15 was excluded to allow for heifer familiarization to waterer usage. Main effects included in the model were endophyte, exit velocity, and their interaction, with denominator degrees of freedom calculated using the Kenward-Roger method. Data was analyzed within period.

Average daily gain was analyzed using the GLM procedure of SAS (9.4) and calculated as daily gains within 3 periods: d 1 to 29 (P1 and P2), 29 to 57 (P3), and 57 to 78 (P4), as well as for the whole experiment. Main effects were endophyte, exit velocity treatment, and their interaction.

Experiment 2

Leptospirosis pomona titer responses, ADG, and G:F were analyzed using the GLM procedure of SAS (9.4, Cary, N.C.). Prior to analysis, titer responses were natural log transformed to provide a normalized data set, thereby meeting the assumptions of ANOVA. The models for these three variables included endophyte treatment, exit velocity, and their interaction. The interaction terms endophyte x week and exit velocity x week were pooled with the error term for ADG and G:F models after initial analysis ($P \geq 0.58$). Water intake was analyzed by period (P1 and P2), whereas ADG and G:F were analyzed for the entire experimental period (d1 to 57).

Lymphocyte production of IFN-γ measures were natural log transformed to provide a normalized data set, and water intake data was averaged by week. Water intake, humoral, and PBMC data were divided into two phases, which corresponded with the experimental timeline. Data was analyzed using the mixed procedure of SAS (9.4) using the repeated option with a first-order autoregressive covariance structure and heifer specified as the subject. The Kenward Roger method was used to calculate denominator degrees of freedom, and the model included endophyte, exit velocity, their interaction, week, endophyte x week, and exit velocity x week. After initial analysis, endophyte x week and exit velocity x week were pooled with the error term for the PBMC model in both periods, as no interactions with week were detected ($P \geq 0.37$).

Ruminal temperature was analyzed for amplitude, acrophase, and mesor parameters for each heifer using MATLAB Release 2013b (The MathWorks, Inc., Natick, MA). The three variables were then analyzed using the mixed procedure of SAS (9.4) with repeated options using the first-order autoregressive covariance structure and heifer specified as the subject. Denominator degrees of freedom were calculated using the

Kenward Roger method, and the model included endophyte, exit velocity, their interaction, and week.

For all analyses, significance was set a $P < 0.10$, and trends considered at $0.10 < P < 0.15$.

Results

Experiment 1

Room Temperature

Room temperature humidity index (Fig. 2.1) fluctuated on d 22 through d 50 as designed, creating an environment noted to decrease steer growth performance (Hahn, 1999) during the day and allowing for cooling in the evening to simulate mid-summer ambient conditions in central Kentucky. Similarly, temperatures remained constant during the designated thermoneutral periods, allowing for a THI conducive to optimal steer growth performance (Hahn, 1999), thus providing an appropriate climate for collection of baseline measurements and to determine the time required for animals to return to baseline levels after removal of the increased THI and E+ treatment.

Peripheral blood mononuclear cells

Results from the analysis of harvested PBMCs are presented in Table 2.5 and Figures 2.2, 2.3, and 2.4. A weak correlation was observed between weaning exit velocity and baseline (P1 of EXP 1 and d1 of EXP 2) total lymphocyte IFN-γ production (R^2 = 0.09; P = 0.05; Fig. 2.2) and the proportion of lymphocytes producing IFN-γ (R^2 = 0.22; P = 0.03; Fig. 2.3). The average amount of IFN-γ produced by lymphocytes during the baseline periods was not related to weaning exit velocity (P = 0.062; Fig. 2.4).

There were no effects of exit velocity, endophyte, or their interaction on lymphocyte proportions, average production, or total production of IFN-γ during P1 or P2 (P ≥ 0.18). The proportion of lymphocytes producing IFN-γ was approximately 45% higher in heifers on the E- diet during P3 (P = 0.03). In P4, this proportion was approximately 40% greater in high exit velocity heifers in the E- treatment group (P =

0.07) compared with the other treatments and total production of IFN-γ was approximately 41% lower in low exit velocity heifers (P = 0.10). Also during this period, average lymphocyte production was 24% higher in E+ heifers (P < 0.01) and tended to be greater in high exit velocity heifers (P = 0.12). Interactions between endophyte and exit velocity treatments were not detected during any period for total or average lymphocyte production of IFN-γ (P ≥ 0.15).

Water Intake

Average daily water consumption (Table 2.6) was not affected by exit velocity or endophyte treatments, or their interaction. Across all treatments, average water intake increased numerically during P3 and returned to baseline levels during P4 (Fig. 2.5).

Body Weight

There were no differences detected for average daily gain between treatments during any period. Results for this measure of growth performance gain are presented in Table 2.7.

Experiment 2

Environmental thermal heat index

Data on environmental conditions within the pen room are present in Fig. 2.6. Due to data logger failure, temperature and humidity was recorded by a primary monitoring system on only 39 days of the 56-d experimental period. Data were collected during this time with a secondary back-up system with less resolution, but sufficient to ensure that temperatures were within control limits. During the study, on d1 through d20,

mechanical failure occurred with the air handling system, allowing ambient conditions to deviate from the designed protocol. However, as is evident in Fig. 2.6, the temperature humidity index (THI) during the heat and endophyte period (P1) was above that ascribed by Hahn (1999) to cause perturbations to homeostasis in cattle. Similarly, most of the days during the thermoneutral period (P2) were below this THI level. The last two days on the graph, as well as d 52 (data not shown due to data logger failure), are above this threshold due to mechanical failure and a county-wide power failure, respectively, resulting in the air handler to be temporarily offline. With the exception of these 3 days, temperatures during P2 were constant and remained below the THI threshold as intended.

Ruminal temperature

Ruminal temperature data are presented in Table 2.8. Differences in ruminal amplitude and mesor, or average, temperatures were not detected during P1 ($P \geq 0.20$), but an endophyte x exit velocity interaction was observed for both responses during P2 ($P = 0.08$ and 0.06, respectively). The differences were confined to the E+ group, with low exit velocity heifers experiencing approximately 0.19°C lower mesor, whereas high exit velocity heifers coming off of the E+ diet experienced approximately 80% greater amplitudes in diurnal ruminal temperature cycles. Conversely, no differences in acrophase were observed for either period, although a trend was detected for exit velocity effects in P1 ($P = 0.12$), with high exit velocity heifers on the E+ diet experiencing peak circadian body temperature approximately 2.24 hours after the other three groups.

Cell-mediated and humoral immunity

All three measures of cell-mediated immunity (proportion of gated cells producing IFN-γ, average and total lymphocyte production of IFN-γ), as measured by flow cytometry, differed by week in P1 and P2, apart from average lymphocyte production of IFN-γ in P1 (Table 2.9). Baseline and P1 measures of IFN-γ did not differ due to endophyte and exit velocity treatments or their interaction ($P \geq 0.18$). Similarly, no treatment effects were observed in the proportion of lymphocytes producing IFN-γ in either period. During P2, average lymphocyte production of IFN-γ was 15% and 17% greater in E+ ($P < 0.01$) and high exit velocity ($P = 0.05$) heifers, respectively, in this period as well. Total lymphocyte production of IFN-γ by E- low exit velocity heifers was approximately 40% of observed production by the other three treatment groups ($P = 0.08$).

Titer responses (Table 2.10) were not affected by either treatment or their interaction ($P \geq 0.52$). As this was a one-time measurement, no time effects were analyzed.

Average daily gain and gain:feed

Despite restricted intake, measures of animal growth performance (Table 2.10) were not influenced by endophyte or exit velocity treatments ($P \geq 0.11$).

Water intake

In both periods, water intake was affected by day ($P < 0.01$; Table 2.11) and a trend ($P = 0.16$) was detected in P2 for an interaction between endophyte treatment and day (Fig. 2.7). Exit velocity x day effects are shown in Fig. 2.8. Similarly, main effects of

endophyte and exit velocity treatments affected consumption throughout the entire study ($P < 0.01$). In both periods, heifers on the E- treatment diet and heifers designated as high exit velocity consumed less water than the other respective treatments. However, an interaction of the two treatments was not observed in either period.

Discussion

An animal's ranking within the exit velocity spectrum remains relatively constant over time (Curley Jr et al., 2006; Bruno et al., 2016). Thus, the first measurement of exit velocity should provide a reliable indication of how temperamental a calf is relative to its herdmates. Therefore, collection of exit velocities at weaning and allocating calves as either high or low exit velocity provided a base from which to test the effects of this measure of temperament on the various responses measured in these experiments.

Cell-mediated Immunity

Examination of the relationship between weaning exit velocity and baseline proportion of lymphocytes producing IFN-γ, average lymphocyte production of IFN-γ, and total lymphocyte production of IFN-γ in cattle is, to our knowledge, unreported in the literature. Our results indicate the presence of a weak correlation between total lymphocyte production of IFN-γ and weaning exit velocity driven primarily by a relationship between proportion of IFN-γ producing lymphocytes and weaning exit velocity. A relationship between exit velocity and average lymphocyte production of IFN-γ was not apparent. Overall, this relationship suggests a linkage between high exit velocity and differentiation of naïve $CD4^+$ T cells to the T_H1 subtype. This further

suggests that higher exit velocity animals are potentially better 'poised' for defense against viral and/or intracellular pathogens.

During P2 of EXP 1, when increased THI was applied in the absence of endophyte treatments, heifer total lymphocyte IFN-γ production did not differ between exit velocity treatments. Others (Minton and Blecha, 1990; Coppinger et al., 1991; Lacetera et al., 2002) have reported similar observations of increased ambient temperature failing to induce changes in cell-mediated immunity.

Average lymphocyte production of IFN-γ responses to endophyte and exit velocity treatments remained consistent between the two experiments, although in Experiment 1 this parameter only tended to differ between exit velocity treatments. In both experiments, heifers on the E+ treatment had greater average lymphocyte production of IFN-γ compared with E- heifers following the endophyte treatment period. Because ergovaline, the major ergot alkaloid produced by the endophytic fungus in tall fescue (Lyons et al., 1986), is a dopamine agonist (Ben-Jonathan, 1985; Lamberts and Macleod, 1990) and D2-like dopamine receptors have been reported on peripheral lymphocytes and natural killer cells (McKenna et al., 2002) one could hypothesize a direct effect of alkaloids on lymphocyte IFN-γ response. However, increased concentrations of dopamine has been shown to increase IL-10 production (Besser et al., 2005; Sarkar et al., 2010), and to down-regulate expression of non-receptor tyrosine kinases lck and fyn, resulting in decreased secretions of IFN-γ (Ghosh et al., 2003; Sarkar et al., 2010). Additionally, in the present studies, differences were detected subsequent to, rather than consequent with exposure, suggesting a mechanism that is more complicated than a direct agonist effect on lymphocytes.

Total lymphocyte production of IFN-γ was also similar between experiments in the period following endophyte treatment exposure. In EXP 1, differences were relegated to the exit velocity treatment only, with relatively greater total production observed in the high exit velocity treatment. However, in EXP 2, exit velocity effects on total lymphocyte production of IFN-γ were only observed in the E- treatment, with greater total production again observed in high exit velocity heifers within this endophyte treatment. Although not statistically different, total production of this cytokine was numerically higher in the E+ treatment as well during this period. Together, the exit velocity effects in both experiments followed a similar pattern to that observed during the baseline period for this measure of lymphocyte production of IFN-γ.

The tendency of EXP 1 heifers on the E+ endophyte treatment to have lower total lymphocyte IFN-γ production during the endophyte exposure period appears to be largely driven by a decrease in the proportion of lymphocytes producing this cytokine, indicating a negative effect of E+ seed on lymphocyte differentiation. One possible mechanism for this is attributable to the presence of the dopamine agonist ergovaline that was present only in the E+ fescue seed, as dopamine has been noted to increase T_H2 differentiation and decrease proliferation and cytotoxicity in humans (Nakano et al., 2009; Sarkar et al., 2010), which would attribute to a lower amount of IFN-γ production, such as that observed in the E+ heifers.

During P4 of the current experiment, the baseline exit velocity pattern was re-established, with high exit velocity heifers maintaining greater proportions of IFN-γ producing lymphocytes than low exit velocity heifers, although a statistical difference

was only detected among E-, as compared with E+, heifers. A similar trend was observed in EXP 2 during the period following endophyte exposure, but only among E- heifers.

Future research exploring these relationships is needed, but a different mitogen may need to be incorporated to clarify the observed endophyte and exit velocity interactions. PMA + ionomycin preferentially activates T_H1 responses and maximizes the amount of IFN-γ produced by cells *in vitro*, but down-regulates production of IL-10 and produces a greater number of dead cells compared with PHA, potentially increasing the variability observed with subsequent responses (Baran et al., 2001). For exploratory research investigating the potential effects of endophyte on IFN-γ production, as well as examining relationships between exit velocity and this cytokine, the use of a mitogen which maximizes the production of the cytokine of interest provides an indication of the effect of a given treatment. However, it does not necessarily provide the most accurate measure of the treatments effect on cytokine production due to the increased variability associated with this mitogen compared with others. Thus, the current two experiments indicated a potential relationship between exit velocity and lymphocyte production of IFN-γ as well as an influence of endophyte upon this relationship. In order to home in on a more accurate measure of these effects, future research may need to include the use of another mitogen that does not preferentially stimulate IFN-γ production, thereby potentially decreasing the variability of the measurements and allowing for a more accurate examination of the influence of exit velocity and endophyte on lymphocyte differentiation and production patterns.

Humoral Immunity

Unlike lymphocyte production parameters, humoral immunity, as measured by vaccination titers to *Lepto. pomona*, did not differ between endophyte treatments during P2 of EXP 2. This finding is inconsistent with previous observations of influences on the humoral response of E+ on grazing steers in Chapter 3 and by others (Dawe et al., 1997; Rice et al., 1997), but is consistent with a prior study using a seed-fed model with steers to examine this response (Dew, 1989). To our knowledge, the present study was the first to investigate the influence of E+ consumption on vaccination titer responses in heifers. Thus, one possibility for the difference in observed effects of E+ consumption on subsequent titer antibody concentrations between the current study and others may be due to a gender effect, as male and female sex hormones have been previously demonstrated to differentially affect B cell differentiation during mitogen stimulation (Sthoeger et al., 1988). However, this potential gender effect may not be the only contributing factor to the differences between experiments observed.

In each of the aforementioned studies (i.e. Dawe et al., 1997; Rice et al., 1997, Chapter 3), steers were grazed on either E+ or E- pastures, which did not allow for measurements of total intake. In the present study intakes were similar between treatments. Additionally, this study was shorter in duration than the three grazing studies mentioned and utilized heifers in place of steers. As mentioned previously, decreased intakes are commonly associated with cattle consuming E+ feedstuffs (Schmidt et al., 1982; Paterson et al., 1995; Spiers et al., 2012). Increased titer responses to vaccination against horse red blood cell and keyhole limpet haemocyanin have been reported to be greater in calves fed on a lower plane of nutrition (Pollock et al., 1994). As intake was controlled in the present study with no observed effects of endophyte treatment on

humoral immunity, and the other studies did not control for intake, it is possible the observed endophyte effects on humoral immunity in those three studies were a result of differences in plane of nutrition between treatments rather than an alkaloid effect per se.

Water Intake and Ruminal Temperature

Hyperthermia in cattle has been reported following exposure to endophyte-infected feeds (Spiers et al., 1995). As the ergopeptine alkaloids target vascular endothelium (Thompson et al., 1950; Strickland et al., 1996; Oliver, 1997; Al-Tamimi, 2002), it has been suggested the hyperthermia experienced by cattle consuming E+ diets is a result of vasoconstriction, leading to shifts in heat dissipation mechanisms (Dyer, 1993; Oliver et al., 1993; Browning Jr and Leite-Browning, 1997; Al-Tamimi, 2002). In Experiment 2, water intake was greatest in the E+ low exit velocity group during both periods. Ruminal temperatures were numerically lower in this group in both periods although statistical differences were only detected in P2, when the SEM were about 40% lower than in P1. Thus, it appears that differences in H_2O consumption may explain the small differences in mean ruminal temperatures among treatments and that the combination of alkaloid consumption and heat exposure may increase variation in mean temperature measurements.

Treatment differences evident in Experiment 2 were not detected in Experiment 1. Reasons for differing effects of treatment on water consumption the two experiments are unclear. Environmental conditions were controlled within similar levels in both experiments and diets were of similar composition.

The highest ruminal temperature amplitudes were observed in the high exit velocity heifers that received the E+ diet. Recognizing that these were ruminal, and not core body temperatures, it's possible these responses were related to differences in water intake, as consumption was greater in low exit velocity heifers on the E+ diet for both periods. However, the difference did not manifest itself until after the increased THI period. The increased average and peak ruminal temperatures observed during the thermoneutral period in E+ high exit velocity heifers may reflect a change in the ruminal environment that extended beyond the exposure period. One potential mechanism that may explain the increased water consumption in E+ calves is an increased liquid passage rate. In sheep, liquid passage rate has been demonstrated to increase with increasing levels of ergovaline supplementation, and was accompanied by increased water intake (Hannah et al., 1990). Increased osmolality, which is a contributing factor to increased passage rates, has been observed in mice consuming an E+, compared with an E-, diet (Barger and Tannenbaum, 1998). In cattle, increased dry matter content in the rumen has been reported for steers consuming an E+ diet, indicating a potential increase in liquid passage rates (Foote et al., 2013). However, passage rates were not examined in either of these experiments and are mentioned here only as a potential explanation for the increased water intake among E+ heifers. Thus, to ascertain if these differences are present in cattle consuming toxic endophyte diets, future research is warranted.

Average Daily Gain

It is well recognized that consumption of endophytic alkaloids can decrease dry matter intake (Schmidt et al., 1982; Paterson et al., 1995; Spiers et al., 2012). Daily

rations offered in both experiments were restricted based upon metabolic body weight, allowing for evaluation of endophyte effects on performance without the potentially confounding effects of intake. Interestingly, during EXP 2, both the trend observed in feed efficiency and the difference in average daily gain between endophyte treatments indicate increased performance of E+ heifers over the whole experimental period. However, in EXP 1, no differences in ADG due to endophyte treatment were observed during any part of, or over the entire, experiment. One explanation for the numerical differences in gain between endophyte treatments in EXP 2 may be that water consumption was greater in E+ cattle throughout the experiment. The difference in daily water consumption more than accounts for the 0.10 kg/d difference in "growth" between treatments.

Conclusion

These experiments demonstrated that a positive relationship may exist between total lymphocyte production of IFN-γ and the proportion of lymphocytes producing IFN-γ with weaning exit velocity in the absence of treatment effects, indicating that the rate of $CD4^+$ differentiation to a T_H1 subtype may be greater in high exit velocity heifers as compared with low exit velocity animals. In opposition to our original hypothesis of additive effects, these data provide evidence that certain immunological responses to alkaloid consumption, coupled with heat exposure, can be modified by animal temperament. Additionally, it was of interest that lymphocyte responses were most prominent subsequent to removal of alkaloid/heat-exposure treatments. This in turn has

potential implications for health responses during the early feedlot phase for cattle previously grazing endophyte-infected pastures.

Table 2. 1. Experiment 1 feedstuff composition of diet[a]

Feedstuff	% DM
Fescue Seed[b]	12.50
Cottonseed Hulls	31.10
Cracked Corn	39.00
Soybean Meal	11.00
Molasses	4.00
Vitamin and TM Supplements[c]	0.88
Melangesterol Acetate[d]	1.52

[a]Heifers were fed at 1.8 x NEm

[b]Endophyte-infected seed was supplemented to provide 10µg ergovaline/ergovalinine per kg BW

[c]Inclusion of supplement ensured adequate levels of Ca (0.41%) and P (0.28%), vitamins A (0.74 IU/kg), D (0.11 IU/kg), E (4.73 IU/kg), and trace amounts of Mg (0.25%), Cl (0.44%), K (0.87%), Na (0.20%), S (0.15%), Co (0.43 ppm), Cu (19.67 ppm), I (0.68 ppm), Fe (164.02 ppm), Mn (72.07 ppm), Se (0.10 ppm), and Zn (53.53 ppm) to meet dietary mineral and vitamin requirements for growing heifers (NRC, 2016).

[d]MGA was provided in a proprietary medicated supplement at a rate of 0.50 $lb \cdot hd^{-1} \cdot d^{-1}$

Table 2. 2. Experiment 1 chemical composition of endophyte-infected and endophyte-free seed[a]

	E- Seed	E+ Seed
Dry Matter (%)	91.60	90.60
Crude Protein (%)	15.00	14.30
Neutral Detergent Fiber (%)	31.30	26.70
Acid Detergent Fiber (%)	22.10	13.20
NEm (Mcal/kg)	1.90	1.93
NEg (Mcal/kg)	1.26	1.29
Ergovaline + Ergovalinine (ppm)	0.00	3.59

[a]Values are presented on a dry matter basis

Table 2. 3. Experiment 2 feedstuff composition of diet[a]

Feedstuff	% DM
Fescue Seed[b]	8.11
Cottonseed Hulls	32.0
Cracked Corn	38.9
Soybean Meal	11.9
Molasses	4.0
Vitamin and TM Supplement[c]	1.6
MGA Supplement[d]	3.6

[a]Heifers were fed at 1.8 x NEm

[b]Endophyte-infected seed was supplemented to provide 10µg ergovaline/ergovalinine per kg BW

[c]Inclusion of supplement ensured adequate levels of Ca (0.43%) and P (0.28%), vitamins A (0.73 IU/kg), D (0.11 IU/kg), E (4.47 IU/kg), and trace amounts of Mg (0.27%), Cl (0.47%), K (0.95%), Na (0.22%), S (0.16%), Co (0.46 ppm), Cu (21.46 ppm), I (0.74 ppm), Fe (181.62 ppm), Mn (84.14 ppm), Se (0.10 ppm), and Zn (54.85 ppm) to meet dietary mineral and vitamin requirements for growing heifers (NRC, 2016)

[d]MGA incorporated into ground corn

Table 2. 4 Experiment 2 chemical composition of endophyte-free and endophyte-infected seed[a]

	E- Seed	E+ Seed
Dry Matter (%)	91.0	89.9
Crude Protein (%)	14.3	14.10
Neutral Detergent Fiber (%)	27.3	24.10
Acid Detergent Fiber (%)	11.2	11.2
NEm (Mcal/kg)	1.52	1.55
NEg (Mcal/kg)	0.92	0.95
Ergovaline + Ergovalinine (ppm)	0.01	5.51

[a]Values are presented on a dry matter basis

Table 2. 5 Experiment 1 lymphocyte intracellular production of IFN-γ in response to endophyte and exit velocity treatments

		E-		E+			P-Values						
		Low	High	Low	High	SEM	Endophyte	Exit Velocity	Endo x EV	Covariate[a]	Day	Endo x Day	EV x Day
Proportion Producing IFN-γ[b]													
	P1[e]	2.57	2.51	2.53	2.57	0.176	0.97	0.96	0.80	<0.01	0.18	0.16	0.01
	P2[f]	4.60	5.95	4.41	3.68	0.786	0.22	0.68	0.28	0.38	-	-	-
	P3[g]	3.32	4.27	2.49	2.74	0.392	0.03	0.13	0.44	0.87	0.86	0.66	0.74
	P4[h]	2.70[i]	4.73[k]	2.68[i]	3.21[i]	0.338	0.07	<0.01	0.07	0.31	<0.01	0.89	0.65
Avg. IFN-γ Produced[c,d]													
	P1[e]	2.77	2.74	2.74	2.80	0.050	0.76	0.70	0.42	<0.01	<0.01	0.58	0.04
	P2[f]	2.52	2.40	2.49	2.30	0.106	0.54	0.18	0.75	0.08	-	-	-
	P3[g]	2.46	2.36	2.36	2.17	0.110	0.22	0.23	0.69	0.01	0.02	0.80	0.89
	P4[h]	1.96	2.16	2.28	2.28	0.062	<0.01	0.12	0.15	<0.01	0.29	0.32	0.73
Total IFN-γ Produced[c,d]													
	P1[e]	3.63	3.61	3.56	3.62	0.075	0.74	0.78	0.61	<0.01	<0.01	0.20	<0.01
	P2[f]	4.09	3.99	3.98	3.71	0.245	0.48	0.47	0.74	0.86	-	-	-
	P3[g]	3.66	3.54	3.29	3.34	0.166	0.14	0.80	0.62	0.04	0.21	0.59	0.83
	P4[h]	2.97	3.36	3.32	3.62	0.194	0.18	0.10	0.82	0.06	0.37	0.37	0.69

[a]Average of P1 values included as a covariate in the statistical model
[b]Units expressed as a percentage of total lymphocytes analyzed
[c] Interferon-γ measured is produced by lymphocytes isolated from whole blood
[d]Means are natural log transformed, untransformed values were expressed in arbitrary units
[e]Baseline data collection period, thermoneutral and endophyte treatments not applied, d1 to 21. n = 12 heifers
[f]Increased temperature humidity index (THI) period, endophyte treatments not applied, d22 to 28. n = 12 heifers
[g]Increased THI and endophyte treatment period, d29 to 50. n = 12 heifers
[h]Thermoneutral and Post-endophyte period, d51 to 78. n = 12 heifers
[i,k]Means with different superscripts are different

Table 2. 6 Experiment 1 water consumption as a proportion of body weight in response to endophyte and exit velocity treatments[a]

	E-		E+			P-Values			
	Low	High	Low	High	SEM	Week	Endo	EV	Endo x EV
P1[b]	4.19	4.30	4.24	4.23	0.135	-	0.92	0.73	0.69
P2[c]	4.49	4.72	4.45	4.38	0.139	-	0.22	0.60	0.34
P3[d]	4.57	4.69	4.43	4.50	0.173	0.33	0.39	0.59	0.88
P4[e]	4.36	4.52	4.26	4.26	0.233	0.16	0.48	0.74	0.75

[a]Means are natural log transformed. Original units were mL/kgBW. n = 12 heifers
[b]Baseline period, thermoneutral and no endophyte treatment, d15 to 21 only
[c]Increased temperature humidity index (THI) period, no endophyte treatment applied, d22 to 28
[d]Increased THI and endophyte exposure period, d29 to 50
[e]Thermoneutral and post-endophyte exposure period, d51 to 78

Table 2. 7 Experiment 1 heifer average daily gain in response to endophyte and exit velocity treatment

	E-		E+			P-Values		
	Low	High	Low	High	SEM	Endo	EV	Endo x EV
d1 to 28[a]	1.57	1.17	1.79	1.07	0.398	0.88	0.20	0.71
d29 to 50[b]	2.01	2.31	2.27	2.07	0.164	0.94	0.78	0.17
d51 to 78[c]	1.79	1.79	1.73	1.59	0.213	0.54	0.75	0.75
Total[d]	1.66	1.70	1.79	1.52	0.151	0.88	0.47	0.34

[a]Baseline thermoneutral period and first week of increased temperature humidity index (THI), no endophyte treatment. n = 12 heifers
[b]Endophyte and increased THI period. n = 12 heifers
[c]Post-endophyte and post-increased THI period. n = 12 heifers
[d]Average gains across the entire experimental period. n = 12 heifers

Table 2. 8 Experiment 2 ruminal temperature responses to endophyte and exit velocity treatments in heifers by period[a]

		E-		E+			P-Values			
		Low	High	Low	High	SEM	Endophyte	Exit Velocity	Endo*EV	Hour
Amplitude[b]										
	P1[d]	0.17	0.15	0.15	0.17	0.026	0.92	0.91	0.51	<0.01
	P2[e]	0.16[f]	0.17[f]	0.15[f]	0.27[g]	0.029	0.10	0.03	0.08	<0.01
Acrophase[c]										
	P1[d]	6.57	4.95	4.67	7.64	1.429	0.79	0.64	0.12	0.61
	P2[e]	7.33	6.26	7.01	5.01	1.096	0.48	0.17	0.68	<0.01
Mesor[b]										
	P1[d]	38.52	38.58	38.48	38.62	0.077	0.97	0.20	0.61	<0.01
	P2[e]	38.45[f]	38.45[f]	38.32[g]	38.51[f]	0.046	0.48	0.07	0.06	<0.01

[a]n = 12 heifers

[b]Means are presented as °C

[c]Means are presented as hour

[d]P1 = Heat stress period, endophyte treatments applied, d1 to 28

[e]P2 = Thermoneutral period, no endophyte treatment applied, d29 to 57

[f,g]Means within the same row that are different are different

Table 2. 9 Experiment 2 heifer lymphocyte production of IFN-γ in response to endophyte and exit velocity treatments, by period

		E-		E+			P-Values[i]			
		Low	High	Low	High	SEM	Endophyte	Exit Velocity	Endo*EV	Day
Proportion Producing IFN-γ[a,b]										
	Baseline[d]	0.800	1.232	0.787	0.907	0.3117	0.61	0.41	0.63	-
	P1[e]	0.767	1.246	1.204	1.043	0.2168	0.60	0.48	0.18	<0.01
	P2[f]	0.884	1.429	1.470	1.335	0.1915	0.24	0.32	0.12	0.02
Average Production IFN-γ[a,c]										
	Baseline[d]	1.520	1.894	2.025	1.646	0.2633	0.64	0.99	0.20	-
	P1[e]	1.515	1.707	1.602	1.683	0.1735	0.86	0.45	0.76	0.16
	P2[f]	1.301	1.575	1.684	1.796	0.0871	<0.01	0.05	0.37	<0.01
Total Production IFN-γ[a,c]										
	Baseline[d]	2.320	3.130	2.810	2.550	0.4699	0.93	0.58	0.30	-
	P1[e]	2.281	2.953	2.805	2.726	0.3140	0.65	0.37	0.27	<0.01
	P2[f]	2.195[g]	3.001[h]	3.146[h]	3.132[h]	0.2015	0.03	0.09	0.08	<0.01

[a]Means are natural log transformed. Interferon-γ produced by lymphocytes isolated from whole blood.

[b]Means are expressed as percentages

[c]Means are presented as arbitrary units

[d]d1 baseline lymphocyte measurements. n = 11 heifers

[e]P1 = Heat stress period, endophyte treatments applied, d1 to 28. n = 12 heifers

[f]P2 = Thermoneutral period, no endophyte treatment applied, d29 to 57. n = 12 heifers

[g,h]Means with different superscripts within the same row are different

[i]Interactions of day with endophyte and exit velocity were dropped from model after initial analysis ($P \geq 0.43$)

Table 2. 10 Experiment 2 Lepto. pomona titer, average daily gain, and gain:feed ratio responses to endophyte and exit velocity treatment in heifers[a]

	Endophyte		Exit Velocity			P-Values		
	E-	E+	Low	High	SEM	Endophyte	Exit Velocity	Endo*EV
L. pomona[b]	7.49	7.38	7.26	7.61	0.365	0.83	0.52	0.52
ADG[c]	0.40	0.50	0.41	0.49	0.037	0.11	0.19	0.58
G:F	0.072	0.090	0.073	0.088	0.0079	0.16	0.24	0.68

[a]n = 12 heifers

[b]Means are natural log transformed

[c]Means are in kg/day

Table 2. 11 Experiment 2 average weekly water consumption in response to endophyte and exit velocity treatments by period

	E-		E+			P-Values					
	Low	High	Low	High	SEM	Endo	Exit Velocity	Endo*EV	Week	Endo*Week	EV*Week
P1[ab]	0.091	0.076	0.104	0.093	0.0055	0.02	0.06	0.75	0.42	0.56	0.67
P2[ac]	0.082	0.066	0.093	0.076	0.0040	0.02	<0.01	0.90	0.04	0.40	0.53

[a]Means are liters consumed/kgBW. n = 12 heifers

[b]Heat stress period, endophyte treatments applied, d1 to 28

[c]Thermoneutral period, no endophyte treatment applied, d29 to 57

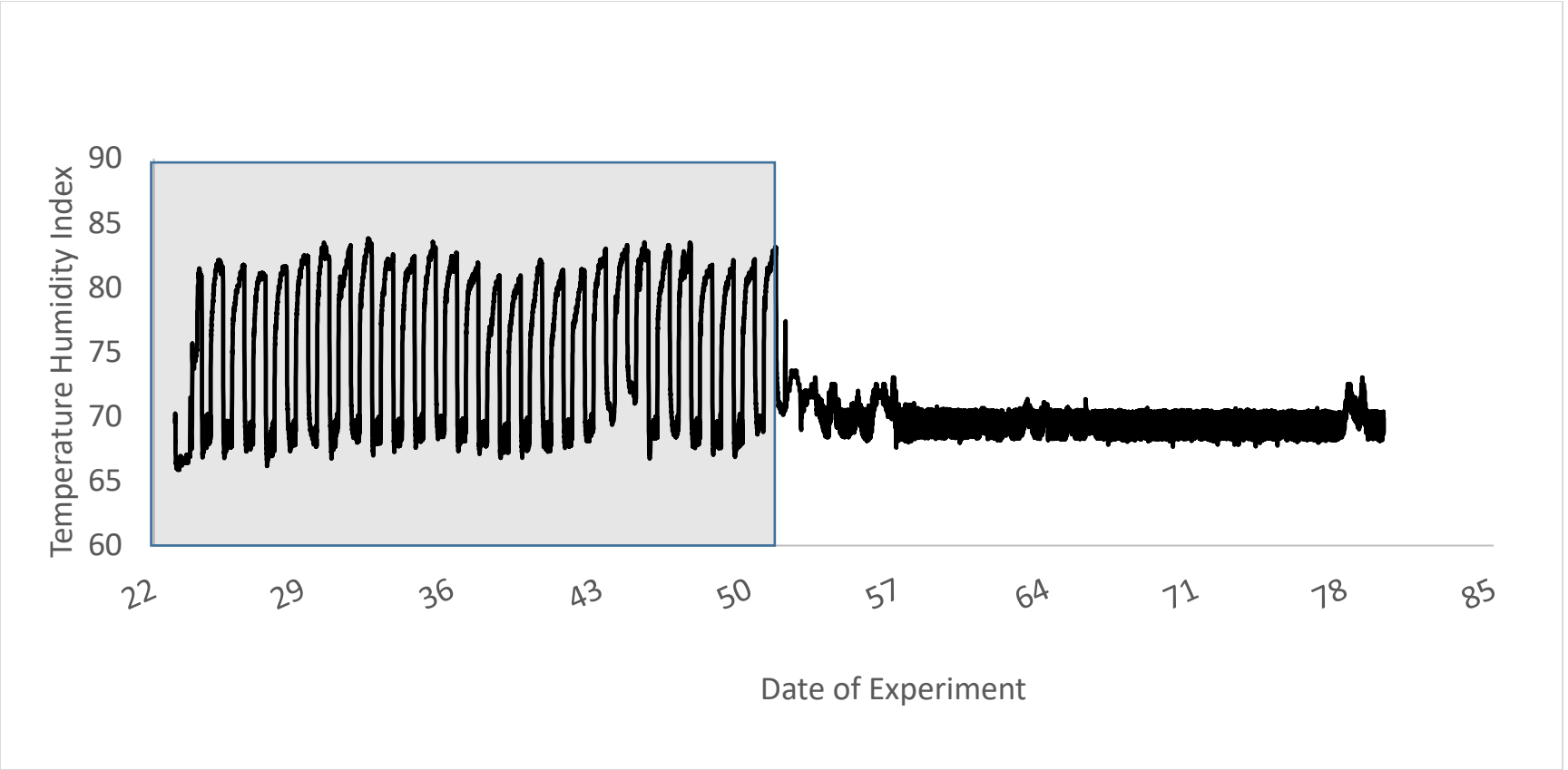

Figure 2. 1 Experiment 1 temperature humidity index within the animal room. This graph depicts the fluctuations in the temperature humidity index (solid line) across time, beginning on d 22, when the heat stress period began, and continuing until the last day of the experiment (d78). Data logger was installed at the beginning of the heat stress period as a secondary system to ensure the air handling system was following the designated heat cycle. Prior to this installation, room temperature was set to a constant 22°C, as is depicted above for days 51 to 78. Also depicted are the three levels of heat stress, as described by Hahn (1999): Alert = red line, Danger = blue line, Emergency = yellow line. Shaded area represents the heat stress period.

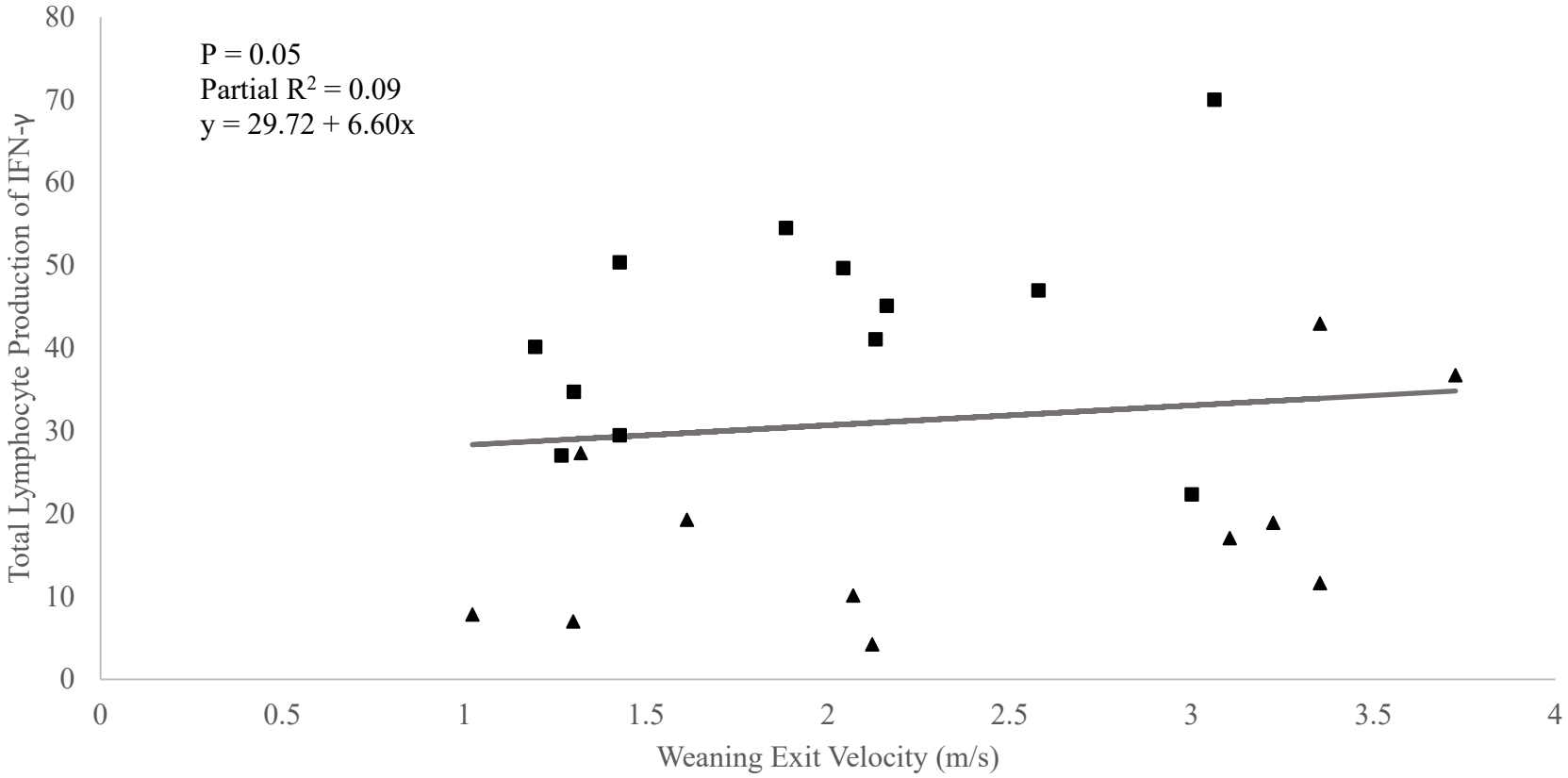

Figure 2. 2 Regression of weaning exit velocity against total lymphocyte production of interferon-γ during 2 experiments utilizing heifer calves. Experiment 1 (n = 12 heifers) data is a three-week baseline average of total lymphocyte production of IFN-γ beginning one week after calves were placed in barn. Experiment 2 (n = 11 heifers) data was from a single collection on the day calves were placed in the barn. One data point was removed from experiment 2 data due to an insufficient number of cells harvested from the sample. PBMCs were isolated from whole blood collected weekly via jugular venipuncture, treated with transport inhibitor brefeldin A, and stimulated with phorbol 12-myristate 13-acetate + ionomycin. Experiment 1 (■). Experiment 2 (▲).

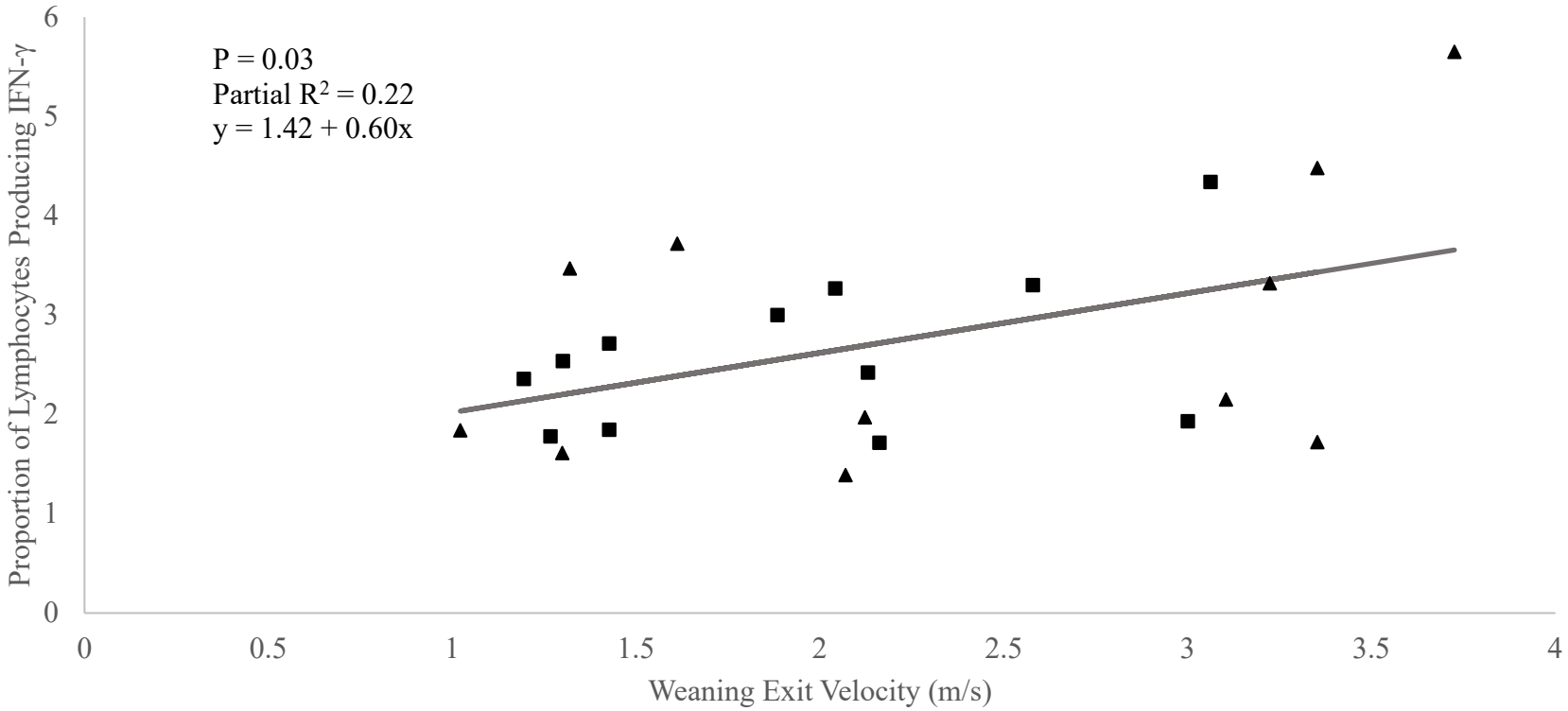

Figure 2. 3 Regression of weaning exit velocity against the proportion of lymphocytes producing interferon-γ during 2 experiments utilizing heifer calves. Experiment 1 (n = 12 heifers) data is a three-week baseline average of the proportion lymphocytes producing interferon-γ values beginning one week after calves were placed in barn. Experiment 2 (n = 11 heifers) data was from a single collection on the day calves were placed in the barn. One data point was removed from experiment 2 data due to an insufficient number of cells harvested from the sample. PBMCs were isolated from whole blood collected weekly via jugular venipuncture, treated with transport inhibitor brefeldin A, and stimulated with phorbol 12-myristate 13-acetate + ionomycin. Experiment 1 (■). Experiment 2 (▲).

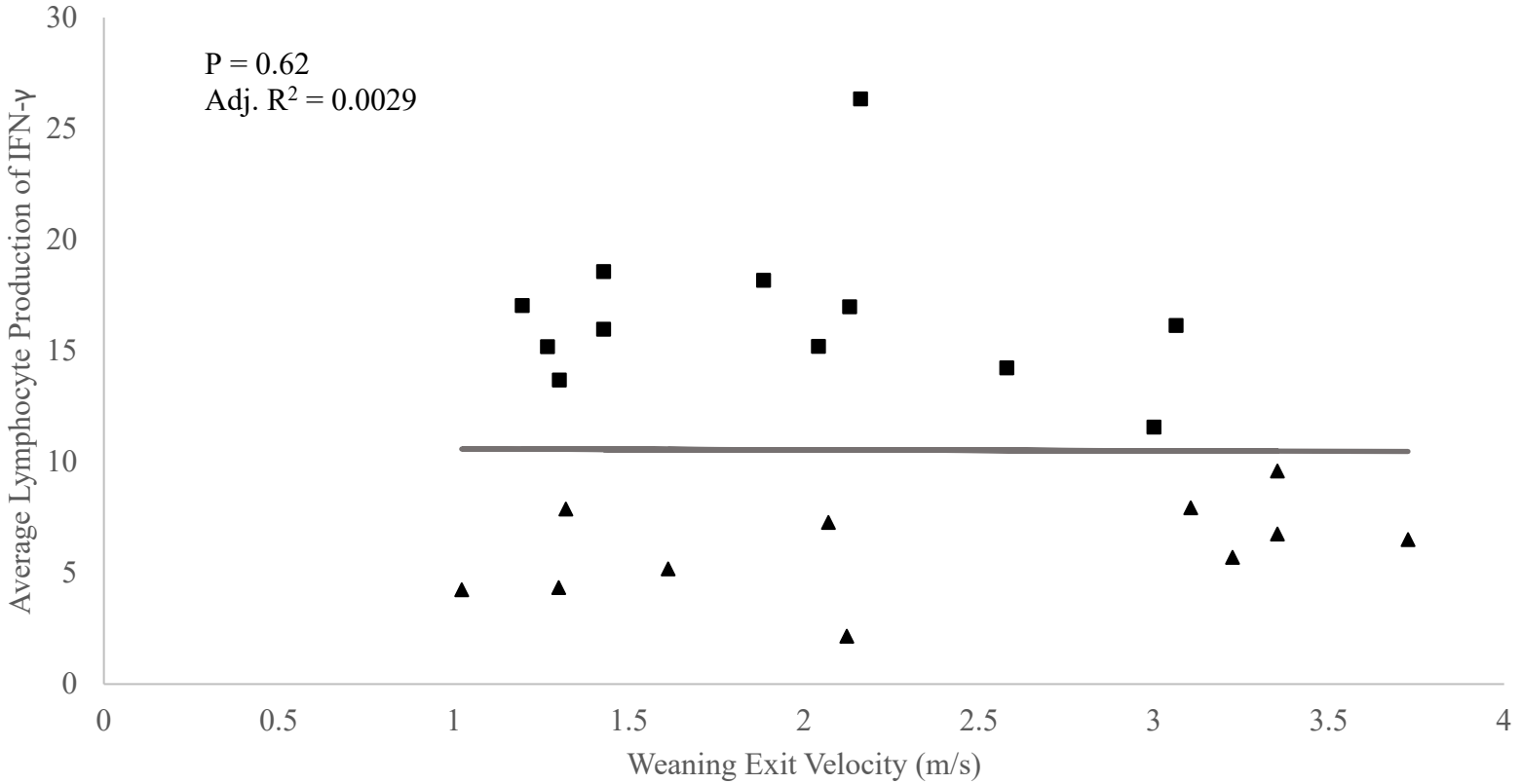

Figure 2. 4 Regression of weaning exit velocity against average lymphocyte production of interferon-γ during 2 experiments utilizing heifer calves. Experiment 1 data (n = 12 heifers) is a three-week baseline average of the average lymphocyte IFN-γ production beginning one week after calves were placed in barn. Experiment 2 (n = 11 heifers) data was from a single collection on the day calves were placed in the barn. One data point was removed from experiment 2 data due to an insufficient number of cells harvested from the sample. PBMCs were isolated from whole blood collected weekly via jugular venipuncture, treated with transport inhibitor brefeldin A, and stimulated with phorbol 12-myristate 13-acetate + ionomycin. Experiment 1 (■). Experiment 2 (▲).

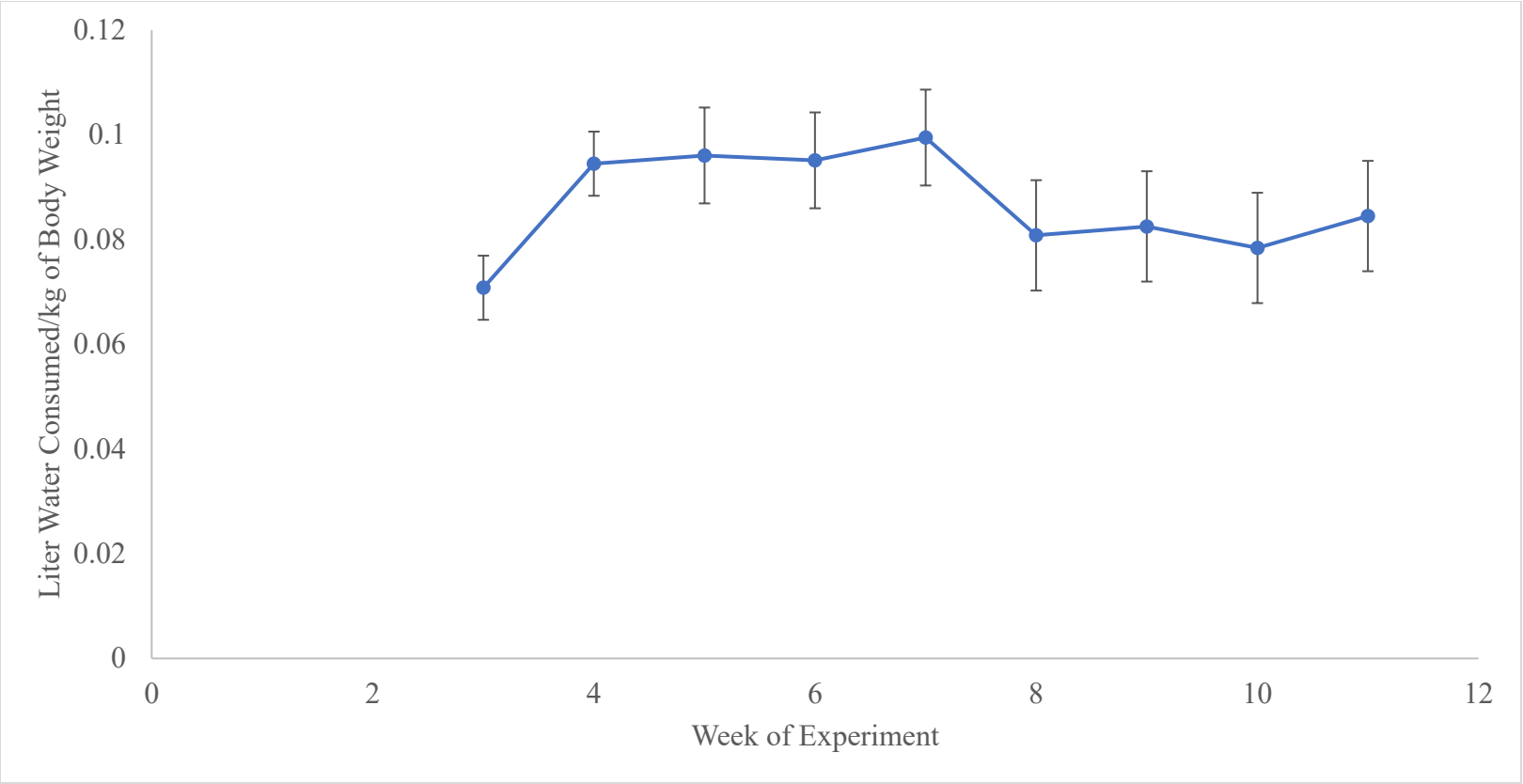

Figure 2. 5 Experiment 1 water intake, as a percentage of body weight, over time. Consumption was calculated as liters/kg of body weight, and natural log transformed to meet the assumptions of ANOVA. Week 3 was part of the baseline period, in which all heifers (n = 12) were fed E- seed in daily rations, and room temperature remained at thermoneutral (22.2°C). Week 4 included only the application of heat stress (33.3°C during day, 22.2°C at night), with all heifers remaining on E- seed as part of their daily rations. Week 5 to 7 included half of the heifer receiving E- seed, and the other half E+ seed, with heat stress treatment still in place. Weeks 8 to 11 all heifers were returned to the E- seed, with room temperatures returning to thermoneutral. Intakes did not differ between treatments.

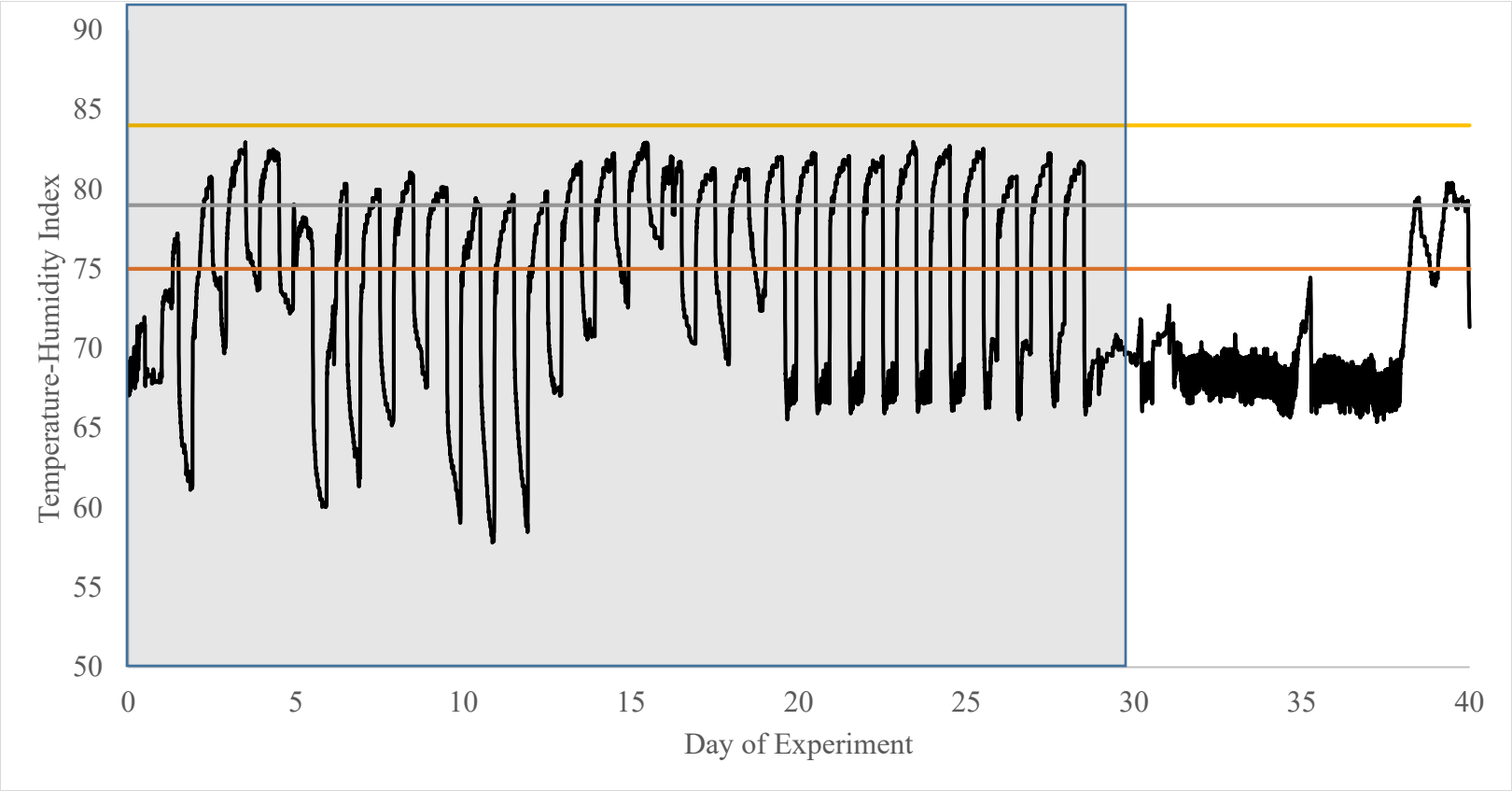

Figure 2. 6 Experiment 2 temperature humidity index of pen rooms during an experiment examining cell mediated immune responses to endophyte and exit velocity treatments. Heifers were subjected in the first 28 d of the experiment to heat stress and endophyte treatment. The heat stress component (d0 to d29; represented by shaded portion) was removed on d29 and rooms remained at thermoneutral for the duration of the study. Due to logger failure, only 39 of 56 days within the experimental period are available for graphical representation. Additionally, mechanical failure throughout the experiment caused a deviation from the designed THI protocol. However, during the heat stress period (d0 to d29), temperatures cycled above the danger and below the alert heat stress thresholds described by Hahn (1999). Alert = red line, Danger = blue line, Emergency = yellow line.

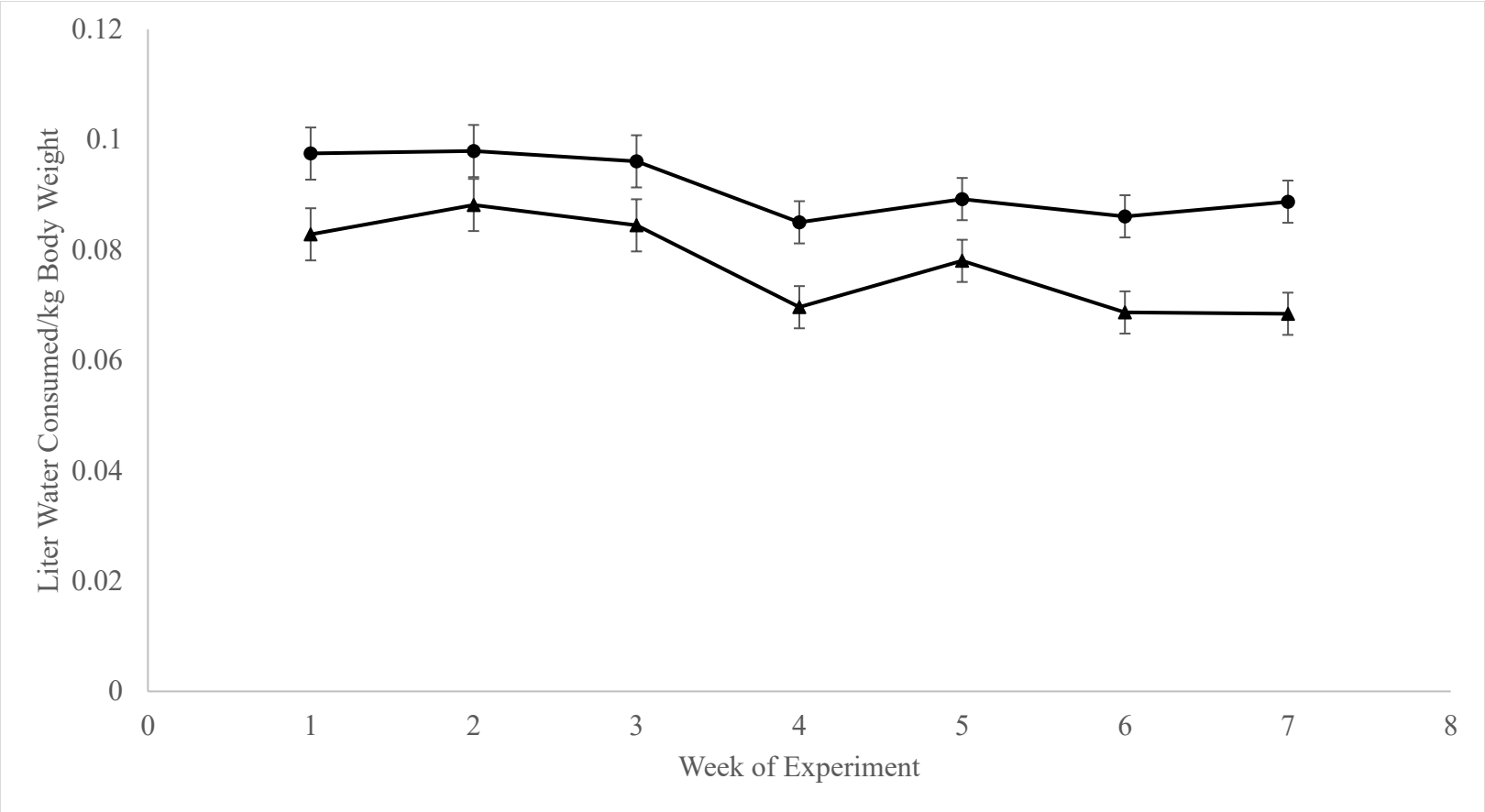

Figure 2. 7 **Experiment 2 heifer water consumption, as a percentage of body weight, by exit velocity designation.** *The experiment was divided into 2 periods of 28 days each. Period 1 consisted of a cyclic heat stress model (22°C to 33°C) and application of endophyte treatment diets. Period 2 was removal of toxic endophyte from E+ group and constant thermoneutral temperatures (22°C). Heifers (n = 12) were evaluated at weaning for exit velocity measures, and heifers from the high and low extremes were selected for this experiment. Differences were observed in both periods for endophyte ($P < 0.01$) and exit velocity ($P < 0.01$) treatments. High exit velocity = ▲. Low exit velocity = ●*

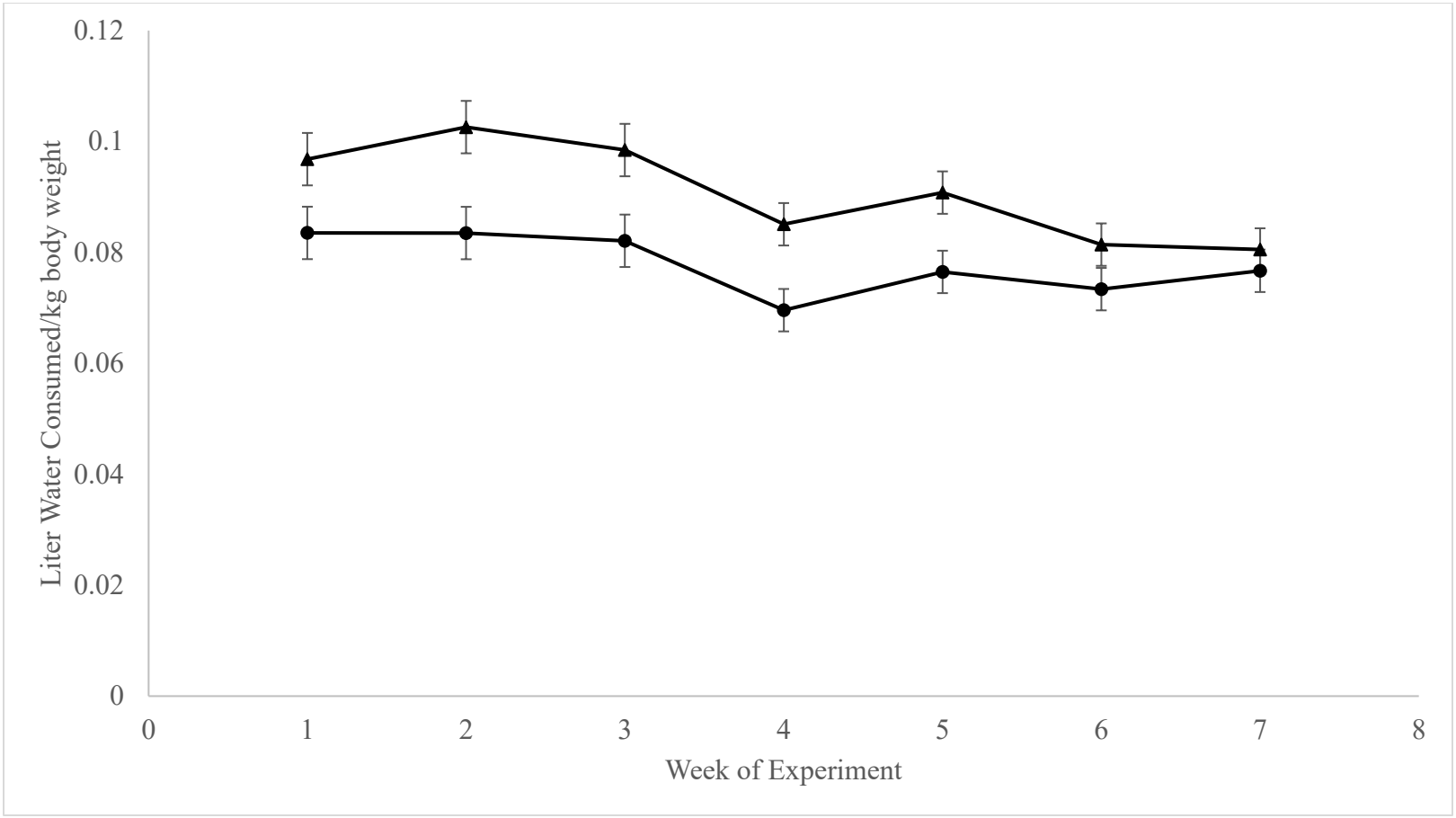

Figure 2. 8 Experiment 2 heifer water consumption, as a percentage of body weight, by endophyte treatments. The experiment was divided into 2 periods of 28 days each and utilized 12 heifers separated into 2 endophyte (E+/E-) and 2 exit velocity (low/high) treatments. Period 1 consisted of a cyclic heat stress model (22°C to 33°C) and application of endophyte treatment diets. Period 2 was removal of toxic endophyte from E+ group and constant thermoneutral temperatures (22°C). Differences were observed in both periods for endophyte ($P < 0.01$) and exit velocity ($P < 0.01$) treatments. E+ = ▲. E- = ●.

Chapter 3: Influence of temperament and fescue toxicosis on steer grazing and finishing growth, immune responses, and carcass characteristics

Abstract

Mixed breed steers (n=120) from 3 sources were weighed and evaluated for exit velocity (EV). Steers were assigned an EV treatment (high/low; based on relative ranking in measured exit velocities within source), blocked by source, and assigned to either endophyte-infected (E+) or endophyte-free (E-) tall fescue pastures (n=20 pastures; 6 animals/pasture) blocked by source with equal representation of EV treatments in each pasture. Pasture groups were randomly assigned to a control or glucomannan supplement. Following grazing, steers were transitioned to drylot pens (n=40 pens) for finishing after 110d on pasture and harvested at approximately 681 kg. Drylot pen assignment was established by placing steers of the same EV treatment within each pasture in the same pen, and steers were fed a corn-based diet finishing diet. Blood was collected on d110, 124, and 138 for evaluation of peripheral lymphocyte interferon-γ (IFN-γ) production, with additional blood collected on d138 to measure *Leptospirosis pomona* titer response. Finishing and carcass data was analyzed as a 2x2 factorial (endophyte and EV treatments). E+ steers had lower grazing ($P<0.01$) and higher finishing ($P=0.07$) average daily gains and finishing gain:feed ratios. Low EV steers consumed more dry matter ($P=0.06$) but had lower gain:feed ratios ($P<0.01$) over the finishing period. E+ steers had higher titer responses to *Lepto. pomona* ($P=0.09$), but no differences between endophyte or EV treatments were observed for peripheral lymphocyte IFN-γ production ($P\geq0.25$). Analysis of carcass data detected higher KPH for E+ steers ($P=0.05$), whereas low EV steers had higher final yield grades ($P=0.10$). No other carcass differences were detected.

These data indicate previous exposure to E+ tall fescue may increase growth performance of steers in the feedlot setting and exit velocity may be a useful tool to predict feedlot eating and growth behavior.

Keywords: endophyte, steer, exit velocity, lymphocyte, vaccination

Introduction

Exit velocity has been considered one of the most practical and objective measures of temperament in cattle (Ferguson et al., 2006). This behavioral measure has been found to be related to both immunological function (Altman, 2015; Bruno et al., 2018) and feedlot growth performance (Burrow and Dillon, 1997; Voisinet et al., 1997; Petherick et al., 2002). However, less is known about relationships between exit velocity and performance during grazing (Ferguson et al., 2006), especially with pastures containing toxic fescue.

Cattle grazed on toxic endophyte (*Epichloë coenophiala)*-infected tall fescue pastures experience a syndrome known as fescue toxicosis. Animals suffering from this syndrome can exhibit several symptoms including decreased feed intake and average daily gain (ADG), elevated respiration rate and body temperature, rough hair coat, and loss of circulation to extremities (Stuedemann et al., 1985; Aldrich et al., 1993). However, prior exposure to tall fescue pastures has been reported to improve (Cole et al., 2001; Duckett et al., 2016) or have no effect (Parish et al., 2013) upon subsequent feedlot growth performance, whereas research examining feedlot cell-mediated and humoral immunity during post-fescue grazing periods is limited, especially when considering potential modulating effects related to temperament measures like exit velocity.

Therefore, the objective of this experiment was to evaluate the relationship of exit velocity on average daily gain and immune function during and following a period of grazing either endophyte-infected or non-toxic tall fescue.

Materials and Methods

All methods were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animal Background

Mixed-breed beef steers (n=120; BW = 304 ± 33 kg) were purchased by order buyer from three sources. Upon arrival at the University of Kentucky's Oran C. Little Research Unit, cattle were weighed and evaluated for exit velocity using previously published methods (Bruno et al., 2016). Prior to the beginning of the grazing period, steers were backgrounded for 28 days on grass hay and mineral supplement. During this time, bacterial and viral vaccinations (Bovi-Shield Gold 5, Zoetis, Florham Park, NJ; Once PMH, Merck Animal Health, Summit, NJ; Somubac, Zoetis; Ultrachoice 7, Zoetis; Autogenous Pinkeye, Central KY Vet Center), and an injection of anthelmintic (Dectomax, Zoetis), were administered.

Treatment Assignment

Treatments were arranged in a 2 x 2 x 2 factorial arrangement in a split plot design with two whole-plot factors and one sub-plot factor. Whole plot factors included

fescue/endophyte association (non-toxic vs toxic; E- and E+, respectively) and supplement type (control vs glucomannan) and were assigned to pasture groups (n = 6 steers/pasture). Supplements were identical in composition (Table 3.1), with the glucomannan supplement additionally including a proprietary glucomannan additive. The subplot factor (experimental unit = individual animals) was exit velocity, which was assigned using the measurements obtained from steers upon arrival at the research unit. Source groups were stratified by exit velocities and split into two equal halves to form high and low exit velocity (EV) treatment groups. These exit velocity treatment designations were used to balance pastures for potential temperament effects so that each pasture had 3 low EV steers and 3 high EV steers. There were no main effects or interactions involving supplement type for any response variable. Thus, supplement type was removed from the statistical model for all results, such that the final model was a split plot design without the originally modeled 2 x 2 factorial arrangement in the whole plot.

Cattle were assigned to either novel endophyte (Lacefield MaxQ II; Phillips, 2016) or endophyte-infected pastures. Animal groups were balanced for body weight, blocked by source, randomly assigned to pastures, and grazed for 110 days. Pastures used in this study were 1.52 hectares in area. Supplement treatments were randomly assigned within pasture pairs. Stocking density was set at 1200 kg initial BW/hectare (6 animals/pasture), a moderate to low stocking rate for this location (Sollenberger and Vanzant, 2011).

Following the grazing period, steers were moved to a dry lot and housed in 2.4 x 14.6 m pens, with 3 animals per pen. Steers were placed in pens, grouped by exit velocity

designation from the same grazing pasture, and remained until completion of the finishing period (167d), at which point they were removed for slaughter. Thus, the experimental unit was pen (3 steers), which maintained the integrity of the design structure from the grazing phase. Data from the receiving period were a focal point in this study, as we hypothesized prior exposure to E+ fescue may exacerbate potential changes in cell-mediated immunity associated with the sudden change in environment and diet. Steers were started on a typical receiving ration (Table 3.2) and transitioned to a finishing diet over the 28d receiving period. Due to decreased consumption during the first week, steers remained on the initial diet for 2 weeks. Following this two-week period, dietary energy concentrations were increased weekly over the next three weeks by decreasing the proportion of corn silage while increasing proportions of cracked corn and distiller's dried grains. Feed bunks were checked once daily and managed to ensure steers had ad libitum access to rations, which were prepared each morning.

Pasture analysis

One week prior to the grazing period, 5 subsamples per pasture were collected using an X pattern across each pasture, with sampling sites chosen at random and subsamples combined to form one pasture sample. After collection, samples were frozen at -20°C, freeze dried in a Botanique Model 18DX48SA freeze drier (Botanique Preservation Co., Peoria, AZ), ground through a 1-mm screen on a Wiley Mill Model 4 (Thomas Scientific, Swedesboro, NJ) and analyzed using a high-performance liquid chromatograph with a florescence detector to quantify ergovaline and ergovalinine concentrations, as developed by Yates and Powell (1988) and modified as in Aiken et al.

(2009). Ergovaline and ergovalinine were identified by excitation at 310 and detection at 420 nm. Samples were specifically for analyzed ergovaline and its isomer, ergovalinine, as these alkaloids are believed to be the causative agents of fescue toxicosis and have been utilized in several studies investigating this phenomenon (Thompson and Stuedemann, 1993).

Body Weight Measurement

Body weight was measured at approximate 4-week intervals throughout the grazing season (d0, 32, 61, 89, 110), 14-day intervals during the first 28d of the finishing period (d 110, 124, and 138), and on approximately 28d intervals throughout the rest of the finishing period (d166, 194, 235, 256, 277). Potential error associated with gut fill differences was diminished during the grazing season by removing steers from pasture the night before each weigh day and placing them in dry lot pens without access to feed or water for approximately 16 hours prior to weighing. During the receiving and finishing periods, steers did not receive daily rations until after body weight had been collected, although access to water was not restricted during these periods. Steers were finished to an average body weight of 680 kg. Due to differences in growth rates, and to ensure all carcasses were similar in size, steers were harvested in 3 groups balanced across treatments over a 7-week period.

Blood Collection and Analysis

Blood was collected via jugular venipuncture for all analyses of blood parameters. During the grazing period, 10 mL serum samples were collected from each steer into

spray-coated silica Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) on d32 and d110 for prolactin analysis, which was conducted by the Schrick laboratory at the University of Tennessee. Serum samples were also collected utilizing the same methodology on d110 and d138 (d0 and d28 of the finishing period) to evaluate baseline and titer responses against an administered *Leptospirosis pomona* vaccine (L5 SQ, Merck).

In addition, during the receiving period, samples were collected from a subset of high exit velocity steers (n = 20; 1 from each pasture) on days 0, 14, and 28, to evaluate lymphocyte interferon-gamma (IFN-γ) production. Because the number of samples that could be simultaneously processed was limited, blood samples for lymphocyte analysis were restricted to only high exit velocity animals, in an effort to delineate effects due to supplement type, none of which were ultimately significant. Although our subsetting strategy greatly reduced the likelihood of detecting potential exit velocity effects, these effects were evaluated within the tested subgroup by using exit velocity as a continuous, rather than categorical, variable.

PBMC Isolation and Stimulation

Peripheral blood mononuclear cells (PBMC) were isolated and stimulated from the heparinized whole blood samples using a modified protocol adapted from the methods of Breathnach et al. (2006). Briefly, the three tubes collected from each sample were combined in 1-50mL centrifuge tube and spun at 800 x g for 30 minutes using a slow brake, with the resulting buffy coat transferred to a new tube and rinsed with 10 mL of warm PBS. This cell solution was layered over 10 mL Ficoll-Paque Plus^{TM} solution

(Amersham Biosciences, Piscataway, NJ), and spun at 500 x g for 30 minutes using a slow brake. Cells were harvested, placed in a new tube containing 20 mL PBS, and centrifuged at 500 x g for 10 minutes using a fast brake. Cells were resuspended in 5 mL PBS, and to this suspension 10 mL D_2O and 10 mL RPMI were added, with tubes topped off with PBS to achieve a final volume of 45 mL. These tubes were centrifuged at 300 x g for 10 minutes using a fast brake, and all subsequent spins were performed using these specifications. Supernatant was dumped from tubes, and tubes were again topped off to 45 mL using PBS to resuspend and wash cells. Tubes were centrifuged, supernatant dumped, and 10 mL of PBS was used to resuspended cells. A subsample of 100 µL was obtained from each sample and mixed with 900 µL of PBS for quantifying PBMC concentration in each sample using a Vicell Counter-XR (Beckman Coulter, Miami, FL) for use in determining the volume necessary for plating samples in duplicate at 1 x 10^6 cells/mL. The appropriate volume from each tube was transferred to a 15 mL tube and centrifuged. Resulting supernatant was dumped from each tube, and cells were resuspended with 4 mL cRPMI (10% fetal bovine serum, 1% penicillin-streptomyciin-glutamine, 0.1% 2-mercaptaethanol, and 88.9% RPMI). Cells were plated in 2 wells of a 24 well plate at 1 x 10^6 cells/mL.

Samples were arranged on a 24 well plate so that duplicate samples were adjacent to each other in the same row, which served as control and stimulated samples. All wells were supplemented with 2 µL of brefeldin A (BFA), and 10 µL of phorbol 12-myristate 13-acetate (PMA)/ionomycin was added only to stimulated sample wells. Sample plates were placed in an 5% CO_2 incubator for 4 hours at 37°C. 200 µL was removed from each incubated well, transferred to a 96 well plate, and spun at 500 x g for 5 minutes, with

resulting supernatant dumped. 100 µL of 2% paraformaldehyde was added to each well, and plates were wrapped in aluminum foil and placed in a 4°C refrigerator overnight.

Intracellular staining and flow cytometry

Staining of PBMCs for IFN-γ and flow cytometer protocols were as described by Breathnach et al. (2006). Briefly, the morning after cells were fixed in paraformaldehyde, plates were centrifuged at 500 x g for 5 minutes and supernatant dumped. Cells were rinsed with 150 µL saponin buffer (1% fetal bovine serum, 0.1% saponin, and 0.1% sodium azide), and plate was centrifuged at 500 x g for 5 minutes, with resulting supernatant dumped. Intracellular staining was performed by reconstituting mouse IgG1 anti-bovine IFN-gamma FITC conjugated antibody in saponin buffer using a 10 µg/mL formulation. After addition of the antibody, plates were incubated on ice for 30 minutes. Plates were centrifuged at 500 x g for 5 minutes and saponin buffer was used to resuspend PBMCs. Plates were centrifuged, supernatant dumped, and each well received 200 µL FACS buffer. An Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) was used to identify and analyze the lymphocyte population subset of isolated PBMCs, with forward and side scatter parameters limited to 30,000 gated events. Stimulated samples were compared to control samples, which were gated at 1%, for determination of the proportion lymphocytes producing IFN-γ and the average production of IFN-γ by the lymphocyte population. These two parameters were multiplied together to approximate the total IFN-γ produced by the analyzed lymphocyte population (Darrah et al., 2007).

Prolactin Analysis

Plasma samples collected on d32 and d110 of the grazing period were analyzed for prolactin concentrations by radioimmunoassay (Bernard et al., 1993) in the Schrick laboratory at the University of Tennessee. The intra- and inter-assay CVs for samples were 7.73% and 3.23%, respectively.

Leptospirosis pomona

Serum blood samples were subjected to a microscopic agglutination test for detection of *Leptospirosis pomona* antibodies (Bruno et al., 2017). Animals without seronegative baseline titers on d110 were excluded from further humoral response analyses (n=12).

Fescue Tolerance Genetic SNP Analysis

On d110, whole blood from each animal was applied to individual sample cards and sent to AgBotanica, LLC (Columbia, MO) for T-Snip™ score analysis, which is a proprietary test purported to be a predictor of animals' growth performance when grazed on endophyte-infected pastures. T-Snip™ analyzes bovine DNA to determine tolerance to endophyte-infected tall fescue based on multiple genetic markers. Higher score values are associated with higher fescue tolerance.

Receiving and Finishing Dry Matter Intake

Dry matter intake was determined on a per-pen basis by subtracting the weekly dry matter weight of refused feed from the total dry matter offered for the week. Ration

ingredients were sampled weekly, and refused feed was pooled within block and subsampled in triplicate each week into previously weighed trays, weighed, and placed in a forced air-drying oven at 55°C for 24 hours for DM determination.

Statistical Analysis

Data collected during the grazing period were analyzed as a split plot with endophyte pasture type (E-/E+) as the whole plot treatment and exit velocity (high/low) as the subplot treatment. When a RCBD whole plot structure was used with the split plot design, variance estimates for the subplot (residual) error for most of the ADG responses were not different from zero, resulting in non-positive definite covariance matrix estimates. Ultimately, the issues stemmed from the fact that the blocking factor accounted for a trivial portion of the variance. Thus, block was removed from the whole plot in these instances. Data were analyzed using the mixed procedure of SAS (9.4, Cary, N.C.). The Kenward-Roger method was used to calculate the denominator degrees of freedom, and block was included as a random effect. Response variables investigated included average daily gain (interval and whole period; ADG) and prolactin concentrations.

Receiving and finishing period data were also analyzed as a randomized complete block design using the mixed procedure of SAS, with the denominator degrees of freedom calculated using the Kenward-Roger method. Unlike with the grazing period, there was no sub-plot for these two periods as pens comprised the experimental unit for all treatment factors. Main effects were endophyte pasture type (E+/E-) and exit velocity (high/low). Response variables for both periods included ADG, dry matter intake (DMI), and gain to feed ratio (G:F), with two steers removed from analyses of these variables

due to poor growth (consistent outlier across first 3 weigh periods; n = 1) and morbidity (death due to anaplasmosis; n = 1). The receiving period additionally included *Leptospirosis pomona* titer responses and measures of lymphocyte proportions and production of IFN-γ. Antibody titer data were analyzed as described for ADG, DMI, and G:F, but included all 120 steers in the analysis because no sign of poor growth or morbidity were observed for any steer during the 28-d vaccination period. The lymphocyte data included observations for a total of 20 steers (as previously described) and exit velocity was incorporated as a covariate rather than a treatment.

Carcass data was analyzed as a randomized complete block design using the mixed procedure of SAS, and the Kenward-Roger denominator degrees of freedom method. Response variables included hot carcass weight, dressing percentage, marbling score, yield grade, backfat thickness, kidney pelvic heart fat percentage, and ribeye area. Due to differences in growth rates between the blocks, three shipment dates were required to ensure all animals were finished at slaughter. Due to slower growing animals within the first three blocks, 4 animals from these blocks were excluded from analysis as they had not reached the targeted 680 kg finished weight by day of shipment for their respective blocks.

Significance for all response variables across all periods was set at $P < 0.10$, and trends at $0.10 < P < 0.15$.

Results

Grazing Period

Pasture data and prolactin responses

Total ergovaline + ergovalinine concentrations are presented in Table 3.3. E+ pastures contained higher concentrations of these two isomers than E- pastures ($P < 0.01$).

Prolactin concentrations were approximately 28% higher for E- steers on d32 and d110 ($P < 0.01$; Table 3.4). Influences of exit velocity on prolactin concentrations, however, were not as consistent. On d 32, high exit velocity steers tended to have lower plasma concentrations ($P = 0.12$), but this trend was not evident on d110 ($P = 1.00$).

Average daily gain

The effects of endophyte, exit velocity, and their interaction on ADG are presented in Table 3.5. For the overall grazing period, E- steers experienced greater ADG compared to E+ steers ($P < 0.01$; 0.58 vs 0.45 ± 0.054 kg/d). Animals designated as low exit velocity tended to have greater ADG throughout the grazing period ($P = 0.14$; 0.54 vs 0.49 ± 0.056 kg/d, respectively), with no interactions between endophyte and exit velocity treatments detected for this period ($P = 0.68$).

To test if differences in ADG may be attributable to genetic differences in toxic endophyte tolerance, the T-Snip™ values were evaluated as a covariate in analyses for ADG responses. These values proved to be ineffective in explaining any growth-related response differences due to endophyte, and no correlation was observed between T-Snip™ values and ADG for either endophyte treatment (Fig. 3.1; $P = 0.38$ and 0.48 for pooled- and independent-slope models, respectively, data not shown).

Receiving Period

Cell-mediated and humoral responses

The three lymphocyte IFN-γ production measurements were not affected by either endophyte or exit velocity treatments ($P \geq 0.34$; Table 3.6). However, a time effect was detected for both response variables across the 4 weeks ($P < 0.01$) in which the proportion of lymphocytes producing IFN-γ increased linearly over time, whereas average lymphocyte production of this cytokine were quadratic during the same time period (Figures 3.2 and 3.3, respectively; total IFN-γ production by lymphocytes is shown in Fig. 3.4). Conversely, *Lepto. pomona* titers were higher for E+ steers, relative to E- steers, during the receiving period ($P = 0.09$; Table 3.7). No differences in titer responses were observed between exit velocity designations ($P \geq 0.44$).

Performance data

The first 28 days after removal from pasture were analyzed separately to observe potential residual effects of grazing toxic endophyte upon responses to sudden environmental and dietary changes. Effects of endophyte, exit velocity, and their interaction were not significant during the receiving period for ADG (Table 3.7; $P \geq 0.16$). However, compared with E+ steers during this period, E- steers consumed approximately 0.37 $kg^{-1} \cdot hd^{-1} \cdot d^{-1}$ more dry matter ($P = 0.03$), but were less efficient in growth efficiency (gain:feed = 0.215 vs 0.244 ± 0.0096 for E- and E+, respectively; $P < 0.01$). Effects of exit velocity on DMI were also observed during this 4-week period, as high exit velocity steers had consumed approximately 0.53 $kg^{-1} \cdot hd^{-1} \cdot d^{-1}$ less dry matter than low exit velocity steers ($P < 0.01$) but gain to feed did not differ between the two treatments ($P = 0.94$). DMI was also examined as a percentage of body weight, with high

exit velocity steers consuming approximately 0.0006 kg·kgBW^{-1}·d^{-1} less dry matter during this 28d period, indicating the observed influence of exit velocity on DMI was independent of body weight.

Finishing Period

Effects of both endophyte and exit velocity treatments were observed throughout the finishing period. Steers previously grazed on E+ pastures maintained a numerically higher ADG during each weigh interval of the finishing period, resulting in an overall 0.09 kg/d greater daily gain for the whole finishing period (Table 3.7; P = 0.07). Steers previously grazed on E+ pastures also experienced a 0.08 greater G:F ratio than their E- counterparts (Table 3.7; P < 0.01), but total dry matter intake and dry matter as a percentage of body weight were not different (P ≥ 0.74) between endophyte treatments.

High exit velocity animals consumed approximately 0.41 kg·hd^{-1}·d^{-1} and 0.0005 kg·kgBW^{-1}·d^{-1} less dry matter than low exit velocity steers for the whole finishing period (P = 0.05). Differences in absolute DMI were largely driven by effects during the 2nd and 3rd weigh intervals, as DMI was approximately 0.37 and 0.63 kg·hd^{-1}·d^{-1}, respectively, greater in low exit velocity steers (P = 0.03), although treatment differences were not detected when DMI was examined as a percentage of body weight (P ≥ 0.15). Exit velocity also affected whole finishing period gain efficiencies, with high exit velocity animals outperforming low exit velocity steers by approximately 0.007 kg of body weight gained per kilogram of feed (P = 0.01). However, no differences in ADG (P = 0.72) were observed between exit velocity treatments.

Carcass Data

All carcass data is presented in Table 3.8. Despite the performance differences between treatments during the finishing period, animal carcass measurements were not notably affected by endophyte or exit velocity. Steers previously grazed on E+ pastures maintained higher kidney, pelvic, heart fat percentages ($P = 0.05$), but did not differ from E- steers in backfat measurements ($P = 0.20$) or marbling scores ($P = 0.80$). Similarly, low exit velocity steers had higher yield grades ($P = 0.04$) but did not differ from high exit velocity steers in other carcass measurements ($P \geq 0.19$).

Discussion

Grazing Period

Circulating prolactin concentrations can be utilized as a physiological indicator of exposure to ergot alkaloids, with lower concentrations of prolactin observed following consumption of these toxins (Schillo et al., 1988). In this study, prolactin concentrations analyzed on d32 and d110 of the grazing period corroborated expectations from pasture alkaloid concentration, with steers grazing E+ pastures having lower serum prolactin concentrations.

The data collected during the grazing period agree with most reports of animals grazing tall fescue pastures. Average daily gain for the whole grazing period was approximately 0.13 kg lower in E+, as compared with E-, steers, which is consistent with reports from others (Coffey et al., 1990; Tully, 1992; Parish et al., 2003; Watson et al., 2004) and a common sign of fescue toxicosis (Schmidt and Osborn, 1993; Waller, 2009).

Fescue toxicosis signs are typically more apparent during periods of increased temperature (Thompson and Stuedemann, 1993). Over the first three weigh intervals, the

THI steadily increased, overall, to its apex just below the emergency heat stress point, and thus above the alert and danger levels, described by Hahn (1999). This was particularly evident during the 3rd interval which coincided with the highest THI values for the entire grazing season, when weight gains in both treatments decreased by 33 to 53% compared with the 2nd interval. Prior to the start of the 4th weigh interval, the THI decreased to near the alert, or lowest, heat stress level (Hahn, 1999), and remained relatively constant through the duration of grazing. During this time, growth rates rebounded to levels similar to those observed for the second interval for both endophyte treatment groups.

Immunological Responses

Humoral immune response, in the form of *Leptospirosis pomona* antibody production, was impacted by endophyte treatment, with increased production in E+ steers. Despite the consistency of this result with other post-endophyte bovine titer responses (Dawe et al., 1997; Rice et al., 1997), investigation of a mechanistic explanation has yet to be provided. One possibility is this increased humoral immunity may be driven by serotonergic responses due to consumption of endophytic alkaloids. Serotonin treatment, in the presence of LPS, was reported to stimulate B cell proliferation in rats and mice, with similar effects observed using the serotonin agonist 8-OH-DPAT (Iken et al., 1995). Ergovaline has been reported to bind with certain serotonin receptor subtypes and induce vasoconstriction in cattle via alteration of serotonin receptors (Dyer, 1993; Klotz et al., 2007). Thus, consumption of this alkaloid by E+ steers may have contributed to the observed increased titer response against *Lepto. Pomona*.

It is also possible the increased titer responses in E+ steers were driven by a previous decrease in the plane of nutrition. Pollock et al. (1994) reported calves fed a greater amount of milk substitute each day prior to weaning had lower vaccination titers to horse red blood cell (administered post-weaning), indicating a previous lower plane of nutrition may increase vaccine efficacy, even after the nutrient restriction period has ceased. The steers grazing E+ pastures, experienced lower ADG than E- steers, indicating that E+ steers were on a lower plane of nutrition. To determine if the increased humoral responses of cattle previously grazed on E+ pastures is a direct result of alkaloid exposure, lower plane of nutrition, or a combination of the two, future research is needed.

In contrast with humoral immune responses, cell-mediated responses were unaffected by endophyte treatment in this study. This finding, on the surface, conflicts with results from other studies conducted by our lab examining relationships between post-endophyte and lymphocyte IFN-γ production (Chapter 2). However, there are three key differences between the current study and those two experiments. First, this study utilized steers whereas Chapter 2 investigated this relationship in heifers. It is unclear what role sex hormones may play in this relationship, if any. Secondly, total DMI was controlled and equivalent between E- and E+ treatment groups in the experiments described in Chapter 2, but was not controlled (and likely differed between endophyte treatments, as reflected by ADG) during the grazing period of the current experiment. Third, in Chapter 2, heifers were fed a known concentration of ergovaline + ergovalinine each day, potentially increasing the ability to detect differences between endophyte treatments. In the present study, average pasture concentrations were obtained at the beginning of the grazing season. However, these concentrations are known to fluctuate

throughout the year (Rogers et al., 2011) and the amount of grass the consumed, including the total amount of alkaloid ingested each day, was not measured in the current experiment. Therefore, to further investigate the relationship, or lack thereof, between E+ consumption and lymphocyte IFN-γ production in steers, future research should be conducted in a manner which better accounts for alkaloid consumption.

Finishing Period Growth Performance

Effects of endophyte treatment on gain efficiency, DMI, and ADG during the first 28 days of the finishing period indicated steers grazed on toxic endophyte pastures may have been experiencing a compensatory response following the restricted growth observed during the grazing period. The increased absolute dry matter intake by E- steers over the first 28d of the finishing period was a result of the relatively larger size of these animals compared with E+ steers during this weigh interval, as there was no difference between endophyte treatments when DMI was analyzed as a percentage of body weight.

Periods of compensation, such as that observed for the E+ steers during the second weigh interval, have been noted to be commonly preceded and accompanied by increased gain efficiency in animals previously under nutrient restriction relative to those who never experienced the restriction (Ryan et al., 1993). In that study, the compensatory response in both sheep and cattle subsequent to a period of severe nutrient restriction was observed first as an increased gain efficiency and then later with increased DMI. However, it was noted this increased DMI lasted through the duration of the experiment for calves, but not sheep, to which the authors attributed sheep with a more severe nutrient restriction (i.e. greater weight loss), thereby encouraging a more rapid and

efficient response. The authors concluded this indicates that the degree to which compensation occurs in the animal is dependent upon the severity of the restriction experienced. In the current study, which had a much lower degree of restriction than that noted by Ryan et al. (1993), E+ steers experienced improved gain efficiencies during the receiving period (d110 to 138), with a tendency from d138 to 166 to consume a greater amount of dry matter (as a percentage of body weight). This indicated that compensatory mechanisms, although softened in comparison to the aforementioned study, may have been present during the first 56d in the feedlot, attributing to the overall difference in ADG observed for the finishing period.

Periods of nutrient restriction have also been associated with decreased liver and gastrointestinal tract weights (Murray et al., 1977; Johnson et al., 1987; Carstens et al., 1991). The restricted growth of these organs, once the nutrient restriction has been alleviated, induces a greater deposition of protein, as opposed to fat, in these animals (Carstens et al., 1991). Therefore, it is possible the improved growth efficiency of E+, compared with E-, steers during the feedlot period of the present study may have resulted from an increased protein deposition due to decreased liver and gastrointestinal tract sizes arising from the decreased growth of these steers during the grazing period. This mechanism for compensation may assist in further explaining the observed titer responses, which were measured during the same period. Following a period of malnutrition, children supplemented with 175 cal/kg and 4 g of protein had greater typhoid immunization responses than was observed prior to supplementation and in the group supplemented with 100 cal/kg and 1 g of protein (Suskind et al., 1976). If compensatory responses were present in E+ steers during the time of vaccination in the

current study, as indicated by growth efficiency and daily gain responses, relative nutrient availability could have been increased in these calves, which may have contributed to the increased titer responses.

Finishing period gain efficiency and DMI were also influenced by exit velocity treatment, as animals with greater exit velocities maintained higher growth efficiencies but numerically lower intakes than their low exit velocity herdmates. Despite numerically lower DMI (on a %BW basis) across the finishing period for animals designated as high exit velocity, there was not a corresponding difference in ADG. Although the observed reduction in DMI in the current study is consistent with previous reports (Burrow and Dillon, 1997; Petherick et al., 2002; Cafe et al., 2011), the increase in efficiency differs from others who have reported decreased (Burrow and Dillon, 1997; Petherick et al., 2002), or no difference (Francisco et al., 2012) in gain efficiencies in high, as opposed to low, exit velocity animals.

Carcass Data

Consumption of E+ pastures has been noted to influence lipid metabolism in cattle, with E+ cattle maintaining lower serum cholesterol concentrations during grazing than calves on E- pastures (Bond et al., 1984a; Stuedemann et al., 1985; Rice et al., 1997). In the current study, previous exposure to endphyte-infected pasture had no apparent effect on fat deposition, with the exception of KPH fat. Differences in KPH due to endophyte treatment have not been noted following a finishing period in other studies (Duckett et al., 2001; Realini et al., 2005). Although a detectable difference between endophyte treatments was approximately 0.06%, or about a 3% magnitude of difference,

this minute increased KPH value in the carcasses of E+ steers is likely of minimal physiological importance.

Conversely, the observed differences in yield grade between exit velocity groups presents an intriguing treatment effect. Yield grade is calculated using an equation that weights the measured carcass characteristics of backfat, KPH, ribeye area, and dressing percentage, with the final number is rounded down to the nearest whole number for reporting (USDA, 1997). Despite the appearance of a consistent relationship between exit velocity and yield grade in the present study (i.e. no interaction with endophyte treatment and lower yield grade associated with increased exit velocity), the main driver of this relationship differed between endophyte treatment groups. In E- steers, the change in yield grade was associated with lower backfat whereas in E+ steers, the relationship between exit velocity and calculated yield grade was related to a change in ribeye area. However, neither of these carcass characteristics were affected by either endophyte or exit velocity treatments, indicating these differences were very subtle, yet distinct enough to affect overall yield grade.

Conclusions

The results from this study indicate that both toxic endophyte and animal exit velocity may influence growth performance during grazing and finishing. From a finishing perspective, this data indicates that exit velocity may be a useful predictor of gain efficiency and feed intake in cattle. Previous toxic endophyte tall fescue consumption improved titer response to a bacterial vaccination. However, it is unclear whether this effect was due to a previous decreased plane of nutrition or a direct effect of an alkaloid upon humoral immunity. Future investigation of the mechanism for this

increased responsiveness to vaccination may be useful in formulating and implementing new management strategies to reduce incidence of morbidity among newly received feedlot cattle.

Table 3. 1 Composition of supplements[a] provided to steers during 110d grazing period.

Ingredient	Inclusion Rate
Salt	85.00%
Magnesium	4.00%
Copper	2000 ppm
Selenium	50 ppm
Zinc	4000 ppm
Manganese	7500 ppm
Iodine	125 ppm
Cobalt	15 ppm
Vitamin A	200,000 IU/lb

[a]Glucomannan treatment supplement contained 30 g/kg proprietary glucomannan additive.

Table 3. 2 Diet composition of 167d finishing period (post-grazing)

	% of DM			
	Weeks 1-2	Week 3	Week 4	Weeks 5-24
Corn Silage	70	50	35	10.0
Distiller's Dried Grains	10	20	25	25.0
Cracked Corn	10	20	30	27.5
High Moisture Corn	0	0	0	27.5
Supplement[a]	10	10	10	10.0

[a]Supplement contained macro and trace minerals and vitamins formulated to support ADG of 1.93 kg/d, along with urea, monensin, and tylosin, in a ground corn carrier.

Table 3. 3 Presence of ergovaline, ergotamine, and related isomers in E+ and E- pastures.

	Endophyte Treatment			P-Value
	E-	E+	SEM	Endo
Ergovaline + Ergovalinine[a]	43	425	38.2	<0.01
Ergotamine + Ergotaminine[a]	0.00	0.00	0.00	-

[a]Values represent the sum of isomers, in ppb

Table 3. 4 Influence of endophyte infected tall fescue and exit velocity (EV) on serum prolactin concentration in steers (n = 120) during 110d grazing period.

	E-		E+			P-Value		
	Low	High	Low	High	SEM	Endo*EV	Endo	EV
Day 32[a]	4.44	4.57	3.41	3.06	0.171	0.49	<0.001	0.12
Day 110[a]	3.82	3.93	2.82	2.71	0.150	0.41	<0.001	1.00

[a]Means are presented as natural log-transformed, original units in ng/mL

Table 3. 5 Influence of endophyte-infected tall fescue and exit velocity (EV) on steer (n = 120) daily gains during a 110d grazing period[a]

	E-		E+			P-Values		
	Low	High	Low	High	SEM	Endo*EV	Endo	EV
d0 to 32	0.29	0.24	0.13	0.06	0.103	0.87	0.13	0.24
d32 to 60	0.85	0.82	0.76	0.72	0.100	0.97	0.21	0.44
d60 to 89	0.58	0.51	0.34	0.36	0.072	0.27	0.07	0.44
d89 to 110	0.82	0.72	0.76	0.71	0.048	0.56	0.31	0.11
d0 to 110	0.61	0.55	0.47	0.43	0.059	0.68	<0.01	0.14

[a]Means are presented as kg/d

Table 3. 6 Influence of endophyte infected tall fescue on steer (n = 20) peripheral lymphocyte IFN-γ production during the first 28d of the finishing period, with exit velocity (EV) as a covariate.

	Endophyte Treatment			P-Value				
	E-	E+	SEM	Endo*EV	Endo*Week	Endo	EV[e]	Week
Proportion of lymphocytes producing IFN-γ[a,b]	1.83	2.00	0.125	0.25	0.54	0.34	0.42	<0.01
Average lymphocyte production of IFN-γ[a,c]	10.31	10.39	0.087	0.74	0.59	0.82	0.67	<0.01
Total lymphocyte population production of IFN-γ[a,d]	12.14	12.40	0.162	0.29	0.68	0.40	0.62	<0.01

[a]Means are presented as log-transformed
[b]Percent of peripheral blood mononuclear cells expressing Interferon-γ
[c]Mean fluorescence intensity of peripheral blood mononuclear cells expressing Interferon-γ
[d]Product of lymphocyte proportion and average production of Interferon-γ values
[e]Only high exit velocity steers were selected for these analyses. Thus, exit velocity was included as a covariate rather than a treatment effect

Table 3. 7a Influence of endophyte infected tall fescue and exit velocity (EV) on steer (n = 118) growth performance and humoral immune responses during a 167d finishing period following summer grazing.

	E-		E+			P-Value		
	Low	High	Low	High	SEM	Endo*EV	Endo	EV
ADG (kg)								
d110-138	1.86	1.66	1.89	1.86	0.109	0.40	0.27	0.24
d138-166	1.55	1.62	1.76	1.75	0.117	0.71	0.10	0.73
d166-194	2.44	2.29	2.35	2.39	0.101	0.26	0.93	0.48
d194-235[a]	2.05	2.25	2.14	2.23	0.107	0.42	0.61	0.02
d235-256[b]	1.61	1.78	1.83	1.73	0.171	0.20	0.39	0.72
d156-177[c]	1.41	1.19	1.31	1.51	0.176	-	-	-
d0-Finish[d]	1.95	1.95	2.02	2.05	0.073	0.78	0.07	0.72
DMI (kg)								
d110-138	8.52	7.82	7.98	7.70	0.207	0.18	0.04	<0.01
d138-166	11.94	11.29	12.01	11.42	0.435	0.92	0.72	0.03
d166-194	12.68	11.79	12.39	12.00	0.336	0.38	0.88	0.03
d194-235[a]	12.79	12.38	12.55	12.59	0.326	0.39	0.96	0.50
d235-256[b]	11.83	11.93	11.45	11.23	0.272	0.47	0.03	0.80
d156-177[c]	10.81	11.14	10.59	10.94	0.196	--	-	-
d0-Finish[d]	11.66	11.07	11.42	11.18	0.267	0.41	0.74	0.06
DMI (as %BW)								
d110-138	0.0212	0.0205	0.0209	0.0204	0.00048	0.74	0.32	0.04
d138-166	0.0265	0.0264	0.0277	0.0265	0.00050	0.20	0.17	0.16
d166-194	0.0250	0.0245	0.0252	0.0246	0.00047	0.87	0.68	0.15
d194-235[a]	0.0218	0.0221	0.0221	0.0222	0.00034	0.76	0.56	0.52
d235-256[b]	0.0190	0.0199	0.0194	0.0192	0.00039	0.15	0.64	0.29
d156-177[c]	0.0175	0.0179	0.0173	0.0178	0.00047	--	-	--
d0-Finish[d]	0.0228	0.0225	0.0230	0.0226	0.000242	0.82	0.33	0.14

Table 3. 8b Influence of endophyte infected tall fescue and exit velocity (EV) on steer (n = 118) growth performance and humoral immune responses during a 167d finishing period following summer grazing.

	E-		E+			P-Value		
	Low	High	Low	High	SEM	Endo*EV	Endo	EV
G:F								
d110-138	0.217	0.212	0.236	0.252	0.0096	0.29	<0.01	0.55
d138-166	0.133	0.143	0.147	0.148	0.0104	0.51	0.20	0.42
d166-194	0.198	0.194	0.191	0.201	0.0062	0.22	0.97	0.56
d194-235[a]	0.163	0.182	0.170	0.179	0.0065	0.24	0.57	<0.01
d235-256[b]	0.136	0.150	0.160	0.148	0.0156	0.21	0.28	0.94
d156-177[c]	0.130	0.107	0.124	0.149	0.0200	--	-	--
d0-Finish[d]	0.169	0.176	0.177	0.184	0.0044	0.92	<0.01	0.01
Lepto. pomona[e,f]	2.89	2.81	3.05	2.98	0.118	0.96	0.09	0.44

[a]Includes all 5 blocks in analyses
[b]Includes only 2 blocks in analyses
[c]Only 1 block included for the means. No statistics available
[d]Analysis of full finishing period for all blocks
[e]Means presented are log transformed
[f]Data was collected during first 28 days of finishing period

Table 3. 9 Influence of endophyte infected tall fescue and exit velocity on steer (n = 118) carcass data.

	E-		E+			P-Value		
	Low	High	Low	High	SEM	Endo*EV	Endo	EV
Hot Carcass Weight[a]	389.8	378.2	383.7	382.0	5.03	0.34	0.82	0.19
Yield Grade	3.24	2.99	3.24	3.10	0.100	0.55	0.54	0.04
Ribeye Area[b]	87.0	87.1	86.6	89.5	1.34	0.30	0.45	0.26
Marbling	475.0	447.2	460.6	469.1	16.69	0.23	0.80	0.52
Backfat[c]	1.32	1.19	1.35	1.35	0.074	0.27	0.20	0.22
Kidney, Pelvic, Heart Fat[d]	1.88	1.91	1.95	1.96	0.045	0.69	0.05	0.54
Dressing Percentage[d]	62.78	62.59	62.73	62.60	0.405	0.92	0.95	0.61

[a]Values presented in kilograms
[b]Values presented in cm^2
[c]Values presented in cm
[d]Values presented as %

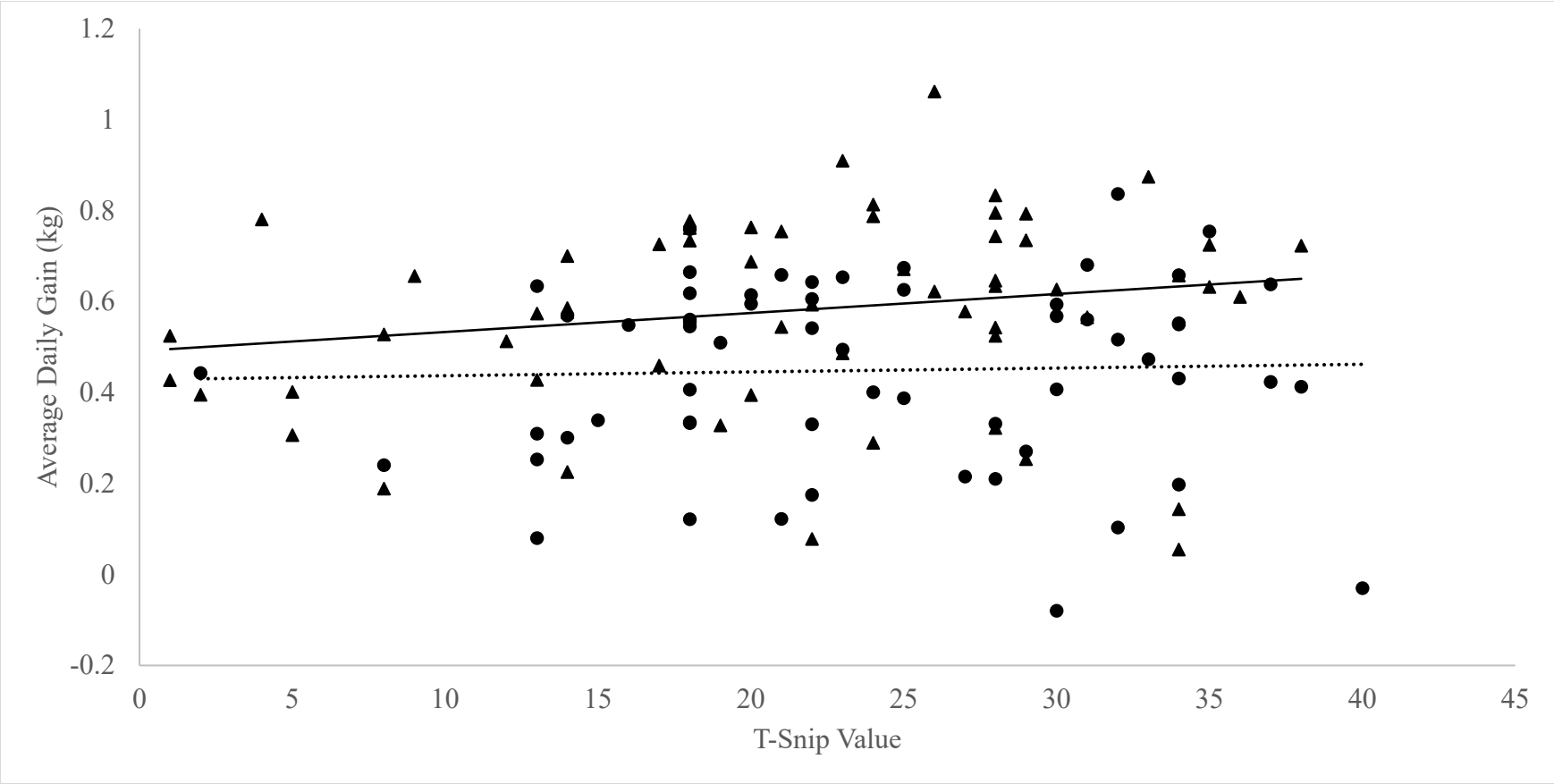

Figure 3. 1 T-snip scores from steers (n = 120) grazing E+ or E- pastures for 110d. Scores range from 0 to 50, with higher values suggested to be associated with greater toxic fescue tolerance. Trendlines, based on endophyte treatments, indicate T-snip scores were not a good determinant for toxic fescue tolerance. The R^2 for E- (▲, solid line) steers was 0.0351, whereas it was 0.0011 for E+ (●, dashed line) steers, indicating that no correlation between expression of the T-snip gene and average daily gain on pasture existed. Analysis of variance, with inclusion of T-snip in the model as a covariate, yielded the following : endophyte (P < 0.01) , exit velocity (P = 0.16), endophyte x exit velocity (P = 0.70), T-snip (P = 0.38).

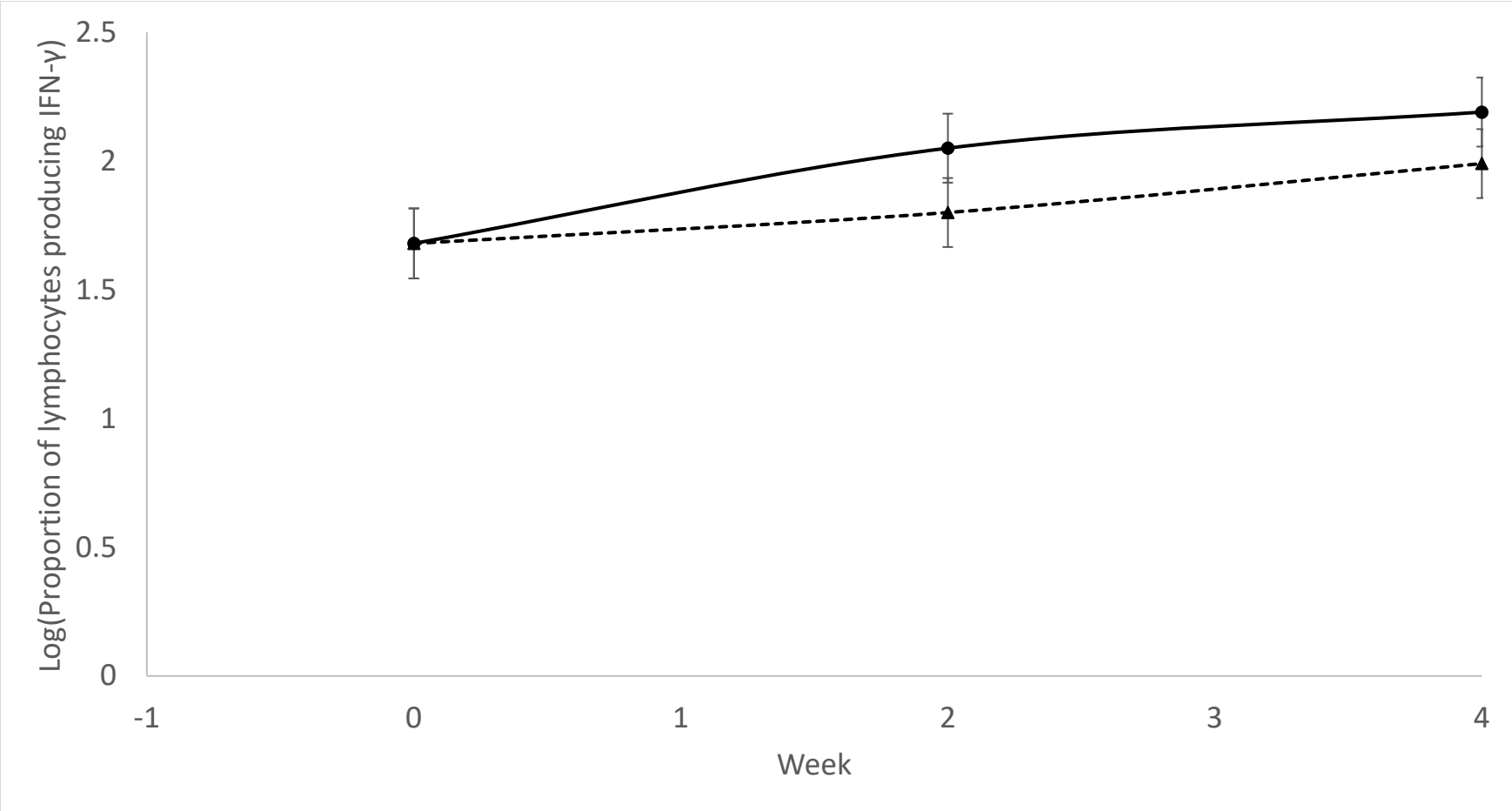

Figure 3. 2 Proportion of lymphocytes expressing interferon-γ by week in steers (n = 20) previously grazed on endophyte infected or novel endophyte tall fescue pastures during receiving phase. Only high exit velocity steers (n = 1/paddock) were utilized for this analysis. Endophyte treatment did not influence the proportion of lymphocytes expressing interferon-γ (P = 0.34), but these proportions did fluctuate over the 4-week collection period (P < 0.01). An interaction between endophyte treatment and week was not detected (P = 0.54). E+ = ●. E- = ▲.

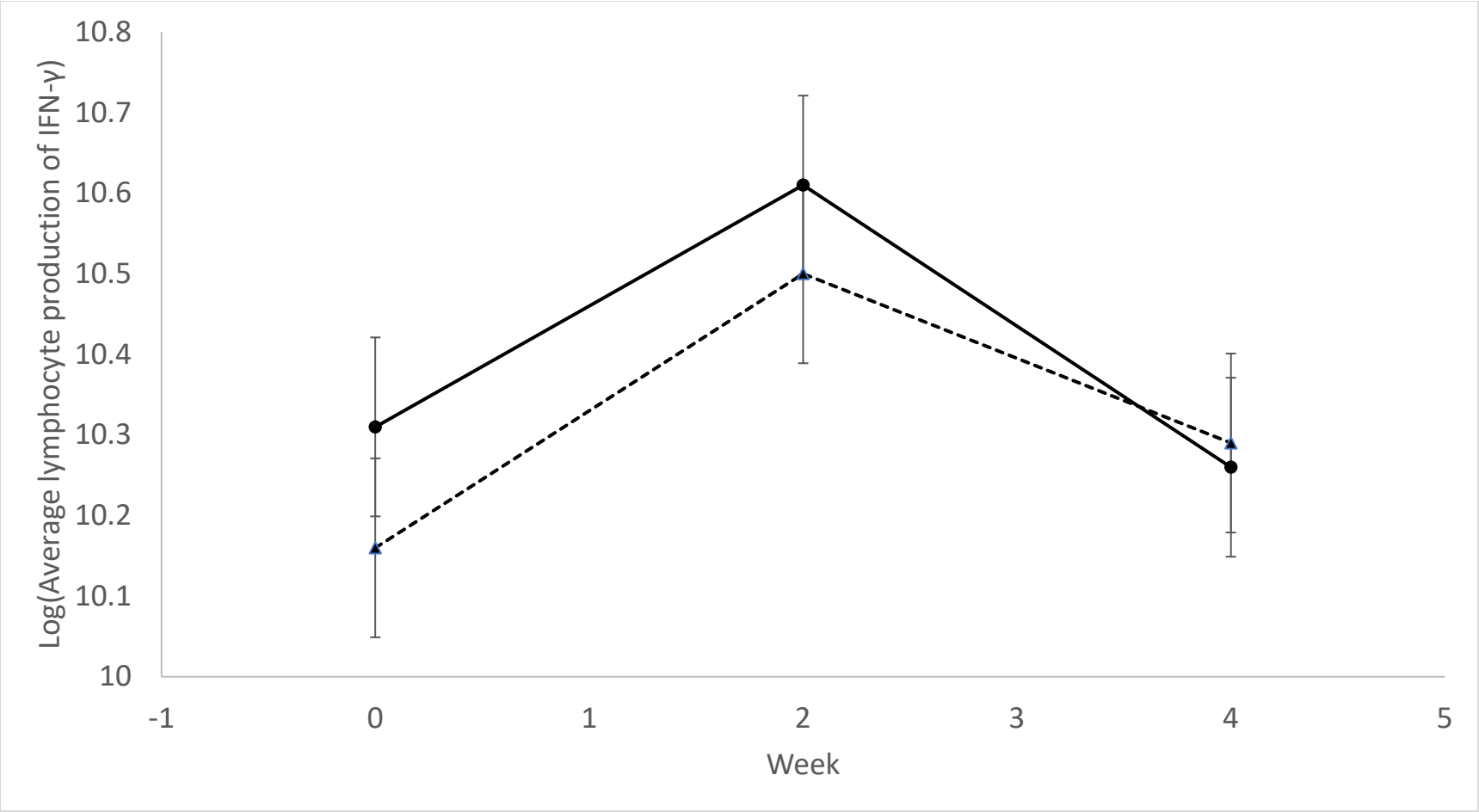

Figure 3. 3 Average lymphocyte production of interferon-γ by week in steers (n = 20) previously grazed on endophyte infected or novel endophyte tall fescue pastures during receiving phase. Endophyte treatment did not influence the average lymphocyte production of interferon-γ (P = 0.82), but average production of this cytokine by lymphocytes did fluctuate over the 4-week collection period (P < 0.01). Only high exit velocity steers (n = 1/paddock) were utilized for this analysis. An interaction between endophyte treatment and week was not detected (P = 0.59). E+ = ●. E- = ▲.

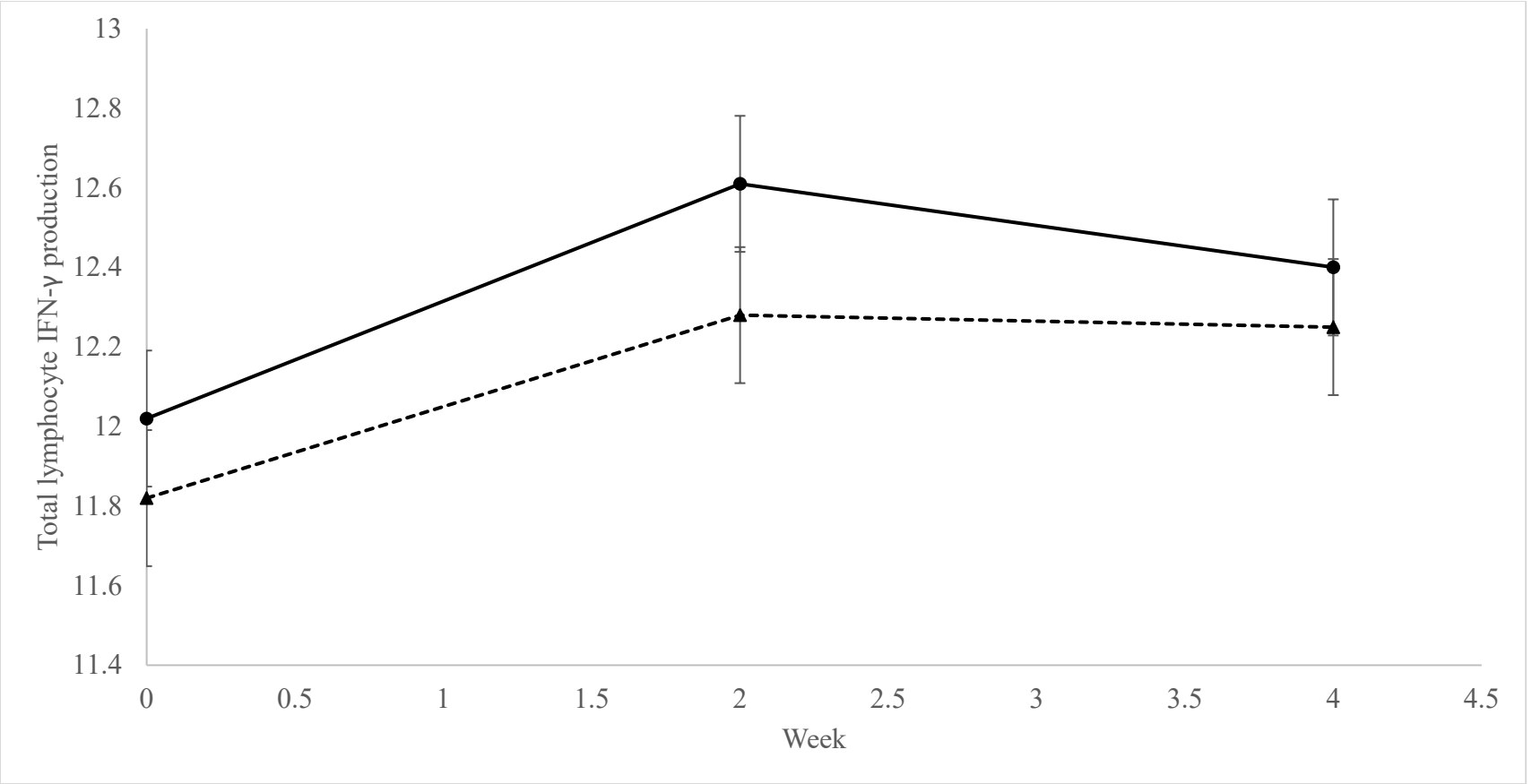

Figure 3. 4 Total lymphocyte interferon-γ production by week in steers (n = 20) previously grazed on endophyte infected or novel endophyte tall fescue pastures during receiving phase. Total IFN-γ production was calculated as the product of the average production of IFN-γ and the proportion of lymphocytes producing IFN-γ. Only high exit velocity steers (n = 1/paddock) were utilized for this analysis. Endophyte treatment did not influence the total lymphocyte production of interferon-γ (P = 0.40), but total production of this cytokine by lymphocytes did fluctuate over the 4-week collection period (P < 0.01). An interaction between endophyte treatment and week was not detected (P = 0.68). E+ = ●. E- = ▲.

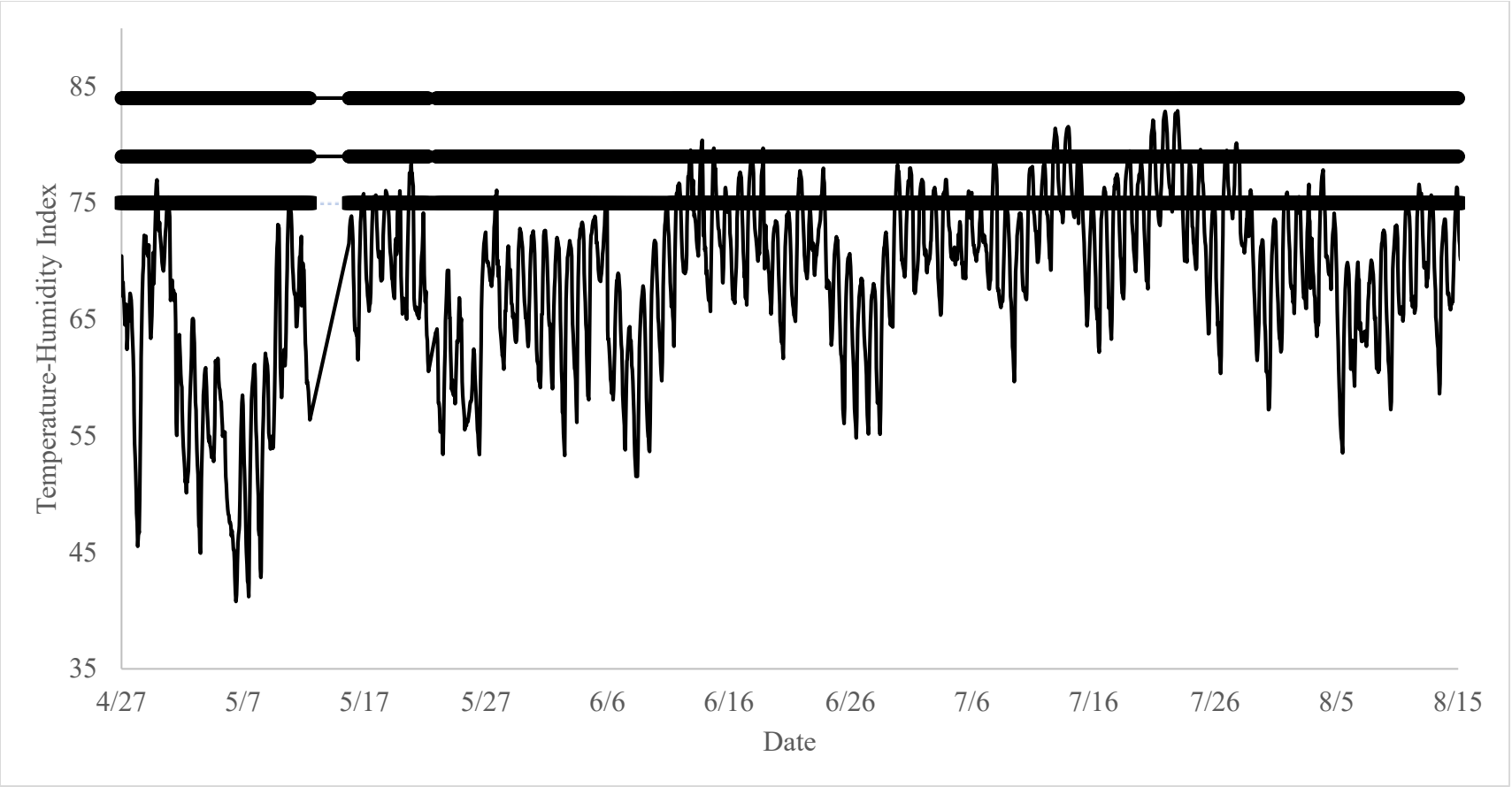

Figure 3. 5 Environmental temperature-humidity index during the 110d grazing period. The fluctuating line represents the ambient THI in pastures, which were adjacent to the NOAA weather station used to collect these data. There are missing data due to station malfunction from 5/11 (d15) at 1300 to 5/14 (d18) at 1600 and 5/21 (d25) at 0700 to 5/21 (d25) at 2000. The three straight lines represent the three levels of heat stress described by Hahn (1999): Alert (bottom, THI = 75), Danger (middle, THI = 79), and Emergency (top, THI = 84).

Chapter 4: Influence of handling and transportation on systemic cytokine production

Abstract

Two experiments were conducted to investigate the effects of stress on immunological responses of beef cattle. Calves were evaluated at weaning for exit velocity. In experiment 1 (EXP 1), 18 steer and 18 heifer Angus calves were selected as the highest (n=9/sex) and lowest (n=9/sex) exit velocities measured in purebred Angus calves born that year (n=85). Exit velocity treatment (high and low) was assigned in both experiments based on these groupings. Calves were subjected to a 4-week halter-breaking program to evaluate the effect of human handling on interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-12 (IL-12) expression changes. Calves were tied to railings and exposed to human contact 5 times per week for 4 weeks. Three mL of blood was collected via jugular venipuncture once weekly for RT-PCR analysis of the aforementioned cytokines. EXP 1 data was analyzed as ΔΔCT values. Experiment 2 (EXP 2) examined the effect a 500-mile transport on isolated peripheral lymphocyte production of IFN-γ, as determined by flow cytometry, in the steers from EXP 1 using the same high/low exit velocity treatments. Plasma blood samples were collected via jugular venipuncture on d0, d1, 2, 3, 7, 19, and 28, relative to start of transport. From these samples, PBMCs were isolated, counted, stimulated, stained for IFN-γ, and analyzed using flow cytometry. EXP 2 data was analyzed as proportion of lymphocytes producing IFN-γ, average lymphocyte production of IFN-γ, and total production of IFN-γ. Cattle in both experiments were fed a corn silage diet. During EXP 1, relative expression of TNF-α was higher ($P<0.01$) and IFN-γ lower ($P=0.03$) in high

exit velocity calves. IFN-γ (P=0.07), IL-6 (P=0.02), IL-10 (P<0.01), and IL-12 (P=0.04) relative gene expression linearly increased over time, irrespective of treatment. In EXP 2, proportions of lymphocytes producing IFN-γ were higher (P=0.10) and average lymphocyte production of IFN-γ was lower (P=0.02) in high exit velocity steers, and these two responses offset each other so that total lymphocyte production of IFN-γ was not affected by exit velocity treatment (P=0.77). The results of these experiments indicate that relative cytokine expression may be affected by exit velocity and improve over time as calves adjust to stressors. Additionally, weaning exit velocity and transportation may not interact to influence the total production of IFN-γ by lymphocytes, but may affect the $CD4^+$ differentiation and individual cell production capabilities.

Keywords: lymphocyte, cattle, cytokine, IFN-γ, stress

Introduction

The average US beef calf will experience multiple transition periods in its lifetime such as weaning, transportation, new locations, new diets, and exposure to new pen mates (Loerch and Fluharty, 1999). This amount of change requires the calf to constantly adjust to new surroundings, with some coping better than others (Koolhaas et al., 1999). This constant transition requires an unavoidable human-calf interaction, with some form of handling observed at every level of production. One method utilized to desensitize calves to human presence is halter breaking, which constitutes an intense handling situation as calves are coerced into a gradual acclimatization to human interaction. The process of handling cattle has been reported to be ineffectual on average daily gain, but increase white blood cell counts (Petherick et al., 2009b) and plasma concentrations of the acute

phase protein ceruloplasmin (Francisco et al., 2012), indicating a potential effect of acclimating cattle to human handling on immune function.

Cattle previously exposed to human handling, compared with cattle novel to handling, are reported to have lower exit velocities at the end of the handling period (Petherick et al., 2009a; Francisco et al., 2012). Measures of temperament, particularly exit velocity, have been noted to be associated with an animal's immune function (Oliphint, 2006; Burdick et al., 2011). Recent studies from our laboratory demonstrated weaning exit velocity has an inverse relationship with systemic interferon-γ (IFN-γ) concentrations (Altman, 2015), and a direct relationship with rectal temperatures (Altman et al., 2016) during the first 24 hours following an LPS injection. In both experiments of Chapter 2, total and average lymphocyte production of IFN-γ were increased in higher exit velocity heifers following the endophyte exposure and increased temperature humidity index period in comparison with lower exit velocity heifers. Collectively, these studies suggest exit velocity, as a measure of temperament, may be related to production of immune system components in cattle.

However, examination of changes in IFN-γ, as well as other key cytokines involved with regulation of innate and adaptive immune responses, during periods of intense human handling remain unreported. If such relationships existed, they could be utilized through evaluation of exit velocity, and other measures of temperament, to evaluate cattle for potential future incidence of and recovery from morbidity.

Similar to handling, transportation of cattle from one location to another may lead to a decrease in the immune status of the animal (Swanson and Morrow-Tesch, 2001). This, coupled with exposure to new pathogens, can lead to increased morbidity/mortality

within a herd during the feedlot receiving period. Examining the relationship between transport-related stress and measures of temperament, namely weaning exit velocity, may improve understanding of morbidity and mortality rates during the feedlot receiving period. In turn, strategies may be developed to identify and isolate 'more susceptible' animals in an effort to more accurately target interventions and improve health management strategies for feedlot cattle.

Therefore, the objective of these experiments was to determine if systemic cytokine production differed between high and low calf exit velocity in response to handling and transportation events. In EXP 1, this constituted an examination of relative gene expression of cytokines involved with activating innate responses and modulating differentiation of $CD4^+$ T cells. It was hypothesized these expression patterns would increase as calves became more accustomed to human presence, but that this increase would be lessened in calves with greater flight responses. In EXP 2, calves were evaluated for lymphocyte IFN-γ production before and after a simulated transport from sale barn to feedlot. It was hypothesized transportation would exacerbate the level of immunosuppression experienced by high exit velocity calves to a greater degree than in low exit velocity calves, and that these responses would be particularly evident in the period immediately following the transportation period.

Materials and Methods

All methods were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Experiment 1

Eighteen Angus steers and eighteen Angus heifers, born on the University of Kentucky Oran C. Little Animal Research Center Beef Unit, were selected from their herd mates based on exit velocity measured at weaning. All calves (n = 85) born in the fall of 2016 were evaluated for weaning exit velocity using a laser trip system (Bruno et al., 2017; FarmTek, North Wylie, TX) following release from a headgate. The start and stop lasers were set 1.68 m apart, with the first laser approximately 0.5 m in front of the head gate to ensure calves did not prematurely start the timer prior to release from the head gate. At this time, vaccinations against viral and bacterial pathogens (Bovi-Shield Gold 5, Zoetis, Florham Park, NJ; Once PMH, Merck Animal Health, Summit, NJ; Somubac, Zoetis; Ultrachoice 7, Zoetis) were boostered and each calf's weaning weights was recorded. Calves were selected for inclusion in this study from the extreme ends (within sex) of the weaning exit velocities evaluated. The exit velocity ranges for each group are as follows: all Angus calves measured = 0.68 to 4.73 m/s; high exit velocity heifers = 3.13 to 3.98 m/s; low exit velocity heifers = 0.91 to 2.26 m/s; high exit velocity steers = 2.86 to 4.73 m/s; low exit velocity steers = 0.68 to 1.35 m/s. The range of these measured exit velocities is consistent with those reported by others (Fell et al., 1999; Hall et al., 2011).

A baseline blood sample was collected at weaning (d-15 for heifers, d-21 for steers) via jugular venipuncture into a Tempus Tube (Thermo Fisher Scientific, Waltham, MA) for use as a reference sample in later analyses of relative gene expression. The weaning period lasted 2 weeks, during which human interference was limited. Following the weaning period, calves underwent a 4-week halter breaking period. During this time, calves were placed in a head gate for collection of blood samples and placement of

halters. Blood samples were collected weekly on d1, 8, 15, and 22 via jugular venipuncture into Tempus Tubes. Samples were frozen at -20C until further analyzed. Following release from the head gate, calves were placed in pens of three, tied to railings, and exposed to human interaction (i.e. brushing and rubbing) for 2 hours a day, 5 days a week.

RNA Extraction and RT-PCR Analysis

Following collection, Tempus® tubes (ThermoFisher) were placed in a -20°C freezer until analyzed. Samples were grouped by collection date for thawing and resulting mRNA fragments were isolated following the manufacturer's instructions.

Cytokine gene expression was determined using an Applied Biosystems 7500 sequence detection system (Applied Biosystems, Foster City, CA) following the methods of Adams et al. (2008). Briefly, 0.5 µg total RNA was diluted in nuclease-free water to a final volume of 49.5 µL, added to 30.5 µL reverse transcription master mix and 16 µL $MgCl_2$. Reactions occurred at 42°C for 15 minutes and 95°C for 5 minutes in a thermocycler, with resulting cDNA diluted to 1:1 in nuclease-free water to allow measurement of multiple genes simultaneously using real time PCR. Incubated PCR reactions occurred at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each reaction well contained 20 µL master mix, 6.25 µL nuclease-free water, and 5 µL cDNA template, with all samples analyzed in duplicate. Changes in gene expression were calculated by relative quantitation using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), with each animal's weaning sample used as the

control sample for that animal (Schmittgen and Livak, 2008). The equations used were as follows:

$\Delta\Delta C_T = (C_T \text{ of cytokine} - \text{Average } C_T \text{ of GADPH})_{\text{Day of interest}} - (\text{Average } C_T \text{ of cytokine- Average } C_T \text{ of GADPH})_{\text{Weaning}}$

Relative quantity of cytokine expression = $2^{-\Delta\Delta CT}$.

Cytokine gene expression in each sample was analyzed using TaqMan™ primer-probe sets (Thermo Fisher, Waltham, MA) for GADPH (for use as a housekeeping gene; Bt03210913_g1), interleukin-6 (IL-6; Bt03211905_m1), interleukin (IL-10; Bt03212727_m1), interleukin-12 (IL-12; Bt03213922_m1), interferon-γ (IFN-γ; Bt03212723_m1), and tumor necrosis factor-α (TNF-α; Bt03259156_m1) expression using inventoried TaqMan™ assays (Applied Biosystems, Foster City, CA). Weaning samples were evaluated for potential exit velocity treatment effects to ensure any observed differences during the halter breaking period. From this data set, 2 calves were excluded from the TNF-α analysis, and 1 calf from IL-6 and IFN-γ analyses, as they were deemed to be outliers using the inter-quartile method (JMP 13, SAS, Cary, NC). Animals removed from weaning samples analyses were excluded from subsequent analyses involving the cytokine from which they were excluded. None of the cytokines differed in expression at weaning between exit velocity treatments ($P \geq 0.20$; data not shown).

Experiment 2

The 18 Angus steers used in EXP 1 (462 ± 43.3 kg BW; 398 ± 13.7 days old), were used for EXP 2. One truck was utilized in transporting all 18 steers approximately

805 km, simulating a typical journey from a central KY sale barn to Midwest US feedlots. Steers were placed on a 36 ft trailer at 0800 and returned to the UKARC around 1800 the same day. Animal location within the trailer was balanced between high and low exit velocity treatments to prevent location bias. Shipping hours were selected to simulate average conditions experienced by calves during a typical shipping period in the fall. Following the transportation phase, steers were placed in small group pens (n = 3; 6 steers/pen) based on starting body weight and balanced for exit velocity treatments. Calves remained in these pens throughout the duration of the study and were fed a typical receiving transition diet (Table 4.1). Pens were cleaned each week to minimize fecal material or urine present in the pen. Fresh bedding, in the form of wood shavings, was provided to maintain a clean, dry area for the calves. Steer body weight were recorded at each blood collection (days 0, 1, 3, 8, 15, and 29).

Blood Sampling

Blood samples were collected on days 0, 1, 3, 8, 15, and 29 from each steer via jugular venipuncture into 3-15 mL Vacutainer (Bectin-Dickson; Franklin Lakes, NJ) tubes with Na-heparin added prior to blood collection. Samples were collected prior to morning feeding. Plasma was harvested from these samples and used to isolate peripheral blood mononuclear cells (PBMC).

Evaluation of Lymphocyte IFN-γ Production

In vitro cell mediated immunity was assessed through isolation of PBMCs from each sample using a protocol modified from the methods of Breathnach et al. (2006).

Briefly, upon arrival to the lab, all three blood tubes from each animal were composited into a 50 mL centrifuge tube, then centrifuged at 800 x g for 30 minutes using a slow brake. The resulting buffy coat was transferred to a new tube, rinsed with 10 mL of warm PBS, and layered on top of 10 mL Ficoll-Paque Plus^{TM} solution (Amersham Biosciences, Piscataway, NJ) in another 50 mL centrifuge tube. Samples were spun at 500 x g for 30 minutes using a slow brake. Cells were harvested and transferred to another 50 mL centrifuge tube containing 20 mL PBS and spun at 500 x g for 10 minutes using a fast brake. Supernatant was dumped out of this tube and 5 mL PBS was used to resuspend cells, with 10 mL D_2O and 10 mL RPMI were added to this suspension. Warm PBS was used to top off all tubes to a final volume of 45 mL PBS when needed. Tubes were placed in a centrifuge and spun at 300 x g for 10 minutes with a fast brake, and these specifications were used for all subsequent spins. The resulting supernatant was dumped from the tube, cells were resuspended in 45 mL of PBS, and tubes were centrifuged. Supernatant was dumped from the tubes, and and 10 mL PBS was used to resuspended cells. A subsample of 100 µL was removed from each tube and added to 900 µL of PBS for quantification of cells in each sample using a Vicell Counter-XR (Beckman Coulter, Miami, FL), with resulting data for calculating the volume needed to freeze duplicate samples at 2.5 x 10^7 cells/mL. The appropriate volume from each tube was obtained by removing excess cell solutions from each tube, with a subsequent centrifuge spin. Resulting supernatant was dumped from each tube, and cells were resuspended with in a freeze media consisting of 50% RPMI (Gibco, Grand Island, NY), 40% fetal bovine serum (Sigma-Aldrich, St. Louis, MO; FBS), and 10% DMSO (Sigma). Solutions were

transferred into in 1.5 mL microcentrifuge tubes and placed in liquid nitrogen until all samples were collected.

All time points from the same animal were analyzed together. On the day of analysis, samples were thawed in warmed 10 mL cRPMI (10% FBS (Sigma), 1% penicillin-streptomycin-glutamine, 0.1% 2-mercaptaethanol, and 88.9% RPMI (Gibco)) and PBMCs re-enumerated on a Vicell Counter-XR (Beckman Coulter, Miami, FL), and plated, stimulated, and stained following the methods described by Breathnach et al. (2006). Briefly, animal samples were split into two subsamples on a 24 well plate to form a control and stimulated well for each animal. To prevent secretion of synthesized IFN-γ, 2 µL of brefeldin A (BFA) was added to each well, and an additional 10 µL of phorbol 12-myristate 13-acetate (PMA)/ionomycin was added to each of the stimulated sample wells to induce IFN-γ production. Sample plates were placed into an incubator for 4 hours at 5% CO_2 and 37°C. Following incubation, 200 µL was removed from each sample well, placed in a 96 well plate, and centrifuged at 500 x g for 5 minutes, and the resulting supernatant was dumped. Cells were fixed in 100 µL of 2% paraformaldehyde and placed in a 4°C refrigerator overnight.

Intracellular staining and flow cytometry analysis of the PBMCs was completed following the methods of Breathnach et al. (2006). Briefly, isolated cells were centrifuged at 500 x g for 5 minutes, resuspended in 150 µL saponin buffer (1% fetal bovine serum, 0.1% saponin, and 0.1% sodium azide), and recentrifuged at 500 x g for 5 minutes. Cells were stained with 10 µg antibody/mL of mouse IgG1 anti-bovine IFN-gamma FITC conjugated antibody (MCA1783F, Bio-Rad, Hercules, CA) in a 99% saponin buffer solution, and then incubated on ice for 30 minutes. Following incubating,

plates were spun at 500 x g for 5 minutes, and cells were resuspended in saponin buffer. Plates were recentrifuged at 500 x g for 5 minutes, and 200 μL FACS buffer was added to each well.

Isolated PBMCs were passed through an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) and evaluated using forward and side scatter parameters, with data collected from the first 30,000 events. From this data, lymphocyte populations were evaluated for proportion producing IFN-γ and average production of IFN-γ. In addition, these two parameters were multiplied together to provide an approximation of total IFN-γ produced by the lymphocyte population (Darrah et al., 2007). PMA-ionomycin stimulated samples were compared with control samples from each steer to ascertain the magnitude of response.

Statistics

Experiment 1

Weaning sample data was analyzed as ΔC_T values using Proc Mixed of SAS (9.4), with gender included as a fixed blocking effect in the model statement. The denominator degrees of freedom were calculated using the Kenward Roger method, and main effects in the model statement included exit velocity treatment and gender. From the 36 data points in each data set, 2, 1, and 1 data points were removed from TNF-α, IL-6, and IFN-γ, respectively.

Data from the halter breaking period was analyzed as $\Delta\Delta C_T$ values rather than relative quantity (RQ) values, as these were normally distributed and RQ values were log distributed, thus meeting the assumptions of ANOVA. Data points were deemed to be

outliers using the inter-quartile method (JMP 13, SAS, Cary, N.C.). Of the 143 data points in each data set, there were 12, 4, 7, and 9 data points removed from TNF-α, IL-12, IL-6, IL-10, and IFN-γ data sets, respectively, prior to statistical analysis, which utilized the mixed procedure of SAS (9.4) with repeated measures. The autoregressive 1 covariance structure was selected, with animal ID as the subject. Animal gender was included in the model statement as a blocking effect. Main effects of the model included week, exit velocity treatment, week x exit velocity treatment, and gender, with denominator degrees of freedom calculated using the Kenward Roger method. Significance was set at $P < 0.10$, and trends considered between $0.10 < P < 0.15$.

Experiment 2

This experiment was conducted as a completely randomized design with repeated measures, with calf as the experimental unit. Calves were evaluated for measures of lymphocyte production of IFN-γ (proportion of lymphocytes producing IFN-γ, average lymphocyte production of IFN-γ, and total lymphocyte production of IFN-γ) and average daily gain. All data was tested for normality using JMP Pro (13.0, Cary, N.C.) prior to analysis with SAS (9.4, Cary, N.C.).

Average daily gain responses were analyzed using the GLM procedure of SAS (9.4), with weaning exit velocity treatment as the main effect. Animals were penned by body weight, and data initially analyzed with pen as a block. However, block was determined to be non-significant ($P = 0.55$) and uninfluential to exit velocity analysis and was subsequently dropped from the model.

The lymphocyte IFN-γ production data were analyzed using the mixed procedure of SAS (9.4), with the Kenward-Roger method used to estimate denominator degrees of freedom. The covariance structure specified was first-order autoregressive. The repeated term was sample date, with subject specified as animal ID. Average production of IFN-γ responses were natural log transformed to provide a normalized data set for analysis, thus meeting the assumptions of ANOVA. The proportion of lymphocytes and total lymphocyte production of IFN-γ data did not require similar transformations. Significance was set as $P \leq 0.10$, and trends considered to be $0.10 < P \leq 0.15$.

Results and Discussion

Experiment 1

Relative expression was determined to increase across time (Table 4.2), regardless of exit velocity treatment, for IFN-γ ($P = 0.02$, Fig. 4.1), IL-6 ($P = 0.02$, Fig. 4.2), IL-12 ($P = 0.04$, Fig. 4.3), and IL-10 ($P < 0.01$, Fig. 4.4). Across the 4-week halter-breaking period, the expression of all 4 cytokines increased by a magnitude of approximately 1. The similar direction of change among these cytokines agrees with current literature that relates IL-6, IL-12, and IFN-γ production changes, and agrees with the interrelationships associated with the cytokines. Interleukin-6, which can contribute to both anti-inflammatory and pro-inflammatory responses (Scheller et al., 2011), has been reported to induce synthesis of IL-10 (Steensberg et al., 2003). Similarly, IL-12 is used by T cells to stimulate production of IFN-γ (Lynch, 2010), which goes on to activate macrophages, increasing production of pro-inflammatory cytokines, and thus decreasing the effectiveness of already present IL-10. In contrast, IL-10 is a major anti-inflammatory

cytokine involved in many immune responses to counteract the effects of pro-inflammatory cytokines to prevent excessive damage to tissues during the immune response (Murphy et al., 2012). Increasing concentrations of IL-10 have been noted to have a suppressive effect on IL-12 (Murphy et al., 2012), which may subsequently influence IFN-γ and IL-6 expression. Therefore, the similar fold-increase of expression among these 4 cytokines may represent a return to normal levels following weaning and be unrelated to the handling to which the calves were subjected. Conversely, no relationship between exit velocity and of relative cytokine expression was observed for IL-6 ($P = 0.61$), IL-12 ($P = 0.19$), and IL-10 ($P = 0.73$).

The increasing relative expression of these cytokines over time suggests expression may have been suppressed initially at weaning and was lessened as acceptance of their new environment occurred. One possible mechanism for this observed pattern, which was not measured in this study, relates to glucocorticoid and catecholamine production (Wiegers et al., 2005). Glucocorticoids and catecholamines have been noted to suppress immune function, particularly cytokine synthesis, through inhibition of NF_KB translocation (Auphan et al., 1995), with catecholamines additionally reported to suppress macrophage activation (Tracey, 2002).

Of the 5 cytokines measured, only TNF-α expression did not change relative to the weaning sample. In response to weaning, O'Loughlin et al. (2011) reported that TNF-α whole blood expression increased during the first 24h and stayed elevated over the next 7d. A similar response was observed in that study for IFN-γ, but to a much larger magnitude. This would suggest that in unstimulated blood samples, such as those used in the current experiment, changes in TNF-α are less responsive to changes in the calf's

environment than IFN-γ. However, there was an increased amount of TNF-α in high, as compared with low, exit velocity calves ($P < 0.01$; Table 4.2). This cytokine enhances vasculature epithelium expression of adhesion molecules used by the immune system for recruitment of leukocytes, chemokines, and cytokines to the site of infection (Murphy et al., 2012). Collectively, this may indicate high exit velocity calves have an enhanced ability to convalesce infected and/or damaged tissue, potentially leading to a more rapid recovery and diminished opportunity for the infection to spread

In addition to increased relative TNF-α expression, high exit velocity calves had lower relative expression of IFN-γ (Table 4.2; $P = 0.06$) during the experimental period. This is consistent with our previous investigation of this relationship following a lipopolysaccharide challenge (Altman, 2015). Interestingly, when glimpsed across time (Fig. 4.5), the magnitude of difference between treatments increased, particularly over the last two weeks, suggesting that if changes in expression of this cytokine are due to acclimation, then high exit velocity calves did not acclimate to human handling during the 4-week period.

It is interesting that IL-12 relative expression was not similarly affected as this cytokine is used by the immune system to stimulate production of preferentially differentiated $CD4^+$ T cells into the T_H1 subtype, a major producer of IFN-γ (Seder et al., 1993; Lynch, 2010). As exit velocity treatment did not appear to influence IL-12 expression, it may be hypothesized that the established exit velocity relationship with IFN-γ mRNA expression was independent of IL-12 production. There are feasible mechanisms through which this type of production may occur. For example, a change in IL-4 production without a subsequent change in IL-12 could influence relative expression

of IFN-γ, as these two cytokines have been shown to stimulate T_H2 and T_H1 differentiation, respectively (Manetti et al., 1993). A shift in favor of a T_H2 response would result in decreased IFN-γ production without influencing IL-12 concentrations.

Experiment 2

Average Daily Gain

The main objective of this study was to identify the potential relationship between exit velocity and lymphocyte production of IFN-γ following a typical transit between sale barn and feedlot. To facilitate estimation of the daily amount of feed needed for each pen, which was calculated on a kg feed/kgBW basis and managed as described by Bruno et al. (2017), animals of similar body weight were penned together, with each pen balanced to ensure treatments were equally represented. As in Bruno et al. (2017) and Chapter 3, both of which utilized a similar diet to the one in the present study, ADG did not differ between exit velocity groups in the current study during the first 28d in the feedlot setting.

Peripheral Blood Mononuclear Cells

Analysis of all three measures of lymphocyte production of IFN-γ (proportion of lymphocytes producing IFN-γ, average lymphocyte production of IFN-γ, and total production of IFN-γ) is critical to gaining a full understanding of the effect of a given treatment on an animal's cell-mediated immune response. Changes in the proportion of lymphocytes expressing IFN-γ provide a measure of the magnitude of response to treatment by the cellular population, whereas average lymphocyte production of the

cytokine describes the quality or potency of cellular changes to the treatment (Darrah et al., 2007). By multiplying these two values together to obtain the total production by lymphocytes of IFN-γ, a conclusion of how much a treatment influences the total functional response, on a systemic level, may be drawn (Darrah et al., 2007). The results from this experiment illustrate why inclusion of only one of these variables may lead to an inaccurate analysis as each variable may have a different relationship with factors of interest.

Total lymphocyte production of IFN-γ ($P = 0.03$; Chart 4.6) and the proportion of lymphocytes producing IFN-γ ($P < 0.01$, Chart 4.7), but not average lymphocyte production of IFN-γ ($P = 0.76$, Chart 4.8), initially decreased following transportation and returned to levels similar to baseline by the end of the 28d receiving period. This may indicate that transportation initially depresses differentiation of $CD4^+$ cells into the T_H1 subtype, consequently causing an overall suppression of total lymphocyte production of IFN-γ without affecting the rate of synthesis of this cytokine by already present $CD8^+$ and $CD4^+$ T_H1 lymphocytes, regardless of exit velocity. However, our study did not include a group of non-transported calves to confirm the observed decrease was due to transportation and not some other, unknown factor.

The influence of exit velocity treatments on lymphocyte production of IFN-γ (Table 4.3) was inconsistent across each of these response variables. An approximate 25% increase in the proportion of lymphocytes producing IFN-γ ($P = 0.10$) was observed with high, as compared with low, exit velocity steers. The opposite was observed in average lymphocyte production of IFN-γ ($P = 0.02$), as low exit velocity animals had approximately 14% greater production levels than high exit velocity animals. As a result

of the opposing responses of lymphocyte proportions and average production of IFN-γ, total lymphocyte IFN-γ production did not differ between exit velocity treatments (P = 0.77). However, despite the overall absence of IFN-γ production differences between exit velocity treatments, these results indicate that exit velocity may work simultaneously through two separate mechanisms to influence production of this cytokine. The diverging relationships of lymphocyte proportions and average production of IFN-γ with exit velocity suggests that in response to intracellular bacteria and viruses, calmer steers may be more efficient with already present IFN-γ producing lymphocytes.

Another potential explanation for the diverging treatment effects may be the mitogen used, as PMA + ionomycin is used to preferentially activate and maximize the amount of IFN-γ produced by cells *in vitro* by activating T_H1 responses and down-regulating IL-10 production (Baran et al., 2001). As the objective of this experiment was to examine potential relationships between lymphocyte IFN-γ production and exit velocity, our methodology required a mitogen that would maximize IFN-γ production, thereby providing a stronger test of this potential relationship. However, as this mitogen specifically targets only one aspect of cell-mediated immunity (i.e. T_H1 responses), it may exaggerate any observed treatment effects. Thus, to further investigate this relationship, future research should utilize a mitogen that does not preferentially activate T_H1 responses, such as phytohaemagglutinin or concavalin A, to explore any potential differences in other cytokines which may inhibit IFN-γ production and develop a mechanistic model which may explain the observed relationships observed with exit velocity in this experiment.

Conclusion

Results from these experiments indicate relationships between exit velocity and cytokine production may be present in cattle during periods of handling and transportation. The decreased expression of IFN-γ in high, relative to low, exit velocity cattle confirms our previous observations of a relationship between this cytokine and measure of temperament. The relatively higher expression of TNF-α in high exit velocity calves may indicate an increased ability to induce changes in vascular epithelium, specifically in expression of adhesion molecules used to recruit other immune mediators to the site of inflammation, potentially expediting the convalescence of the injured tissue. Together, these two responses may indicate that exit velocity shows promise as a measure for evaluating the ability of calves to resist intracellular bacterial and viral pathogens as well as eradicate and alleviate pathogen tissue infiltration and damage.

The absence of an overall net effect on lymphocyte production of IFN-γ between treatments suggests exit velocity may not be a good predictor of morbidity following transportation periods. However, these results also indicated exit velocity may be a measure of temperament that could be used to describes changes in lymphocyte differentiation and production, specifically as they relate to IFN-γ production. Exploration of these pathways was beyond the scope of this study but warrants further investigation.

Table 4. 1 Diet composition of 28-day receiving period following 805 km transport for 18 Angus steers

	% of DM			
	Week 1	Week 2	Week 3	Week 4
Corn Silage	70	70	50	35
Distiller's Dried Grains	10	10	20	25
Cracked Corn	10	10	20	30
Supplement[1]	10	10	10	10

[1]Supplement contained macro and trace minerals and vitamins formulated to support ADG of 1.93 kg/d, along with urea, monensin, and tylosin, in a ground corn carrier.

Table 4. 2 Influence of exit velocity on the ΔΔCT of cytokines in Angus steers (n = 18) and heifers (n = 18), blocked by gender, during a 4-week halter breaking period.

	Exit Velocity[a,b]			P-Value				
							Contrasts	
	Low	High	SEM	EV	Week	EV*Week	Linear	Quad
IFN-γ	-0.18 (1.13)	0.39 (0.77)	0.209	0.06	0.07	0.30	0.02	0.95
IL-6	-0.41 (1.33)	-0.24 (1.18)	0.228	0.61	0.01	0.49	<0.01	0.63
IL-12	-0.78 (1.72)	-0.32 (1.25)	0.244	0.19	0.04	0.70	0.02	0.86
TNF-α	0.43 (0.74)	-0.16 (1.12)	0.131	<0.01	0.99	0.64	-	-
IL-10	-0.89 (1.86)	-0.79 (1.73)	0.228	0.73	<0.01	0.98	<0.01	0.68

[a]Values are presented as ΔΔCT with associated errors. Statistical analysis was conducted on these values, as they were normally distributed.
[b]Values in parentheses are the transformed 'relative quantity' (RQ) values

Table 4. 3 Cell Mediated Immunity by exit velocity of 18 Angus steers in response to 805 km transport

	Exit Velocity Treatment			P-Value		
	Low	High	SEM	EV	Day	EV x Day
Proportion of lymphocytes producing IFN-γ[a]	2.21	2.77	0.240	0.10	<0.01	0.94
Average lymphocyte production of IFN-γ[b,c]	7.41	7.26	0.042	0.02	0.76	0.61
Total lymphocyte production of IFN-γ[c]	3800	3991	443.5	0.77	0.03	1.00

[a]Means are expressed as a percentage
[b]Means are natural log transformed, original units were arbitrary
[c]Means are expressed in arbitrary units. Calculated as the product of the proportion of lymphocytes producing IFN-γ and the average lymphocyte production of IFN-γ

Table 4. 4 Average daily gain by exit velocity of 18 Angus steers over 28 days following 805 km transport

	Exit Velocity Treatment			P-Value
	Low	High	SEM	EV
ADG (kg)	2.10	2.08	0.152	0.92

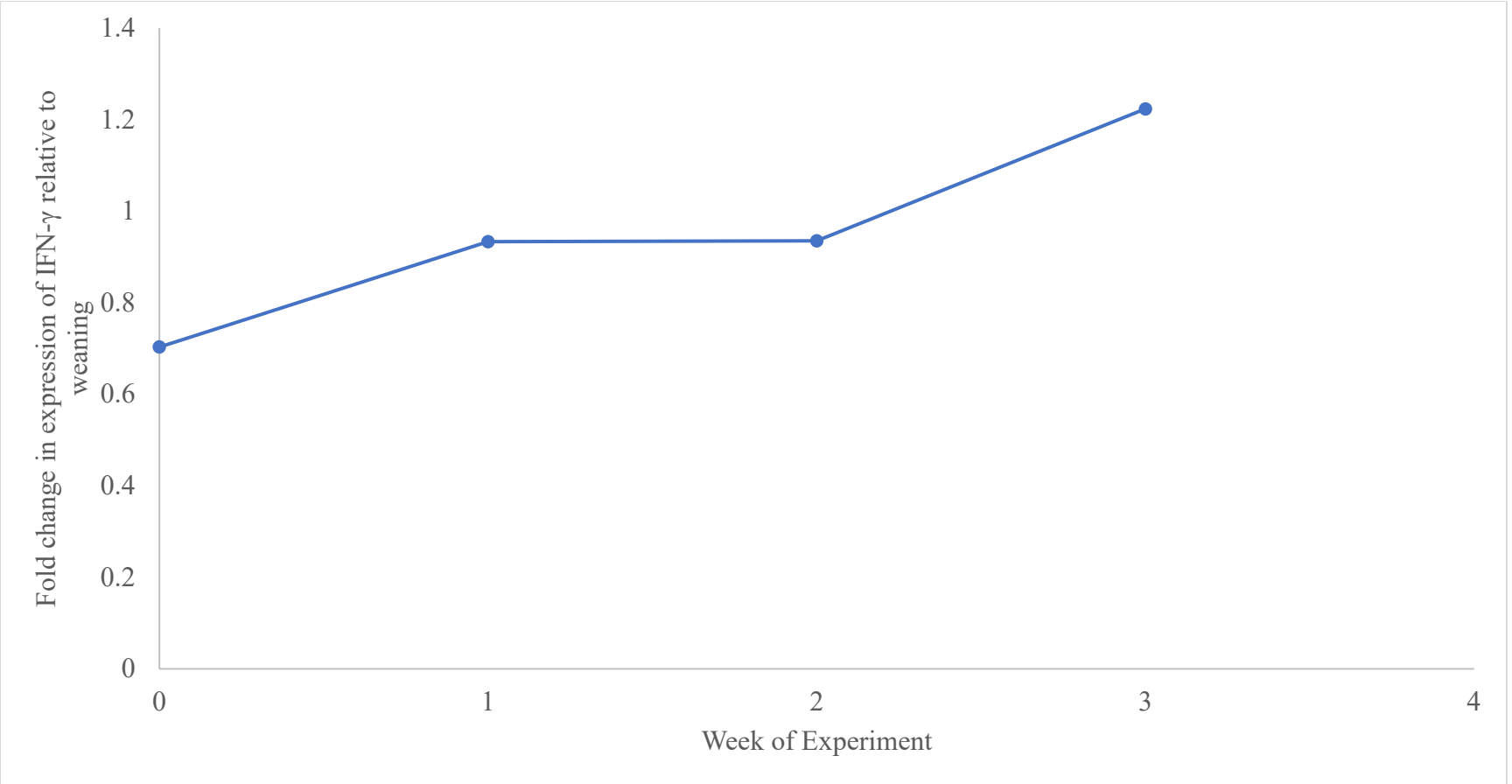

Figure 4. 1 Fold change in expression of Interferon-γ, relative to weaning, in Angus steers and heifers (n = 18/sex) during a 4-week halter-breaking *period. Data was analyzed using the ΔΔCT method of Livak and Schmittgen (2001). Values presented are relative to the values obtained at weaning, with each animal serving as its own control. GADPH was used as the reference gene for this study. Calves were separated into high and low exit velocity treatments based upon previous measurements at weaning, but no exit velocity treatment x time interactions were detected. A time effect (P = 0.06) was detected for interferon-γ, with a linear relationship (P = 0.02) detected.*

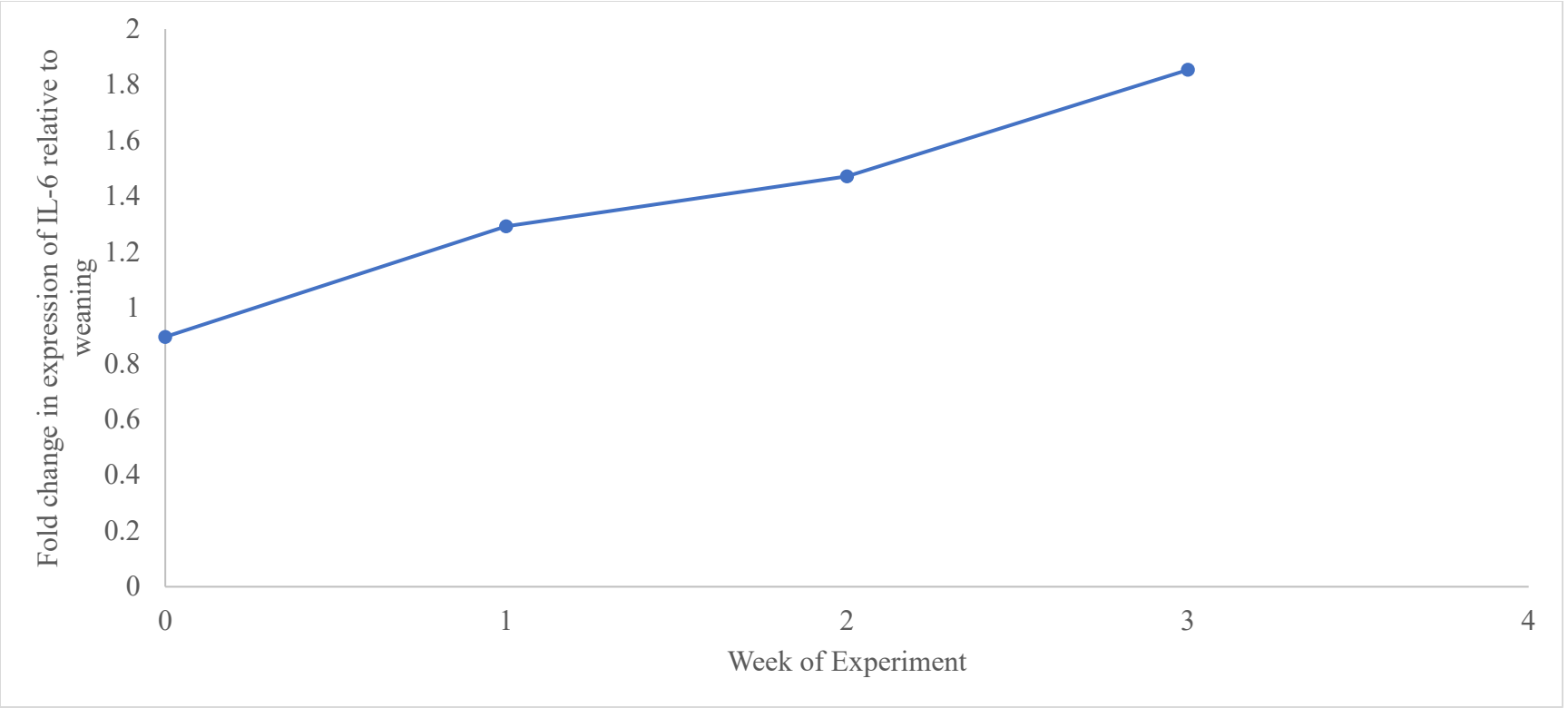

Figure 4. 2 Fold change in expression of Interleukin-6, relative to weaning, in Angus steers and heifers (n = 18/sex) during a 4-week halter-breaking period. Data was analyzed using the ΔΔCT method of Livak and Schmittgen (2001) and converted to relative quantity of expression (RQ) following analysis to illustrate the fold-change expression over time. Data was not analyzed using RQ values as they were log distributed and the ΔΔCT values were normally distributed. Values presented are relative to the values obtained at weaning, with each animal serving as its own control. GADPH was used as the reference gene for this study. Calves were separated into high and low exit velocity treatments based upon previous measurements at weaning, but no exit velocity treatment x time interactions were detected. A linear time effect (P = 0.01) was detected for interleukin-6, with a linear relationship (P < 0.01) detected.

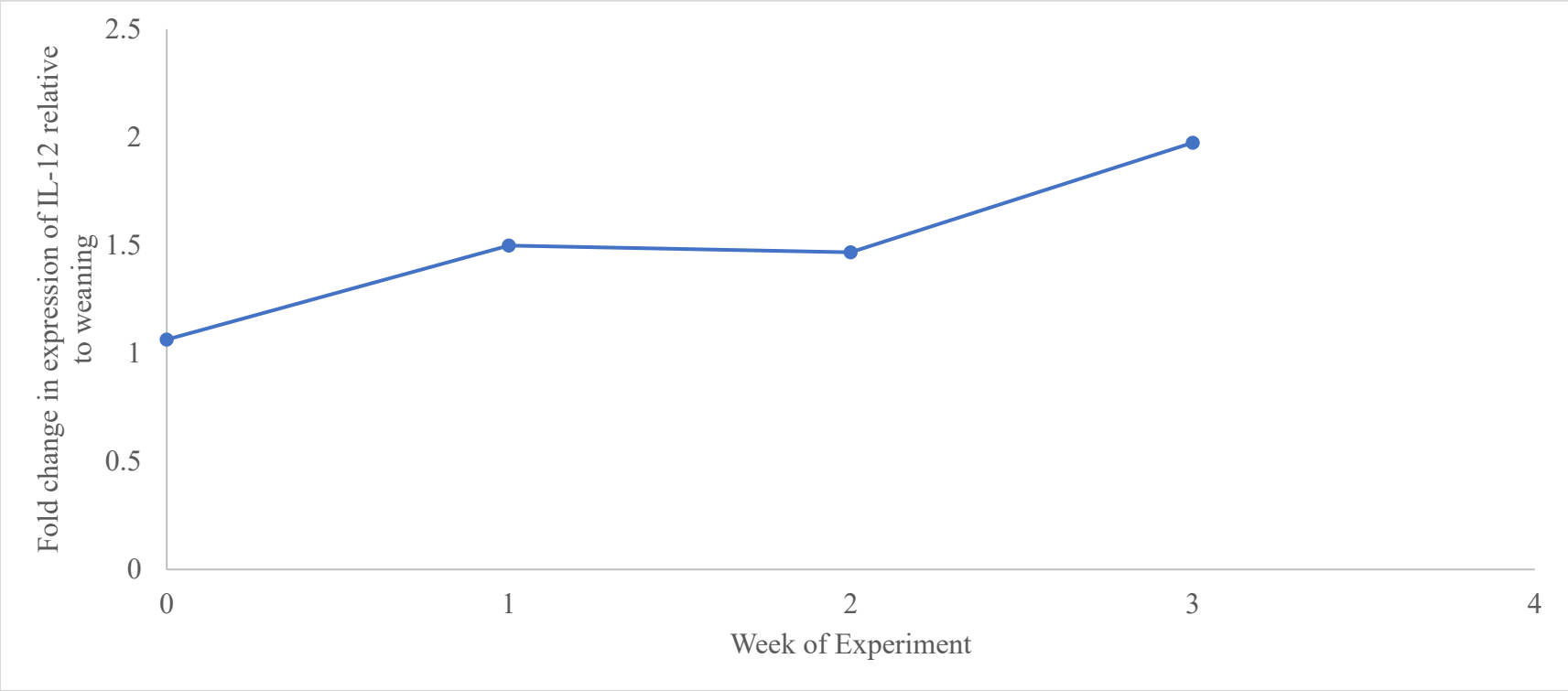

Figure 4. 3 Fold change in expression of Interleukin-12, relative to weaning, in Angus steers and heifers (n = 18/sex) during a 4-week halter-breaking period. Data was analyzed using the ΔΔCT method of Livak and Schmittgen (2001) and converted to relative quantity of expression (RQ) following analysis to illustrate the fold-change expression over time. Data was not analyzed using RQ values as they were log distributed and the ΔΔCT values were normally distributed. Values presented are relative to the values obtained at weaning, with each animal serving as its own control. GADPH was used as the reference gene for this study. Calves were separated into high and low exit velocity treatments based upon previous measurements at weaning, but no exit velocity treatment x time interactions were detected. A linear time effect (P = 0.04) was detected for interleukin-12, with a linear relationship (P = 0.02) detected.

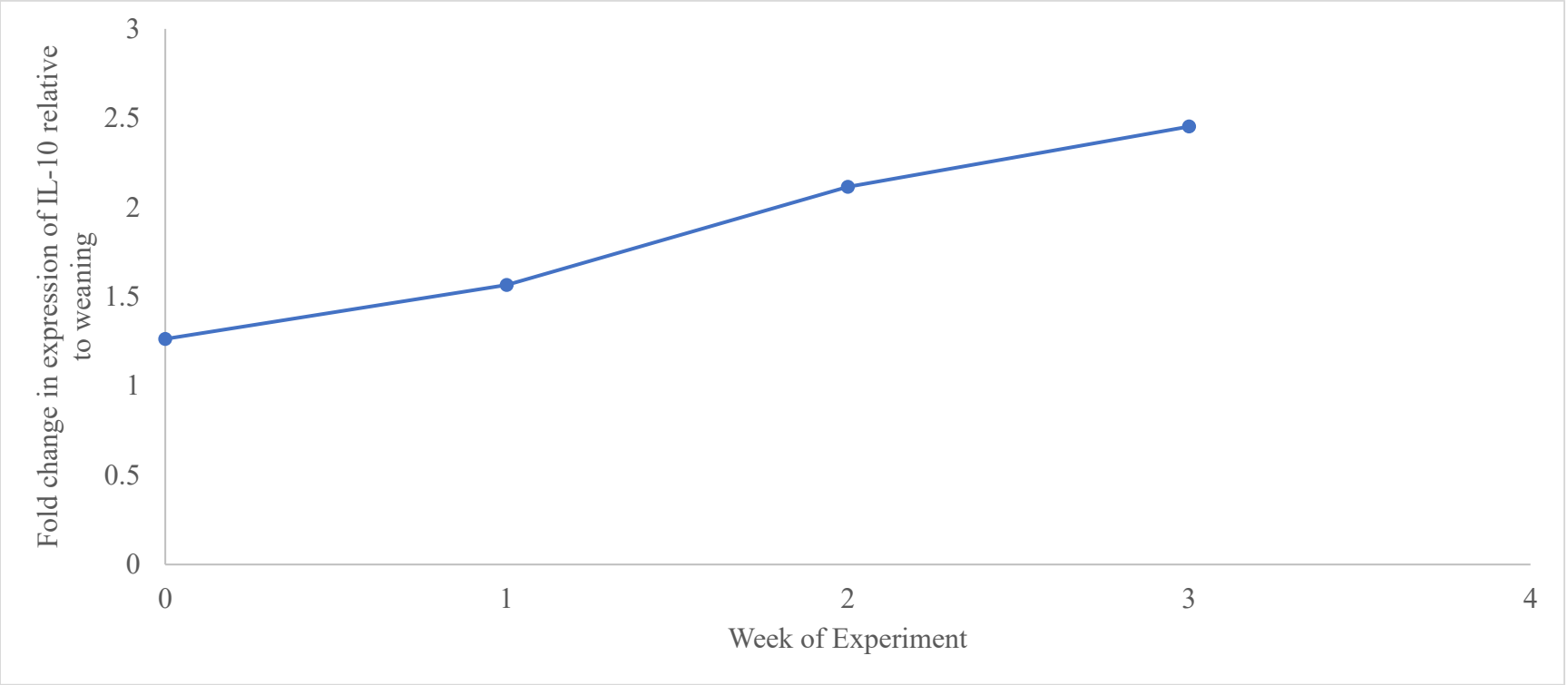

Figure 4. 4 Fold change in expression of Interleukin-10, relative to weaning, in Angus steers and heifers (n = 18/sex) during a 4-week halter-breaking period. Data was analyzed using the ΔΔCT method of Livak and Schmittgen (2001) and converted to relative quantity of expression (RQ) following analysis to illustrate the fold-change expression over time. Data was not analyzed using RQ values as they were log distributed and the ΔΔCT values were normally distributed. Values presented are relative to the values obtained at weaning, with each animal serving as its own control. GADPH was used as the reference gene for this study. Calves were separated into high and low exit velocity treatments based upon previous measurements at weaning, but no exit velocity treatment x time interactions were detected. A linear time effect (P < 0.01) was detected for interleukin-10, with a linear relationship (P < 0.01) detected.

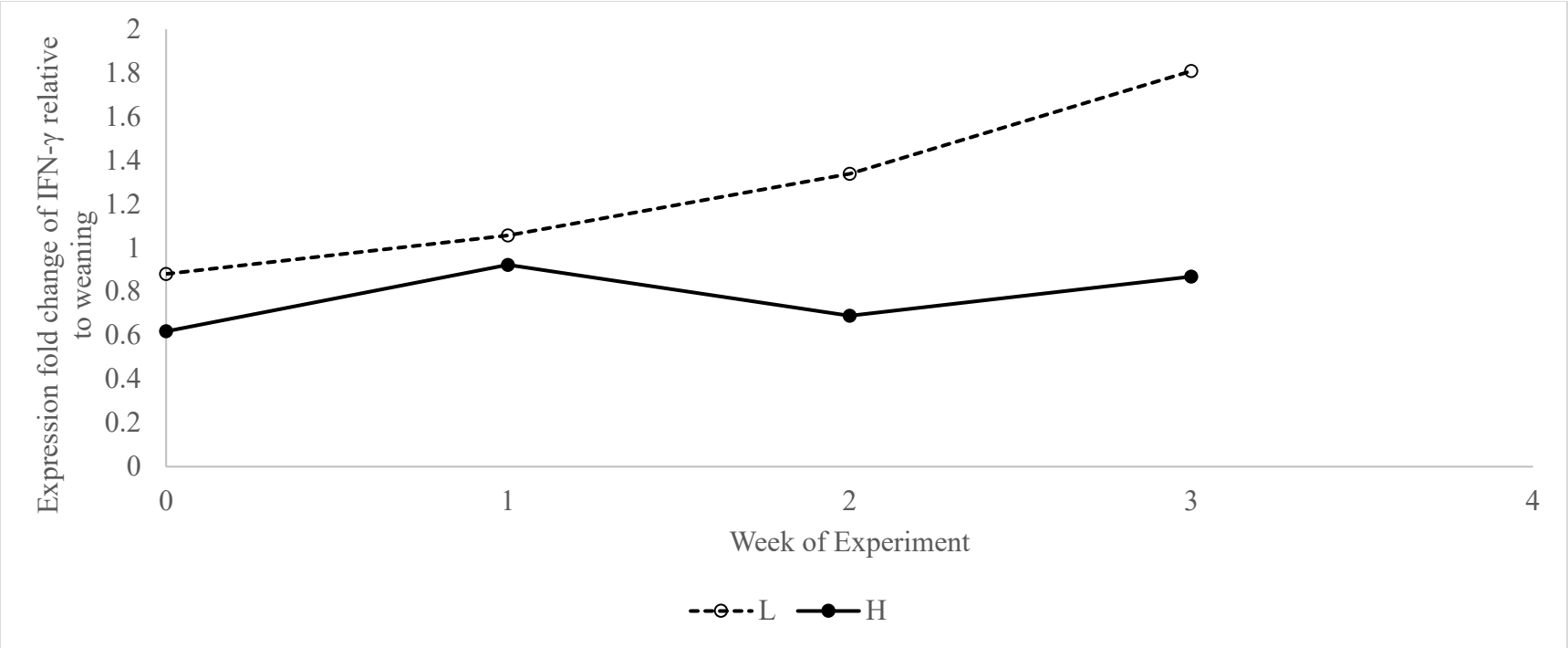

Figure 4. 5 Fold change in expression of Interferon-γ, by exit velocity and relative to weaning, in Angus steers and heifers (n = 18/sex) during a 4-week halter-breaking period. Data was analyzed using the ΔΔCT method of Livak and Schmittgen (2001) and converted to relative quantity of expression (RQ) following analysis to illustrate the fold-change expression over time. Data was not analyzed using RQ values as they were log distributed and the ΔΔCT values were normally distributed. Values presented are relative to the values obtained at weaning, with each animal serving as its own control. GADPH was used as the reference gene for this study. Calves were separated into high and low exit velocity treatments based upon previous measurements at weaning, with greater increase in IFN-γ expression relative to samples obtained at weaning detected for low exit velocity calves. An exit velocity treatment x time interaction was not detected (P = 0.39), but this graph demonstrates the low exit velocity treatment was the only one to change expression of this cytokine, indicating any acclimation to handling may have been limited to this group. Responses are shown by high (●) and low (◦) exit velocity treatments.

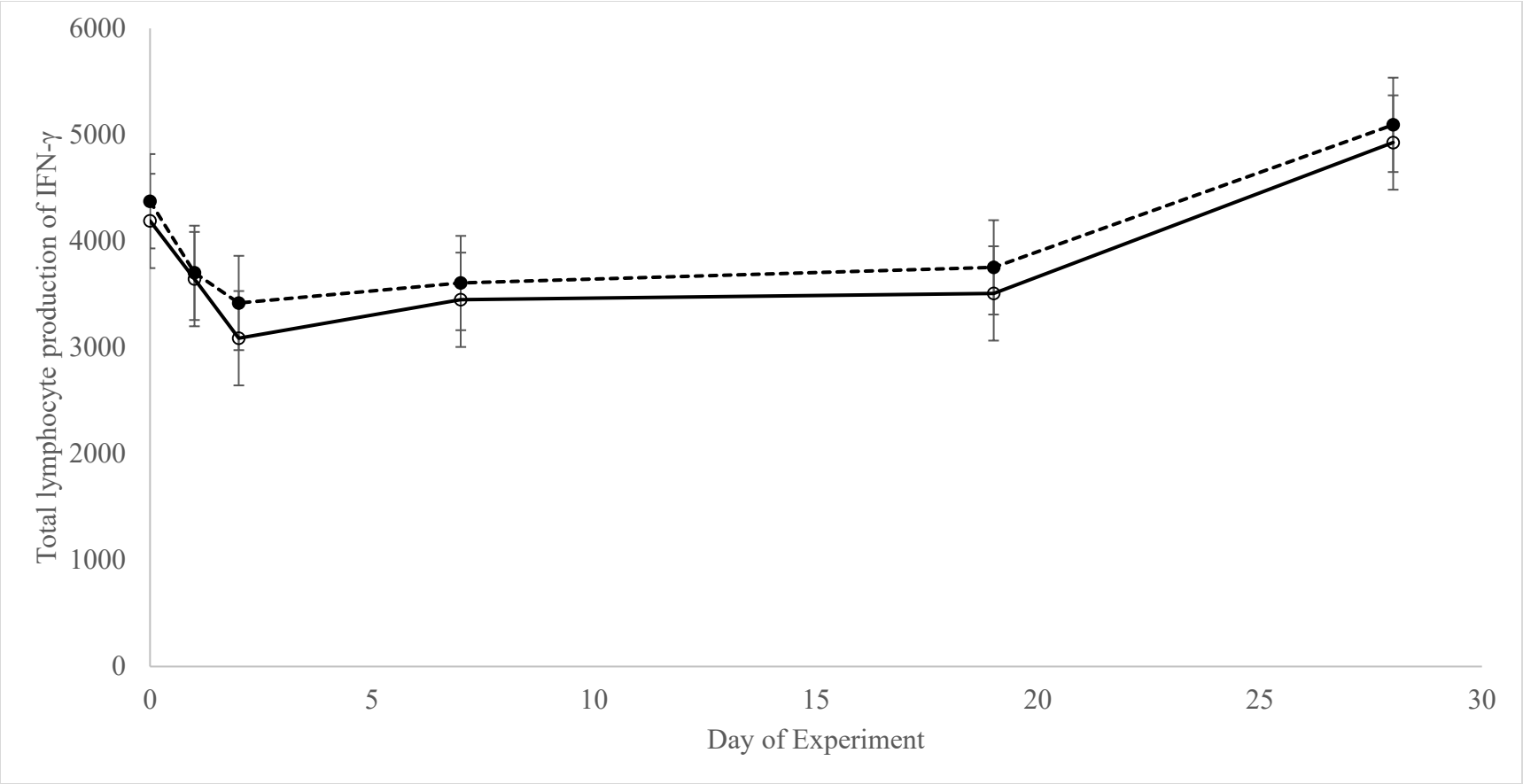

Figure 4. 6 Influence of 805 km transport on the total lymphocyte production of interferon-γ in 18 Angus steers. Six plasma samples were obtained from 18 steers during a 28d experiment on d0 (prior to transport), d1 (after transport), d2, d7, d19, and d28. From these samples, peripheral blood mononuclear cells were isolated, counted, stimulated in vitro *using brefeldin-A and phoribol myristate-13-acetate, and stained for interferon-γ. Sample analyses were gated around the lymphocyte population on a flow cytometer. A time effect (P = 0.03) was detected for this variable, but no differences were observed between exit velocity treatments (P = 0.77). Responses are shown by high (●) and low (◦) exit velocity treatments.*

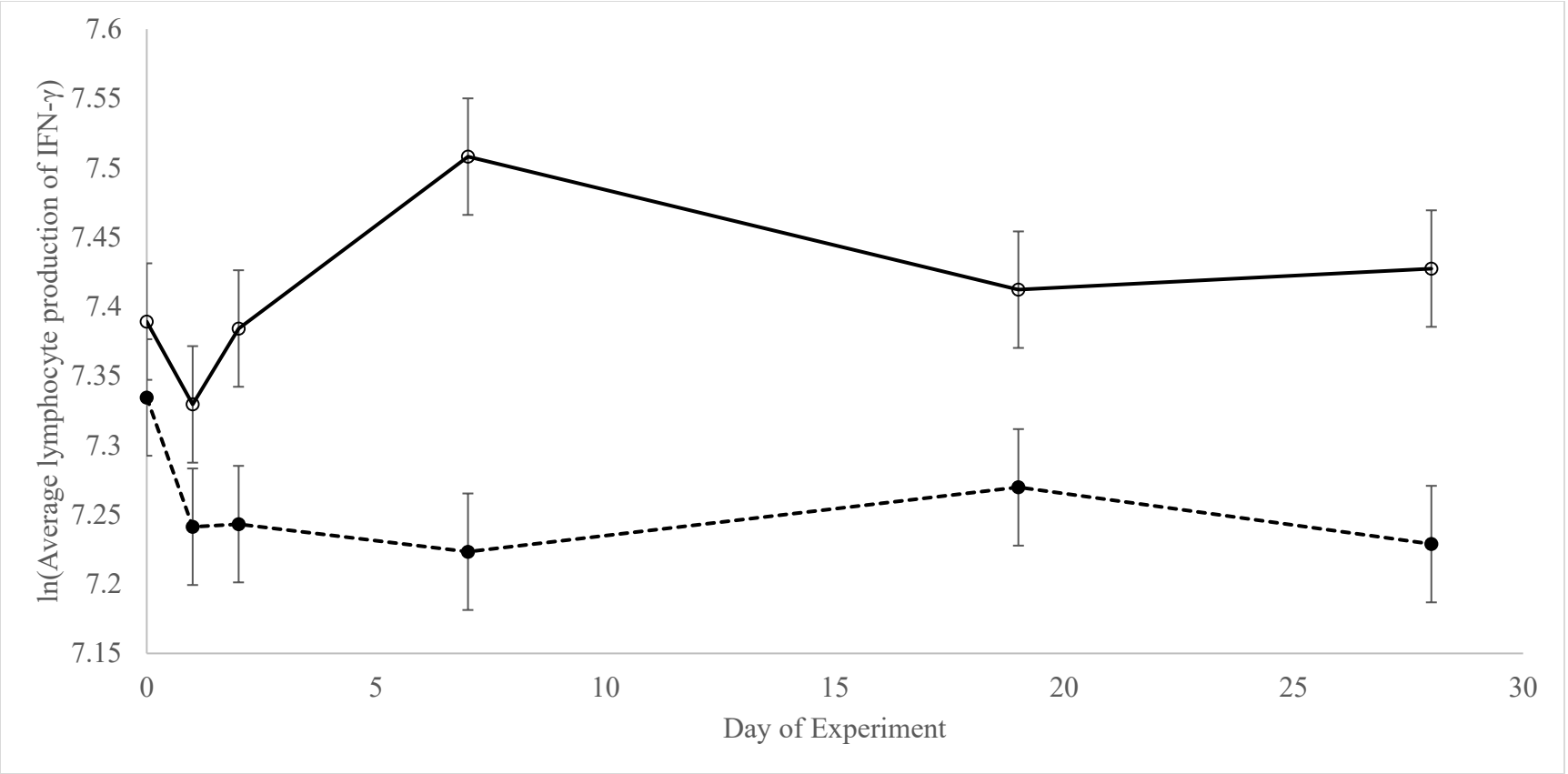

Figure 4. 7 Influence of 805 km transport on the average lymphocyte production of interferon-γ in 18 Angus steers. Six plasma samples were obtained from 18 steers during a 28d experiment on d0 (prior to transport), d1 (after transport), d2, d7, d19, and d28. From these samples, peripheral blood mononuclear cells were isolated, counted, stimulated in vitro *using brefeldin-A and phoribol myristate-13-acetate, and stained for interferon-γ. Sample analyses were gated around the lymphocyte population on a flow cytometer. No time effect (P = 0.76) was detected for this variable, but high exit velocity steers were found to have lower average lymphocyte production of interferon-γ (P = 0.02) than steers during the experimental period. Responses are shown by high (●) and low (○) exit velocity treatments.*

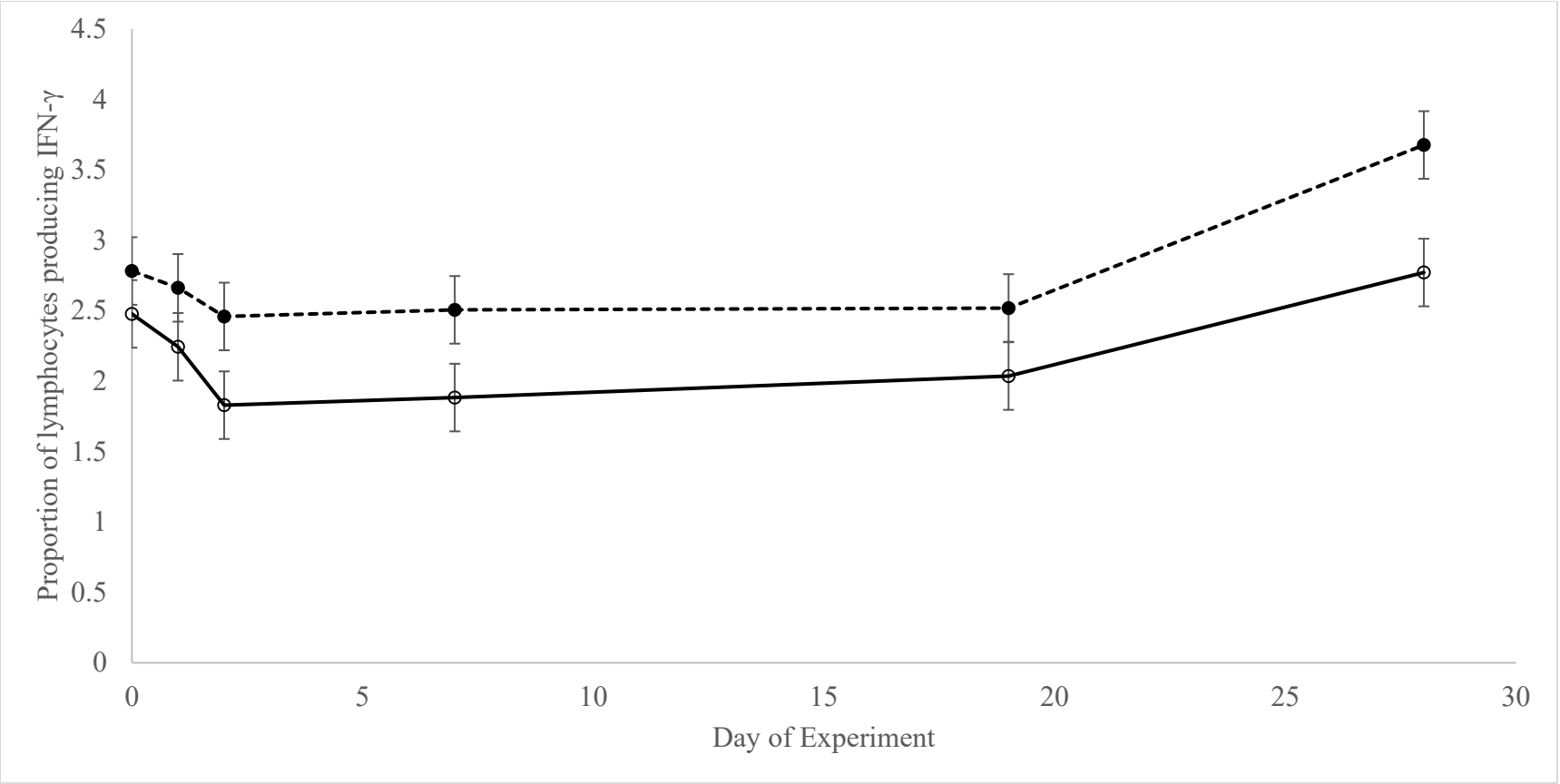

Figure 4. 8 Influence of 805 km transport on the proportion lymphocytes producing interferon-γ in 18 Angus steers. Six plasma samples were obtained from 18 steers during a 28d experiment on d0 (prior to transport), d1 (after transport), d2, d7, d19, and d28. From these samples, peripheral blood mononuclear cells were isolated, counted, stimulated in vitro *using brefeldin-A and phoribol myristate-13-acetate, and stained for interferon-γ. Sample analyses were gated around the lymphocyte population on a flow cytometer. A time effect (P < 0.01) was detected for this variable, and high exit velocity steers were found to have higher proportions of lymphocytes producing IFN-γ (P = 0.10) than steers during the experimental period. Responses are shown by high (●) and low (◦) exit velocity treatments.*

Chapter 5: Conclusions

The relationship between animal temperament and various measures of performance has been evidenced by others across species. In cattle, measures of temperament, particularly exit velocity, have been examined and utilized in several studies to estimate future growth performance. Several other experiments have demonstrated a relationship between these behavioral traits and components of the immune system in a variety of species. Collectively, these former studies have indicated exit velocity may be useful as a potential indicator of growth performance and immunological responses.

In this dissertation, high exit velocity steers experienced greater growth efficiency than low exit velocity steers following a period of grazing endophyte-infected and endophyte-free pastures. Although this result was most pronounced during the first 28d in the feedlot, this relationship with exit velocity was detected for the entire finishing period. However, average daily gain and dry matter intake (as a percentage of body weight) did not share a similar relationship with exit velocity over this period. Similarly, this relationship does not appear to be consistently reported in literature. In light of this, it would be premature to conclude exit velocity is a useful predictor of future growth performance at this time, but the appearance of this relationship in this dissertation warrants further investigation in future studies.

The relationship between exit velocity and systemic immune responses was also examined in this dissertation and determined to exist in the absence of endophyte and heat treatments and under a controlled environmental setting. During this pre-treatment period, total lymphocyte production of interferon-γ (IFN-γ) was observed to increase with increasing exit velocity, apparently largely due to changes in the proportion of lymphocytes producing IFN-γ

rather than a change in production rates. The presence of such a relationship gives credence to our theory that exit velocity could be utilized to estimate measures of cell-mediated immunity, particularly as this relationship was reestablished following cessation of the heat and endophyte treatments.

Application of treatments associated with commonly encountered management practices did not appear to affect IFN-γ lymphocyte production in cattle with differing exit velocities. When calves were exposed to increased environmental heat conditions during the first study, lymphocyte production of IFN-γ did not differ between exit velocity treatments. This absence of a divergence in lymphocyte production of IFN-γ between these two treatment groups continued during the endophyte-infected treatment period. Similarly, total production of this cytokine by lymphocytes did not differ between exit velocity treatments following a 10h, 805 km transportation period. However, this nonexistence of an exit velocity effect on total lymphocyte production of this cytokine resulted from the offsetting relationships of average lymphocyte production of IFN-γ and the proportion of these lymphocytes producing IFN-γ with this measure of temperament. This difference became more obvious later in the post-transport period, as these differences were manifested to a greater extent, indicating potential relationships between differentiation and production capabilities of lymphocytes with exit velocity that could be masked by exposure to transportation. Cumulatively, these results indicate that application of these conditions (i.e. transport, endophyte, and heat) may diminish the ability to discern an effect of exit velocity on lymphocyte production of IFN-γ, with subsequent removal of animals from these conditions enhancing the visibility of this relationship.

Contrasting with these responses were the measures of whole blood gene expression of IFN-γ collected during a period of acclimation to human handling. Relative to samples obtained at weaning, expression of IFN-γ did not change in high exit velocity calves, whereas it increased over time in low exit velocity calves. This may indicate high, as compared with low, exit velocity calves are slower to acclimate to change, potentially suppressing cell-mediated immune responses, as evidenced by the discrepancy in IFN-γ expression between exit velocity treatments, and increasing the susceptibility of these calves to damage associated with intracellular pathogen infection. When viewed in conjunction with baseline lymphocyte measures from the first study of this dissertation, these results suggest that IFN-γ production may be directly related to exit velocity obtained at weaning.

Interestingly, the relationship of exit velocity with cell-mediated immunity did not correspond to measures of humoral immunity. In neither the seed or the grazing experiments was exit velocity observed to influence titer responses to *Lepto. pomona* vaccination. However, a difference between studies was observed with endophyte treatment. Although this could be related to a difference in gender, the increased growth response of steers previously grazed on endophyte-infected pastures as well as other literature examining titer responses following periods of nutrient restriction, suggest the increased humoral response of these steers may have been a result of compensatory responses rather than a direct effect of endophyte consumption. Thus, future research investigating potential effects of endophyte-infected tall fescue consumption on concurrent or subsequent vaccination responses should utilize a model which allows for examination of nutrient restriction in combination with endophyte treatments.

Collectively, these findings indicate a potential relationship of exit velocity with systemic IFN-γ expression. The incorporation of this cytokine with many cell-mediated immune system activities indicate the potential relationship of IFN-γ with exit velocity described in this dissertation should be further investigated to ascertain the mechanisms through which these relationships are manifested. Exploration of these mechanistic pathways may assist in developing improved management strategies for maximizing herd health, most prominently in the feedlot setting.

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