Impact of Endophyte-Infected Tall Fescue Seed on the Acute Phase and Metabolic Responses of Cattle During an Immunological Challenge

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IMPACT OF ENDOPHYTE-INFECTED TALL FESCUE SEED ON THE ACUTE PHASE AND METABOLIC RESPONSES OF CATTLE DURING AN IMMUNOLOGICAL CHALLENGE

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, KY
2015

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ABSTRACT OF THESIS

IMPACT OF ENDOPHYTE-INFECTED TALL FESCUE SEED ON THE ACUTE PHASE AND METABOLIC RESPONSES OF CATTLE DURING AN IMMUNOLOGICAL CHALLENGE

Acute phase responses (APR) and circadian rhythm of body temperature (CRT) changes to a minor, chronic stressor (endophyte) and major, acute stressor, lipopolysaccharide (LPS) were evaluated in two experiments (E1: 22 heifers; E2: 24 heifers). Vaginal (E1 and E2) and rectal (E2) temperature probes recorded body temperature. Temperature was analyzed by cosinor analysis in each of 4 Phases: pre-endophyte (P1), endophyte exposure, pre-LPS (P2), post-LPS spike 1 (P3), post-LPS spike 2 (P4). During P1 of E1, amplitude (P=0.05), but not mean temperatures (P>0.10), differed across days (P=0.05). Endophyte affected E2 vaginal temperature amplitudes in P2 (P=0.07) and P4 (P=0.08) without influencing mean temperatures. Significant treatment*weaning exit velocity (wEV) interactions occurred for E2 mean rectal temperatures during P1-P3 (P≤0.10). During E1, post LPS, endophyte depressed glucose (P=0.05) and increased creatinine (P<0.01) and decreased TNF-α (P<0.01) at 24h. During E2, post-LPS, IFN-γ decreased (P=0.02) with increasing wEV. Endophyte increased serum BUN concentrations with slow wEV and increased BUN with fast wEV (P=0.04) post-LPS in E2. Endophyte increased NEFA (P<0.01) and IL-6 (P=0.02) from 1-8 hours post-LPS during E2. Results
demonstrate modulation of CRT and APR by both endophyte and wEV, and demonstrate effectiveness of cosinor analysis in detecting CRT responses.

Keywords: endophyte, LPS, CRT

Alexander W. Altman
December 9, 2015
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Due to its prevalence in the Southeastern United States, tall fescue (\textit{Festuca arundinacea}) has been of interest to researchers for many years. Both positive and negative benefits occur for cattle producers utilizing endophyte-infected tall fescue. Saikkonen et al. (1998) examined over 200 research papers and found most research regarding grass endophyte-herbivore interactions dealt with either tall fescue or perennial ryegrass, much of which concluded that ingestion of endophytes impacts herbivores negatively. The suggestion of resistance to grazing by herbivores due to the presence of endophytic fungi as one of the benefits experienced by the host plant has also been postulated (Cheplick and Clay, 1988). However, literature is limited concerning the impact of this fungus on cattle immunological response to infection.

**History of Tall Fescue and Endophyte**

Tall fescue is believed to have developed during the early years following the most recent ice age in Western Europe. Seeds were brought from that region to the United States by the pioneers and settlers (Borrill, 1976). Since that time, tall fescue has become one of the United States’ most commonly grown cool season forages (Jackson et al., 1984). The most prevalent variety of tall fescue, Kentucky 31, was first identified in Menifee County, Kentucky. Tall fescue is now widely distributed across the United States due to its adaptability, ease of establishment, resilience, productivity, and
appearance (Stuedemann and Hoveland, 1988). Tall fescue is a very popular pasture grass, with an estimated 20% of the beef herd in the United States exposed to this grass during their lifetime (West and Waller, 2007).

Since its rise in popularity, tall fescue has been the source of several research studies. Wild-type fescue presents producers with several negative effects on animal production, particularly during the hotter times of the year, a phenomenon referred to as fescue toxicosis. Early observations revealed that the effects of fescue toxicosis on livestock were not the same in every tall fescue pasture. Some herds that grazed on tall fescue pastures presented no symptoms of fescue toxicosis, whereas others suffered from poor performance. To determine the cause of these symptoms, researchers (Bacon et al., 1977) examined pastures from five different states (Georgia, Kentucky, Maryland, Missouri, and Virginia). Within each state, equal numbers of herds grazing the tall fescue pastures presented either symptoms or no signs of fescue toxicosis. After examining samples from each of the pastures, the group concluded that the fungal endophyte *Epichloe typhina* was, at least in part, responsible for the effects of fescue toxicosis.

Since that time, researchers have found that fescue related problems can be attributed to *Neotyphodium coenophialum*, a fungus related to *E. typhina* but lacking a sexual reproductive stage (Latch, 1997). This lack of a reproductive stage leads to variability in level of endophyte infection in progeny, with occurrences of endophyte-free (E-) seeds resulting from endophyte-infected (E+) tall fescue plants ((Welty et al., 1994) as reported by (Saikkonen et al., 1998)). Since its identification, an estimated 90% of tall fescue pastures in the US have been tagged as *Neotyphodium coenophialum* infected ((Sleper and West, 1996) as reported by (Waller, 2009)).
Endophyte

The term endophyte comes from the Greek words endo (translated as within) and phyte (translated as plant.) Therefore, endophyte refers to any organism growing within a plant, regardless of whether it is symbiotic or parasitic in nature. The endophyte fungus (N. coenophialum) is distributed unequally throughout the plant, concentrated heavily in the leaf sheaths, seeds, and crown. The fungi benefits from the plant through nutrition, protection, survival, and improved dissemination (though plant seed) (Siegel et al., 1987). Endophyte toxicity and viability within tall fescue seeds have been shown to be decreased by ammoniation (Simeone et al., 1998), exposure to heat, chemicals (fungicides), high temperature/low moisture storage (Siegel et al., 1987), light and air (Garner et al., 1993).

Endophyte-Infected Fescue Problems

Fescue toxicosis is an all-encompassing term used to describe the many adverse effects of the endophyte toxins to the animal, which include (but are not limited to) fescue foot, poor performance, summer slump, and fat necrosis (Bush et al., 1979). These adverse effects experienced by the animal provide tall fescue fields with a certain amount of tolerance to overgrazing (Siegel et al., 1987). Ergot alkaloids were identified as the causative agents of problems associated with grazing endophyte-infected tall fescue in the late-1980’s by Solomons et al. (1989) through comparing the contractile response of the dorsal pedal vein in cattle to various alkaloids. These researchers found no contractile response in veins treated with loline alkaloids. Conversely, ergotamine showed the
highest contractile response of the alkaloids tested. Ergotamine was also shown to be a more effective inhibitor of norepinephrine (used by the vasculature for contractile response) compared to the lolines. This was a revolutionary discovery as the prevailing theory of the time attributed the adverse effects of fescue experienced by livestock to plant produced perloline and related alkaloids, rather than ergot alkaloids.

Ergovaline, a known dopamine agonist, is the most abundant ergopeptine produced by endophytic fungi present in tall fescue (Jones et al., 2003). Average ergovaline concentration ranges in tall fescue seed are 0.1 to 6.0 µg/g (Belesky et al., 1988; Porter, 1995). It has been suggested that this concentration can be reduced through the utilization of heavy, continuous grazing, which has been shown to reduce the effects of fescue toxicosis compared to rotational grazing due to the minimization of seed head formation. The use of continuous grazing as a management strategy may be particularly useful when herd numbers are low, as animals selectively graze seed heads (Schmidt and Osborn, 1993).

There are three disorders observed in cattle and associated with consumption of endophyte-infected tall fescue: fescue foot, bovine fat necrosis, and summer slump.

**Fescue Foot**

First coined in 1949 by I.J. Cunningham in New Zealand, fescue foot is characterized by the formation of dry gangrene in the feet (where the name originates from), ears, and tail tip and can be attributed to vasoconstriction caused by exposure to E+ tall fescue. The affected areas, in severe cases, can experience sloughing (Garner and Cornell, 1978). Early symptoms of grazing high concentrations of E+ tall fescue are
similar to those observed with sick cattle in general: arched back, weight loss/reduced rate of gain, and rough hair coat. These clinical signs can appear in early as 3 to 7 days after initial exposure during grazing and may include a characteristic red line at the coronary band of the hind foot, swelling in extremities accompanied by skin discoloration, and lesions on sides of legs and around feet (Bush et al., 1979). Other symptoms experienced by the animal may include a dull hair coat, scours, lameness, tranquilized appearance, and attempts to remain cool in warm weather. Closer examination may reveal shivering in cool weather, elevated temperatures, elevated respiration rate, elevated pulse rate, and occasional absence of rumen motility. A tremor starting in the hindquarters and moving forward may also be observed, with a cough sometimes occurring when the tremor passes through the chest cavity (Bush et al., 1979). Fescue foot is more likely to occur in cool regions/climates due to exacerbating effects on already present vasoconstriction (Ball, 1997).

Fat Necrosis

Also referred to as lipomatosis (Waller, 2009), fat necrosis is a condition associated with endophyte-infected tall fescue consumption primarily affecting the abdominal cavity in cattle. The necrotic fat lesions range in size from small nodules embedded within normal fat deposits to large, irregularly shaped masses (Townsend et al., 1991). These lesions can cause constriction of the reproductive and digestive organs (Waller, 2009).

To determine the effect of endophyte-infected tall fescue consumption on cattle fat composition, Townsend et al. (1991) placed cattle on three different levels of
endophyte-infected pastures. At the conclusion of the grazing period, tissue samples from subcutaneous adipose, kidney adipose, semitendinosus muscle, and longissimus muscle were collected from three steers in each treatment and analyzed for fat content. The researchers found consumption of endophyte related to lower proportions of unsaturated fat (palmitoleic acid and oleic acid) and higher proportions of saturated fat (stearic acid) content. This finding is in agreement with that of Rumsey et al. (1979), who had previously found that saturated fatty acids, particularly stearic acid, are in high proportions in the peritoneal fat tissues when necrotic fat lesions are present.

Another contributing factor to the incidence of fat necrosis in animals is grazing nitrogen enriched pastures. Stuedemann et al. (1985) showed that as amount of nitrogen fertilizer applied to pastures increased, occurrence of fat necrosis in animals also increased. This agrees with the increased occurrence of fat necrosis in high poultry producing areas, where spreading of high nitrogen-containing onto pastures is common (Ball, 1997).

**Summer slump**

Arguably the most researched condition associated with endophyte-infected tall fescue consumption, summer slump is a debilitating condition plaguing the beef industry. Occurring during warm weather, summer slump is characterized by reduced average daily gain and feed intake, intolerance to heat, excessive salivation, non-shedding rough hair coat, elevated body temperature, and endocrine imbalance (Schmidt and Osborn, 1993). Onset of summer slump also leads to changes in behavior, which include more time spent in the shade and water during the day and increased grazing frequency at night.
(Bond et al., 1984; Schmidt and Osborn, 1993). Consumption of endophyte-infected feeds can also be detrimental to overall animal health. In addition to differences in body temperature, long rough coats in summer, and decreased gain, Saker et al. (1998) observed decreased immunocompetence in the form of runny noses and increased monocyte phagocytic activity (P < 0.01) in steers consuming an E+ compared with an E- diet.

Losses attributed to summer slump in the early 1990’s by the beef industry were estimated to be over $600 million due to growth ($365 million) and reproductive ($365 million) associated problems (Hoveland, 1993). These figures were later shown to underestimate the extent to which summer slump impacts the industry (Allen and Segarra, 2001) due to a failure to account for losses attributed to immunological compromise (Saker et al., 1998). Hoveland also failed to present any predicted losses experienced in the stocker industry other than a mention that producers generally avoid E+ tall fescue pastures due to low average daily gains. Allen and Segarra (2001) estimated this loss to be $0.34/stocker/day, or a combined total of $12.33 million/day for the entire U.S..

Poor weight gains associated with varying levels of E+ have been observed in several states across the Southeastern US ((Hoveland et al., 1984) as reported by (Schmidt and Osborn, 1993)). Combined results of 12 independent studies conducted across the US, within the major tall fescue growing zone (mid IL to west border of MO, top two-thirds MS, to eastern third NC) detected no differences in ADG when comparing between low (pastures with ≤ 5% E+) and moderate E+ (pastures with ≥20 to ≤ 35% E+) in spring, summer, or spring+summer. However, differences were observed when
comparing moderate to high E+ (pastures with ≥50 to ≤ 97% E+), and all levels of E+ with clover across all seasons, with the exception of comparing low E+ pastures to low and moderate E+ with clover pastures during the summer grazing period (Thompson et al., 1993). These results indicate that dilution can reduce the intake of endophyte-infected tall fescue. Incorporating legumes as a means of dilution can also increase gains experienced by cattle on E+ feeds vs. cattle fed diets consisting of E- feeds without legumes (Coffey et al., 1990). Incorporation of warm-season grasses also provide a good dilution strategy. Ball (1997) asserts fescue toxicosis is more severe in cooler regions (i.e. Missouri) during hot, summer months compared to the warmer regions (i.e. Alabama) due to less warm-season grass dilution in pastures.

Despite the negative impact endophyte has on cattle, the effects of summer slump are not permanent, nor do they have a long lasting effect after animals are removed from endophyte-infected feeds. Compensatory gain is experienced during the feedlot phase by cattle previously fed endophyte-infected diets (Coffey et al., 1990). This compensatory gain has been shown to be equal (Piper et al., 1987) or better (Cole et al., 1987) in cattle consuming high E+ than low E+ diets.

\textit{Intake, Digestibility, and ADG}

Ergovaline is a known dopamine agonist that has been implicated in the reduced intake of cattle fed endophyte-infected tall fescue. Decreased OM intakes have been observed in cow-calf pairs grazing E+ pastures, compared to pairs on E- pastures, during hotter months (Peters et al., 1992). Chestnut et al. (1991), using E+ (tall fescue) and E- pastures, also reported higher intakes for cattle grazing E- pastures. However, intake may
have been confounded for this study, as estimates were made using the cage and strip methodology (Linehan (1952) as reported by Chestnut et. al (1991)) as well as implementation of a put-and-take grazing system to maintain a consistent grass height within each pasture.

Comparisons of E+ vs. E- intake differences were also examined by Aldrich et al. (1993) using a total of 36 head of cattle in two separate experiments. Experiment 1 utilized 12 Angus heifers fed either an E+ (381 ppb ergovaline) or E- diet. Diets were identical with the exception of the type of tall fescue seed. Heifers were placed in an environmentally controlled area and exposed to increasing temperatures (22°C to 32°C) and humidities (50% to 60%) throughout the day in an effort to establish diurnal patterns. Diets were offered twice a day and orts were collected, weighed, and subsampled daily. There were no differences in intake between the two treatments. However, in their second experiment using similar dietary treatments (E+ = 285 ppb ergovaline) with 24 Holstein steers, decreased intakes for E+ treated steers when temperature and humidity (60% for all steers) remained constant. Steers were placed on either an E+ or E- diet, with each treatment group subdivided into 22°C and 32°C environments. The authors reported greater intakes for E- steers and concluded the difference may have been a result of an inability to efficiently dissipate body heat. This conclusion is supported by the results of Bond et al. (1984), which stated cattle exposed to E+ pastures spend more time grazing at night and less during the heat of the day when environmental temperatures are greater than cattle on E- pastures.

However, heat dissipation problems may not fully explain decreased intakes. Ergot alkaloids were reported to have dopaminergic properties as early as 1978 ((Muller-
Schweinitzler and Weidmann) as reported by Thompson et al. (1993)). Maas et al. (1982), using goats, showed intravenous infusion of dopamine inhibited ruminal contractions. Similarly, administration of domperidone, a dopamine antagonist, to heifers caused feed intake of E+ fescue to be more similar to that seen with E- fescue (Jones et al., 2003). These findings suggest that the mechanism for intake may lie beyond problems associated with overheating.

Schmidt et al. (1982) used 12 crossbred steers in July and August (34 to 38°C daytime temperatures) to determine digestibility differences (among other variables) between E+ and E- feedstuffs. Steers were assigned to one of four treatment diets: E+ chopped tall fescue hay, E- chopped tall fescue hay, E+ tall fescue seed, or E- tall fescue seed. In vitro dry matter disappearance was determined for all four diets using the two-stage Tilley and Terry (1963) procedure, with the inoculum used for IVDMD determinations collected from an animal not adapted to fungus-infected tall fescue. The results of the study found E+ tall fescue was more digestible than E- tall fescue diets. An in vivo trial conducted by Hannah et al. (1990) using sheep observed similar results. The group used sheep to show a relationship between temperature and digestibility of endophyte-infected feedstuffs. Three experiments were conducted using the same diet with varying levels of ergopeptines present. The first experiment examined the effect of ergovaline levels (0, 1.5, 3.0 pmm) at approximately 27°C and 50% humidity and the second occurred at 20°C and 60% humidity. The third study examined effects of three different levels of ergopeptines (0 ppm ergovaline, 1.5 ppm ergovaline, and 20 ppm ergotamine tartrate) fed at two different temperatures and humidities (27°C and 60% vs. 34°C and 50%, respectively). The combined results of the three experiments indicated
that increasing concentrations of endophyte fed during warm weather decreases the animal’s ability to digest feed. The results of this study correlate with the conclusions from Aldrich et al. (1993) mentioned above.

Average daily gain, like digestibility, is also negatively impacted by the presence of endophyte (Jackson et al., 1984; Paterson et al., 1995; Schmidt et al., 1982). Crawford et al. (1989), using the results of their Missouri study in combination with results from 7 other studies, estimated effects of endophyte infection level on ADG using the following equations:

Endophyte-Infected Fescue (EIF, %) = (# infected tillers/total # tillers)*100

The authors found that average daily gain (ADG) averaged across three years for the spring-summer period was negatively correlated to EIF, with a relationship of approximately a 0.10 lb/d decrease in ADG for each 10% increase in EIF. The use of their overall equation relied upon, in part, the Schmidt et al. (1982) estimate of ADG for cattle on endophyte free fescue falling between 1.4 to 1.6 lb/day. This observed relationship was observed only with spring-summer, not fall, grazing (Williams et al., 1984; Crawford et al., 1989).

**Prolactin**

Prolactin is an important regulatory hormone named initially for its function as a stimulator of mammary growth and lactogenesis (Trott et al., 2008), but is now known to have several functions within the body. Primarily secreted by the lactotrophs in the
anterior pituitary, prolactin is also synthesized and secreted by other tissues in the body (Freeman et al., 2000).

Endophytic toxins and daylength modulate prolactin concentrations. High endophyte-infected fescue significantly decreases prolactin concentrations in the pituitary compared to low endophyte-infected fescue (Schillo et al., 1988). For this reason, prolactin has become a primary research marker for fescue toxicosis. Prolactin is down-regulated by the D₂ dopamine receptor (Lamberts and Macleod, 1990). Ergovaline, a known dopamine agonist (Jones et al., 2003), has been shown to inhibit prolactin release (Strickland et al., 1992; Strickland et al., 1994). Photoperiod has a positive correlation with the concentration of serum prolactin (Bourne and Allen Tucker, 1975).

Prolactin, among other known roles, has also been classified as a cytokine (Bouchard et al., 1999). The prolactin receptors are part of the class 1 cytokine receptor superfamily (Bole-Feysot et al., 1998) whose members include receptors for several interleukins, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, and erythropoietin. Prolactin binding sites have been located in lymphoid tissues such as the spleen, thymus (nurse cells and epithelial cells), macrophages, and lymphocytes (T-cells and B-cells), as well as in the liver on hepatocytes (Bole-Feysot et al., 1998).

The role of prolactin within the immune system remains somewhat unclear. Whereas most agree prolactin has a role in the regulation of the immune system, this hormone has been shown to be both immunostimulatory and immunosuppressive (Bouchard et al., 1999). Bernton et al. (1988) used mice injected with one of three pathogens (*Mycobacterium bovis*, *Listeria monocytogenes*, or inoculated
Proprionibacterium acnes) to determine the effect of prolactin on immune response. The mice were given daily injections of bromocryptine (a known dopamine antagonist). Resulting lymphocyte proliferation correlated directly with serum prolactin levels, with significant suppression of lymphocyte proliferation observed after 48 hours of bromocryptine treatment. The authors concluded prolactin is essential to lymphocyte responses to antigenic stimuli.

Others have observed direct correlation of cytokine production to increasing or decreasing prolactin levels. Dimitrov et al. (2004) collected blood from 15 men and observed concentrations of interferon-γ, tumor necrosis factor-α, and interleukin-2 to increase with increasing doses of prolactin. Similarly, an increase in synthesis of pro-inflammatory cytokines tumor necrosis factor-α and interleukin-12 have been observed when peripheral blood mononuclear cells were stimulated with prolactin and lipopolysaccharide (Brand et al., 2004).

However, Moreno-Carranza et al. (2013) used normal, wild-type (PRLR +/+), and prolactin-receptor knock-out (PRLR -/-) mice to show the immunosuppressive effect of prolactin. The mice were partially hepatectomized and examined for IL-6 production and liver regeneration. The group reported that mice incapable of producing prolactin had a higher mortality rate than other mice in the study. Their conclusion was that prolactin promoted survival in the wild-type and normal mice by working through mechanisms to downregulate IL-6 production and upregulate the suppressor of cytokine signaling-3 (SOCS3) mechanism.

Blood Flow
Endophyte-infected tall fescue diets have been found to reduce blood flow to core and peripheral body tissues in cattle through vasoconstriction, resulting in higher rectal temperatures and greater heat transfer inefficiencies (determined by finding the difference between rectal and skin temperatures (Rhodes et al., 1991)). Contractile responses to N-acetyllylloline, lysergic acid, ergotamine, and ergovaline relative to a norepinephrine response were determined and compared using cross-sections of the cranial branch of the saphenous vein from mixed breed cattle of both genders. Ergovaline resulted in a much higher contractile response than the others, but the authors mentioned that an additive effect may exist (Klotz et al., 2006; Klotz et al., 2007; Klotz et al., 2008).

**Immunology**

Immunology can best be defined as the study of the body’s defense against infection. The body’s response to infection can be divided into two major systems: innate and adaptive. The innate response, which includes the release and activities of cytokines, is a non-specific response to a pathogen that is considered the body’s first line of defense during an immunological attack (Gruys et al., 2005). The adaptive response is specific to the pathogen and develops over the individual’s lifetime as a result of exposure to pathogens. This response utilizes B cell and T cell lymphocytes to attack the antigen using a highly specific, developed response. Depending upon the pathogen and the body’s previous experiences, the adaptive response may also utilize immunological memory, providing the immune system with a more rapid response than is achieved during novel infection (Carroll and Forsberg, 2007).
One key component to a normal immune response is the production and utilization of cytokines. Cytokines are small proteins (about 25 kDa) released by various cells throughout the body in response to an activating stimulus. These proteins induce responses of their own when bound to specific receptors. Cytokines can act in autocrine, paracrine, or endocrine fashion, depending on their stability and role in the immune response (Murphy, 2011), a particularly important feature during immunological responses (Xing et al., 1998). Although the release of these immunological components benefit the host in combating the pathogens circulating throughout the body, their presence can also negatively impact the animal’s normal growth, metabolic, and reproductive functions (Whitlock et al., 2008).

Many different types of cytokines exist within the body, each with a multifunctional and diverse role in the overall immune response. Of primary importance during the initial immune response are pro-inflammatory cytokines. Upon release, these cytokines activate inflammatory cells and components of the vascular system, leading to the production of more cytokines and inflammatory mediators (Gruys et al., 2005). Three of the more important pro-inflammatory cytokines are tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-6 (IL-6) (Carroll and Forsberg, 2007). These three, among other roles, initiate a process known as the acute phase response (APR). This process is responsible for fever (a key regulator of infection), production of acute phase proteins, increase in white blood cell count, and a contributing factor to behavioral changes such as sleep patterns and intake (food and water) (Carroll and Forsberg, 2007). Increased circulating concentrations of IL-6, TNF-α, and IFN-γ have been associated
with decreased voluntary food intake and increased tissue degradation (Carroll et al., 2009).

**Tumor Necrosis Factor-alpha (TNF-α)**

Tumor necrosis factor-α (TNF-α), the prototype member of the tumor necrosis factor family, begins as a membrane bound, trimeric cytokine, but can be released from the membrane as needed (Murphy, 2011). This cytokine is mediated by members of the tumor necrosis factor receptor superfamily to promote production of pro-inflammatory cytokines as well as induce caspase-mediated apoptosis (Watts, 2005). Interleukin-8 (IL-8) is a chemokine used for cytokine recruitment to inflamed tissue. TNF-α has been shown to help enhance IL-8 secretion from polymorphonuclear (PMN) cells in the presence of LPS, as well as increase phagocytosis, degranulation, and oxidative burst activity of bovine PMN (Sohn et al., 2007). Additionally, TNF-α is involved in pro-inflammatory and anti-inflammatory events through stimulation of cytokine production through stimulation of IL-4, interferon-γ, and IL-10 production (Whitlock et al., 2008). In an LPS model, TNF-α response occurs during the first 4-6 hours after administration of the endotoxin, after which the cytokine’s circulating concentrations return to baseline levels (Whitlock et al., 2008). TNF-α induces endothelial cells to modify gene regulation and surface expression of intercellular adhesion molecules (ICAMs), enabling neutrophils and other circulating leukocytes to slow their rate of flow as they pass, and ultimately migrate into, the tissue of interest (Baumann and Gauldie, 1994).

However, despite all of its benefits to the body, when left unchecked, this cytokine can cause autoimmunity. TNF-α is used to locally contain infections through the
stimulation of epithelial cells to express blood clotting proteins, preventing the pathogen from invading the surrounding tissues or entering the bloodstream. TNF-\(\alpha\) release into the bloodstream can be detrimental to the animal as it stimulates vasodilation, leading to a drop in blood pressure and increases permeability of blood vessels, ultimately resulting in plasma volume decreases, causing the animal to experience septic shock. Presence of TNF-\(\alpha\) in the bloodstream also increases clotting protein expression, leading to an inability to clot properly and occluded bloodflow. Improper coagulation and loss of plasma volume leads to kidney, liver, heart, and lung failure (Murphy, 2011).

**Interferon-gamma (IFN-\(\gamma\))**

The interferon family derives its name from the ability to interfere with virus replication. IFN-\(\gamma\), a member of the interferon family, is first secreted by natural killer cells and is used in the activation of macrophages during an immune response (Murphy, 2011). This secretion is considered an important controlling factor of some infections prior to a secondary release from CD8 cytotoxic T-cells (Murphy, 2011) and is believed to be an indicator of disease severity (Bozza et al., 2008). Interferon-\(\gamma\) has been shown to increase translational efficiency in macrophages, resulting in increased production of pro-inflammatory cytokines such as TNF and IL-6 while decreasing production of anti-inflammatory cytokines such as IL-10 (Su et al., 2015). Additionally, and more pertinent to an LPS model, IFN-\(\gamma\) disrupts the inhibitory feedback loop of TLR4 signaling through suppression of HES1 and HEY1, thereby augmenting IL-6 and IL-12 production and potentiating the inflammatory response (Hu et al., 2008).
**Interleukin-6 (IL-6)**

Interleukin-6, a member of the interleukin family, is the primary stimulator of most acute-phase proteins and the chief stimulator of the acute phase response (Murphy, 2011). Inhibiting IL-6 has been shown to cause a greater than 95% reduction in hepatocyte-stimulating activity with LPS-stimulated monocyte- or IL-1/TNF-α stimulated fibroblast-conditioned medium (Gauldie et al., 1992). Whereas increased IL-6 has been correlated with worsened prognosis (Nakajima et al., 1997), this cytokine has also been noted as a particularly important anti-inflammatory agent, used to control local and systemic acute inflammatory responses (Xing et al., 1998). Gauldie et al. (1992), concluded that IL-6, in addition to its pro-inflammatory role, is an important anti-inflammatory used by the body to prevent excess tissue damage and facilitate a return to homeostasis during and after an immune response. Circulating levels of IL-6 are particularly important in regulating production of TNF-α (Petersen and Pederson, 2006), which has been demonstrated using an LPS model, with IL-6 depressing production of TNF-α during an LPS challenge (Schindler et al., 1990). The difference in IL-6 activity (pro- vs anti-inflammatory) is due to modulation of JAK/STAT pathways based upon presence or absence of mediators produced by simultaneously activated signal transduction pathways (Bode et al., 1999).

**Lipopolysaccharide (LPS)**

The term lipopolysaccharide is used to describe purified bacterial extracts which are, within reason, free of detectable contaminants, particularly protein (Hitchcock et al.,
An essential component for bacterial viability, LPS is readily found in the cellular membrane of gram-negative bacteria (Rietschel et al., 1994) and is classified as an endotoxin (Williams, 2013). Endotoxins, which can be further defined as bacterial poison (Beutler and Rietschel, 2003), are believed to be the most potent and multivalent molecules of bacterial origin in their capacity to elicit responses from immune cells (Rietschel et al., 1994). Lipopolysaccharide is an amphiphilic molecule (Williams, 2013) that is essential to bacterial viability (Rietschel et al., 1994). LPS is the biologically toxic component of endotoxin (Williams, 2013), which can be further defined as bacterial poison (Beutler and Rietschel, 2003). Lipopolysaccharide is released from the outer membrane when bacteria multiply and also when they die and lyse (Rietschel et al., 1994). Detection of this endotoxin is initiated through toll-like receptor 4. The binding of LPS by this receptor triggers a signaling cascade, which can result in the activation of pro-inflammatory cytokine genes by transcription factor NFκB in the nucleus of immune cells (Akira and Takeda, 2004), which is further amplified by the presence of prolactin (Brand et al., 2004).

Changes in cytokine concentrations affect the entire body, not just immunological components. Metabolic and physiological responses to LPS differ depending on the parameters of the study (i.e. diets, genes, temperament) and derivative of LPS used, but generalized reactions are observed in each study. One of the most easily observed is fever, which is caused by the increase in pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β (Murphy, 2011).

LPS challenge studies have also resulted in observed metabolic changes. During endotoxemia, animals typically experience a hyperglycemic period followed by a
hypoglycemic period (Spitzer et al., 1989) caused by increases in IL-6 and TNF-α, both of which will lead to the animal experiencing insulin resistance (Rotter et al., 2003). Non-esterified fatty acid (NEFA) concentrations are also increased by TNF-α (Kushibiki et al., 2000) and IL-6 (Pedersen et al., 2003) due to stimulation of lipolysis and hepatic lipid synthesis. Likewise, creatinine and blood urea nitrogen (BUN) concentrations are elevated during sepsis (Yura et al., 2009).

**Endophyte and Immune Function**

Immune system compromise has been observed to be related to consumption of endophyte-infected tall fescue. The immune system uses the spleen as a collection point for antigens within the bloodstream, making it a key component of the body defense against blood-borne pathogens (Murphy, 2011). After collection, antigens are taken up either by B-cell receptors (which in turn cause B-cells to differentiate into plasma cells) or dendritic cells within the spleen (which then present themselves to the T-cell rich areas of the spleen) (Murphy, 2011). To study the effects of endophyte-infected tall fescue seed on immune response, rats were placed on either E+ or E- diets (Simeone et al., 2008). Serum titer response to immunization in addition to spleen cell flow cytometry analysis and response to mitogens, was examined. Rats consuming the endophyte-infected diet experienced lower spleen weights and T-cell/B-cell responses than rats on the endophyte-free group. Similarly, Saker et al. (1998) found that monocyte and total leukocyte counts were greater ($P < 0.05$) in beef steers consuming E- vs. E+ diets over a three year study. Endophyte presence was also shown to affect tetanus vaccination efficacy, with
suppressed antibody response detected in cattle consuming endophyte-infected fescue diets (Dawe et al., 1997).

Filipov et al. (1999), using 8 Angus steers (4 per treatment), observed differences in immune and metabolic responses between cattle on E+ and E- diets during an LPS challenge. Greater serum TNF-α and cortisol concentrations were observed for both cytokines in E+, compared with E-, heifers following administration of LPS. Additionally, a time x treatment interaction was observed for serum growth hormone, with concentrations higher in E- heifers at 1 hour and in E+ heifers at 2 hours post-LPS.

To fully understand the results of this experiment, there are several points that must be considered. First, the study utilized animals that had been grazing either E+ or E- pastures for 8 months prior to the study. During that period, the authors did not measure or estimate intake. Also, pasture composition was not reported, only the concentration of ergot alkaloids of the E+ and E- pastures (3.3 and 0.3 mg/kg, respectively). Steers assigned to the E+ treatment had decreased prolactin concentrations and average daily gain when compared with E- treated steers. Due to this difference in ADG, which occurred over a prolonged period, as well as a lack of data on specific alkaloid intake, it is difficult to separate the direct effects of alkaloids from the resulting effects of restricted growth rates. Additionally, environmental data was provided only for the day LPS was administered, not for the grazing period. As mentioned above, some of the problems associated with endophyte-infected tall fescue are exacerbated during hot weather and absent during mild weather.

A second study (Filipov et al., 2000) was conducted the following year to examine the effect of ergotamine on the acute phase response of cattle using an LPS
challenge model. Sixteen Angus steers were utilized, with 4 steers per treatment.

Intravenously administered treatments, consisting of a two injection series (injection 1 + injection 2), were as follows: control (saline + saline), ergotamine tartrate + saline, saline + LPS, and ergotamine tartrate + LPS. All treatments were administered by injection at the same time, with the first substance in each treatment given at time -30 min and the second substance listed for each treatment given at time 0. Ergotamine tartrate was administered at 40 µg/kgBW, saline at 2.7 mL, and LPS at 0.2 µg/kgBW. Based upon depressed responses of haptoglobin, TNF-α, and thromboxane B₂ in ergotamine + LPS treated steers (compared with LPS only treated steers), the authors concluded that ergotamine tartrate had a significant effect on the response of cattle to immunological challenges, but the question remains as to how this data applies to endophyte consumption. The steers were injected with a single, large dose of ergotamine tartrate, not ergovaline (the primary ergot alkaloid produced by N. coenophialum), minutes prior to the LPS challenge. The two compounds do act similarly in some regards, but it remains unknown if their effects on the immune system are similar or if the responses observed in this study were representative of what would be experienced by cattle grazing E+ tall fescue. Additionally, it is unknown how ergovaline is absorbed and metabolized by the animal after consumption. Thus, intravenous administration is likely to present concentrations of active components to receptors that are markedly different from concentrations that would occur when alkaloids are consumed in the diet. Thus, significant questions remain about the influence of endophytic alkaloids on the immunological response of cattle when those alkaloids are consumed at levels consistent with normal production conditions.
Conclusion

Connections between decreased animal performance and endophyte presence have been studied extensively. Likewise, there are several studies which have examined the role of cytokines during an immune response. However, published research examining the relationship between endophyte consumption and resulting immune responses is limited. As immunological responses are specific to the pathogen present and differ between animals and environments, further research is needed in this area for cattle. The research cited above indicates a link between endophyte and immune responses. Cattle on endophyte-infected tall fescue pastures during warm weather present symptoms similar to those observed during a pathogenic infection. Additionally, prolactin has been reported several times to contribute to immune responses. Concentrations of this hormone are depressed in cattle consuming E+ diets, which may or may not impact the ability of that animal to respond to immunological attack. Others have examined T cell and B cell responses to endophyte consumption. Whereas it is informative that consumption of endophyte alters adaptive immunity responses, more information is needed on the modulations to innate immunity response. These components are critical during the acute phase of infection and begin to occur before the producer becomes aware that an animal is sick and may require intervention. Any suppression of these early responses to infection could lead to the animal becoming more susceptible to further infection. If the mechanisms of endophyte toxicity are to be fully understood, all aspects of this toxin’s influence on the body must be investigated, including any immune system alterations.
CHAPTER 2 – COSINOR ANALYSIS OF CIRCADIAN RHYTHM OF BODY TEMPERATURE

INTRODUCTION

Measurement of body temperature has historically been a focal point of animal research, but little analysis on daily rhythmicity has been performed in livestock (Piccione et al., 2003; Piccione and Refinetti, 2003). Homeostatic regulation of core temperature in mammals is maintained at around 37-39°C, with fluctuations seen throughout the day where the highest body temperature is often recorded during the active part of the day and the lowest during the inactive part of the day (Refinetti and Menaker, 1992). Therefore, describing the mean temperature of animals may not adequately describe all of the possible important alterations in body temperature.

“Circadian rhythm of body temperature” (CRT) refers to the phenomenon of a repeatable pattern of body temperature observed over consecutive approximate 24-hour periods (Refinetti, 2009). This pattern can be described using a mathematical modeling approach referred to as cosinor analysis, which provides measures for all parameters of CRT (Nelson et al., 1979). These parameters are defined as mesor (roughly defined as the mean), amplitude, acrophase (timing of the peak temperature relative to some fixed time point), and frequency (Nelson et al., 1979). Cosinor analysis provides measures for mesor, amplitude, and acrophase, but typically involves an assumed frequency (e.g. 24 h for normal circadian rhythms; Refinetti and Menaker, 1992).

In controlled experiments, use of cosinor analysis allows for a more complete description of possible treatment effects on body temperature. For example, a change in
amplitude may occur with a given treatment without any change in mesor, or mean
temperature. Likewise, treatments may influence the acrophase with or without any
alteration to the mesor or amplitude. Possibilities such as these require use of analytical
methods that are more sophisticated than simple analysis of mean temperature and
cosinor analysis has been commonly used to assess CRT in a wide variety of animals
(Refinetti, 2009).

In this experiment, we examined the impact of endophyte-infected tall fescue on
the febrile response of cattle prior to and during the acute phase response to an LPS
challenge. These exogenous factors had the potential to act as minor and major disruptors
of CRT, respectively. The objective of this study was to evaluate the use of cosinor
analysis for assessment of body temperature alterations induced by both endophytic
alkaloid exposure and endotoxin administration.

MATERIALS AND METHODS

All procedures used in these two experiments were approved by the University of
Kentucky Institutional Animal Care and Use Committee.

Animals and Experimental Design

Two experiments were conducted in which a total of 46 heifers were subjected to
LPS challenges concurrent to consuming either an endophyte-infected (E+) or endophyte-
free (E-) diet. Heifers were halter-broken prior to the start of the experiments. On day 1
of each experiment, heifers were placed into 3.0 x 3.7 m stalls with ad libitum water
supplied through permanently mounted waterers. Heifers were adapted to stalls for 11
days prior to receiving treatment diets. Heifers were maintained in a thermoneutral
environment at 22.3°C ± 1.3°C, which was verified through the use of room temperature loggers (HOBO 64K Pendant UA-001-64; Onset Computer Corporation; Bourne, MA). A 14 h:10 h light:dark cycle was established with lights coming on at 0600 h and turned off at 2000 h daily. The LPS challenge was administered on day 22 at 1000 h. Blood samples were collected via jugular vein catheters, which were placed the day prior to challenge (day 21). Collection times started at 0800 h, with samples taken every 30 minutes until 1800 h. A final blood sample was collected 24 hours after the start of the start of the challenge (1000 h on day 22). Blood samples were used for cytokine and metabolite analyses, which are reported separately (Altman et al., 2016).

**Experiment 1.** This experiment used twenty-two Angus heifers (292 ± 9.0 kg) and was conducted in two periods. The first period occurred in October 2012 and utilized 12 purchased heifers. The second period (December 2012; 54 days after period 1) utilized 3 purchased heifers and 7 heifers born and raised on the University of Kentucky C. Oran Little Research Center (LRC). Purchased heifers arrived on the research farm 26 days prior to the start of period 1. Home-grown heifers were weaned 275 days prior to the start of period 2. All heifers were vaccinated for respiratory diseases (Bovi-Shield Gold 5, Zoetis, Florham Park, NJ) and *Haemophilus Somnus* (Somubac, Zoetis, Florham Park, NJ). Heifers also received vaccinations against clostridial diseases (purchased heifers: Ultrabac 7, Zoetis, Florham Park, NJ, home grown heifers: Vision 7, Merck, Whitehouse Station, NJ) and *Manneheimia haemolytica type* type A1 (purchased heifers: One Shot, Zoetis, Florham Park, NJ; home-grown heifers: Vista Once SQ, Merck, Whitehouse Station, NJ). Safeguard (Merck, Whitehouse Station, NJ) was applied as a deworming agent. Home-grown heifers were also vaccinated against pinkeye (Autogenous Pinkeye,
MVP Laboratories, Omaha, NE). Within each period, half of the heifers were randomly assigned within weight strata to one of the two treatment diets. One heifer was treated for an infection occurring in the hind leg, but administration of medication ceased 1 day prior to heifers starting treatment diets.

Experiment 2. Sixteen Angus heifers (323 ± 34.5 kg; 301 ± 14 days old) and eight Hereford-Angus heifers (333 ± 19.5 kg; 310 ± 7 days old), all from the LRC, were stratified by sire breed, weight, and temperament (based upon weaning exit velocity), and randomly assigned within strata to an endophyte-infected (E+) or endophyte-free (E-) diet. Heifers were weaned at 183 (± 13) days of age, 119 days prior to the start of the experiment. Weaning exit velocity was determined using an infrared laser “trip wire” system (Polaris 3845A-MI1043; Farmtek, Inc., Wylie, Texas) in which the heifers were released from the head gate, passed through the first laser (0.46 m from head gate) starting the timer, and then through the second laser (1.68 m from first) to stop the timer. All heifers were vaccinated for clostridial diseases (Ultrabac 7, boostered with One-Shot Ultra 7; Zoetis, Florham Park, NJ), *Haemophilus Somnus* (Somubac, Zoetis, Florham Park, NJ), respiratory diseases (Bovi-Shield, Zoetis, Florham Park, NJ) and pinkeye (Solidbac, Zoetis, Florham Park, NJ).

**Treatments and Diets**

Diets (Tables 2.1, 2.2, and 2.3), were fed at 1.8 x NEm requirements and balanced to meet protein and mineral requirements for 0.75 kg/d growth rates (NRC, 2000). Pretreatment diets (Table 2.1) were total mixed rations consisting of corn silage, soybean meal, and trace mineralized salt for both experiments. The treatment diets (Table 2.2 and
Table 2.3) consisted of a cracked corn/cottonseed hull/fescue seed mix supplemented with soybean meal and trace mineral salt. Endophyte-infected fescue seed contained 15.1% CP, 28.8% NDF, and 2.29 ppm ergovaline + ergovalinine. Endophyte-free seed contained 15.1% CP, 30.6% NDF, and 0 ppm ergovaline + ergovalinine. The E+ diet was fed to provide ergovaline + ergovalinine at a dose of 10µg/kg BW. Heifers began receiving treatment diets on day 12 of each experiment. Heifers in Exp. 2 were additionally fed 0.5 mg melengesterol acetate·hd⁻¹·day⁻¹ in a 0.23 kg supplement during both the pre-treatment and treatment periods in order to suppress estrus. Refused feed was collected, weighed, and recorded at 0700 each morning. Animals were fed at 0800 daily.

**Temperature Probes**

Two different temperature probes were utilized for determining body temperature of the heifers during the LPS challenge period. Indwelling vaginal probes (Burdick et al., 2012) were placed on day 7 and remained patent until day 24 (48 hours after LPS administration.) Temperature was recorded every 5 minutes beginning on day 2 and concluding on day 24. Rectal temperature probes (Reuter et al., 2010) were utilized in Exp. 2 in addition to the vaginal probes for comparison with vaginal temperature response. The rectal probes were placed on day 10, removed on day 20 to allow for acquisition of caudal vein measurements (reported in Altman et al., 2016), then replaced on day 21 (the day before the LPS challenge), and removed a final time on day 24. The rectal probes also recorded temperature readings every 5 minutes.
Calculations and Statistical Analysis

Temperature data were analyzed using cosinor analysis (Refinetti et al., 2007). Each experiment was divided into four phases for analysis. Phase I, the pre-endophyte phase, encompassed temperature measurements from just after placement of temperature probes (day 7 of each experiment for vaginal probes, and day 10 of Exp. 2 for rectal probes) until the day that endophyte treatments were administered (day 12), resulting in a total of 5 days of vaginal probe data in both experiments and 2 days of rectal probe data in Exp. 2. Phase II, the post-endophyte, pre-LPS challenge phase, included a total of 9 days (day 12 through day 21). The lengths of Phases III and IV were determined from graphical analysis of the biphasic temperature response subsequent to LPS challenge. Phase III was comprised of a 9 h window encompassing the first temperature spike, commencing 1 h prior to LPS challenge, and continuing until 7 h post LPS challenge. Phase IV described the second post-LPS temperature spike, and included 14 h, commencing 9 h post-LPS and ending 22 h post-LPS. For Phases I and II, which were comprised of multiple days, curve parameters were determined (described below) separately for each day for each heifer and subjected to repeated measures analysis. Conversely, Phases III and IV were comprised of single curves for each heifer, such that repeated measures analysis was not conducted on the curve parameters. Cosinor analysis was performed using the linear least squares method of Nelson et al. (1979) with MATLAB Release 2013b (The MathWorks, Inc., Natick, MA), using a 24 h period for all curves in Phases I and II, a 9 h period for Phase III, and a 14 h period for Phase IV. Although data were collected at 5 minute intervals, hourly mean temperature values were used for curve fitting. In order to facilitate comparison between rectal and vaginal
temperature measures in Exp. 2, measures from all temperature probes were normalized by incubating probes in a 50 °C water bath for 58 hours at the conclusion of the experiment. Temperature measurements for each probe were then normalized to the mean temperature measured for all probes during the water bath exposure. Vaginal temperature data for one heifer on the E(-) treatment in Exp. 1, and one heifer on the E(-) treatment in Exp. 2, and rectal temperature data for one heifer on the E(+) treatment in Exp. 2 were unavailable due to temperature probe failure. Additionally, in Exp. 2, rectal probes were removed on d 20 (toward the end of Phase II) to facilitate ultrasonic measures of caudal vein areas, and replaced 27 hours later.

Cosinor analysis permits a more detailed assessment of alterations in body temperature across a time period as compared with analysis of body temperature data without regard to its circadian rhythm. Curve parameters determined in this analysis included the mesor, or midline estimating statistic of rhythm (in this case, with equidistant measurement intervals, equal to the mean temperature), amplitude of oscillation, and the phase of the maximum relative to a fixed reference time, known as the acrophase (Refinetti et al., 2007). In some cases, least squares fits of these parameters provided poor fits to the data. Data were excluded from analysis when adjusted $R^2$ values were less than 0.40. Figure 2.1 shows representative charts to give a graphical illustration of the difference in curve fits with various $R^2$ values. Table 2.4 shows the number of curves which were excluded from analysis based on this criterion.

For Exp. 1, there were two extraneous sources of variation that were accounted for in the statistical model using a single blocking factor. Variation due to period (2 periods) and heifer source (purchased or home-grown) were combined to create three
blocks: block 1 encompassed period 1, purchased heifers (n = 12), block 2 was comprised of purchased heifers in period 2 (n = 3), and block 3 included the home-grown heifers from period 2 (n = 7). Because this was a preliminary experiment in which we were attempting to gain perspective on the impact of different sources of variation, block was included in the model as a fixed effect, to permit estimation of block means. For the multi-day data collected in Phases I and II, data were analyzed as repeated measures using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). The model included block, treatment, day, and the treatment x day interaction, and the Kenward Roger method was used to estimate denominator degrees of freedom. The repeated term was day, subject was specified as individual animal, and the R-matrix covariance structure was specified as first-order autoregressive. On two separate days in Phase II, the number of observations available for parameter estimation was limited (n ≤ 3) for the E-treatment, so data for those two days were not included in the statistical analysis. Data from Phases III and IV were comprised of single measures for each parameter (for each animal). Thus, the responses in those phases were analyzed using the Mixed procedure of SAS with a model containing the fixed effects of block and treatment.

For experiment 2, data were similarly analyzed with the exception that there were two sources of extraneous variation accounted for in the model. Sire breed (Angus vs. Hereford) was included as a fixed block effect. Additionally, weaning exit velocity (wEV) was included as a covariate. For covariate analysis, we first fit an unequal slopes model. For variables for which this model fit (wEV x treatment P < 0.10), we compared treatment effects at each of three levels of wEV, corresponding to the minimum, mean, and maximum observed wEV values for these heifers. In the absence of significant wEV
x treatment interactions, we evaluated a common slope model for the covariate. When the covariate was found to have a significant effect on a response variable, we estimated the slope of the response. Thus, for Phases I and II (those with repeated measures), cosinor parameters were evaluated using repeated measures analysis with the Mixed procedure of SAS with the same options as specified for Exp. 1. Terms in the model statement (for the common slope model) included sire breed, wEV, treatment, day, and the treatment x day interaction. For Phases III and IV, without repeated measures, the GLM procedure of SAS was used (for ease of estimating effects within unequal slopes models; no differences in P-values existed between Proc Mixed and Proc GLM). Terms in the common slope model included sire breed, treatment, and wEV. In all cases, the unequal slope model differed from the common slope model by including the wEV x treatment interaction term in the model. On three separate days in Phase II, the number of observations available for parameter estimation was limited (n ≤ 3) for the E- treatment, so data for those two days were not included in the statistical analysis.

Outliers were identified as individual observations whose studentized residuals (after fitting the appropriate model) exceeded the third quartile or fell below the first quartile by more than 1.5 x the interquartile range. Generally, these situations occurred when animals were experiencing transient febrile responses that were unrelated to treatments. Thus, their data was removed from analysis for these short periods of time.

The difference between rectal and vaginal temperatures (RV delta) was initially analyzed using the cosinor analysis method mentioned above, with detection of only mesor differences between the two temperature measures. The absence of effects for amplitude or acrophase indicated that any meaningful differences between measurement
sites would be accounted for by differences in absolute temperature measurements. Therefore, RV delta was analyzed using repeated measures on hourly temperature differences. The model was the same as the previously described repeated measures model for Exp. 2. The wEV x treatment interaction independent slope model was significant for all phases. However, the nature and magnitude of the effects was essentially identical for all phases. Because no treatments were applied during Phase I, the interaction was considered an artifact. Thus, the common slope model was determined to be more appropriate to explain these responses.

RESULTS

Fit of cosine curves. The number of fitted curves within each phase of each experiment are shown for each dietary treatment in the first two columns of Table 2.4. Each curve represents a single day for an individual animal (in Phases I and II), or a single 9 h (Phase III) or 14 h (Phase IV) window for each animal. Thus, the total number of curves fit in each phase of each experiment varies with the number of days and the number of animals with valid data. We used an R² value of 0.40 as a cutoff for inclusion of curve parameters for statistical analysis (with the idea that the resulting parameters were unreliable with R² ≤ 0.4). The second pair of columns in Table 2.4 indicates the number of curves that were excluded based on this criteria. The third pair of columns in Table 2.4 show the number of excluded curves as a percentage of the total curves that were fit, and the final pair of columns indicates the relative strength of the cosine fit (R²) of the curves that were used in the statistical analysis. Though it is inappropriate to conduct statistical analysis on the R² or RMSE values resulting from these least-squares fits, patterns of poor fits could be helpful in identifying limitations to cosinor
rhythmometry in this experimental model. In general, difficulties in fitting the observed data were prevalent in Phase II in both experiments (29 to 42% of fitted curves failed to meet our R² criterion of 0.4), with no consistent differences noted between dietary treatments or between rectal and vaginal temperature measurements. In general, cosinor analysis provided good fits of the post-LPS temperature spikes (R² from 88 to 94%; Phases III and IV), moderately good fits (R² from 68 to 78%) during the pre-endophyte period (Phase I), and poor fits during Phase II (R² from 61 to 69% for the curves which met the inclusion criterion), when the animals were transitioning from corn-silage based diets to the cracked corn/cottonseed hull/fescue seed diets.

_Treatment, block, and day effects._ In Exp. 1, no treatment x day interactions were detected (P ≥ 0.30; Table 2.5). Mean vaginal temperatures (represented by the mesor) were unaffected (P = 0.19) by day in Phase I, although there was an effect of day (P < 0.01) during Phase II. Across this nine day phase, mean temperatures varied maximally from 38.58 to 38.69 °C, with a maximum change across consecutive days of 0.06 °C (Fig. 2.5). Endophyte treatment did not affect (P > 0.66) mean vaginal temperature until the second post-LPS febrile response (Phase IV; Fig. 2.6), when temperature was 0.28 °C greater with exposure to endophytic alkaloids (P = 0.08). Differences among blocks were detected (P ≤ 0.04) in Phases II and IV which were attributable to heifer source (difference of ≤ 0.04 °C between periods for similarly-sourced heifers; difference of 0.25 °C in Phase II and 0.74 °C in Phase IV between the two sources of heifers, data not shown).

Unlike mean temperature, the amplitude of the daily temperature did differ (P = 0.05) across days in Phase I, though the largest change was from 0.24 to 0.30 °C from d 1
to d 2 (Fig. 2.4). Amplitude was not different (P ≥ 0.11) among blocks or treatments during any phase.

Prior to application of the endophyte treatment (Phase I), acrophase was not different (P ≥ 0.19) among animals destined for different treatments, or across days, although it did differ among blocks (P < 0.01). In this case, heifers in period 1 demonstrated a daily peak temperature approximately 150 min later that those in period 2 (data not shown). During Phase II, the acrophase varied from 0020 h to 0140 h across days (P = 0.06; Fig. 2.5), with no detectable pattern in the variation among days. The first post-LPS temperature spike (Phase III) was delayed by 14 minutes (P = 0.09; Fig. 2.6) with E+, though differences between blocks (P < 0.01) were about 1.5 times that magnitude, and predominantly influenced by heifer source, as opposed to period of the study (data not shown). Neither treatment nor block effects influenced the timing of the Phase IV peak temperature (P ≥ 0.43). This appeared to be largely due to greater variation among animals (i.e. larger SEM) in the timing of their second, as compared with their first post-LPS temperature spike.

In Exp. 2 (Table 2.6), the influence of model effects on CRT responses differed between rectal and vaginal temperature measurements. The only treatment x day interaction detected for Exp. 2 occurred during Phase I for rectal acrophase response (P = 0.02). However, Phase I occurred before exposure of heifers to treatment diets, indicating that this interaction was a chance occurrence. Temperature profile differences among days were limited to Phase I, during which heifers experienced shifts in vaginal temperature mesor (P < 0.01), amplitude (P = 0.10), and acrophase (P = 0.01) among
days. Conversely, rectal temperature parameters did not differ \( (P \geq 0.25) \) among days. However, only 2 days’ measurements were available for Phase I rectal temperatures.

Sire group effects were most notable during the first post-LPS temperature spike (Phase III), when vaginal temperature mesor \((39.53 \text{ vs } 39.27 \pm 0.067^\circ C, \text{ respectively})\) and amplitude \((1.01 \text{ vs } 0.80 \pm 0.061^\circ C, \text{ respectively})\) and rectal temperature amplitude \((0.92 \text{ vs } 0.76 \pm 0.058^\circ C, \text{ respectively})\) were found to be greater \((P \leq 0.07)\) in Hereford-sired, as compared to Angus-sired, heifers. Conversely, during Phase I, the amplitude of vaginal temperatures was approximately 0.05 \(^\circ C\) greater \((P = 0.04)\) in Angus-, as compared with Hereford-sired heifers. No other sire breed effects were detected \((P > 0.13)\).

Interactions between weaning exit velocity and endophyte treatment were predominantly observed with rectal, as compared with vaginal, temperature profiles. Interactions for rectal temperature mesor were detected \((P \leq 0.10)\) in Phases I, II, and III. For each of these, mesor increased with increasing exit velocity in \(E^+\) animals, and decreased with increasing exit velocity in \(E^-\) animals, such that differences between endophyte treatments were only detected \((P \leq 0.09)\) at the highest exit velocities (Fig. 2.2 depicts the general nature of these interactions during all phases). Because this interaction was detected prior to initiation of endophyte treatments (Phase I), it must be considered a serendipitous occurrence during that phase. Additionally, because the nature and magnitude of the effects was consistent through all three phases, this effect must be attributed to chance in all of these cases. During Phase IV, both vaginal \((P = 0.06)\) and rectal \((P = 0.07)\) temperature amplitudes were influenced by such an interaction. In contrast to the response with the mesor, the nature of these interactions was such that
increasing wEV was associated with increasing amplitude for E-, and with decreasing amplitude for E+ heifers (Fig. 2.3).

Weaning exit velocity influenced Phase I (P = 0.01) and Phase III (P = 0.10) vaginal mesor temperatures, with temperature increasing 0.18°C and 0.14°C (Phase I and III, respectively) for every 1 m/s increase in exit velocity. Weaning exit velocity also shifted the rectal temperature acrophase during Phase I, (P = 0.08), where every 1 m/s increase in wEV was associated with a 0.88 h delay in the time of peak temperature response.

There were no effects of endophyte treatment that occurred independently of interactions with wEV or day.

**DISCUSSION**

**Appropriateness of model**

The underlying model was designed with the intent of producing subclinical physiological effects of alkaloid toxicity. Thus, the model served as a good platform for evaluating the utility of cosinor rhythmometry to detect disruption of body temperature regulation in the presence of relatively small alkaloid-induced effects on mean body temperature.

Effects of LPS on mean temperature have been well characterized (Whyte et al., 1989; Soszynski et al., 1991; Steiger et al., 1999; Waldron et al., 2003; Hulbert et al., 2011), thereby providing a good model to evaluate whether additional information can be
gleaned by applying cosinor analysis separately to each of the two febrile periods that typically follow LPS administration.

**Limitations of this study**

Although cosinor analysis affords opportunity to gain some insight into the influence of potential disruptors of CRT, the physiological significance of many of these responses is unknown at present. Overall, endophyte treatments had relatively minor effects on body temperature responses. The results of this study thus provide an opportunity to evaluate potential usefulness of cosinor analysis for detecting subtle effects due to treatment. Small changes in the magnitude or timing of the temperature response may ultimately provide greater insight into the complex biological mechanisms that interact to influence body temperature regulation.

**General Description of CRT**

Temperature profiles during Phase I for both experiments are in agreement with existing data for cattle under thermoneutral conditions. Mean temperatures for both experiments were between 38.7 to 38.9°C, which is in close agreement with the normal range of 38.3 ± 0.5°C reported for cattle (Merck, 1991). Amplitudes of the temperature curves were also similar between experiments, but remained below the typical range of 0.3 to 0.5°C described by Piccione and Caola (2002). It should be noted that this range is an approximation the authors used to describe the relative consistency of core body temperature, which can be influenced by environmental changes. Because the heifers in this study were kept in a thermoneutral environment, temperature oscillations should be expected to remain below what is observed in cattle living in less controlled
environments. Using lactating dairy cows housed in a thermoneutral environment, Lefcourt et al. (1999) observed temperature amplitudes of 0.34 ± 0.15°C, which are similar to those observed in this study. Acrophase was the only measured curve parameter to differ substantially between the two experiments, with temperature peaks in Exp. 2 occurring 1 hour later than those observed during Exp. 1. However, timing of the cosine wave’s zenith in both experiments falls within the range observed by Lefcourt et al. (1999), who found zenith to occur between 2300 and 0200 hr. These later zeniths may be due to differences in light/dark cycles and feeding time, which were approximately 1 and 2 hours, respectively, behind those used in the current study. Similar results were observed by Scott et al. (1983), with zenith occurring between 2200 and 0100 hr, although light/dark cycles and feeding times from that study are unavailable for comparison.

Factors influencing CRT

Results from Exp. 1 indicated possible extraneous sources of variation not fully accounted for in the methodology. During the last two phases of this experiment, there was some evidence of endophytic alkaloid effects. However, these effects were overshadowed by more significant differences between blocks.

Natural circadian rhythms are innate and controlled by endogenous factors in addition to the light/dark cycle (Piccione and Caola, 2002). Seasonal variation may have partially contributed to observed block effects, particularly acrophase differences during the acclimation phases (Phase I) of Exp. 1. Estrus was another potential contributing factor to differences in block effects, as occurrence of this physiological event has
previously been reported to coincide with sharp rises in body temperature (Piccione et al., 2003). By splitting Exp. 1 into two periods spaced two months apart, these factors potentially influenced results. Designing Exp. 2 to be conducted within a single period, and incorporation of MGA to suppress estrous allowed for greater control of these factors. Additionally, the design of Exp. 2 explicitly addressed variation from other extraneous sources, including weaning exit velocity (as a measure of temperament) and genetics, leading to greater confidence in obtained results.

A high degree of control over external sources of variation was emphasized in the transition from Exp 1 to Exp 2. Differences in SEM of response variables between the two experiments can provide some insight to the degree to which this was accomplished. The most notable reductions in SEM from Exp. 1 to Exp. 2 were for vaginal temperature mesor and amplitude in Phase III (i.e. LPS response). However, the SEM for acrophase increased between Exp. 1 and Exp. 2. The implications are that close attention to controlling potential extraneous influences on one response (e.g. temperature response to LPS) may not reduce variation, or may increase variation, in other CRT-associated parameters. Investigators will thus need to prioritize response variables of interest when designing studies to evaluate CRT responses.

Light, Temperature, and Animal Handling. Major factors acting as synchronizing agents for circadian rhythms include light:dark cycles, feeding time, and ambient temperature (Piccione and Caola, 2002). With minor exceptions (noted below) all of these factors were kept constant throughout these two experiments. Mechanical issues that led to "out of control" temperatures did not appear to have major influence on measured responses. Temperatures largely remained below the upper critical temperature
(Hahn, 1999) for the heifers in this experiment. Temperature rhythms were reasonably consistent during Phase I of Exp. 1, with no differences detected among days for mean temperatures. However, day-to-day differences were observed in the amplitude of the temperature response. While the underlying mechanism for this response cannot be determined from the present study, it is informative that cosinor analysis was able to detect a modest (0.06°C) increase in amplitude from the first to second day of temperature measurement. For studies conducted in less controlled environments, identification of such shifts could represent important responses to the environment.

Results from Exp. 1 also revealed a shift in acrophase across days during Phase II, largely driven by a 3 h shift on the last day of this phase. This delayed acrophase may have been an artifact induced by handling of the animals for jugular catheterization late in the last day of Phase II. Rather than exclude that day from the analysis, however, the ability to detect such a shift is further evidence of a potential benefit of cosinor analysis.

*Source/prior treatment of animals.* Results from Exp. 1 indicated that animals from different sources (UK raised vs. purchased heifers) experienced different magnitude and timing of febrile responses to LPS, regardless of treatment, as well as substantial differences in mean vaginal temperatures prior to LPS challenge. Home-raised heifers did not have endophyte exposure prior to the start of the experiment, but the endophyte exposure status of the purchased heifers was unknown. We are unaware of any published studies examining the effects of repeated exposures to endophyte-infected fescue. This is probably due to inconsistency with current management practices, which generally do not include transferring growing animals on and off diets containing endophyte-infected feeds. In light of this, it is unknown whether observed differences in heifer origin were
due to prior endophyte exposure or other environmental or genetic factors. Potential differences in environmental factors were accounted for in the methodology for Exp.2 by using heifers from a single source.

Diet effects. The most notable perturbation in CRT was the apparent influence of a dietary change (while keeping formulated energy and protein delivery consistent) on the maintenance of sine-wave-like temperature rhythms during Phase II compared with the rhythms observed in Phase I. The major event in this phase was the change from a corn silage based diet to a cottonseed hulls/corn/fescue seed diet. This abrupt change in diet appears to have disrupted the consistency of the daily temperature rhythm, with the disruption experienced equally by both treatment groups (Table 2.4). Disruptions to the CRT due to dietary changes have previously been observed in sheep (Maloney et al., 2013). However, it is important to note that their study examined the effect of dietary energy on CRT, whereas, in the present study, diets were formulated for consistent energy and protein profiles across Phases I and II. In both studies, amplitudes and overall goodness of fit changed in response to dietary alterations. Although feeding behavior was not specifically monitored in this study, it is possible that the shift in diet resulted in altered consumption patterns, which in turn may have influenced CRT. Timing of meals is a well-recognized entraining agent in mammals (Piccione and Caola, 2002). Although feeding time was consistent throughout both experiments, alterations in consumption patterns would be expected to have effects and could explain disruption of daily biological rhythms (Damiola et al., 2000). The ability to detect such responses supports the use of cosinor rhythmometry as a tool for evaluating functional relationships between nutritional factors and body temperature regulation.
Sire breed effects. The results of Exp. 2 revealed some differences between sire breeds for acute phase responses, with Hereford sired heifers experiencing higher amplitudes than Angus sired heifers during Phase III for both rectal and vaginal temperatures and higher mesor for vaginal temperature. Alternatively, during Phase I, the amplitude of the vaginal CRT curves was 0.05 °C greater in Angus- than in Hereford-sired heifers. Differences indicate that sire breed could influence the temperature response of cattle during immunological events. In this case, breed effects that manifested as higher temperature amplitudes under the ‘normal’ conditions of Phase I were associated with dampened amplitude responses to LPS during Phase III.

Effect of endophyte presence on acute phase response to LPS

Endophyte effects were limited to LPS periods (Phases III and IV) in both experiments. Exposure to endophyte led to higher mesor for vaginal temperatures during Phase IV of Exp. 1 and rectal temperatures during Phase III of Exp. 2. Additionally, during Phase III of Exp. 1, peak temperature for E+ heifers was achieved approximately 14 minutes later than in E- heifers. At this time, the physiological importance of these differences is unknown. However, current research indicates the two phases are differentially regulated (Blatteis et al., 2005). The initial (Phase III) febrile response appears to be a result of hepatic responses to LPS presence communicated to the preoptic area of the hypothalamus by the vagus nerve, initiating the febrile response independent of pyrogenic cytokines. Evidence suggests the second LPS peak (Phase IV), is initiated in the preoptic-anterior hypothalamus, maintaining the febrile course resulting from pyrogenic cytokine presence (Blatteis et al., 2005). Data from the current study demonstrate that endophyte exposure can influence the CRT of heifers during
immunological challenge, suggesting that endophyte may modulate overall febrile responses.

**Exit velocity effects**

Weaning exit velocity (measured prior to the start of Exp. 2) influenced CRT and febrile responses of heifers. Detailed mechanisms relating behavioral responses to immunological variables remain elusive. However, these results are consistent with other work showing relationships between immunological responses and temperament (Burdick et al., 2011; Burdick Sanchez et al., 2014; Fell et al., 1999).

Also of interest were significant wEV effects (Exp. 2) in mesor vaginal temperatures during Phase I, with a relationship of each m/s increase in exit velocity correlating to a 0.18°C in body temperature. In the first three phases, wEV x endophyte interactions existed for mesor rectal temperatures. However, because endophyte treatment had not yet been initiated in Phase I, and because of the similarity in significance levels for all three phases, these differences were considered artifacts. Alternatively, a wEV x endophyte interaction of a different nature was observed during Phase IV of Exp. 2 for both rectal and vaginal temperature measures, with increased amplitude in high wEV animals exposed to endophyte. Of particular interest was the similar response detected for both rectal and vaginal measures, with amplitudes increasing in E- heifers (0.22 and 0.24°C, for vaginal and rectal temperatures) and decreasing in E+ heifers (-0.10 and -0.07°C for vaginal and rectal temperatures) for every 1 m/s increase in wEV. This was the only similar response observed during Exp. 2 for rectal and vaginal temperatures, indicating the two measures may produce different
CRT profiles in response to the same stimulus. The nature of this interaction was such that in absence of alkaloid exposure, increased wEV was associated with increased amplitude of the second (or centrally mediated) febrile response. The results of this study indicate that alkaloid exposure may ameliorate this response. Observation of this relationship only during Phase IV suggests important mechanisms involved in both temperament and alkaloid induced effects are centrally located (i.e. hypothalamic) and share some commonality in affected pathways. Alternatively, wEV influenced mean vaginal temperatures during Phase III, regardless of endophyte treatment, suggesting that temperament has additional influence on peripheral or hepatically stimulated febrile responses to immune challenge which are not modified by low level exposure to endophytic alkaloids. Important for our current objectives is that potential insights into such relationships would not have been possible without detailed analysis of the curve parameters (i.e. analysis of only mean temperatures.)

**Differences between rectal and vaginal temperatures**

Revisions to the methodology for Exp. 2 included placement of both rectal and vaginal temperature probes to identify potential differences in temperature measurements between the two locations. It is common to use either rectal or vaginal temperatures in studies with heifers, as these are generally considered to provide similar data. Highly correlated vaginal and rectal temperatures have been found in Holstein cows (Vickers et al., 2010), provided the measurements were taken during the same time period. Burdick et al. (2012) observed similar results using Brahman heifers. However, correlations between mean temperatures do not necessarily imply that other components of CRT (namely amplitude and acrophase) are similar. In a canine study examining the impact of
light/day cycles on CRT responses, Giannetto et al. (2015) reported that dogs exposed to continuous light with no dark cycle (light/light) had vaginal temperatures that lacked rhythmicity patterns, yet rhythmicity was present for rectal temperatures in the same dogs. This loss of rhythmicity was not observed with dogs exposed to other light cycles (light/dark and dark/dark). Thus, there is evidence that, at least in some species, rectal and vaginal temperatures are differentially regulated.

Results from Exp. 2 indicate the two temperature measures do not respond to stimuli similarly. Presence of vasoconstrictive compounds (endophyte) during Phase II (P = 0.05; data not shown) presented measurable differences in the difference between rectal and vaginal temperatures (RV delta). Exit velocity impacted RV delta across all four phases (P ≤ 0.024; data not shown), with differences between the two sites of measurement decreasing approximately 0.3°C for every 1 m/s increase in wEV. Time effects (P ≤ 0.07) were also significant across all four phases (Fig. 2.13), but the largest difference was a 47% decrease in magnitude of RV delta between Phases II and III. The increased similarity between rectal and vaginal temperatures during Phases III and IV, as opposed to Phases I and II, suggests the two measures are more similarly regulated during febrile responses.

Examinations of differences between rectal and vaginal temperatures are limited. Although the mechanisms behind these differences remain unknown, their implications are potentially important. These results indicate that a variety of factors could impact core temperature readings differently by site. This is especially important in regard to relationships with temperament, as a 3 unit increase in wEV was equivalent to almost a full 1.0°C difference between vaginal and rectal temperature measurements.
CONCLUSION

For cosinor rhythmometry to be useful in this application, it needs to provide information about temperature responses that would not be available from simple analysis of mean temperatures. It is noteworthy that the SEM for amplitudes was much smaller than the SEM for mesor, especially during Phases I and II. This points to one benefit of this analytical approach, namely increased power to detect statistical differences between treatments where they might exist. Additionally, differences across days were observed for amplitude and acrophase that would have remained undetected with simple mean temperature analysis for both vaginal and rectal temperatures.

Vickers et al. (2010) noted that while rectal and vaginal temperatures followed a similar pattern, both had slightly different diurnal patterns. Thus, they warned against relying upon only one measurement to draw conclusions. By using cosinor analysis, we were able to detect some subtle differences between vaginal and rectal temperatures during the control period. These observed differences were not limited to temperature means and would have remained undetected without CRT analysis. Additionally, this study provided some indication that vaginal temperatures respond differently to endophyte and endotoxin as compared with rectal temperatures.

Cosinor analysis remains a novel approach for analyzing body temperature in cattle research. The results of this study indicate that use of cosinor rhythmometry in evaluating body temperature can lead to greater insight into the febrile response of cattle during an immunological event. Further investigations into the mechanisms for these responses, such as those occurring within the POA, may enhance overall understanding.
of temperature regulation. Additionally, more studies are needed examining both vaginal and rectal temperatures if differences between the two temperature measures are to be fully understood.
Table 2.1. Ingredient composition of the pre-treatment diets by experiment

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Silage (40% grain)</td>
<td>89.7</td>
<td>86.0</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>9.6</td>
<td>9.2</td>
</tr>
<tr>
<td>Trace Mineral Salt$^a$</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>MGA Carrier (Cracked Corn and Wheat Middlings)$^b$</td>
<td>0.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

$^a$Trace mineralized salt included 92.9% salt, 68 ppm Co, 1838 ppm Cu, 120 ppm I, 9290 ppm Mn, 19 ppm Se, and 5520 ppm Zn.

$^b$To provide MGA at 0.5 mg·hd⁻¹·day⁻¹.
Table 2.2. Ingredient composition of treatment diets (Exp. 1 and 2)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cracked Corn</td>
<td>36.0</td>
<td>34.6</td>
</tr>
<tr>
<td>Cottonseed Hulls</td>
<td>30.0</td>
<td>28.8</td>
</tr>
<tr>
<td>Fescue Seed</td>
<td>20.0</td>
<td>19.2</td>
</tr>
<tr>
<td>Supplement&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Molasses</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>MGA Carrier (Cracked Corn and Wheat Middlings)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Supplement included trace mineralized salt, vitamin premix, limestone, and molasses. Trace mineralized salt provided 92.9% salt, 68 ppm Co, 1838 ppm Cu, 120 ppm I, 9290 ppm Mn, 19 ppm Se, and 5520 ppm Zn. Vitamin premix supplied 1820 IU/kg Vitamin A, 363 IU/kg Vitamin D, and 227 IU/kg Vitamin E.

<sup>b</sup>To provide MGA at 0.5 mg·hd<sup>-1</sup>·day<sup>-1</sup>
Table 2.3. Chemical composition of the treatment diets by experiment

<table>
<thead>
<tr>
<th>Component</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>NEm (Mcal/kg DM)</td>
<td>1.65</td>
<td>1.65</td>
<td>1.67</td>
<td>1.67</td>
</tr>
<tr>
<td>CP</td>
<td>12.9</td>
<td>13.3</td>
<td>15.5</td>
<td>15.5</td>
</tr>
<tr>
<td>NDF</td>
<td>34.3</td>
<td>35.4</td>
<td>32.4</td>
<td>31.2</td>
</tr>
<tr>
<td>ADF</td>
<td>26.2</td>
<td>27.1</td>
<td>24.1</td>
<td>21.7</td>
</tr>
</tbody>
</table>
Table 2.4. Number and percentage of cosine curves for which parameters were excluded from statistical analysis based on lack of fit ($R^2 < 0.40$).

<table>
<thead>
<tr>
<th>Experiment and Phase</th>
<th>Total number of curves fit</th>
<th>Number of curves excluded, of total</th>
<th>Mean $R^2$ for included curves,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
<td>E+</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>55</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td>Phase II</td>
<td>99</td>
<td>90</td>
<td>29</td>
</tr>
<tr>
<td>Phase III</td>
<td>11</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Phase IV</td>
<td>11</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>60</td>
<td>55</td>
<td>3</td>
</tr>
<tr>
<td>Phase II</td>
<td>108</td>
<td>97</td>
<td>45</td>
</tr>
<tr>
<td>Phase III</td>
<td>12</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Phase IV</td>
<td>12</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Rectal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>22</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>Phase II</td>
<td>88</td>
<td>96</td>
<td>37</td>
</tr>
<tr>
<td>Phase III</td>
<td>11</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Phase IV</td>
<td>11</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

*Phases divided into pre-treatment diet (Phase I), treatment diet/pre-LPS challenge (Phase II), LPS challenge period (Phase III), and post LPS challenge period (Phase IV), as described in the text.*
Table 2.5. Influence of endophyte-infected fescue seed on vaginal temperature response in each of four phases of an experiment that included an LPS-challenge (Exp. 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dietary treatment</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>Mesora, C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>38.71</td>
<td>38.75</td>
</tr>
<tr>
<td>Phase II</td>
<td>38.63</td>
<td>38.65</td>
</tr>
<tr>
<td>Phase III</td>
<td>39.59</td>
<td>39.52</td>
</tr>
<tr>
<td>Phase IV</td>
<td>39.27</td>
<td>38.99</td>
</tr>
<tr>
<td>Amplitude, C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>0.28</td>
<td>0.27</td>
</tr>
<tr>
<td>Phase II</td>
<td>0.21</td>
<td>0.18</td>
</tr>
<tr>
<td>Phase III</td>
<td>0.89</td>
<td>0.86</td>
</tr>
<tr>
<td>Phase IV</td>
<td>0.67</td>
<td>0.49</td>
</tr>
<tr>
<td>Acrophase, h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I</td>
<td>21.89</td>
<td>22.15</td>
</tr>
<tr>
<td>Phase II</td>
<td>23.48</td>
<td>23.75</td>
</tr>
<tr>
<td>Phase III</td>
<td>13.36</td>
<td>13.13</td>
</tr>
<tr>
<td>Phase IV</td>
<td>24.41</td>
<td>23.90</td>
</tr>
</tbody>
</table>

*aMesor represents average temperature observed

b Phases divided into pre-treatment diet (Phase I), treatment diet/pre-LPS challenge (Phase II), LPS challenge period (Phase III), and post LPS challenge period (Phase IV), as described in the text.

cAcrophase represents time of peak temperature relative to midnight.
Table 2.6. Influence of endophyte-infected fescue seed on vaginal and rectal temperature responses in each of four phases of an experiment that included an LPS-challenge (Exp. 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dietary treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>Mesora&lt;sup&gt;a&lt;/sup&gt;, C</td>
<td>Trt</td>
<td></td>
</tr>
<tr>
<td>Vaginal Phase I</td>
<td>38.82</td>
<td>38.74</td>
</tr>
<tr>
<td>Phase II</td>
<td>38.65</td>
<td>38.71</td>
</tr>
<tr>
<td>Phase III</td>
<td>39.40</td>
<td>39.39</td>
</tr>
<tr>
<td>Phase IV</td>
<td>39.00</td>
<td>38.94</td>
</tr>
<tr>
<td>Rectal Phase I</td>
<td>39.08</td>
<td>38.89</td>
</tr>
<tr>
<td>Phase II</td>
<td>38.86</td>
<td>38.77</td>
</tr>
<tr>
<td>Phase III</td>
<td>39.52</td>
<td>39.38</td>
</tr>
<tr>
<td>Phase IV</td>
<td>39.12</td>
<td>38.94</td>
</tr>
<tr>
<td>Amplitude, C</td>
<td>Trt</td>
<td></td>
</tr>
<tr>
<td>Vaginal Phase I</td>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>Phase II</td>
<td>0.17</td>
<td>0.19</td>
</tr>
<tr>
<td>Phase III</td>
<td>0.84</td>
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</tr>
<tr>
<td>Phase IV</td>
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</tr>
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</tr>
<tr>
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<td>0.18</td>
</tr>
<tr>
<td>Phase III</td>
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<td>0.89</td>
</tr>
<tr>
<td>Phase IV</td>
<td>0.48</td>
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</tr>
</tbody>
</table>
Table 2.6. Influence of endophyte-infected fescue seed on vaginal and rectal temperature responses in each of four phases of an experiment that included an LPS-challenge (Exp. 2 continued).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dietary treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Probability of a greater F-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td><strong>Acrophase&lt;sup&gt;c&lt;/sup&gt;, h</strong></td>
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<tr>
<td>Phase I</td>
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</tr>
<tr>
<td>Phase IV</td>
<td>23.82</td>
<td>23.55</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mesor represents average temperature observed

<sup>b</sup>Phases divided into pre-treatment diet (Phase I), treatment diet/pre-LPS challenge (Phase II), LPS challenge period (Phase III), and post LPS challenge period (Phase IV), as described in the text.

<sup>c</sup>Acrophase represents time of day relative to midnight.
Figure 2.1. Representative least-squares cosine fits of temperature data, demonstrating fits that were above ($R^2 = 0.50$, Fig. a), at ($R^2 = 0.40$, Fig. b), and below ($R^2 = 0.30$) the cutoff value of 0.40 for inclusion of curve parameters in statistical analysis.
Figure 2.2. Representative graph of interaction between weaning exit velocity and endophyte treatment. This graph corresponds to the interaction observed for rectal mesor temperatures during Phase I. The three points used for reference are the slowest (0.73 m/s), average (1.67 m/s), and fastest (2.89 m/s) weaning exit velocities measured. The solid line represents E+ heifers and the dash line represents E- heifers. Differences between E+ and E- were only significant ($P = 0.06$) at the greatest value of weaning exit velocity.
Figure 2.3. Representative graph of interaction between weaning exit velocity and endophyte treatment. This graph corresponds to the interaction observed for vaginal temperature amplitude during Exp. 2, Phase IV. The three points used for reference are the slowest (0.73 m/s), average (1.67 m/s), and fastest (2.89 m/s) weaning exit velocities measured. The solid line represents E+ heifers and the dash line represents E- heifers. Differences between E+ and E- were only significant (P = 0.09) at the greatest value of weaning exit velocity.
Figure 2.4. Mean daily temperature profiles observed for each treatment group during Phase I of Exp. 1. This experiment examined only vaginal temperature profiles. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves per treatment per day (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.5. Mean daily temperature profiles observed for each treatment group during Phase II of Exp. 1. This experiment examined only vaginal temperature profiles. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment per day (n) are found at the top (E+) and bottom (E-) of the graph. Data for 10/19 and 10/23 were excluded from statistical analysis due to low n for the E- treatment (n=3, and n=2, respectively).
Figure 2.6. Mean temperature profiles observed for each treatment group during Phases III and IV of Exp. 1. This experiment examined only vaginal temperature profiles. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.7. Mean daily vaginal temperature profiles observed for each treatment group during Phase I of Exp. 2. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment per day (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.8. Mean daily rectal temperature profiles observed for each treatment group during Phase I of Exp. 2. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment per day (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.9. Mean daily vaginal temperature profiles observed for each treatment group during Phase II of Exp. 2. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment per day (n) are found at the top (E+) and bottom (E-) of the graph. Data for 7/19, 7/22 and 7/23 were excluded from statistical analysis due to low n for the E- treatment (n=3).
Figure 2.10. Mean daily rectal temperature profiles observed for each treatment group during Phase II of Exp. 2. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment per day (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.11. Mean vaginal temperature profiles observed for each treatment group during Phases III and IV of Exp. 2. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.12. Mean daily rectal temperature profiles observed for each treatment group during Phases III and IV of Exp. 2. Depiction of the light/dark cycle on the graph occurs with white areas corresponding to the light portions of the day and shaded areas corresponding to dark portions of the day. The number of curves used per treatment (n) are found at the top (E+) and bottom (E-) of the graph.
Figure 2.13. Effect of time on difference between rectal-vaginal temperature profiles observed for each treatment group during all four phases of Exp. 2. Different shades used to differentiate between each phase, with darker shades corresponding to either Phase I or III and lighter shades for Phase II or IV. The white area between Phases II and III is the time period when rectal probes were removed, meaning no differences between vaginal and rectal temperatures could be determined.
CHAPTER 3 – ALTERATIONS OF IMMUNE AND METABOLIC COMPONENTS DURING THE ACUTE PHASE RESPONSE

Introduction

Tall fescue (*Festuca arundinacea*) is the most commonly used cool season forage in the Southeastern United States due in part to its adaptability (Pendlum et al., 1980). Most tall fescue pastures are infected with an endophytic fungus (*Neotyphodium coenophialum*), which provides the plant with increased hardiness for better survivability (Latch, 1997). While this fungus has been shown to improve forage stands, its presence in feed can be detrimental to animal performance (Stuedemann and Hoveland, 1988). Fescue foot, bovine fat necrosis, and ‘summer slump/syndrome’ are disorders observed in cattle consuming endophyte-infected tall fescue diets (Schmidt and Osborn, 1993).

Several studies have examined the impact of endophyte-infected tall fescue on immunological responses. Simeone et al. (1998) and Saker et al. (1998) determined that presence of endophyte in feed can alter immune cell proliferation. Similarly, diets containing endophyte have been shown to suppress antibody response to tetanus vaccination (Dawe et al., 1997).

Despite evidence indicating a potential immunological compromise due to consumption of endophyte-infected feeds, published work examining the impact of endophyte on the acute phase response in cattle is limited. Filipov et al. (1999) used lipopolysaccharide (LPS) to determine the effects of endophyte presence in feed on the immunocompetence of cattle. Attributing cause and effect from that study is difficult because cattle grazed E+ vs. E- pastures for approximately 8 months prior to LPS challenge. Endophyte consumption has been shown to depress intake and growth.
(Schmidt et al., 1982), so it is unclear whether immunological responses were a direct consequence of endophytic alkaloids, or an indirect response mediated through lower nutritional plane. Thus, to ascertain whether endophyte toxicity has a direct effect on acute phase responses, a different experimental model is necessary. The objectives of the present experiments were to determine the influence of exposure to endophytic alkaloids, independent of effects on intake or growth rate, on the acute phase response of cattle.

**Materials and Methods**

All procedures used in these experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee.

**Animals and Experimental Design**

Two experiments were conducted in which 46 heifers were subjected to LPS challenges subsequent to consuming either an endophyte-infected (E+) or endophyte-free diet (E-). Heifers were halter-broken prior to the start of the experiments. On day 1 of each experiment, heifers were placed into 3.0 x 3.7 m individual stalls with ad libitum water supplied through permanently mounted waterers. Heifers were adapted to stalls for 11 days prior to the start of the treatment diets. Heifers were maintained in a thermoneutral environment at 22.3°C ± 1.3°C, which was verified through the use of room temperature loggers. A 14h:10h light:dark cycle was established with lights coming on at 0600h and turned off at 2000h daily. Administration of LPS occurred on d 22 at 1000h. Collection of blood samples occurred via jugular vein catheters, placed the day prior to challenge (d 21). Collection times started at 0800h, with samples taken every 30 min until 1800h. A final blood sample was obtained 24 h after the start of the challenge.
Sickness behavior scores (SBS) were recorded in conjunction with blood sample collection for the first 8 h following LPS administration.

**Experiment 1.** This experiment used twenty-two Angus heifers (292 ± 9.0 kg) and was conducted in two periods. The first period occurred in October 2012 and utilized 12 purchased heifers. The second period (December 2012) utilized 3 purchased heifers and 7 heifers born and raised at the University of Kentucky C. Oran Little Research Center (LRC). Non-purchased heifers were weaned 275 days prior to the start of period 2. Purchased heifers arrived on the research farm 26 days prior to the start of period 1. All heifers were vaccinated for respiratory diseases (Bovi-Shield Gold 5, Zoetis, Florham Park, NJ) and *Haemophilus Somnus* (Somubac, Zoetis, Florham Park, NJ) regardless of origin. Purchased heifers received vaccinations against clostridial diseases (Ultrabac 7, Zoetis, Florham Park, NJ) and *Manneheimia haemolytica type A1* (One Shot, Zoetis, Florham Park, NJ). Safeguard (Merck, Whitehouse Station, NJ) was applied as a deworming agent. Heifers raised at the LRC were vaccinated against clostridial diseases using Vision 7 (Merck, Whitehouse Station, NJ), *Manneheimia haemolytica type A1* using Vista Once SQ (Merck, Whitehouse Station, NJ), and pinkeye (Autogenous Pinkeye, MVP Laboratories, Omaha, NE). Within each period, half of the heifers were randomly assigned to one of the two treatment diets, with both groups balanced for weight. One heifer was treated for an infection occurring in the hind leg, and administration of medication ceased 1 d prior to heifers starting treatment diets.

**Experiment 2.** Sixteen Angus heifers (323 ± 34.5 kg; 301 ± 14 days old) and eight Hereford-Angus heifers (333 ± 19.5 kg; 310 ± 7 days old), all from the LRC, were stratified by sire breed, weight, temperament (based on weaning exit velocity), and
randomly assigned within strata to an endophyte-infected (E+) or endophyte-free (E-) diet. Heifers were weaned at 183 (± 13) days of age, 119 days prior to the start of the experiment. Weaning exit velocity was determined using an infrared laser “trip wire” system (Polaris 3845A-MI1043; Farmtek, Inc., Wylie, Texas) in which the heifers were released from the headgate, passed through the first laser (0.46 m from headgate) starting the timer, and then through the second laser (1.68 m from first) to stop the timer. All heifers were vaccinated for clostridial diseases (Ultrabac 7, boostered with One-Shot Ultra 7; Zoetis, Florham Park, NJ), Haemophilus Somnus (Somubac, Zoetis, Florham Park, NJ), respiratory diseases (Bovi-Shield, Zoetis, Florham Park, NJ) and pinkeye (Solidbac, Zoetis, Florham Park, NJ). Hair coat scores were determined by two trained observers and based on a 1 to 4 scale, with 1 being a slick coat and 4 being a winter coat. Scores were applied at the conclusion of the experiment. Caudal vein areas were obtained in both groups using ultrasound techniques before and after heifers were exposed to treatment diets following the methods of by Aiken et al. (2007).

**Treatment Diets**

Diets (Tables 3.1, 3.2, and 3.3), were fed at 1.8 x NEm requirements and balanced to meet protein and mineral requirements for 0.75 kg/d growth rates (NRC, 2000). Pretreatment diets (Table 1) were total mixed rations consisting of corn silage, soybean meal, and trace mineralized salt for both experiments. The treatment diets (Table 3.2 and Table 3.3) consisted of a cracked corn/cottonseed hull/fescue seed mix supplemented with soybean meal and trace mineral salt. Endophyte-infected fescue seed contained 15.1% CP, 28.8% NDF, and 2.29 ppm ergovaline + ergovalinine. Endophyte-free seed contained 15.1% CP, 30.6% NDF, and 0 ppm ergovaline + ergovalinine. The E+ diet was
fed to provide ergovaline + ergovalinine at a concentration of 10µg/kg BW. Heifers began receiving treatment diets on day 12 of each experiment. Heifers in experiment 2 were additionally fed 0.5 mg melengesterol acetate·hd⁻¹·day⁻¹ in a 0.23 kg supplement during both the pre-treatment and treatment periods in order to suppress estrous starting on day 1 of each experiment. Refused feed was collected, weighed, and recorded at 0700 each morning. Animals were fed at 0800 daily.

**Blood Sampling**

Heifers were fitted with jugular vein catheters (sterile Tygon tubing; US Plastics, Lima, OH, USA) on d 22 to allow for ease of blood collection and decreased stress response during the LPS challenge period. On d 23, 10 mL blood samples were collected into Sarstedt tubes (Sarstedt, Newton, NC, USA) with no additive starting at 0800 h (2 hours prior to LPS administration) and every half hour until 1800 h (8 h after LPS administration.) A final sample was collected 24 h after LPS was administered (1000 h, d 23). Prior to each blood sample, approximately 3 mL of waste (composed of heparinized saline in the intravenous line and a small amount of blood) was drawn from the catheter to ensure no heparinized saline was included in the sample. After each blood sample was taken, 10 mL of saline was given as a fluid replacement followed by 2.5 mL of heparinized saline to prevent clotting in the catheter. Lipopolysaccharide (LPS) was administered at a dosage of 5µg/kg BW. Blood samples sat at room temperature for 30 minutes before centrifugation at 4500 x g for 15 min at 4°C. Serum was harvested from the blood samples and then frozen at -80°C.

**Sample Analysis**
Treatment diets were analyzed for ergovaline and ergovalinine content using HPLC as described by Aiken et al. (2007).

Serum samples were analyzed in duplicate for concentrations of blood urea nitrogen (BUN; both experiments), β-hydroxybutyrate (BHB; Exp. 1 only), non-esterified fatty acid (NEFA; both experiments), glucose (both experiments), insulin (Exp. 1 only), creatinine (Exp. 1 only), cortisol (both experiments), tumor necrosis factor-alpha (TNF-α; both experiments), interleukin-6 (IL-6; both experiments), and interferon gamma (IFN-γ; both experiments).

The three cytokines (TNF-α, IL-6, and IFN-γ) were analyzed together using a multiplex sandwich ELISA (Searchlight® Bovine Cytokine 3 Array kit; Aushon Biosystems, Billerica, MD, USA) following the manufacturer’s instructions. Cytokine intra-assay coefficients of variation (CV) for experiment 1 and 2 were 5.19% and 3.47% (TNF-α), 5.70% and 2.77% (IFN-γ), and 4.23% and 7.03% (IL-6). Cytokine inter-assay CV for experiment 1 and 2 were 21.0% and 15.75% (TNF-α), 7.89% and 5.32% (IFN-γ), and 20.8% and 2.40% (IL-6). Limit of detection was 0.5, 0.1, 3.3 pg/mL for TNF-α, IFN-γ, and IL-6, respectively (Carroll et al., 2013).

Serum glucose concentrations were analyzed using the Autokit Glucose (Wako Diagnostics, Richmond, VA, USA) enzymatic assay with a modified protocol for fitting a 96-well plate. The protocol, briefly described, called for 2 µL of either serum or standard and 300 µL of prepared working solution to be added to each well. Each plate was incubated at 37°C for 5 minutes before reading at 505 nm using a plate reader (Biotek Powerwave 340; Biotek Instruments, Winooski, VT, USA). Sample absorbances were
compared with a standard curve to determine glucose concentrations. Intra- and inter-assay CV were 10.1% and 7.89% for Exp. 1, and 9.84% and 9.42% for Exp. 2, respectively. Limit of detection was 3.8 mg/dL (Sanchez et al., 2014).

Serum creatinine concentrations were determined using a colorimetric assay (Quantichrom™ Creatinine Assay Kit; Bioassay Systems, Hayward, CA, USA) following manufacturer’s instructions. Intra- and inter-assay CV for creatinine were 3.76% and 10.77%, respectively. Limit of detection was 0.1 mg/dL.

Serum BUN concentrations were determined using a Detect X Urea Nitrogen (BUN) Colorimetric Detection Kit (K024-H1; Arbor Assays, Ann Arbor, MI, USA). Following the manufacturer’s instructions, sample concentrations were compared to a standard curve comprised of known concentrations of BUN. Intra- and inter-assay CV for experiment 1 and 2 were 3.84% and 4.87% for Exp. 1, and 14.82% and 16.60%, for Exp. 2, respectively. Limit of detection was 0.065 mg/dL.

Serum insulin concentrations were analyzed using a sandwich type immunoassay (ALPCA Insulin (Bovine) ELISA; ALPCA Diagnostics, Salem, NH, USA) following the manufacturer’s instructions. Intra- and inter-assay CV for insulin were 4.88% and 11.47%, respectively. Limit of detection was 0.1 ng/mL.

A single-antibody radioimmunoassay (DSL-2100; Diagnostic Systems Labs, Webster, TX, USA) with rabbit anticortisol antiserum-coated tubes was used to determine serum cortisol concentrations following manufacturer’s instructions. Intra- and inter-assay CV for Exp. 1 were 9.35% and 5.40%, and 16.56% and 8.80% for Exp. 2, respectively. Limit of detection was 1.20 ng/mL (Sanchez et al., 2014)
Serum concentrations of BHB were analyzed using a colorimetric assay (K632-100; Biovision Incorporated, Milpitas, CA, USA) according to the manufacturer’s instructions. Intra- and inter-assay CV for BHB were 1.60% and 5.39%, respectively. Limit of detection was 0.01 mM (Burdick et al., 2014).

Samples were analyzed for serum NEFA concentrations using the enzymatic HR Series NEFA-HR assay (Wako Diagnostics) following the manufacturer’s instructions. Intra- and inter-assay CV for experiment 1 were 17.83% and 11.69%, and 16.57% and 13.38% for Exp. 2, respectively. Limit of detection was 0.0014 mM (Burdick et al., 2014).

Statistics

All data were analyzed as repeated measures using the Proc Mixed method of SAS (SAS, Inc., Cary, NC, USA). Variables which were non-normally distributed were determined to be log-distributed. These variables were log transformed prior to statistical analysis and included NEFA, IFN-ɤ, and TNF-α for both experiments, insulin, glucose, BUN, and BHB from Exp. 1, and IL-6, cortisol, and SBS from Exp. 2. For Exp. 1, there were two extraneous sources of variation that were accounted for in the statistical model using a single blocking factor. Variation due to period (2 periods) and heifer source (purchased or home-grown) were combined to create three blocks: block 1 encompassed period 1, purchased heifers (n = 12), block 2 was comprised of purchased heifers in period 2 (n = 3), and block 3 included the home-grown heifers from period 2 (n = 7). Because this was a preliminary experiment in which we were attempting to gain
perspective on the impact of different sources of variation, block was included in the model as a fixed effect, to permit estimation of block means. For Exp. 1, the model included block, endophyte treatment, sample time and the treatment x time interaction. For Exp. 2, the model included sire breed as a block effect, weaning exit velocity (wEV) as a covariate, endophyte treatment, sample time and the treatment x time interaction. For data from both experiments, the between/within method was used to estimate denominator degrees of freedom, the repeated term was sample time, subject was specified as individual animal, and the R-matrix covariance structure was specified as first-order autoregressive.

**Results**

The results are presented separately, by experiment, for pre- and post-LPS phases in Tables 3.4, 3.5, 3.6 and 3.7. Hair coat score and caudal vein data from Exp. 2 are in Table 3.8.

**Experiment 1**

*Cytokines and cortisol.* No differences due to block (P > 0.18), treatment (P > 0.25), or time x treatment (P > 0.73) were detected during the pre-LPS period for any of the immunological variables (Table 3.4). Differences existed among sampling times (P < 0.01) for all three cytokines and cortisol during both the pre- and post-LPS periods (Table 3.5). Block (P = 0.01), as well as time x treatment interaction (P < 0.01) effects were detected for TNF-α (Fig. 3.1) in the post-LPS period. TNF-α concentrations for both treatments increased until 30 minutes after endotoxin administration, then decreased until 8 h after challenge. TNF-α concentrations for E+ heifers returned to baseline levels by 24
hours post-LPS, whereas E- heifer TNF-α levels remained slightly elevated at 24 hours relative to baseline concentrations. Endophyte-free treated heifers had greater TNF-α concentrations at 6.5, 7.5, and 24 h post-LPS than E+ heifers (P ≤ 0.05). IFN-γ and IL-6 concentrations increased for both treatments during the post-LPS period until 4 hours after challenge (P < 0.01), with levels similar to baseline observed by 24 h post-LPS (Fig. 3.2 and 3.3, respectively). A block effect (P < 0.01) was detected for cortisol during the post-LPS period. Cortisol concentrations (Fig. 3.4) increased for both treatments until 5 hours post-LPS and returned to baseline by 24 hours. No treatment (P ≥ 0.17) differences were observed for any of the cytokines in the post-LPS period.

Metabolites. No time x treatment interactions were detected for any of the metabolites or insulin during the pre- or post-LPS periods (Fig. 3.5-3.11). A block effect was observed for insulin during the pre- (P < 0.01), but not the post- (P = 0.22), LPS period. During the post-LPS period, glucose (P = 0.05) was lower in E+ vs. E- heifers. Non-esterified fatty acid concentrations (P = 0.01) were greater for E+ compared to E- heifers during the pre-LPS period, but no difference was detected during the post-LPS period (P = 0.94). Creatinine was also higher in E+ heifers (P < 0.01) during the post-LPS period. Creatinine and BHB decreased and BUN increased (P ≤ 0.10) from -2 h to 0 h during the pre-LPS period. Similarly, all metabolite concentrations were observed to differ due to time (P < 0.01) during the post-LPS period. Insulin, glucose, and NEFA increased for E+ and E- heifers until 2 hours post-LPS. In both treatment groups, insulin returned to baseline levels at 4 hours post-LPS, whereas glucose and NEFA levels dropped below baseline concentrations. NEFA concentrations returned to pre-LPS levels at 4 hours, whereas glucose concentrations remained below baseline until 24 hours. Serum BUN
concentrations fluctuated around baseline throughout the trial period, with no differences observed between treatments. Creatinine and BHB concentrations decreased in both treatment groups until 2 hours post-LPS. Creatinine levels continued to decrease throughout the trial period whereas BHB concentrations returned to levels similar to baseline around 8 hours for E+ and E- heifers.

Experiment 2

In experiment 2, time was observed to impact concentrations for all variables measured during both pre- and post-LPS periods with the exception of IL-6 during the pre-LPS period (Table 3.6 and 3.7).

Cytokines. Sire and wEV x endophyte treatment effects were not detected for any of the cytokines or cortisol (P ≥ 0.24). All cytokine data was log transformed in order to eliminate skewness and normalize data for ANOVA.

TNF-α was not detected in any of the pre-LPS samples (Fig. 3.12). TNF-α concentrations for both treatments increased until 30 minutes after LPS administration, then decreased around 4 hours post-LPS to levels similar to those observed at 0 h. No differences were detected for sire (P = 0.40), wEV (P = 0.75), time x treatment (P = 0.40), or treatment (P = 0.85) during the post-LPS period.

IFN-γ (Fig. 3.13) was not observed to vary between treatments during the pre- (P = 0.63) or post-LPS (P = 0.35) periods. There was a change in IFN-γ concentrations for both treatment groups post-LPS (P = 0.09), with both treatments increasing until 4 hours after LPS administration, then returning to baseline by 24 hours. Weaning exit velocity
had a significant (P = 0.02) effect on IFN-γ, with a 1.47 pg/mL decrease for every 1 m/s increase in exit velocity.

IL-6 concentrations (Fig. 3.14) did not vary between E+ and E- treatments during the pre-LPS period (P ≥ 0.80), but were higher (P = 0.02) in E+ compared to E- heifers (4786 vs. 3521 ± 342 pg/mL, respectively) during the acute phase response. Concentrations of IL-6 for both treatments increased until 5 hours post-LPS and returned to baseline by 24 hours. No sire or wEV differences were detected for either period (P ≥ 0.27).

Cortisol concentrations (Fig. 3.15) decreased slightly across the pre-LPS phase (P < 0.01) and increased post-LPS (P < 0.01) until 1.5 hours after LPS was given, returning to baseline levels by 24 hours. No differences (P ≥ 0.23) between treatment groups or sire breeds, or related to wEV were observed for either LPS period.

Metabolites. All metabolites increased during the post-LPS period. Glucose (Fig. 3.16) concentrations, for both treatments, peaked at 1 h post-LPS before decreasing below baseline levels, with nadir reached at 8 h. Glucose concentrations returned to levels similar to baseline by 24 hours. This response of glucose to LPS administration is consistent with previous studies (Spitzer et al., 1989. NEFA (Fig. 3.17) concentrations decreased from -2 h to 0 h during the pre-LPS period. After LPS administration, NEFA concentrations increased during the first 30 minutes, then decreased until 2 h post-LPS, before slowly increasing until 24 hours, at which point both treatment groups were detected to have elevated NEFA levels relative to baseline.
Concentrations of BUN (Fig. 3.18) increased until 2 hours post-LPS, with E+ heifers still elevated above baseline values at 24 hours. NEFA values were log transformed to normalize data distribution. No differences were observed between E+ and E- heifers pre-LPS for glucose (P = 0.24), NEFA (P = 0.70), or BUN (P = 0.70) concentrations. Similarly, no differences were detected post-LPS for glucose (P = 0.52). NEFA was found to have a treatment x time interaction (P = 0.04) during the post-LPS period, with heifers on the E+ diet having greater NEFA concentrations at 1, 3, 4.5, 5.5, 6.5, 7, 7.5, and 8 h than E- treated heifers. Post-LPS BUN (P = 0.06) concentrations were detected to have a wEV x treatment interaction (P = 0.04; Fig. 3.19). Concentrations of BUN increased with increasing exit velocity for E- treated heifers, whereas increasing exit velocity resulted in decreased BUN concentrations for E+ treated heifers.

**Sickness Behavior.** Sickness behavior scores were not recorded prior to administration of LPS and there were no visual signs of distress or sickness during that period. Post-LPS sickness behavior scores increased for both groups (P<0.01; Fig. 3.20) until 1.0 hours after LPS administration. Sickness behavior scores returned to baseline at 4.0 hours post-LPS. No differences were detected between E+ and E- treatments. Similarly, no effects due to sire, wEV, wEV x treatment, or treatment x time (P ≥ 0.69) were observed.

**Caudal Vein and Hair Coat Score.** E+ heifers experienced an overall greater change in caudal vein diameter (Table 3.8), with an observed decrease as opposed to the increase observed in E- heifers (P< 0.01). A difference between sire breeds (P = 0.07) was also observed, with a greater decrease occurring in purebred heifers. No differences in hair coat scores were detected due to treatment (P = 0.28) or sire breed (P = 0.91).
Discussion

The purpose of this study was to develop a model for studying the relationship between endophyte presence in the diet and immunological responses of cattle. Similar studies utilizing different models (Filipov et al., 1999; Filipov et al., 2000) provided useful insights into potential effects of endophyte exposure on the bovine immune response during an LPS challenge. However, those two studies obtained somewhat contradictory results. With summer-long grazing of E+ and E- pastures, endophyte presence stimulated a pro-inflammatory response (Filipov et al., 1999), whereas with bolus injection of ergotamine, alkaloid exposure inhibited the inflammatory response (Filipov et al., 2000). Neither of those studies demonstrated a conclusive link between endophytic alkaloid exposure and acute phase responses. The first study was confounded with potential differences in intake (both quality and quantity) between treatment groups, which may have contributed to some of the observed differences attributed to endophyte exposure. Maintaining plasma glucose levels reduces the effect of increasing stress hormone levels and changes in immunity (Venkatraman and Pendergast, 2002). Glucose also serves as an important energy source for macrophages (Bishop et al., 1999), which are important for production of pro-inflammatory cytokines such as IL-6 and TNF-α (Murphy, 2011). Differences in dietary fat consumption may also alter the immune system, as incorporation of high fat diets with exercise has been observed to decrease IL-6 concentrations in humans (Venkatraman and Pendergast, 2001). The second study by Filipov et al. (2000) used a bolus injection of a non-endophytic alkaloid, which may or may not similarly impact immune function when compared with endophytic alkaloids.
Results from Exp. 1 indicated only minor endophyte effects prior to LPS. Only NEFA were altered by endophyte, with about a 0.02 mM depression in serum NEFA concentrations with E+ as compared with E-. No other response variables were influenced (P > 0.10) by endophyte prior to the LPS challenge. During the acute phase response, serum glucose concentrations were depressed by about 8 mg/dL with E+, whereas creatinine concentrations were slightly increased (by 0.09 mg/dL) with E+ compared with E-. Additionally, effects of endophyte on TNF-α varied across time, with differences detected in baseline data prior and subsequent to the acute phase response, but with very little difference between E+ and E- during the response period. If the differences in baseline levels of TNF-α were a consequence of endophyte exposure, the finding that peak TNF-α was unaffected is not unprecedented., Adams et al. (1996) found that overall cytokine response to an immune challenge did not differ between chronically ill and uninfected, healthy cattle. However, it is likely that the small differences in baseline TNF-α in this experiment were artifacts, particularly given results from Exp. 2, discussed below.

Possible extraneous sources of variation not fully accounted for in the methodology of Exp. 1 may have influenced the detection of endophyte effects. Analysis indicated significant block effects (heifer source and/or period) for some of the response variables which could be at least partially eliminated by conducting such experiments in a single period, and minimizing differences in previous background of experimental animals. Additionally, slight differences in dietary protein (E+ vs. E-, 13.1 vs 14.0% DM) could have contributed to responses, particularly BUN, that were attributed to endophyte treatment. Furthermore, dissimilarity in disposition among the heifers suggested that
differences in temperament among animals may have added additional variability. Literature examining temperament effects on metabolic parameters in cattle is limited. However, certain trends appear consistent in the few available published studies. Increasing cortisol levels are known to cause an increase in blood glucose (Munck and Koritz, 1962) and NEFA (Samra et al., 1996) levels. Fell et al. (1999) demonstrated correlations between flight time and relative cortisol levels. Similarly, Hulbert et al. (2011) found cortisol and glucose levels greater in temperamental, compared with more docile bulls. As flight responses were not measured in Exp.1, differences may have confounded treatment responses. Weaning exit velocity, as a measure of temperament, was incorporated as a covariate in Exp. 2.

Estrus impacts the state of energy balance experienced by the animal, with glucose removal from plasma occurring more quickly during estrus than anestrus (Richards et al., 1989). Potential differences in timing of estrus may have also influenced vaginal temperature (reported separately in Altman et al., 2016). In order to account for this possible source of variation among the heifers, melengesterol acetate (MGA) (Brown et al., 1988) was added to the diet during experiment 2.

In Exp. 2, the primary physiological indicator of endophyte effects was the influence of the E+ diet on change in caudal vein area. By 8 d after initiation of endophyte treatments, heifers receiving E+ had experienced a decrease in caudal vein area of 2.4 mm², whereas caudal vein diameter in E- heifers had increased by 1.7 mm². This difference of 4.1 mm² was comparable to differences in caudal vein area observed by Aiken et al. (2009), who examined caudal vein areas of heifers placed on one of three diets to provide ergovaline at 0, 0.39, and 0.79 µg/g diet (compared with 0.55 µg/g diet
provided in the present study.) The alkaloid dose in the present study was determined with the intent of providing ergovaline at levels similar to those that would be consumed by animals grazing endophyte infected fescue. It was anticipated that many of the classical signs of fescue toxicosis would not be evident at this dose level, particularly at thermoneutral conditions (Aldrich et al., 1993). Thus, the presence of this vasoconstrictive effect is an important physiological indicator of the presence of alkaloid effects in this study.

Treatment effects on metabolites in Exp. 2 were limited to NEFA (time x treatment P = 0.04) and BUN (wEV x treatment P = 0.04) concentrations subsequent to LPS injection. Additionally, IL-6 was generally greater (time x treatment P < 0.01) with E+ during the acute phase response. The time x treatment interaction for NEFA largely occurred late in the acute phase response. The observed response pattern of rapid increase from 0.5 to 1 h, rapid decrease from 1 to 2 h, and gradual increase until 24 h for NEFA concentrations subsequent to LPS treatment in the current study with both treatments was similar to that observed in other studies (Bernhard et al., 2012; Burdick Sanchez et al., 2014). Pedersen et al. (2003) indicated that IL-6 is a potent stimulus for lipolysis. Interleukin-6 is, among other things, the chief stimulator of the acute phase response. The corresponding time courses of IL-6 and NEFA responses (Fig. 3.14 and 3.17, respectively) in the present study are consistent with IL-6 as a mediator of the NEFA response. Potential mechanisms relating endophyte exposure to increases in IL-6 are unknown, but these data do support a pro-inflammatory role of endophytic alkaloids. Results of the present study, however, suggest a much more limited proinflammatory role of endophytic alkaloids as compared with the prior studies which either confounded
direct effects with long term effects on intake and growth (Filipov et al., 1999) or used large bolus doses of ergotamine as a model compound (Filipov et al., 2000).

It has been shown that the acute phase inflammatory response results in accelerated muscle protein degradation and increased hepatic acute phase protein synthesis, with at least 60% of the amino acids used for synthesis derived from body protein degradation (Johnson, 1997). Interestingly, endophyte exposure during Exp. 2 appeared to alter a relationship between protein metabolism and wEV. Blood urea nitrogen concentrations in the E- heifers were positively correlated with increases in exit velocity. This pattern was reversed with heifers on the E+ treatment, with increasing flight responses associated with decreased BUN concentrations (Fig. 3.20). The mechanism underlying the interaction between endophyte exposure and temperament is unclear, but these results indicate that metabolic responses associated with the acute phase are responsive to a complex regulatory pathway that can be influenced by diverse stimuli.

Lipopolysaccharide immune responses are initiated through toll-like receptor 4. The binding of LPS by this receptor triggers a signaling cascade, which can result in the activation of pro-inflammatory cytokine genes by transcription factor NFkB in the nucleus of macrophages (Akira and Takeda, 2004; Brown, 2008). Interferon-γ functions as a part of the immune system through control of infections in the early stages of immune response via release of nitric oxide (NO), an effective anti-microbial and anti-tumor agent. During the acute phase response, infected macrophages produce IL-12 and IL-18, cytokines used in natural killer cell activation, which proceed to produce IFN-γ (Murphy, 2011). Relating measurements of temperament in cattle to immunological
responses is still a novel concept with limited research available. In the present study, increases in wEV were associated with decreased IFN-γ production during the post-LPS period. A similar observation has previously been reported in human trials (MacMurray et al., 2014). In that study, patients were categorized by personality and temperament as one of four types: harm avoidance, novelty seeking, reward dependence, and persistence. Harm avoidance, which the authors described as a tendency to respond to aversive stimuli by inhibiting behavior, correlated with expression of the low IFN-γ producing allele.

**Conclusion**

The primary goal of this study was to evaluate the immunological impact of endophyte-infected tall fescue on cattle. Controlling alkaloid consumption and eliminating differences in dry matter intake through incorporation of respective alkaloid doses in total mixed rations with fescue seed, which were fed at restricted levels, resulted in a greater insight into the relationship between alkaloid consumption and subsequent cytokine and metabolite responses during the acute phase response compared with previous studies. Specifically, in the present study, NEFA and IL-6 responses were similar by treatment across time, indicating a relationship between the two during immunological challenge. Blood urea nitrogen was influenced by both flight response and endophyte treatment, with endophyte consumption suppressing BUN concentrations at higher exit velocities. Caudal vein area measurements were an important marker for confirmation of alkaloid effects, particularly given the sub-pharmacological doses that were evaluated in these experiments. Additionally, we determined that many of the important cytokine and metabolic responses are affected by genetics, temperament, and other factors that need to be considered in the experimental design. Others (Hannah et al.,
1990; Aldrich et al., 1993) have shown effects of fescue toxicosis to be exacerbated at elevated temperatures, indicating administration of LPS at thermoneutral temperatures may mask some of the potential treatment effects. Thus, future experiments evaluating the APR at elevated ambient temperatures may reveal different responses than these, which were obtained under thermoneutral conditions.
Table 3.1. Ingredient composition of the pre-treatment diets by experiment

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Silage (40% grain)</td>
<td>89.7</td>
<td>86.0</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>9.6</td>
<td>9.2</td>
</tr>
<tr>
<td>Trace Mineral Salta</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>MGA Carrier (Cracked Corn and Wheat Middlings)b</td>
<td>0.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

\(^a\)Trace mineralized salt included 92.9% salt, 68 ppm Co, 1838 ppm Cu, 120 ppm I, 9290 ppm Mn, 19 ppm Se, and 5520 ppm Zn.

\(^b\)To provide MGA at 0.5 mg·hd\(^{-1}\)·day\(^{-1}\)
Table 3.2. Ingredient composition of treatment diets (Exp. 1 and 2)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cracked Corn</td>
<td>36.0</td>
<td>34.6</td>
</tr>
<tr>
<td>Cottonseed Hulls</td>
<td>30.0</td>
<td>28.8</td>
</tr>
<tr>
<td>Fescue Seed</td>
<td>20.0</td>
<td>19.2</td>
</tr>
<tr>
<td>Supplement&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Molasses</td>
<td>4.0</td>
<td>3.8</td>
</tr>
<tr>
<td>MGA Carrier (Cracked Corn and Wheat Middlings)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Supplement included trace mineralized salt, vitamin premix, limestone, and molasses. Trace mineralized salt provided 92.9% salt, 68 ppm Co, 1838 ppm Cu, 120 ppm I, 9290 ppm Mn, 19 ppm Se, and 5520 ppm Zn. Vitamin premix supplied 1820 IU/kg Vitamin A, 363 IU/kg Vitamin D, and 227 IU/kg Vitamin E.

<sup>b</sup>To provide MGA at 0.5 mg·hd<sup>-1</sup>·day<sup>-1</sup>
Table 3.3. Chemical composition of the treatment diets by experiment

<table>
<thead>
<tr>
<th>Component</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Percent of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEm (Mcal/kg DM)</td>
<td>E+</td>
<td>E-</td>
<td>E+</td>
</tr>
<tr>
<td>1.65</td>
<td>1.65</td>
<td>1.67</td>
<td>1.67</td>
</tr>
<tr>
<td>CP</td>
<td>12.9</td>
<td>13.3</td>
<td>15.5</td>
</tr>
<tr>
<td>NDF</td>
<td>34.3</td>
<td>35.4</td>
<td>32.4</td>
</tr>
<tr>
<td>ADF</td>
<td>26.2</td>
<td>27.1</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21.7</td>
</tr>
</tbody>
</table>
Table 3.4. Effects of endophyte exposure on cytokines, hormones, and metabolites prior to LPS (0.5µg/kg BW) administration (Exp. 1).

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary Treatment</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>ln TNF-α (pg/mL)</td>
<td>-0.26</td>
<td>0.87</td>
</tr>
<tr>
<td>ln IFN-γ (pg/mL)</td>
<td>1.81</td>
<td>2.13</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>55.63</td>
<td>57.98</td>
</tr>
<tr>
<td>Cortisol (ng/mL)</td>
<td>8.35</td>
<td>8.84</td>
</tr>
<tr>
<td>ln Insulin (ng/mL)</td>
<td>-0.69</td>
<td>-0.60</td>
</tr>
<tr>
<td>ln Glc (mg/dL)</td>
<td>4.39</td>
<td>4.41</td>
</tr>
<tr>
<td>ln NEFA (mM)</td>
<td>-2.48</td>
<td>-2.29</td>
</tr>
<tr>
<td>ln BHB (mM)</td>
<td>0.23</td>
<td>0.28</td>
</tr>
<tr>
<td>ln BUN (mg/dL)</td>
<td>0.11</td>
<td>-0.004</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.37</td>
<td>1.32</td>
</tr>
</tbody>
</table>
Table 3.5. Effects of endophyte exposure on cytokines, hormones, and metabolites subsequent to LPS (0.5µg/kg BW) administration (Exp. 1).

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary Treatment</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>ln TNF-α (pg/mL)</td>
<td>4.07</td>
<td>4.28</td>
</tr>
<tr>
<td>ln IFN-γ (pg/mL)</td>
<td>3.85</td>
<td>4.01</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>7144</td>
<td>8044</td>
</tr>
<tr>
<td>Cortisol (ng/mL)</td>
<td>60.26</td>
<td>56.42</td>
</tr>
<tr>
<td>ln Insulin (ng/mL)</td>
<td>0.07</td>
<td>0.26</td>
</tr>
<tr>
<td>ln Glc (mg/dL)</td>
<td>4.14</td>
<td>4.26</td>
</tr>
<tr>
<td>ln NEFA (mM)</td>
<td>-2.20</td>
<td>-2.21</td>
</tr>
<tr>
<td>ln BHB (mM)</td>
<td>-0.003</td>
<td>0.11</td>
</tr>
<tr>
<td>ln BUN (mg/dL)</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.24</td>
<td>1.15</td>
</tr>
<tr>
<td>SBS</td>
<td>1.32</td>
<td>1.19</td>
</tr>
</tbody>
</table>
Table 3.6. Effects of endophyte exposure on cytokines, hormones, and metabolites prior to LPS (0.5µg/kg BW) administration (Exp. 2).

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary Treatment</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>lnIFN-γ (pg/mL)</td>
<td>-1.75</td>
<td>-1.18</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>90.4</td>
<td>76.9</td>
</tr>
<tr>
<td>lnCort (ng/mL)</td>
<td>2.33</td>
<td>2.13</td>
</tr>
<tr>
<td>GLC (mg/dL)</td>
<td>82.76</td>
<td>85.80</td>
</tr>
<tr>
<td>lnNEFA (mM)</td>
<td>-2.69</td>
<td>-2.69</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>9.46</td>
<td>9.56</td>
</tr>
</tbody>
</table>
Table 3.7. Effects of endophyte exposure on cytokines, hormones, and metabolites subsequent to LPS (0.5µg/kg BW) administration (Exp. 2).

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary Treatment</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>lnTNF-α (pg/mL)</td>
<td>0.03</td>
<td>-0.03</td>
</tr>
<tr>
<td>lnIFN-γ (pg/mL)</td>
<td>1.41</td>
<td>1.84</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>4786</td>
<td>3521</td>
</tr>
<tr>
<td>lnCort (ng/mL)</td>
<td>3.58</td>
<td>3.63</td>
</tr>
<tr>
<td>GLC (mg/dL)</td>
<td>69.03</td>
<td>70.37</td>
</tr>
<tr>
<td>lnNEFA (mM)</td>
<td>-2.56</td>
<td>-2.81</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>9.33</td>
<td>9.46</td>
</tr>
<tr>
<td>lnSBS</td>
<td>0.17</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Table 3.8. Effects of endophyte exposure on Exp. 2 hair coat scores and change in caudal vein diameters.

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary Treatment</th>
<th>Probability of a greater F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>Hair Coat</td>
<td>3.32</td>
<td>2.74</td>
</tr>
<tr>
<td>Change in Caudal Vein Area (mm²)</td>
<td>-2.42</td>
<td>1.75</td>
</tr>
</tbody>
</table>
Figure 3.1. Concentrations of tumor necrosis factor-α during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with differences in concentrations (P < 0.10) at a given time point denoted with an “*”. 
Figure 3.2. Concentrations of interferon-γ during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.3. Concentrations of interleukin-6 during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.4. Concentrations of cortisol during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with significance set at $P < 0.10$. 
Figure 3.5. Concentrations of insulin during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.6. Concentrations of glucose during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.7. Concentrations of non-esterified fatty acid during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.8. Concentrations of β-hydroxybutyrate during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.9. Concentrations of blood urea nitrogen during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.10. Concentrations of creatinine during pre- and post-LPS periods of Exp. 1, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.11. Sickness behavior scores for the post-LPS period of Exp. 1, with i.v LPS administration occurring at 0 h. Scores were obtained in 0.5h intervals from -2h to 8h. Data is presented as LSM ± SEM with significance set at P < 0.10. Sickness behavior scores were not obtained prior to LPS administration.
Figure 3.12. Concentrations of tumor necrosis factor-α during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.13. Concentrations of interferon-γ during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Concentrations were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.14. Concentrations of interleukin-6 during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with differences in concentrations (P < 0.10) at a given time point denoted with an “*”. 
Figure 3.15. Concentrations of cortisol during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with significance set at $P < 0.10$. 

![Graph showing cortisol concentrations](image-url)
Figure 3.16. Concentrations of glucose during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with significance set at P < 0.10.
Figure 3.17. Concentrations of non-esterified fatty acid during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with differences in concentrations (P < 0.10) at a given time point denoted with an “*”.
Figure 3.18. Concentrations of blood urea nitrogen during pre- and post-LPS periods of Exp. 2, with i.v LPS administration occurring at 0 h. Blood samples were collected in 0.5h intervals from -2h to 8h, with a final sample collected at 24 h. Data is presented as LSM ± SEM with significance set at P < 0.10.
Fig. 3.19. Graph of interaction between weaning exit velocity and endophyte treatment. This graph corresponds to the interaction observed for Post-LPS concentrations of BUN during Exp. 2, Phase IV. The three points used for reference are the slowest (0.73 m/s), average (1.67 m/s), and fastest (2.89 m/s) weaning exit velocities measured. The solid line represents E+ heifers and the dash line represents E- heifers. Differences between E+ and E- were significant (P = 0.09) at the greatest and lowest values of weaning exit velocity.
Figure 3.20. Sickness behavior scores for the post-LPS period of Exp. 2, with i.v LPS administration occurring at 0 h. Scores were applied in 0.5h intervals from -2h to 8h. Scores were log transformed to normalize distribution. Data is presented as LSM ± SEM with significance set at P < 0.10. Sickness behavior scores were not applied prior to LPS administration.
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