

Assessment of redox markers in cattle

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ABSTRACT: Metabolic redox status may have important implications to cattle health and production. Antioxidants and biomarkers of oxidative stress were evaluated in cattle under three phases of management. Each phase stood alone as a treatment model, and managerial aspects during the phase were evaluated as potential moderators of redox balance. Yearling heifers were used to assess the impact of fescue toxicosis and heat stress on selected markers in study 1. Intravaginal temperatures, ADG, serum prolactin, plasma malondialdehyde, and whole blood Se, along with peripheral blood mononuclear cell glutathione peroxidase, glutathione reductase, and reduced:oxidized glutathione were determined during summer grazing. Results suggested that endophyte consumption does not promote oxidative stress in cattle. Heat stress may alter glutathione redox of white blood cells. In study 2, effects of gradual weaning strategies (anti-suckle nose clip and fenceline wean) and transport were evaluated in calves. Calf weights, Se and malondialdehyde in plasma, along with glutathione peroxidase and glutathione reductase in leukocytes were measured at -7, 0, 1, and 7 days surrounding weaning and transport. Little benefit of gradual weaning was detected, and oxidative stress may have been negligible. In study 3, the influences of grain- and forage-based diets were compared in finishing steers pre- and post-harvest. Total antioxidant capacity and malondialdehyde concentration of plasma, along with serum α -tocopherol, β -carotene, and γ -tocopherol

were measured. Antioxidants and lipid oxidation were assessed in beef. Forages promoting plasma antioxidant capacity may protect cattle against oxidative stress. Antioxidants derived from forages inhibit lipid oxidation in pasture-finished beef.

DEDICATION

I dedicate this work to the memory of my grandfather, Rudolph S. Smith. His attentive instruction instilled within me a passion for agriculture and cattle that will be retained always. His lessons continue to extend beyond the field, into sundry aspects of my life. I

thank God for the gift he was in my life.

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CHAPTER I

INTRODUCTION

Interaction of prooxidants and antioxidants in living systems determines biological redox state. Shifts in redox balance have important implications for animal health and function. Excessive prooxidant species or inadequate antioxidant defenses lead to oxidative stress, a condition that is practically interpreted with reference to resultant functional impairment or onset of disease. Research relating redox status to health and production in cattle is wide-ranging, yet not consolidated into a single literature review. The present thesis compiles such work and furthers the understanding of redox status in cattle by assessing commonly used redox markers in three models significant to beef cattle production in Virginia: endophyte-infected fescue toxicosis, weaning and transport stress, and forage-finished beef.

In study 1, yearling heifers were allotted to endophyte-infected and endophyte-free tall fescue (*Festuca arundinacea* Schreb.) paddocks during periods of elevated temperature-humidity indices. Intravaginal temperature recordings and serum prolactin concentrations were used to confirm physiological impacts of heat stress and endophyte consumption. Markers of glutathione redox balance in peripheral blood mononuclear cells were assessed, blood Se status was compared, and concentrations of lipid peroxidation products in plasma were determined.

Effects of management strategies involving gradual, two-stage weaning were evaluated in study 2. Steers were used to compare anti-suckle nose clips and fenceline weaning techniques to traditional, abrupt weaning. Transport effects on biomarkers were also evaluated when calves were completely separated from dams and shipped to another

location. Lipid peroxidation products along with Se concentrations were determined in plasma. Activities of the antioxidant enzymes glutathione peroxidase and glutathione reductase were assessed in isolated leukocytes, and calf body weights were recorded.

The third study in the current thesis describes effects of forage-finishing systems on antioxidant status and post-mortem lipid oxidation. Prior to harvest, antioxidant markers in steers finished on a traditional grain-based diet were compared to markers in steers finished on pasture systems with various forage bases. Upon harvest, tissue samples from a sub-set of the steers were ground into burger, and lipid oxidation was determined. Correlations between pre- and post-harvest markers were estimated.

CHAPTER II
REVIEW OF LITERATURE
INTRODUCTION

Precipitation, acid-base, and oxidation-reduction are the three most general classes of chemical reactions. Of these, oxidation-reduction, or redox, reactions are prominent, and perhaps most important (Silberg, 2006). Oxidation of a chemical species involves loss of electrons, whereas reduction entails accrual of electrons. Increases and decreases in oxidation numbers of atoms involved in electron transfers arise due to oxidations, and reductions, respectively. The scientific community has recently given a great amount of attention to redox reactions in biological systems. Indeed, omnipresence of specific oxidizing species (prooxidants) and reducing agents (antioxidants) in diverse natural settings highlights their universal importance. Exposure to biologically generated free radicals and reactive oxygen species (**ROS**) are natural occurrences in living organisms. Likewise, coordination of endogenously derived and exogenously obtained antioxidant defense mechanisms in organisms is accepted as being normal. Under ordinary circumstances, prooxidants and antioxidants delicately balance each other, yet when equilibrium is upset, deleterious effects are bound to ensue. Recognizing involvement of ROS in the pathogenesis of a multitude of diseases, biomedical scientists have devoted considerable amounts of research to understanding the phenomenon of oxidative stress (**OS**). Although OS has been previously defined as “a disturbance in the prooxidant-antioxidant balance in favor of the former”, a more useful description might further this interpretation and highlight the importance of tissue damage, or disease processes brought about by such “stress” (Sies, 1991). Extensive reviews covering

oxidant-related stress have been previously conducted (Sies, 1991; Sies and Groot, 1992; Young and Woodside, 2001; Kohen and Nyska, 2002), and comparatively smaller compilations are available for species and situation specific examples of OS. The intent of this literature review is to describe information pertaining specifically to redox in cattle.

IN VIVO REDOX BALANCE AND OXIDATIVE STRESS

Based on the above definition, there are three aspects of OS to consider: 1) free radicals and ROS, 2) antioxidant defense mechanisms, and 3) tissues liable to oxidant-induced damage. Extensive descriptions of these aspects of OS are beyond the scope of this review, but a brief overview is essential for proper understanding of the literature to be later described.

Bi-radical oxygen, along with the superoxide anion, hydroxyl ion, peroxy species, alkoxy species and nitric oxide are the most frequently encountered oxygen based radicals, whereas hydrogen peroxide, organic peroxides, hypochlorous acid, ozone, singlet oxygen, peroxy nitrite and various aldehydes are common non-radical reactive oxygen derivatives (Kohen and Nyska, 2002). For the purpose of simplification, all such species will be referred to within this document as ROS. Additionally, all transition metals in the first row of d-block of the periodic table are extant as elements containing unpaired electrons. For this reason, their valence state is subject to change with the transfer of electrons, and they too can act as prooxidants and generators of ROS (Young and Woodside, 2001).

Both exogenous and endogenously derived ROS contribute to the total load imposed upon antioxidant defenses. Externally derived sources of ROS consist of those

resulting from exposure to molecular oxygen itself, irradiation, pollution, ultraviolet light, wide ranges of xenobiotics, and many kinds of foods. The most significant supply of ROS however, comes from within the body. Mitochondrial respiration, enzymes producing ROS (either directly or as by-products), and respiratory burst of white blood cells all contribute to the endogenous pool of ROS (Kohen and Nyska, 2002; Wallace, 2005; Misra, 2006).

Antioxidant defense mechanisms can best be understood when broken down into two parts: antioxidant enzymes; and low-molecular-weight-antioxidants. Antioxidant enzymes of significance are superoxide dismutase (**SOD**), catalase, glutathione peroxidase (**GSH-Px**) and glutathione reductase (**GR**). Superoxide dismutase (of various forms) catalyzes dismutation of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide and molecular oxygen. In turn, catalase and GSH-Px can break hydrogen peroxide and organic hydroperoxides (GSH-Px) down into water, oxygen, and alcohols. Glutathione reductase works in conjunction with GSH-Px and regenerates the reduced form of low-molecular-weight-thiol, glutathione (**GSH**) (Griffith, 1999). Trace minerals such as Fe (in catalase), Cu, Zn, Mn (in SOD) and Se (in GSH-Px) are essential to the structure and function of many of these antioxidant enzymes (Kleczkowski et al., 2004).

Low-molecular-weight-antioxidants, also commonly referred to as chain-breaking antioxidants, work as scavengers by directly interacting with ROS and free radicals. Common water soluble chain-breaking antioxidants include GSH, albumin, bilirubin, uric acid, ascorbic acid, and thioredoxin, whereas tocopherols, tocotrienols, carotenoids and ubiquinol make up the group of familiar lipid associated antioxidants. Many of these antioxidants are of dietary origin. Similarly, over 4,000 polyphenolic flavonoids such as

catechins, flavones, and isoflavones, have been identified as natural antioxidants in foods (Young and Woodside, 2001).

Although basal levels of ROS have some essential roles within the body, it is their surplus that is associated with degenerative processes and disease. Superfluous oxidizing species result from excessive generation, direct exposure to oxidizing agents, or inadequacy of antioxidant defenses. Either way, pathogenic effects of ROS can generally be traced back to tissue damage. Concern over ROS attack usually involves three major biological polymers: lipids, proteins, and nucleic acids. Consequently, OS research often centers on identification of by-products of damaged polymers in the body. For example, malondialdehyde (**MDA**), often assessed as thiobarbituric acid reactive substances (**TBARS**), is an end-product of lipid peroxidation that is commonly measured.

Another trend in antioxidant and OS research involves the development of assays attempting to discern total antioxidant capacity in different bio-fluids and systems. These tests attempt to account for the high degree of synergism present in the body's antioxidant defenses. Just as diverse prooxidants can be implicated together as the impetus behind OS, antioxidants work through and with each other to prevent tissue damage and disease. Adequate levels of both vitamin E and Se (as a component of GSH-Px) for example, are vital to preventing nutritional degenerative myopathy. Feeding diets insufficient in vitamin E advances the accumulation of TBARS and lipid hydroperoxides (evaluated as ascorbate-induced TBARS) in various muscles of beef calves (Walsh et al., 1993a; Walsh et al., 1993b). When dietary Se is also insufficient, peroxidation of myopathy-prone muscles, like the heart, is also evident (Walsh et al., 1993a). Quantification of a single antioxidant often tells little about whole body defense, making

it necessary to evaluate multiple indicators of redox status. Although measuring multiple antioxidants or biomarkers of stress is helpful, measuring a myriad of variables also tends to be impractical. Consequently, total antioxidant capacity assays are being employed on a routine basis. Some such tests have been used in research with cattle. Matthes et al. (2002) measured electrical potential of calf plasma and calculated its Redox Stability Index based on the Nernst equation. Finding that this index is well-correlated with Se content of muscle tissues after calf harvest, the authors proposed that the Redox Stability Index is an effective tool, highlighting the importance of Se-dependent GSH-Px in antioxidant defense. Castillo et al. (2003) observed dairy cows in peak lactation and cows in the sixth mo of lactation and found that concentration of lipid hydroperoxides is greater in plasma of cows during peak lactation, without a concomitant alteration in trolox equivalent antioxidant capacity (TEAC) of plasma. These researchers suggested that the increase in lipid peroxidation indicates a greater risk for OS in high-producing cows due to increased metabolic demand, and that hydroperoxides are allowed to accumulate due to failure of TEAC. The authors concluded that TEAC methodology is effective in assessing metabolic status of cows. Mandebvu et al. (2003) concurred, reporting that lactating cows have higher TEAC than dry cows. Authors related this finding to the different diets of the animals, as the diet during lactation included high levels of vitamin A and E that were not available to dry cows. Additionally, seasonal effects on plasma TEAC are noted, which may be associated with availability and antioxidant content of forages. As an alternative to total antioxidant capacity assays, some researchers attempt to predict antioxidant status by evaluating total free radical concentration of blood. Bo et al. (1998) used electron paramagnetic resonance

spectroscopy (**EPR**) to show that Se supplementation of deficient cattle not only improves erythrocyte GSH-Px activity, but also decreases overall concentration of free radicals in whole blood. Other total antioxidant capacity assays employed in the bovine model are discussed below, as appropriate. Objective criticisms and comparisons of these and other commonly used techniques are available (Prior and Cao, 1999; Ghiselli et al., 2000).

OXIDANT-RELATED EFFECTS IN THE PERIPARTAL PERIOD

Both demands of labor in the dam, and initiation of pulmonary respiration in the perinatal calf could allow onset of OS. Some research shows that plasma antioxidant status around calving is quite different in cows and calves. Total antioxidant status, as determined by the ferric-reducing ability of plasma (**FRAP**) method, is greater in calves than in dams immediately following parturition (Laszlo et al., 2000; Gaal et al., 2006). Additionally, FRAP is highly-correlated with plasma albumin ($r = 0.96$), and β -carotene ($r = 0.72$), but not well-correlated with plasma α -tocopherol ($r = 0.29$) (Laszlo et al., 2000). Interestingly, in the study by Gaal et al. (2006), plasma TEAC, erythrocyte MDA, and erythrocyte GSH-Px were not different between cows and calves after calving, which may suggest that elevated FRAP in calves is related to high levels of bilirubin and low content of polyunsaturated acids in plasma of newborns. Electron paramagnetic spectroscopy reveals a higher concentration of blood free radicals, along with lower erythrocyte SOD activity in calves, thereby illustrating the potential difficulty encountered in comparing different total antioxidant capacity assays. Regardless, Gaal et al. (2006) maintained that the presence of oxidative stress in perinatal calves is confirmed

by excess free radicals, and suggested that postnatal increases in erythrocyte MDA are brought about by simultaneous increases in blood free radicals and decreases in FRAP.

Inanami et al. (1999) utilized EPR spin-trapping technology to compare superoxide scavenging ability of plasma from periparturient dams with that of postpartum calves. In the hours after birth, they found that cows are better than calves at disposing of radicals. However, superoxide scavenging ability of calves is increased by the 12th h after birth, and continues to increase until peak abilities are established from 3 to 30 d following birth. Associating these findings with elevated TBARS and MDA in the serum of calves after birth, researchers suggested that lowered ceruloplasmin ferroxidase activity, along with lower albumin-associated bilirubin status permit lipid peroxidation immediately after calving. Lipid peroxidation products in calf serum are diminished once antioxidant defenses become functional, a transition that may be aided by the transfer of antioxidants from colostrum. Mature milk has SOD and ceruloplasmin-independent scavenging ability, which is lacking in colostrum (Inanami et al., 1999).

Oxidant-related stress in cows around calving has been characterized apart from calves. As previously suggested, dams can experience OS due to the demands of labor. They can also be hindered by the demands of lactation. Kolb and Seehawer (2000) postulated that oxygen consumption nearly doubles in cows with high milk production, leading to greater internal generation of ROS. Harmon et al. (1997) described a decline in total antioxidant activity in cows on the day of calving, which matched concurrent drops in plasma vitamin E and ceruloplasmin activity. Conversely, other scientists have observed no differences between plasma TEAC, or erythrocyte TBARS and GSH-Px in cows at calving (Gaal et al., 2006). These investigators suggested that only erythrocyte

SOD activity is lowered, while FRAP is enhanced by labor. Elsewhere, follow-up of dry cows postpartum reveals that plasma TEAC actually peaks 1 wk post-calving (Castillo et al., 2005). In the study by Castillo et al. (2005), large individual variations were found for plasma MDA, which was numerically, though not statistically, higher at 1 wk post-calving. Authors proposed that increased TEAC was a mechanism for coping with OS. They pointed out that TEAC is significantly correlated with plasma albumin ($r = 0.64$) in close-up dry cows, whereas MDA is associated with non-esterified fatty acids ($r = 0.61$) in dry cows, and plasma triglycerides ($r = 0.82$) in early lactation cows. Assessing antioxidant potential of peripheral blood mononuclear cells, Sordillo et al. (2007) concluded that the capacity of antioxidants to reduce elemental Cu is decreased 21 d after calving. Incongruous findings like those mentioned above illustrate the lack of practicality in comparing antioxidant status obtained under different experimental conditions, and demonstrate the limitations associated with the majority of total antioxidant capacity assays.

Periparturient decreases in SOD activity mentioned above are interesting to note, as other studies have gleaned divergent information about antioxidant enzymes in transitioning cows. The mRNA expression of SOD in leukocytes declines in first-calf heifers from 22 d before calving, up to 22 d after calving (Colitti and Stefanon, 2006). Colitti and Stefanon (2006) mixed either lycopene or grape polyphenols (as sources of natural antioxidants) in the TMR of close-up heifers. In contrast to what is observed in control and lycopene fed animals, SOD expression in white cells increases from 22 d pre-calving until 15 d post-calving in heifers receiving grape polyphenols. Leukocyte GSH-Px expression was also investigated in this study, but it remained constant throughout the

periparturient period regardless of dietary treatments. Feeding grape polyphenols was advocated as a way to control calving-induced oxidative stress. Previous findings of Stefanon et al. (2005) sharply contrast this notion. Examining actual antioxidant enzyme activity rather than gene expression, these researchers indicated that feeding grape polyphenols leads to lower SOD activity in blood of periparturient cows. They also showed that blood SOD and GSH-Px activity are increased after calving. The only point of agreement between these two similar feeding trials is that tomato lycopene does not alter antioxidant enzyme activity (Stefanon et al., 2005; Colitti and Stefanon, 2006). The contentions between these trials may be related to the fact that leukocyte gene expression was examined in one instance, whereas whole blood enzyme activity was assayed in the other. The usefulness of feeding antioxidant nutraceuticals around calving is questionable, pending further evidence.

Other investigators propose that a homeostatic mechanism controls OS around calving. Bernabucci et al. (2005) followed a group of cows through the periparturient period and found that erythrocyte SOD activity progressively increases during the last 3 wk of pregnancy before declining to dry cow levels in the 1st wk postpartum. Similarly, plasma thiols (representative of GSH) peak 4 d before calving, only to return to baseline levels after parturition. Conversely, intracellular thiols do not increase prior to delivery, but do decrease post-delivery. Other experimental techniques yield different results. Employing techniques reported to be specific for GSH (rather than just thiols), researchers found whole blood GSH to be lowered post-calving (Stefanon et al., 2005; Sordillo et al., 2007). Observations of Sordillo et al. (2007) have important implications regarding OS related to calving. These investigators noted that oxidized glutathione

(GSSG) in whole blood is higher in cows at calving, and that the reduced to oxidized glutathione ratio (GSH:GSSG) is markedly decreased at this time. Such observations indicate a shift in cellular redox potential. Lowered GSH and increased GSSG may explain why lipid hydroperoxides are elevated post-calving. Peripheral blood mononuclear cell GSH-Px activity was also analyzed in cows studied by Sordillo et al. (2007). In conjunction with elevated hydroperoxides, GSH-Px activity was greater after calving; a response that investigators suggested is cytoprotective. Accumulation of lipid peroxides indicates that elevated GSH-Px may not have been sufficient to prevent oxidative damage to body lipids. Still, Bernabucci et al. (2005) noted other seemingly adaptive responses of plasma GSH-Px in periparturient cows. They showed that plasma GSH-Px activity increases steadily, starting 30 d before calving and peaking in the week following calving. Erythrocyte GSH-Px activity behaved differently than other antioxidants in this study, actually decreasing between -4 and +11 d around calving. Comparing antioxidant enzyme responses of animals in different trials may not be appropriate, as trace mineral status, though critical for enzyme activity, is bound to differ according to environment, diet, and physiological demand. For example, the same authors describe incongruent physiological responses of erythrocyte SOD, but similar responses of both plasma GSH-Px and erythrocyte GSH-Px in separate years (Bernabucci et al., 2002, 2005). Ronchi et al. (2000) affirmed parturition-induced responses that are almost identical to those reported by Bernabucci et al. (2005) for plasma thiols, and erythrocyte SOD and GSH-Px. Despite some self-cited contradictory results, Bernabucci et al. (2005) found that plasma ROS (as measured by the d-ROM test) are decreased in days leading up to calving. This decrease was attributed to a compensatory increase in

antioxidant protection. Perhaps the most important inference of Bernabucci et al. (2002; 2005) is that oxidative load on the dam is increased around calving. This assertion is backed by higher concentrations of plasma ROS, and significantly elevated levels of plasma TBARS after delivery in both reports.

OXIDANT-INDUCED RETENTION OF FETAL MEMBRANES

Cows experiencing OS during the periparturient period may be predisposed to a barrage of other disorders. Much research points to failure of antioxidant defenses, or excessive ROS generation, as links to retained placenta (**RP**) (Kankofer, 2002). Insufficient erythrocyte GSH-Px activity in the weeks before calving has been related to RP (Brzezinska-Slebodzinska et al., 1994), yet placentas retained longer than 12 h have greater GSH-Px activity than placentas normally expelled (Kankofer et al., 1996). Superoxide dismutase activity in retained placentomes is puzzling, being greater than normal in fetal sections, but less than normal in maternal parts (Kankofer et al., 1996). Evaluation of whole body antioxidant status, rather than just the condition of involved tissues may be more relevant to understanding the pathogenesis of OS. When plasma from cows that shed placentas before 12 h was compared to that of cows that retained placentas for more than 12 h, plasma from healthy cows was superior at protecting phycoerythrin from degradation by free radicals generated *in vitro* (Stec et al., 1991; Miller et al., 1993). Some of the healthy cows had been supplemented with 1000 IU/d of vitamin E. Those not supplemented had lower plasma α -tocopherol and free radical scavenging ability, along with greater erythrocyte TBARS during the last 6 wk of gestation (Miller et al., 1993). The ability of a sample to protect phycoerythrin against peroxy radicals is described as an effective assay for determination of fast-acting

antioxidants (**FAA**) in plasma (Miller and Madsen, 1994). Cows with below average FAA are 3.4 times more likely to experience RP, but cows supplemented with vitamin E are less likely to suffer RP because 1000 additional IU of vitamin E per day makes cows 218 times more likely to have above average FAA. Cows supplemented with vitamin E are only 2% as likely to have above average red blood cell TBARS (Miller and Madsen, 1994). Other research supports the idea that 1000 IU of supplementary vitamin E per day increases FAA and plasma α -tocopherol, while decreasing TBARS in erythrocytes (Brzezinska-Slebodzinska et al., 1994). Results of this trial again indicate that cows with RP have lower FAA up to 2 wk before calving.

Investigations examining the accrual of oxidized macromolecules in fetal membranes themselves also signify the possibility of ROS related pathogenesis. Proteolysis may be important for proper separation of placentomes, but oxidative alterations of tissue peptides may delay spontaneous discharge. Retained fetal membranes have greater concentrations of the oxidized proteins, formylkinurenine and bityrosine, than placentas normally expelled (Kankofer, 2001b). Along the same lines, TBARS, conjugated dienes, and lipid hydroperoxides are more concentrated in problem placentas (Kankofer, 2001a). Caesarean section may also alter placental redox status, as total antioxidant status is lower in placentas when surgery is required (Kankofer et al., 2006), an observation that may be associated with the prevalence of oxidized tissue (Kankofer, 2001a, b). Either way, products of macromolecule oxidation are generally greater, and total antioxidant capacity lower, in fetal portions of placentomes (Kankofer, 2001b, a; Kankofer et al., 2006). Whether these oxidative insults represent causative agents, or are merely side-products of preexisting afflictions, is debatable.

OXIDATIVE STRESS AND UDDER EDEMA

Udder edema has also been linked to OS. Plasma from healthy heifers is better at scavenging ROS than that from heifers with udder edema (Stec et al., 1991). Similarly, heifers with plasma FAA below average have a 4.7 times greater risk for udder edema, but prepartum feeding of vitamin E reduces the severity of edema (Miller and Madsen, 1994). Importantly, this benefit of vitamin E may only be realized when feedstuffs are of adequate Se status (Miller et al., 1993). The high level of synergism exhibited by dietary Se and vitamin E, and their essentiality to animal health, have been extensively reviewed (Surai, 2006).

ANTIOXIDANT THERAPY FOR MASTITIS

Cooperative effects of vitamin E and Se also prevent mastitis, another ROS linked periparturient disorder. Supplementing cows with 0.74 g vitamin E per day during the dry period decreases the incidence and duration of clinical mastitis (Smith et al., 1984). A Se injection (0.1 mg/kg BW) at 21 d prepartum, on the other hand, has no effect on occurrence, but does lessen the duration of the disorder. When given in conjunction, vitamin E and Se maximally diminish mastitis. Both milk and plasma vitamin E concentrations are lower in mastitic than in healthy cows (Atroshi et al., 1986b). In addition, intracellular killing of *Escherichia coli* by peripheral blood neutrophils is enhanced in cows given prepartum vitamin E supplements, whereas killing of *Staphylococcus aureus* is enhanced in cows supplemented with vitamin E and Se (Hogan et al., 1990). The improved phagocytic ability of neutrophils isolated from Se-sufficient cows is likely related to GSH-Px interaction in post-phagocytic respiratory burst. Extracellular concentrations of hydrogen peroxide are higher in neutrophils harvested

from milk in cows fed a Se deficient diet, yet cellular production of superoxide (vital to the kill mechanism) is not altered by Se status. Moreover, neutrophils obtained from milk of Se-supplemented cows can kill a higher percentage of ingested bacteria (Grasso et al., 1990). Mastitic cows have compromised erythrocyte GSH-Px activity, likely because of the relationship between Se nutrition and GSH-Px function (Atroshi et al., 1986a, b). Ndiweni and Finch (1996) proposed that Se and vitamin E could work together or independently, assuming varying degrees of importance to neutrophils depending upon the circumstances. Although no cumulative effect of the compounds was observed by these authors, *in vitro* addition of both vitamin E and Se enhanced chemotactic migration of polymorphonuclear leukocytes, and vitamin E alone improved phagocytic ability of the cells. Ramadan et al. (2001) also found that supplementing polymorphonuclear cells with Se *in vitro* augments phagocytosis and killing ability, and established that the antioxidant β -carotene helps these abilities. This finding contrasted previous work that had suggested β -carotene has no effect on phagocytosis, but supported the former indication that it boosts killing ability of periparturient phagocytes (Daniel et al., 1991).

The relationship between other low-molecular-weight antioxidants and mastitis has been examined. Healthy cows have greater concentrations of erythrocyte GSH than those hampered by mastitis (Atroshi et al., 1986a). Weiss et al. (2004) induced mastitis with intramammary infusions of *E. coli* and discovered that plasma vitamin C concentrations decrease 39%, whereas milk ascorbic acid decreases by sixty-two percent 24 h later. In addition, they noted that severity of clinical signs is related to magnitude of vitamin C reduction in milk from challenged quarters.

EFFECT OF THERAPEUTIC INJECTIONS ON ANTIOXIDANT STATUS

In mastitic cows, Malinowski et al. (2006) used lysozyme dimer injections in conjunction with antibiotic treatments (Synulox-Pfizer) and noticed that respiratory burst of leukocytes is enhanced over that in cows treated with antibiotics alone. Activity of SOD, GSH-Px, and GR was also numerically, but not statistically, increased in cows injected with lysozyme dimer. Previous work shows that lysozyme dimer shots increase TEAC in pregnant heifers without altering SOD, GSH-Px or GR (Malinowski et al., 2004). On the other hand, a treatment containing 600,000 I.U. vitamin A, 200,000 I.U. vitamin D₃, 1.5 mg/kg vitamin E, and 0.022 mg/kg Se boosts SOD activity by 24 h, and increases GSH-Px activity by 72 h post-injection. The effect on GSH-Px is almost certainly due to improved Se status. Treatment with the antibiotic, tilmicosin, sustains serum concentrations of vitamins A and E in the face of transport stress and dust exposure (Chirase et al., 2001), but vaccinating calves against *Mannheimia haemolytica* does not effect lipid peroxidation or total antioxidant capacity in serum (Chirase et al., 2004).

OXIDATIVE STATUS RELATED TO BODY CONDITION SCORE

Recent developments in human medicine point to obesity as a predisposing factor for OS (Vincent and Taylor, 2006). In a related manner, over-conditioned cattle exhibit sensitivity to oxidant-related phenomena. Transition dairy cows with excessive body condition scores (**BCS**) and marked fluctuations in BCS around transition show signs of increased ROS generation, yet may have compromised antioxidant defenses like SOD, GSH, and erythrocyte thiols (Ronchi et al., 2000; Bernabucci et al., 2005). Only cows with BCS greater than 3 (5-point scale) may be affected, as discrepancies in oxidative

status are not apparent when low-BCS and medium-BCS animals are compared. Oxidant/antioxidant imbalance may be related to metabolic demand and negative energy balance in these cows, as plasma ROS are correlated with plasma β -hydroxybutyrate ($r = 0.40$) and non-esterified fatty acids ($r = 0.32$) (Bernabucci et al., 2005). Post-calving plasma TBARS are also elevated in high-BCS cows, indicating that oxidative damage is occurring. In contrast, O'Boyle et al. (2006) cite no difference in primary lipid peroxidation products (lipid hydroperoxides) between normal- and high-BCS cows. Total antioxidant status of white blood cells, however, is decreased in high-BCS cows. Fat cows also tend to have a lower ratio of reduced to oxidized GSH (GSH:GSSG) in their blood, signifying altered redox balance. In the study by O'Boyle et al. (2006), GSH:GSSG ratio differences were not statistically significant, perhaps because of extremely large variations in individual cows, which were observed. Neither of the previously discussed studies detected any effect of BCS on GSH-Px activity, be it in plasma, red blood cells, or leukocytes (Bernabucci et al., 2005; O'Boyle et al., 2006). Thioredoxin reductase activity, on the other hand, is lower in high-BCS cows (O'Boyle et al., 2006), and thus may be a more sensitive marker of OS than GSH-Px. Thioredoxin reductase requires Se at its active site, and can act as an antioxidant by reducing a wide array of organic peroxides. Sordillo et al. (2007) found that activity of thioredoxin reductase is lower in white blood cells after calving compared to before calving. These authors postulated that post-calving reductions in cellular antioxidant potential and increases in lipid hydroperoxides show the importance of thioredoxin reductase to antioxidant defenses of leukocytes. Mezes et al. (1997) noted that Hungarian flekvieh bulls with greater weaning weights and growth rates during finishing have similar plasma

GSH-Px and erythrocyte catalase activity, but greater plasma MDA concentrations than bulls with lower weaning weights and growth rates during finishing. This may mean that finishing rate and fatness affect the *in vivo* rate of lipid peroxidation.

OXIDANT-RELATED HEPATIC LIPIDOSIS

In addition to stresses predicted above, excessive body fat accretion leads to another condition that has been linked to OS. Sub-optimal antioxidant status in dairy cows with fatty liver disease implicates susceptibility to ROS. Surveys of clinical cases of hepatic lipidosis show that plasma vitamin E is consistently lower in afflicted cows than in healthy cows (Hidiroglou and Hartin, 1982; Mudron et al., 1997; Mudron et al., 1999b). Moreover, vitamin A status can be compromised in these animals. Cows with hepatic lipidosis have lower plasma vitamin A and E at calving, and fail to build stores to appropriate post-calving levels (Hidiroglou and Hartin, 1982). Vitamin E insufficiency could be related to depressed feed intake in sick cows, whereas low vitamin A might be a result of liver damage limiting the organ's storage and mobilization abilities. As might be expected, these findings translate to accumulation of oxidatively damaged products. Cows with fatty livers have greater concentrations of hepatic TBARS (Mudron et al., 1999b), and levels of lipid peroxidation increase with severity of fat infiltration (Mudron et al., 1997). Because TBARS are derived from lipid, it makes sense that TBARS accumulation should be related to total fat available to be oxidized. Indeed, liver triglyceride content correlates well with total TBARS yielding Spearman's coefficients of ($r = 0.38$) and ($r = 0.83$) for the observations of Mudron et al. (1997) and Mudron et al. (1999b), respectively.

REDOX STATUS RELATED TO LACTATION AND CONCENTRATE DIET

Stage of lactation and amount of concentrate in the ration may also affect oxidative status of cows. Mudron et al. (1999a) compared plasma antioxidant status of cows at differing stages of lactation and noted that FRAP is greatest in late lactation cows, yet lowest in dry cows. Importantly, plasma albumin and α -tocopherol are also highest in late lactation cows, which may suggest that they are associated with FRAP. Dry cows had the lowest antioxidant potential in the study by Mudron et al. (1999a). Like Mandebvu et al. (2003), Mudron et al. (1999a) suggested that discrepancies between dry and lactating cows might be related to antioxidant content of diets. This assumption could be supported by the findings of Wachter et al. (1999), which indicate that forage intake is positively associated with plasma antioxidant activity. These authors proposed that plasma antioxidant activity peaks in fresh cows and gradually declines with progression of lactation. Interestingly, they also indicated that there may be a breed component of oxidative status, as Jerseys were found to have plasma antioxidant activity that is consistently higher than Holsteins (Wachter et al., 1999).

Recently, Gabai et al.(2004) investigated effects of altering dietary amounts of roughage and starch throughout lactation. In this trial, cows on a basal diet (24.9% DM starch) at the beginning of lactation either remained on this ration, or were switched to a low-starch ration (21% DM) at 43 DIM, and then a high-starch ration (28.3% DM) at 66 DIM. It was discovered that plasma TBARS, plasma GSH, and erythrocyte GSH-Px activity increase with progression of lactation in all cows. Of utmost importance was the finding that TBARS progression is reduced in cattle when they go on low-starch diets. Induction of erythrocyte GSH-Px activity, on the other hand, is abruptly decreased when

cows are switched to high-starch diets. Gabai et al. (2004) concluded that oxidative load is decreased in cows fed low-starch diets, and that higher levels of plasma insulin in cows fed high-starch diets may contribute to OS.

HEAT INDUCED OXIDATIVE STRESS

Apart from stressors related to calving, periparturient disease, and metabolic demands, some researchers have theorized that environmental factors may strain the delicate oxidant/antioxidant balance important for proper physiological functioning. Lakritz et al. (2002) found that blood GSSG is elevated in heat-stressed cattle. On a similar note, plasma total antioxidant activity decreases when cows are placed in environmentally controlled chambers and exposed to 29.5 °C temperatures for a period of 7 d (Harmon et al., 1997). Harmon et al. (1997) surveyed a herd during periods of varying ambient temperature and concluded that as the temperature humidity index (**THI**) approaches levels dangerous to livestock, total antioxidant activity declines. Other researchers cite no effect of heat stress on plasma antioxidants like α -tocopherol, β -carotene, retinol, or retinyl palmitate (Trout et al., 1998). These scientists found that TBARS are not different in muscles of heat-stressed cows and control cows. Incongruous results obtained from heat chamber trials may relate to the ability of animals to recuperate when allowed to cool down. Even though Trout et al. (1998) subjected cows to much greater temperatures (rising from 30.7 °C in the morning to 38.3 °C in the afternoon), animals were removed from chambers at 1500 h each day, and not put back until 0800 h the next morning.

Other investigators have compared markers of oxidative status in cows transitioning during warmer or cooler seasons. Plasma TBARS, thiols, and GSH-Px are

not altered in cows exposed to elevated summertime THI, but erythrocyte TBARS, SOD, and thiols are greater in cows during the summer than in cows tested during cooler weather (Bernabucci et al., 2002). Erythrocyte GSH-Px activity is also greater in heat-stressed cows, leading the authors to suggest a compensatory response of these cells to oxidant challenge during heat stress. These researchers maintained that red blood cells may be appropriate models of OS due to their highly unsaturated plasma membrane and constant exposure to oxygen interacting with Fe.

TRANSPORT AS A MODEL OF OXIDATIVE STRESS

Exposing cattle to stimuli that are physically and psychologically stressful activates the hypothalamic-pituitary-adrenal axis, leading to elevated levels of glucocorticoids and enhanced susceptibility to disease (Wernicki et al., 2006). The inevitability of livestock transport makes stress associated with transportation an appropriate field of focus. Chirase et al. (2004) observed significantly decreased serum total antioxidant capacity in transported steers, and found that serum MDA concentrations in calves triple after transportation. More importantly, respiratory disease rate is positively correlated with MDA, and calves that die after transport have 44% greater increases in MDA than surviving calves. Amplification of lipid peroxidation in reaction to shipping stress has also been described by Wernicki et al. (2006). These authors reported large increases in plasma TBARS on the 1st through 3rd d after transportation, before observing a gradual decline on the 6th d, and a return to baseline levels on the 9th d post-transport. Plasma cortisol and TBARS were correlated in the calves used for this study. Total antioxidant capacity in the calves tested by Chirase et al. (2004) continued to decline for up to 28 d on a post-transport dry lot. Previous data

evidences the fact that transportation stress reduces serum vitamin E, and that exposure of steers to simulated dust storms further exacerbates the decline (Chirase et al., 2001). Vitamin E supplemented in receiving rations increases ADG and decreases morbidity in transported cattle (Gill et al., 1986; Hays et al., 1987). Similarly, the antioxidant supplement, AgradoTM, may be beneficial in improving the health of received cattle, as it decreases morbidity and average number of medical treatments required for calves received from auction (Stovall et al., 1999). Health benefits are likely related to improved antioxidant status. Despite having lower pre-transit serum vitamin E, steers fed AgradoTM have greater post-transit vitamin E and A status than control steers (McBride et al., 2001). Moreover, these steers consume more feed and have greater post-shipment ADG than their non-supplemented counterparts. The importance of antioxidant status during shipping and receiving can be further substantiated by work demonstrating decreased ADG and increased bovine respiratory disease in conjunction with decreased post-transport concentrations of serum vitamins A and E (Chirase et al., 2001). Preventing similar declines in antioxidant defenses may be vital to warding off shipping fever.

Due to the value of immune cells in combating disease, the oxidative status of leukocytes in response to transport stress is of interest. Leukocyte FRAP is greater in calves after shipping, but OS associated with transport is evidenced by excessive accumulation of leukocyte TBARS (Urban-Chmiel, 2006). Production of ROS is also greater in leukocytes obtained after transport, possibly as a result of enhanced respiratory burst (Urban-Chmiel, 2006). Pregel et al. (2005) concluded that total antioxidant status, assessed as the ability of a sample to reduce Cu, is a useful tool for measuring stress in dairy calves transported by road for 5 h. They observed a considerable increase in

antioxidant capacity of serum after calves were allowed a 2 mo recovery period following transport.

OTHER INVESTIGATIONS OF OXIDATIVE STATUS IN CATTLE

Previously discussed literature involves some of the more common models of OS in cattle. As interest in ROS, antioxidants, and their combined effects on health grows, research expands into diverse fields. Observations that cows with abomasal displacements have compromised vitamin E status (Mudron et al., 1997) might lead investigators to question the role of ROS in this disorder. Elsewhere, analysis of genetic values for somatic cell scores and productive life in Holsteins shows that these traits tend to be positively linked to plasma total antioxidant activity (Wachter et al., 1999).

Oxidative stress research often relates to antioxidant content of diets. Though the importance of adequate antioxidants in feedstuffs is obvious, an interesting twist on nutritional links with OS relates to concentrations of prooxidants in feeds. Consequences of transition metals in animal nutrition have been characterized (Miller and Madsen, 1994), but less is known about outcomes of feeding other easily oxidized diet components. Some investigators have examined effects of polyunsaturated fatty acids (**PUFA**) on muscle myopathy. Recalling aforementioned effects of vitamin E and Se on nutritional degenerative myopathy, it is obvious that severe depletion of these nutrients is a predisposing factor. When oils high in PUFA are also fed, myodegeneration may be eminent. Kennedy et al. (1987) noted that feeding linseed oil high in linolenic acid (18:3) produces severe skeletal myodegeneration and myocardial lesions in calves with subclinical skeletal myopathy. Such diets promote accumulation of an array of lipid peroxidation products in calves already at risk (Walsh et al., 1993b). An in-depth review

of the effects of forage finishing on PUFA content and oxidation of beef products will be carried out subsequently.

A certain fraction of antioxidants (like vitamin E) and minerals (such as Se) are considered essential to oxidant defenses. Time tested, these common markers are assayed over and over in OS research. However, as literature surrounding OS in cattle develops, minerals and antioxidants not considered before may come into focus. Guoyan et al. (1998) proposed that germanium may play a synergistic role with Se in boosting antioxidant functions of dairy cows. Cattle supplemented with Ge have positive correlations between blood Ge and serum GSH-Px and SOD activity, and negative correlations between blood Ge and serum MDA. Zinc is another mineral that has thus far received little attention in studies of cattle redox. Though its essentiality in mitochondrial SOD is unquestionable, different research trials suggest varying effects of dietary Zn. Activity of SOD may be compromised in cattle marginally deficient in Zn, yet if Zn concentration in feedstuffs is excessive, Cu absorption may be limited, in turn also limiting SOD activity (Kleczkowski et al., 2003). Through similar mechanisms, increasing Zn, Mo, and S concentration of beef cattle diets decreases vitamin E in liver, and ceruloplasmin and vitamin C in serum (Kleczkowski et al., 2004). Although vitamin C is infrequently investigated in ruminants, the previous authors found a negative relationship between ascorbic acid content of feedstuffs and Zn status of cattle. Despite the idea that excess vitamin C can be reduced to participate in ROS generating reactions with transition metals, when vitamin C is fed to bulls consuming rations high in Cu, there is no evidence of peroxide generation (Kleczkowski et al., 2003).

Some scientists have linked oxidative status to diseases of parasitemia. More et al. (1989) examined the blood cells of calves infected with anaplasmosis and found that erythrocyte GR and GSH-Px activity are decreased during the post-patent period, whereas SOD activity is lowered in both erythrocytes and leukocytes at this time. Anaplasmosis also causes a significant loss of GSH in red blood cells. Cattle naturally infected with tick-spread *Theileria annulata* also show greater erythrocyte GSH-Px activity, and more red cell lipid peroxidation (Grewal et al., 2005).

Erythrocyte TBARS accumulate in instances of pyrrolizidine alkaloid poisoning in cattle (Bondan et al., 2005). Consumption of plants in the *Senecio* genus leads to erythrocyte fragility that is associated with GSH depletion and lipid peroxidation. Superoxide dismutase activity of red blood cells may be enhanced in instances of toxicity, which leads some to propose a compensatory mechanism stimulated by excessive radical generation (Bondan et al., 2005). An adaptive type of system regulating antioxidant enzyme activity has been mentioned previously in this review, as it is a popular theory used to validate the results of many trials. Although indirect evidence supports the possibility of this notion, schemes predicting precise mechanisms through which it may occur are not well developed. At least one study has indicated that cattle do not respond to peroxidative challenge by inducing the enzymes, GR, glutathione transferase, SOD, and catalase (Walsh et al., 1993a). Future research may be needed to elucidate the theory of antioxidant compensation.

FESCUE TOXICOSIS AND LINKS TO OXIDATIVE STRESS

Cattle grazing wild type endophyte-infected tall fescue (E+) may have altered levels of the dietary antioxidant vitamins A and E. Steers grazing E+ in Mississippi were

found to have lowered vitamin E, whereas steers grazing E+ in Virginia had decreased vitamin E and A (Fike et al., 2001). In this experiment, researchers applied a seaweed (*Ascophyllum nodosum*) based treatment (Tasco) to E+. Although Tasco supplementation seemed to boost vitamin A levels in Virginia steers, the authors concluded that the effect of E+ on vitamin E needed further investigation. Also utilizing Tasco, Allen et al. (2001) came to similar conclusions. In this study, steers that had been grazing endophyte-free tall fescue (E-) had higher serum vitamin E than steers that had been grazing E+ when the animals arrived at the feedlot. Other studies indicate minimal effects of E+ on either vitamin E (Montgomery et al., 2001), or vitamin A (Munyabagisha et al., 1993). Lakritz et al. (2002) reported that while heat stress significantly increases oxidized glutathione (GSSG), the combination of heat stress and E+ not only increases GSSG, but also decreases the reduced form of the antioxidant (GSH). This finding implicates induction of oxidative stress in both heat-stressed, and heat-stressed/E+ exposed cattle. Fescue toxicosis may also have an effect on total antioxidant status. Unpublished data from the University of Tennessee at Knoxville indicates that compared to plasma from steers grazing E-, plasma from steers grazing E+ is more than 7 times as likely to have below average antioxidant capacity (Oliver, 1997). There is currently no evidence of oxidative damage caused by E+. Realini et al. (2005) found no differences in carcass lipid oxidation in steers that grazed E+ versus nil-ergot alkaloid producing novel endophytic (AR542) tall fescue.

LIPID PEROXIDATION IN FORAGE FINISHED BEEF

Thus far, oxidation and antioxidant interactions have been covered as they pertain to animal health. In the subsequent section of this review, attention will be shifted to

redox balance as it influences beef products. Increasing interest in forage finishing beef cattle has recently led to a surge of scientific literature regarding the topic. Ruminant nutritionists and meat scientists have taken special interest in the fact that forage-based production systems can alter fat composition of ruminant tissues, resulting in beef products that possess greater percentages of PUFA. The unique double-bond character exhibited by lipids in these products lends them particular susceptibility to chain propagated autoxidation reactions. Autoxidation of meats leads to formation of secondary oxidation products, which have the potential to negatively impact flavor attributes. Studies investigating effects of forage feeding on tissue antioxidant status have been carried out with the hope that adequacy of dietary antioxidants may play a role in counterbalancing this phenomenon.

Several consistent observations about tissue from forage-fed animals have been made. When compared to concentrate (grain) finished beef, it is evident that forage-finished beef contains greater amounts of conjugated linoleic acid, and omega-3 polyunsaturated fatty acids, with particularly high levels of α -linolenic acid (18:3 n -3) and long chain n -3 fatty acids (Laborde et al., 2002; Steen et al., 2003; Martin and Rogers, 2004; Mir et al., 2004; Noci et al., 2005; Scollan et al., 2005; Scollan et al., 2006).

This phenomenon imparts higher PUFA:SFA (polyunsaturated fat:saturated fat) ratios, and lower n -6: n -3 ratios to tissues of forage-fed animals, effects that have important implications to human nutrition. Although mechanisms behind these findings are not completely defined, the basic principle behind them is associated with fatty acid content of forages. Plant lipids are primarily composed of glycolipids (70-80%) and phospholipids (Harfoot, 1981), which are notably made up of high proportions of α -

linolenic acid (Scollan et al., 2005). Up to 14% of linoleic acid (18:2*n*-6), and 8% of α -linolenic acid (18:3*n*-3) can escape ruminal biohydrogenation (Scollan et al., 2005). Phospholipid fatty acids are less influenced by diet than triacylglycerol fatty acids, but PUFA not saturated in the rumen can be incorporated into both phospholipids and triacylglycerols (Laborde et al., 2002; Noci et al., 2005; Scollan et al., 2006).

Lipids are essential to the palatability of meats (Gray and Pearson, 1994).

Although they are unquestionably responsible for desirable flavors and aromas, upon oxidation they can lead to what has been described as “warmed-over flavor”, “rancidity”, or more generally “meat flavor deterioration” (Gray and Crackel, 1992). Forage-finished beef has been regularly cited as possessing off-flavors that are characteristic of lipid oxidation (Wood et al., 2003; Campo et al., 2006; Scollan et al., 2006). Although propensity of lipids in meat to oxidize depends on numerous factors, the concentration of PUFA may be the most important consideration (Gray and Pearson, 1994).

Even though specific lipid oxidation products in beef can be precisely monitored with chromatographic procedures, TBARS assay is the most widely used test for oxidation in meats. Thiobarbituric acid reactive substances assay is not specific for malondialdehyde or any other single product of lipid oxidation, but the test correlates well to sensory data (Shahidi, 1994), and its simplicity and convenience greatly outweigh its criticisms (Simic et al., 1992). Campo et al. (2006) found that Spearman’s correlations between beef TBARS and perceptions of beef flavor and overall liking were -0.80 and -0.84 respectively, whereas correlations between TBARS and abnormal and rancid flavors were 0.82 and 0.84, respectively.

When ideas about the effect of forage feeding on PUFA in meat and subsequent susceptibility of these lipids to oxidize are taken into context, it is easy to imagine how consumer acceptance of pasture-raised beef could be discouraged. Reverte et al. (2003) froze restructured steaks from cattle that had been finished on either alfalfa pasture or a corn-supplemented diet. They found that after 3 mo of storage, TBARS increase dramatically in steaks from pastured cattle, yet remain relatively unchanged in steaks from corn-fed animals. These results could explain negative correlations observed between grass finishing and sensory acceptability in previous studies (Schroeder et al., 1980; Melton et al., 1982; Larick et al., 1987).

Recent research suggests that inherent antioxidant properties of pasture-based beef may protect it against oxidative degradation regardless of PUFA content. Gatellier et al. (2004) attempted to clarify this phenomenon by examining antioxidant status of meat from cattle finished on pasture, or a cereal grain-based concentrate. Interestingly, efforts to employ broad measures of total antioxidant status were fruitless, as the TEAC method revealed no treatment effects. Examination of total hydroxyl radical scavenging activity of samples did initially indicate that meat from concentrate-fed animals was a better scavenger. However, further research revealed that higher GSH-Px activity in this meat interfered with the assay by neutralizing substrate hydrogen peroxide before it could yield hydroxyl radicals via the Fenton reaction. The concentrate-fed animals were found to have an enhanced Se status, which unquestionably explained the deviation of this Se-dependent enzyme. Similarly, elevated Cu status in pastured animals could explain the increased activity of Cu-based SOD that was observed in their tissues. Similar effects of finishing system on these mineral-dependent antioxidant enzymes are noted elsewhere

(Mercier et al., 2004). Although Gatellier et al. (2004) highlighted the importance of considering mineral concentrations in finishing diets, they most notably reported improved vitamin E status in cattle on forage. Durand et al. (2005) suggested that because large incorporations of PUFA in meat fail to stimulate antioxidant enzyme activity, protection of PUFA depends on chain-breaking antioxidant molecules in meat. Vitamin E protection seems to be particularly important in inhibiting lipid peroxidation.

Oxidation potential of meat is greater when grasses high in vitamin E are used to boost PUFA than when oils low in vitamin E are used (Durand et al., 2005). Like other phenolic compounds with electron withdrawing character at *ortho* and *para*-positions, vitamin E (α -tocopherol) can act to break chain reactions of lipid peroxidation (Yanishlieva and Marinova, 2003). Specifically, vitamin E scavenges peroxy radicals by donating hydrogen to, or forming adducts with, peroxy radicals (Kamal-Eldin et al., 2003). Importantly, the lipophilic nature of this compound allows it to associate with lipid bilayers where a preponderance of muscle PUFA reside. Marked reduction in beef lipid oxidation occurs when feedlot-fed cattle are dosed with α -tocopherol acetate (Arnold et al., 1993b). It takes 12-18 wk to saturate muscle tissue with vitamin E, and once saturated it requires greater than 18 wk for depletion to occur (Arnold et al., 1993a). Cattle finished on forages rich in this antioxidant can build appreciable stores over the time required to reach appropriate harvest weights (Arnold et al., 1993a; Muramoto et al., 2005). Vitamin E deposition is enhanced in cattle consuming more unsaturated dietary lipids (Felton and Kerley, 2004), and forages are known to be rich in linolenic acid. Yang et al. (2002a) reported that cattle grazed on pasture could achieve concentrations of α -tocopherol in their tissues that are at least as high as those produced by vitamin E

supplementation in grain-fed animals. Furthermore, they noted that another antioxidant, β -carotene, is present in considerable amounts in the tissues of grazing animals. This effect has been previously described in forage-fed animals (Simonne et al., 1996; Descalzo et al., 2005; Muramoto et al., 2005), and could potentially confer additional oxidative protection to pasture-raised meat. Other antioxidants present in forage may also offer protection against lipid peroxidation in meat.

Mercier et al. (2004) finished cows outdoors on grass pasture, or indoors on cereal grain-based concentrates and found that despite higher PUFA concentrations, TBARS are lower in meat from cows fed grass. Vitamin E and β -carotene, along with other carotenoids, phytic acid, and flavonoids derived from grass could account for protection against oxidation. Evidence relating specifically to protective effects of vitamin E is best established. Meat from steers raised on grass silages accumulates fewer TBARS under modified atmosphere packaging than meat from steers raised on cereal concentrates (Campo et al., 2006). Such findings are proposed to be due to the relatively low content of vitamin E in concentrate compared to grass silage. Citing the fact that the psoas major (filet mignon) is especially susceptible to oxidative insult upon harvest, Descalzo et al. (2005) collected this muscle from steers of different nutritional backgrounds. Some steers had been grazed on natural Argentinean pasture, while others were individually fed a corn-based diet. Subsets of steers from each group were supplemented with 500 IU of vitamin E per day. Major findings of the study related to the α -tocopherol content of muscle from pasture-fed animals. It was discovered that α -tocopherol in muscle from pasture-fed cattle is twice as high as in corn-fed animals. Supplementation of vitamin E to grain-fed cattle does increase pre-harvest plasma α -tocopherol to levels comparable to

those in grass-fed steers, but it fails to build tissue concentrations of α -tocopherol to levels that match those in grazing steers (Descalzo et al., 2005). Furthermore, in pastured animals, supplementation fails to additionally increase plasma or tissue α -tocopherol concentrations (Descalzo et al., 2005). Meat from grazing cattle is 3 times less oxidized than meat from grain-fed cattle, and vitamin E supplementation has little effect on either group. The finding that β -carotene exhibits trends similar to α -tocopherol in pasture and corn-fed steers prompted researchers to suggest that these antioxidants have a cooperative effect in diminishing lipid oxidation. Analysis of volatile oxidation by-products in meats supports conclusions derived from TBARS as 3-methylbutanyl, hexanal, heptanal, and octanal are all lower in samples from pasture-fed cattle (Descalzo et al., 2005). All of this is apparent despite the fact that pasture-fed animals have higher PUFA than corn fed animals (Descalzo et al., 2005). Steaks from steers finished on pasture exhibit higher linoleic (18:2), linolenic (18:3), arachidonic (20:4), eicosapentaenoic (20:5), and docosapentaenoic (22:5) acid concentrations than those from steers fed a corn silage-based ration (Realini et al., 2004a). Nonetheless, α -tocopherol is higher in muscle from grazing steers, and TBARS levels are consequently lower over 3 wk of display. In contrast to the study by Descalzo et al. (2005), Realini et al. (2004a) indicate that supplementing concentrate-fed cattle with vitamin E can raise their tissue α -tocopherol to levels comparable to those seen in grazing steers. This effect is likely due to a higher level of supplementation (1000 IU/day vs. 500 IU/day) in the study by Realini et al. (2004a). Steaks from supplemented and concentrate-fed cattle oxidized to the same extent as steaks from pastured animals, indicating that vitamin E oxidatively protects PUFA in steaks from pasture-fed cattle (Realini et al., 2004a). Different conclusions can be made

about beef that is ground. Ground beef from cattle finished on concentrate still has higher initial oxidation than ground beef from pastured or concentrate-fed and supplemented animals, but after 8 d of display, ground beef from pastured steers is more oxidized (Realini et al., 2004a). This effect is best explained by the fact that grinding meat disrupts integrity of cellular membranes, which contain high percentages of unsaturated phospholipids. When this occurs, PUFA may be liberated and exposed to oxygen, so that antioxidant components of tissues may not be able to prevail over the susceptibility of PUFA to oxidation.

Similar reasoning may explain the observations of Yang et al. (2002b). Muscle samples were obtained from steers that were forage- or grain-fed in this trial. Within each dietary treatment, steers were supplemented with vitamin E at levels of 0 or 2500 IU/day. When tissues were exposed to aerobic conditions for 7 d, it was found that TBARS values were dramatically higher for all of the treatment groups except the one that had been supplemented and grain-fed. Meat was not ground in this trial, but it was kept anaerobically at 0°C for 47 d prior to analysis. Freezing of muscle may damage tissue membranes and promote effects similar to grinding by releasing unsaturated lipids into aqueous phases of tissue where they are oxidized (Campo et al., 2006). Unfortunately, the precise mechanism by which this may occur is not well studied, and conflicting results have been observed. O'Sullivan et al. (2002) indicated that freezing steaks before TBARS analysis does not necessarily allow oxidizable PUFA to override protective effects of vitamin E. When groups of heifers were allowed to consume grass silage, corn silage, or a 50:50 mixture of silages ad libitum, diet was found to have a significant effect on both α -tocopherol, and linolenic acid (18:3) composition of resulting meat tissue.

Vitamin E content of *longissimus dorsi* samples was found to average 2.08, 2.95, and 3.84 $\mu\text{g/g}$ for heifers fed corn silage, 50:50 silage, and grass silage respectively, whereas linolenic acid (18:3) made up 0.796, 1.37, and 1.78% of fatty acids for the respective samples. The influence of diet over these results was clearly observed, as the corn silage contained only 20.76 ± 4.29 $\mu\text{g/g}$ of α -tocopherol and $5.47\% \pm 0.25\%$ of fatty acids as linolenic acid, while the grass silage had 105.41 ± 10.05 $\mu\text{g/g}$ of α -tocopherol, and $47.31\% \pm 1.02\%$ of fatty acids as linolenic acid. Steaks from corn silage-fed heifers exhibited higher TBARS values than other steaks after 4, 8, and 12 d of display, despite the fact that all samples were frozen prior to analysis. Steaks from heifers fed grass silage yielded TBARS numbers lower than other steaks on display d 8 and 12.

Another study by the same authors (O'Sullivan et al., 2003) compared steers fed various amounts of forage and concentrate to steers offered concentrate only. Post-harvest *longissimus dorsi* samples were obtained and frozen before analysis for α -tocopherol content and oxidative stability. There was a trend for animals consuming higher amounts of herbage to have increased vitamin E status, and TBARS analysis revealed that steaks from concentrate-fed animals were more oxidized over 17 d of storage. Other research backs-up the assertion that beef contains higher concentrations of α -tocopherol and exhibits greater oxidative stability when cattle are fed grass silage rather than concentrates (O'Sullivan et al., 2004).

Some research has been devoted to exogenous supplementation of antioxidants in traditional feedlot diets. Walenciak et al. (1999) included 150 ppm of the antioxidant supplement AgradoTM in finishing diets of steers and found that upon harvest, ground beef from these steers displayed reduced TBARS values in comparison to ground beef

from steers that received no antioxidants. Oxidation values for meat from supplemented steers remained much lower over a 6 d retail display period. O'Grady et al. (2006) included natural antioxidants like tea catechins and rosemary extract in a barely-based concentrate diet being fed to bullocks. Dietary antioxidant supplementation was not effective in this trial, as TBARS analysis of meats revealed no treatment effect on lipid stability. Conversely, direct addition of antioxidants to ground beef is more helpful, as both tea catechins and rosemary extract stifle lipid oxidation throughout a 1 wk storage period (O'Grady et al., 2006). These results support previous evidence regarding the usefulness of free radical scavengers for retarding lipid oxidation in beef products (St. Angelo et al., 1990; Ahn et al., 2002). Although post-slaughter antioxidant treatment of meats may be an attractive tool for battling lipid oxidation, many of the previously discussed studies would suggest that such strategies are less important when animals are finished on forages high in vitamin E. Ahn et al. (2002) proposed that approaches involving dietary vitamin E accretion are more effective at preventing lipid oxidation than those that directly add α -tocopherol to ground beef. This is probably due to the propensity of dietary vitamin E to be incorporated into phospholipid bilayers, which are predisposed to oxidation (Ahn et al., 2002). The lack of need to apply antioxidant treatments to meat from forage-fed animals is exemplified by work in which vitamin C was added to ground beef from animals finished on either tall fescue, or a concentrate diet (Realini et al., 2004b). Over an 8 d display period, vitamin C addition improves oxidative stability of beef from concentrate-fed animals, but beef from grass-fed animals is not altered by vitamin C treatment. This study found that despite its higher PUFA content, TBARS fail to build to appreciable levels in any beef, treated or not treated, from

grass-fed cattle. Vitamin E was not measured, but meat from fescue-fed animals was hypothesized to have had a greater antioxidant capacity, which was theoretically attributed to vitamin E.

Oxidative potential of beef is a multifaceted concept that depends, at the very least, on a delicate balance of antioxidants like α -tocopherol, and prooxidants like PUFA. Considering just one side of this equation seems futile. Much of the current literature indicates that the antioxidant character of forage-finished beef may be able to make up for the propensity of its lipids to oxidize. Vitamin E status seems to be particularly important in warding off oxidation. Still, additional factors like maintenance of cellular integrity and interactions of other antioxidants play important roles (albeit less well-defined roles) in chain lipid peroxidation. Frequently overlooked is the role of total fatty acid content, rather than just fatty acid composition, on accrual of secondary oxidation products. The lifetime physical activity status, as well as the age of cattle at slaughter, are also factors that may impact quality of beef from forage-fed animals. Perhaps these aspects of production can be investigated in context with lipid peroxidation. Future research on forage-raised beef will likely be aimed at discerning more precise relationships between lipid oxidation and antioxidant interactions, vitamin E distribution, and product integrity.

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CHAPTER III

INFLUENCE OF ENDOPHYTE INFECTED TALL FESCUE CONSUMPTION AND HEAT STRESS ON INTRAVAGINAL TEMPERATURES, PLASMA LIPID OXIDATION, BLOOD SELENIUM AND GLUTATHIONE REDOX OF MONONUCLEAR CELLS IN HEIFERS GRAZING TALL FESCUE

ABSTRACT: A grazing experiment was conducted to assess the effects of wild type endophyte-infected tall fescue (E+) consumption and elevated ambient temperatures on intravaginal temperatures, plasma lipid peroxidation, blood Se, and glutathione redox of peripheral blood mononuclear cells. Thirty-four crossbred Angus heifers were allotted by weight to 4 blocks consisting of E+ and endophyte-free tall fescue (E-) pastures. Monthly, in June, July, and August, temperature loggers were fixed into blank controlled internal drug releasers (CIDR) and inserted into a subsample of heifers (n = 16) for 2 d. After 48 h, heifers were weighed, and blood was collected via jugular venipuncture. Peripheral blood mononuclear cells were isolated for analysis of glutathione peroxidase activity, glutathione reductase activity and reduced:oxidized glutathione. Plasma malondialdehyde was evaluated as a marker of lipid peroxidation, and whole blood Se concentration was determined. Serum prolactin was assayed at the end of the grazing period. Data were analyzed using repeated measures with pasture as the experimental unit, and block as a random effect. Heifer ADG was highest in August, and lowest in July ($P < 0.001$). In August, heifers grazing E+ exhibited higher ($P < 0.05$) afternoon intravaginal temperatures and temperature fluctuations than heifers grazing E-. In July

and August, all heifers had higher afternoon temperatures ($P < 0.02$), and lower reduced:oxidized glutathione ($P < 0.0001$) than in June. Glutathione reductase activity of all heifers was greater in June ($P = 0.03$) than in July. Similarly, all heifers exhibited decreased glutathione peroxidase activity ($P < 0.0008$) in July, whereas whole blood selenium was lowered ($P < 0.0001$) in July and August. No treatment or date effects were detected for malondialdehyde, but serum prolactin was low at the end of the grazing period ($P = 0.008$) in heifers stocked on E+. Using these markers, differences in oxidative stress were not detected between heifers consuming E+ or E-. Date effects indicating altered glutathione redox and enzyme activity may have been related to heat stress and nutritional limitations.

Key words: cattle, endophyte, fescue, glutathione, heat stress, oxidative stress

INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb.), the predominant forage for grazing animals in southwest Virginia, is commonly infected with the fungal endophyte *Neotyphodium coenophialum* ([Morgan-Jones and Gams] Glenn, Bacon, and Hanlin; Glenn et al., 1996). Although this fungus acts in a mutualistic way to improve plant hardiness, it also produces alkaloids that are detrimental to the performance of grazing animals (Stuedemann and Hoveland, 1988; Strickland et al., 1993; Omacini et al., 2005). Abnormalities are particularly notable during periods of high temperature-humidity indices, as cattle exposed to elevated ambient temperatures and endophyte alkaloids are predisposed to hyperthermia (Oliver, 1997). In addition, the low-molecular weight antioxidant glutathione may be compromised by fescue toxicosis.

Lakritz et al. (2002) reported a decrease in reduced glutathione (**GSH**), and an increase in oxidized glutathione (**GSSG**) in blood of heat stressed cattle consuming wild type endophyte-infected tall fescue (**E+**). Furthermore, disrupted redox status could promote the accumulation of by-products from oxidant-induced tissue damage. Saker et al. (2004) observed increased lipid hydroperoxides in the plasma of wether lambs fed E+ hay after a period of heat stress, and found that erythrocyte glutathione peroxidase (**GSH-Px**) activity increased linearly during this time. Because immune cell function is impaired in cattle grazing E+ (Saker et al., 1998; Saker et al., 2001), the status of the glutathione redox system in immune cells of cattle stocked on E+ may be of interest. An experiment was conducted to test the hypothesis that fescue toxicosis is exacerbated by an imbalance in the glutathione redox system of leukocytes. Objectives were to evaluate intravaginal temperatures, plasma lipid oxidation, and redox balance of glutathione in leukocytes from cattle grazing E+ in typical conditions, such as those encountered during summertime in Virginia.

EXPERIMENTAL PROCEDURES

Experimental Design

During the summer of 2006, an experiment was conducted at Virginia Polytechnic Institute and State University's Kentland Farm (81°5' West longitude; 37°25' North latitude). Thirty-four crossbred Angus heifers (BW = 329 ± 13.7 kg) were purchased from the same origin and stocked for 3 wk on naturalized pasture composed predominantly of Kentucky bluegrass (*Poa pratensis* L.), orchardgrass (*Dactylis glomerata* L.), tall fescue, red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.). On May 5, 2006 heifers were allotted by weight to 4 pasture blocks. Each

block contained two 1.1 ha Kentucky 31 tall fescue paddocks, one composed of endophyte-free tall fescue (E-), the other consisting of E+. Endophyte-infected and E- stands were originally established in September of 2002, and showed an original stand persistence of 74 and 63%, respectively in 2005 (Rotz, 2006). Routine forage analysis in 2005 and 2006 revealed mean total alkaloid concentrations of 1565 ± 552 and 135 ± 49 ppb for E+ and E- pastures, respectively (Agrinostics Ltd. Co., Watkinsville, GA). Each paddock was subdivided into 6 sections with poly-wire (O'Brien Plastics Ltd., Auckland, NZ) so that heifers could be allowed to strip-graze the paddock over time. Water and a Se-free trace mineral salt mixture (Cargill Inc., Minneapolis, MN) were provided *ad libitum* and heifers were monitored daily. On June 28, 2006, temperature recording data loggers (ACR Systems Inc., Surrey, Canada) were fixed into blank controlled internal drug releasers (CIDR; InterAg, Hamilton, New Zealand) and inserted vaginally into a sub-set of heifers (n = 16; 8 E+ and 8 E-) at 0800. Heifers were weighed and then returned to their assigned paddocks. After 48 h, data loggers were removed and heifers were bled by jugular venipuncture into evacuated tubes containing either heparin or EDTA (Becton Dickinson, Franklin Lakes, NJ). Blood was placed on ice for transport to the laboratory (21 km from site), where it was processed immediately. The sampling protocol was followed 21 d later on July 19, 2006, and 56 d later on August 23, 2006. An additional evacuated tube of blood was also collected in August for analysis of serum prolactin. All animal handling procedures were approved by the Virginia Polytechnic Institute and State University Animal Care and Use Committee.

Ambient Temperature and Temperature Humidity Index

Weather data were collected throughout the grazing period from a WeatherWatch 2000 weather station (Campbell Scientific Inc., Logan, UT) located on-site at Kentland Farm, and daily temperature-humidity indices (**THI**) were calculated according to Amundson et al. (2006). Heifer temperature data were recorded every 15 min, and recordings were subsequently extracted from data loggers using ACR systems Inc. software (Surrey, Canada). For each sampling date, assessment of intravaginal temperatures was carried out by comparing morning and evening average temperatures recorded by data loggers over the 2 d for which they were in place. This was done to appreciate day and night temperature fluctuations that we expected to occur in heat stressed cattle consuming ergot alkaloids (Bourke, 2003). All intravaginal temperatures recorded between 0400 and 0800 on both days preceding sampling were averaged to represent daily minimum temperatures (T_{\min}), whereas all intravaginal temperatures recorded between 1600 and 2000 on both days preceding sampling were averaged to assess daily maximum temperatures (T_{\max}). The daily fluctuation (ΔT) was defined as the difference between afternoon average and morning average.

Sample Processing and Analysis

Blood with EDTA was centrifuged for 5 min at 2,500 x g and the buffy coat was removed and dispersed into 12 mL of lysis buffer (pH = 7.2, 150 mM NH_4Cl , 10 mM NaHCO_3 , 10 mM EDTA) to remove residual erythrocytes. The bovine buffy coat consists almost entirely of mononuclear cells (Carlson and Kaneko, 1973), as confirmed by cell differentials in our laboratory. Blood smears performed on buffy coat leukocytes resuspended in autologous plasma revealed 97% peripheral blood mononuclear cells (**PBMC**). Lysis buffer containing PBMC was incubated for 10 min at room temperature,

and then centrifuged at 470 x g for 10 min to pellet the cells. Cells were then washed in 10 mL of Hank's Balanced Salt Solution (Gibco Industries Inc., Langley, OK), and re-pelleted by centrifugation. Pellets were reconstituted into 1 mL of distilled water, aliquoted into storage vials, and frozen at -70 °C until being assayed for glutathione reductase (**GR**) and GSH-Px activity. For analysis of GSH:GSSG in PBMC isolates, blood was handled via a slight modification of the procedures described above. To assess GSSG separate from GSH, 100 µL of the isolate was added to 10 µL of the thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate, before freezing and storage. Heparinized blood was centrifuged for 5 min at 3,000 x g and 4 °C, and plasma was separated into vials for storage at -70 °C until analysis of malondialdehyde (**MDA**) concentration. Assessment of PBMC GR, GSH-Px, and GSH:GSSG, as well as plasma MDA was carried out with colorimetric assay kits (OXIS International, Portland, OR). Glutathione peroxidase activity was measured using the coupled test procedure (Flohe, 1989) in which the action of GSH-Px is coupled to that of GR, and the change in absorbance at 340 nm is measured as NADPH is oxidized in the presence of substrate *tert*-butyl hydroperoxide. Glutathione reductase activity was determined spectrophotometrically at 340 nm after the oxidation of NADPH in the presence of excess GSSG (Carlberg and Mannervik, 1985). Enzyme activity was expressed in milliunits and defined as the amount of enzyme catalyzing the reduction of one nmol of *tert*-butyl hydroperoxide per min at pH 7.6 and 25 °C for GSH-Px, and as the amount of enzyme catalyzing the reduction of one nmol of GSSG per min at pH 7.6 and 25 °C for GR. All enzyme measurements were adjusted based on the total protein content of the PBMC isolate (Bio-Rad Laboratories, Hercules, CA), which was quantified using

the Coomassie blue method of Bradford (1976). Determination of GSH:GSSG ratio was accomplished by employing a spectrophotometric methodology based on the enzymatic techniques developed by Tietze (1969). Malondialdehyde was evaluated as a marker of lipid peroxidation by recording formation of a spectrophotometrically-detectable carbocyanine dye that results from the reaction of MDA with N-methyl-2-phenylindole at 45 °C (Gerard-Monnier et al., 1998). Whole blood Se concentration was determined with atomic absorption spectrophotometry using a graphite furnace on a Perkin Elmer AAnalyst 800 (PerkinElmer Life and Analytical Science Inc., Wellesley, MA). Blood was diluted 10-fold in distilled water and, after addition of a Pd/Mg nitrate matrix modifier (Van Cauwenbergh et al., 1990), spectra absorption was measured at 196 nm. Serum prolactin was assayed by RIA (Miller et al., 1999).

Statistical Analysis

Data were analyzed using repeated measures PROC MIXED with pasture as the experimental unit, and block as a random effect (SAS Inst. Inc., Cary, NC). Fescue type, date and fescue type x date were main effects. The arh (1) covariance structure was the best fit for data based on Akaike's Information Criteria. Tukey's test was applied *post hoc* to test significant fescue type x date interactions. For gain, initial BW was added to the model as a covariate. Significance was determined at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

One goal of the current experiment was to accurately assess heifer body temperatures at rest. Rectal temperatures collected while cattle are in the squeeze chute may be affected by confounding variables (e.g., exertion required in walking from

paddocks to handling facilities, handling-induced stress, barn environment, and waiting time). For this reason, indwelling data loggers were used to measure intravaginal temperatures over the entire 48 h period preceding blood collection. Because we were concerned that heat stress may be a function of both body heating and body cooling, we chose to analyze average intravaginal temperatures taken from periods when heifers were hottest (1600 to 2000) and coolest (0400 to 0800). Heifers consuming E+ tended ($P = 0.075$) to have greater T_{\max} than heifers consuming E-. Heifers had greater T_{\max} in the hotter months, July ($P < 0.0001$) and August ($P = 0.01$), than in June (Table 3.1). Severity of heat events is often defined by their respective THI. Livestock managers are advised to be on alert when THI range from 75 to 78, whereas THI from 79 to 84 are classified as dangerous conditions for cattle (Hubbard et al., 1999). Although heifers were exposed to THI in the alert zone during all 3 mo, ambient conditions were most severe during July and August, with July THI reaching the danger threshold (Appendix 2). The normal range for bovine core temperatures is 38.0 °C-39.0 °C (Jackson and Cockcroft, 2002). With the exception of the heifers stocked on E- in August, average T_{\max} exceeded these ranges in July and August. Bourke (2003) suggested that 39.5 °C is the critical rectal temperature at which hyperthermia exists in cattle. Although the mean T_{\max} in our heifers was never higher than this value, the observation that average afternoon body temperatures in late summer exceeded the reference range indicated that the animals were heat stressed. During August, there tended to be a fescue x date interaction ($P = 0.095$), with heifers grazing E+ having greater T_{\max} than heifers grazing E- (39.4 ± 0.16 °C and 38.8 ± 0.16 °C, respectively). Differences in morning heifer temperatures were not detected at any sampling dates ($P = 0.430$).

Table 3.1 Least squares means for intravaginal temperature data¹ from heifers grazing Kentucky-31 wild type endophyte-infected (E+), or endophyte-free (E-) tall fescue in June, July, and August

Item	Fescue			Month ²				P-value ³		
	E+	E-	SE	June	July	August	SE	T	M	T x M
T _{max} , ⁴ °C	39.1	38.9	0.08	38.6 ^a	39.4 ^b	39.1 ^b	0.10	0.075	<0.001	0.095
T _{min} , ⁵ °C	37.9	37.7	0.10	37.8	37.7	37.9	0.09	0.28	0.21	0.43
ΔT, ⁶ °C	1.27	1.21	0.07	0.79 ^a	1.66 ^b	1.27 ^c	0.06	0.57	<0.001	0.001

^{a,b}Within a row, means with an uncommon superscript differ, $P < 0.05$.

¹Temperature data for 8 heifers grazing E+ and 8 heifers grazing E- (n = 16).

²June 28, 2006 to June 30, 2006; July 19, 2006 to July 21, 2006; August 23, 2006 to August 25, 2006.

³P-value for treatment (T), month (M), and treatment x month (T x M).

⁴Two-day average intravaginal temperature from 1600 to 2000.

⁵Two-day average intravaginal temperature from 0400 to 0800.

⁶ΔT = (T_{max}) – (T_{min}).

Table 3.2 Mean environmental conditions¹ recorded at Kentland Farm during the June, July and August data collection periods

Climatic condition	Daytime			Nighttime		
	June	July	August	June	July	August
Temperature, °C	25.7	29.3	28.5	14.2	18.1	14.8
Relative humidity, %	68.3	71.2	59.2	100	100	100
THI ²	74.6	80.1	77.3	57.6	64.6	58.6

¹Average ambient conditions while the recorders were in use and recording for two consecutive days; i.e. the two-day average from 1600 to 2000 for daytime values, and the two-day average from 0400 to 0800 for night-time values.

²THI = (0.8 x temperature) + [(% relative humidity/100) x (temperature – 14.4)] + 46.4.

Date and a fescue type x date interaction were detected for ΔT (Table 3.1), with the greatest ΔT in July, least ΔT in June and August ΔT being intermediate. Ambient temperature followed a similar pattern. In August, E+ heifers exhibited greater ($P = 0.046$) ΔT than E- heifers (1.41 ± 0.09 °C vs. 1.14 ± 0.09 °C). Morning heifer temperatures were numerically not different, so the interaction was associated with daytime temperature elevations in T_{\max} . Differences in nighttime and daytime ambient temperatures were also greatest during August (Table 3.2). Average temperatures, maximum temperatures, minimum temperatures, and temperature fluctuations recorded by the temperature loggers over the entire 2 d period are given in Appendix 1.

A date effect on ADG was detected (Table 3.3). Daily gain was greatest ($P < 0.0001$) in all heifers during the month of August, whereas weight loss occurred during July. Cattle gains on E+ are generally negatively influenced by the presence of the endophyte (Stuedemann and Hoveland, 1988; Strickland et al., 1993; Oliver, 1997). However, there was no effect of fescue type on heifer ADG. Elevated ambient temperatures, along with summertime declines in forage availability (Rotz, 2006) may explain the observed BW losses. A decrease in PBMC GSH:GSSG was observed after the first sampling date in heifers grazing E+ and E- (Table 3.3). As the predominant low-molecular-weight thiol in animal cells, GSH serves as a crucial antioxidant to offset environmentally derived oxidative insult. Oxidation of GSH occurs either directly, or through enzymatic means, as free radicals and other reactive oxygen species are scavenged (Wu et al., 2004). The GSH:GSSG in heifer PBMC was lower ($P < 0.0001$) in both July and August, than at the initial sampling (Table 3.3). A lower ratio, which is commonly used as an indicator of cellular redox, indicates either an increased oxidative

Table 3.3 Least squares means for ADG, peripheral blood mononuclear cell reduced to oxidized glutathione ratio, peripheral blood mononuclear cell glutathione peroxidase and glutathione reductase activity, plasma malondialdehyde, and blood Se in heifers grazing endophyte-infected (E+), or endophyte-free (E-) tall fescue in June, July, and August

Item	Fescue		SE	Month ⁶			SE
	E+	E-		June	July	August	
ADG, kg	0.30	0.15	0.095	0.14 ^a	-0.14 ^b	0.66 ^c	0.057
GSH:GSSG ¹	29.7	27.6	2.41	49.0 ^a	19.5 ^b	17.4 ^b	3.23
GR, mU/mg ²	28.4	27.4	1.76	31.1 ^d	24.3 ^c	28.2 ^{de}	2.04
GSH-Px, mU/mg ³	66.6	73.2	4.87	83.2 ^d	53.6 ^c	72.9 ^d	4.01
MDA, uM ⁴	9.5	12.1	1.37	10.8	10.3	11.4	1.09
Selenium, ppb ⁵	87	88	5.9	110 ^a	78 ^b	75 ^b	4.8

^{a,b,c}Within a row, means with an uncommon superscript differ by month, $P < 0.001$.

^{d,e}Within a row, means with an uncommon superscript differ by month, $P < 0.05$.

¹Ratio of reduced (GSH) to oxidized (GSSG) glutathione in peripheral blood mononuclear cells.

²Glutathione reductase activity of peripheral blood mononuclear cells expressed as milliunits per mg of lysate protein.

³Glutathione peroxidase activity of peripheral blood mononuclear cells expressed as milliunits per mg of lysate protein.

⁴Plasma malondialdehyde.

⁵Whole blood Se.

⁶June 30, July 21, and August 25, 2006.

load on the cells, or decreased antioxidant defenses. Failure to detect any fescue effects in conjunction with the date effects suggests that the elevated THI during these months were sufficient to create this outcome regardless of endophyte-toxins. To the best of the authors' knowledge, this is the first report of glutathione redox balance in PBMC from animals grazing E+. Lowered GSH:GSSG could result from depletion of GSH, increased generation of GSSG, or both. Lakritz et al. (2002) indicated that heat stress induced an increase in GSSG in bovine whole-blood, independent of endophyte consumption. Hyperthermia alone can increase the reactivity of oxygen-based radicals (Issels et al., 1986). Settivari et al. (2006) observed that rats consuming E+ fescue seeds downregulated transcription of genes responsible for GSH production *in vivo*. Support for the notion that glutathione production is compromised is supported by the finding that heat stress in combination with E+ fescue consumption depletes total glutathione (GSH + GSSG) concentration in whole blood (Lakritz et al., 2002). Similarly, application of heat stress to cultured bovine lymphocytes reduces the concentration of total intracellular glutathione (Paula-Lopes et al., 2003). Unfortunately, our experimental methods did not allow the determination of the total amount of glutathione (GSH + GSSG). However, GSH:GSSG comparisons can be made to ratios extrapolated from previous work. Whole blood GSH:GSSG ratios ranged from 162 in cows in thermoneutral conditions to 73 in cows exposed to heat stress and E+ fescue (Lakritz et al., 2002). This same ratio in blood of dairy cows of normal vs. high BCS was reported to be 0.66 and 1.9 respectively (O'Boyle et al., 2006). In the latter study, the authors cited extreme variability in GSH:GSSG, which may have been related to a delay in post-sampling addition of the thiol scavenger. In the present study, GSH:GSSG was determined in isolated PBMC.

Although we are not aware of any reports on GSH:GSSG in bovine immune cells, values from the present study are within the range of those cited in canine (15 to 80) PBMC (Zickler et al., 2002), murine (25 to 142) peritoneal leukocytes (Alvarez et al., 2006), and human (18.4 to 22.2) neutrophils (Tauler et al., 2002).

Similar to the GSH:GSSG ratio, GR activity was influenced by date (Table 3.3). Activity of GR was lower ($P = 0.034$) at the July sampling than in June, while GR activity at the August sampling only tended ($P = 0.053$) to be lower than that in June (Table 3.3). The decrease of GR activity is related to the decrease in GSH:GSSG, because GR is important in maintaining normal physiological ratios of GSH:GSSG (Griffith, 1999). Several authors have reported the possibility of an adaptive upregulation of GR with the onset of oxidant-related stress (Schirmer et al., 1989; Townsend et al., 2003). Current observations regarding GSH:GSSG confirm the presence of thermally-induced oxidative stress in bovine PBMC, yet contrary to an adaptive response, a reduction in GR activity was observed. We are unaware of any previous literature regarding GR activity in response to E⁺ consumption.

Several speculations are offered in support of the present data. As evidenced by the negative ADG observed between the June and July sampling dates, forage availability may have become limiting. In addition, heat stress is known to decrease feed intake, and reduce the length of daily grazing time (VanSoest, 1982; Trout et al., 1998). Thus, activity of GR may have been nutritionally related. Food deprived rats have significantly lower activity of erythrocyte GR than rats fed *ad libitum* (Wohaieb and Godin, 1987). Glutathione reductase is an enzyme that depends on flavin adenine dinucleotide as a prosthetic group (Schirmer et al., 1989), and is dependent upon an adequate supply of

riboflavin. In humans, altered riboflavin status can account for dissipation of GR activity (Tessier et al., 1995; Tauler et al., 2006a). Rumen microflora should synthesize adequate riboflavin to render such a hypothesis unlikely in cattle (Santschi et al., 2005; Schwab et al., 2006). An alternative explanation for reduced GR activity in July could be a possible depletion of total glutathione. Total glutathione availability is compromised by heat stress (Paula-Lopes et al., 2003), or reduced energy intake (Tateishi, 1990; Lu, 2000; Wu et al., 2004). Depletion of total glutathione could cause a decrease in the overall concentration of GSSG in spite of increased GSH:GSSG. Oxidized glutathione plays a role in protecting GR against redox inactivation, and a decrease in GSSG concentration can reduce GR activity *in vitro* (Mata et al., 1985; Lopez-Barea et al., 1990). This conjecture would not be supported by the findings of Lakritz et al. (2002), which indicated increases in bovine blood GSSG despite exposure to thermal stress. Still, heifers used in the current experiment were subjected to heat stress and, as previously suggested, may have had limited intake in July, so the possibility that GR was redox inactivated cannot be ruled out.

Activity of PBMC GSH-Px followed a pattern similar to that of GR. Like GR, GSH-Px activity was lower during the month of July compared to June ($P < 0.001$) and August ($P < 0.001$; Table 3.3). Again, present results are contrary to the suggestion that GSH-Px is an enzyme that is induced by oxidant-applied stress (Townsend et al., 2003; Surai, 2006a; Tauler et al., 2006b). Saker et al. (2004) previously observed increases in erythrocyte GSH-Px activity in heat stressed wethers consuming E+ fescue hay, and suggested an endogenously regulated protective response by this antioxidant enzyme. Similarly, after an initial decline, white blood cell GSH-Px activity in the wethers

increased in response to heat stress. Bernabucci et al. (2002) found that plasma GSH-Px activity was not different between transition dairy cows exposed to elevated summertime THI and those in temperate conditions. In contrast, heat stressed cows had erythrocyte GSH-Px activity that exceeded that of the cows transitioning during cooler periods. Conversely, our data exhibited a decrease in PBMC GSH-Px activity in spite of elevated THI. Like GR, the reduced GSH-Px activity may be nutritional in nature. Because of the essentiality of Se in GSH-Px, maintenance of enzyme activity is tightly linked to Se status (Rotruck et al., 1973).

The Se status of heifers was affected by sampling date (Table 3.3). Fescue type had no effect on whole blood Se ($P = 0.86$), but Se concentrations were lower ($P < 0.001$) in both July and August than in June. This alteration in Se availability is possibly related to the decrease in GSH-Px activity that was observed in July. Cattle are Se deficient when the concentration in whole blood is less than 80 ppb (Puls, 1988), and the correlation between sub-adequate GSH-Px activity and Se status is greater in Se-deficient cattle than in Se-adequate cattle (Surai, 2006b). Heifers were given *ad libitum* access to a mineral mixture without Se. Thus, reduced forage DMI or prolonged consumption of low Se forage could have caused the observed decline in Se status. The apparent recovery in GSH-Px activity during the month of August despite a still low Se status is difficult to explain, but may relate to utilization of body Se reserves. When Se availability is adequate, Se-containing amino acids are non-specifically incorporated into many body proteins, such as those making up skeletal muscle. When Se becomes limiting, stress conditions may increase selenoprotein requirements despite decreased Se bioavailability. Surai (2006a) proposed that during such conditions proteosomes are activated which

degrade body proteins, thereby releasing Se-containing amino acids for additional synthesis of selenoproteins like GSH-Px. Proteasome activity may be partially regulated by redox balance, so it is possible that this mechanism could account for the rebound in GSH-Px activity that was observed in the heifers during August. Alternatively, the increase in GSH-Px activity observed at the last sampling could represent an adaptive increase to oxidative stress experienced over the previous two months, with the lag in response due to the life span of the PBMC. Whole blood Se and erythrocyte GSH-Px are highly correlated in cattle, but plasma Se and plasma GSH-Px are not (Scholz and Hutchinson, 1979). The relationship between plasma Se and PBMC GSH-Px activity is not well defined. Association of these two variables may be weakened by the fact that plasma Se is a short-term marker of Se status, whereas PBMC GSH-Px, because of the life span of the cell, is a longer-lived response variable.

No treatment or date effects were detected for the concentration of plasma MDA (Table 3.3). Malondialdehyde is a by-product of lipid peroxidation, and peripheral accumulation indicates oxidative damage of tissues. Regardless of the lowered GSH:GSSG in PBMC, no evidence of oxidative insult could be found using this whole-body biomarker of oxidative stress. Realini et al. (2005) previously showed that tissue from cattle grazing E+ fescue is not at increased risk of post-mortem lipid oxidation. However, it was expected that some consequence of E+ consumption on plasma lipid peroxidation might be observed in the current experiment. Plasma lipid hydroperoxides increased in wether lambs fed E+ hay during a period of heat stress (Saker et al., 2004), but this effect could have been due to the heat, rather than the presence of the endophyte. Bernabucci et al. (2002) noted that transition dairy cows exposed to a hot environment

displayed greater erythrocyte MDA than similar cows exposed to a thermoneutral environment. Interestingly, a difference in plasma MDA concentrations in the same cows was not detected. Similarly, Trout et al. (1998), found that dairy cattle exposed to heat stress via controlled chambers exhibit no evidence of increased MDA concentration in muscle tissue. Likewise, there was no effect of elevated ambient temperatures on lipid oxidation markers in plasma in the present study.

The toxic potential of the paddocks utilized in the present experiment had been evaluated in previous grazing trials. In the year preceding the current study, E+ paddocks had ergovaline and lysergic acid amide concentrations of 330 and 424 $\mu\text{g}/\text{kg DM}$, respectively (Stewart, 2006). Neither ergovaline nor lysergic acid amide were detectable in samples taken from E- paddocks. In 2004 (Boland, 2005) and 2005 (Stewart, 2006), immunoblot testing of tillers (Hiatt et al., 1997) revealed a rate of endophyte infection exceeding 80% in E+ paddocks, whereas endophyte was not detectable in E- paddocks. One consistent physiological response to fescue toxicosis in cattle is a reduction in serum prolactin (Porter and Thompson, 1992; Strickland et al., 1993). Indeed, this effect is so well characterized that it has been routinely used to document that livestock are being impacted by E+ alkaloids (Oliver, 1997). In addition to the historical data provided above, we measured serum prolactin concentrations (intraassay CV 10.5%; interassay CV 9.1%) at the last sampling date to verify the distress produced by E+ consumption. Serum prolactin concentrations were lower ($P = 0.008$) in heifers stocked on E+ (20 ± 3.0 ng/mL) in August than in heifers stocked on E- (167 ± 18.0 ng/mL). Rates of endophyte infection or alkaloid concentrations required to reduce serum prolactin have not been precisely defined. Watson et al. (2004) did report lowered prolactin in cattle grazing

pastures with only 448 ppb of ergot alkaloids, whereas Parish et al. (2003) observed prolactin declines when cattle grazed E+ pastures ranging from 822 to 1208 ppb in alkaloid content. Wide ranges of serum prolactin were reported by these authors depending on season, location, animal gender, and age. In addition, alkaloid concentrations of pastures used in their studies were lower than those of the E+ paddocks (1565 ppb) in the present experiment. The magnitude of difference in serum prolactin from heifers grazing E+ versus E- in the current trial is similar to the magnitude of difference reported in cattle grazing E+ versus nonergot alkaloid-producing endophyte-infected fescue (Nihsen et al., 2004), and similar to the magnitude of change reported when cattle are switched from E- to E+ pastures (Aiken et al., 2006).

IMPLICATIONS

Heifers grazing E+ had lowered serum prolactin, indicating that they were experiencing the physiological effects of fescue toxicosis. Our data suggested no influence of E+ consumption on glutathione redox in PBMC, or accumulation of lipid peroxidation products in plasma. Using the selected biomarkers, we found no evidence that consumption of endophyte toxins promotes oxidative stress in cattle. Our experiment is the first to report GSH:GSSG and GR activity in immune cells from animals grazing E+ under heat stress. Results indicated that cattle experiencing heat stress have altered immune cell redox, which may be exacerbated when nutrition becomes limiting. Forage availability and DMI was speculated to impact antioxidant enzyme activity, but the nature of this influence was not well characterized. Further research will clarify this relationship, and the magnitude of its importance in heat stressed cattle.

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CHAPTER IV

INFLUENCE OF TWO-STAGE WEANING WITH SUBSEQUENT TRANSPORT ON BODY WEIGHT, PLASMA LIPID PEROXIDATION, PLASMA SELENIUM, AND ON LEUKOCYTE GLUTATHIONE PEROXIDASE AND GLUTATHIONE REDUCTASE ACTIVITY IN BEEF CALVES

ABSTRACT: Weaning and transport of calves are stressful events associated with increased susceptibility to infectious disease and injury. Oxidative stress may exacerbate psychological and physiological demands brought about by these practices. Management strategies employing two-stage weaning can alleviate exhibition of stress-linked behaviors. Therefore, a trial was conducted to assess the effects of two-stage weaning and subsequent transport on oxidative stress markers in calves. Thirty-six crossbred Angus steers (initial BW and age of 243 ± 20.8 kg, and 221 ± 19.7 d, respectively) were blocked by weight and allotted to control (C), fenceline (FL) and nose clip (NC) groups. On d -7 of the trial, FL calves were separated from dams by a fence, and NC calves were fitted with anti-suckle devices. Control and NC calves remained with dams until d 0, at which point all calves were permanently removed from dams and transported 172 km. Calves were weighed and bled by jugular venipuncture on d -7, 0 (pre-transit), 1 and 7. Leukocytes were analyzed for glutathione peroxidase and glutathione reductase activity, whereas concentrations of malondialdehyde (MDA) and Se were measured in plasma. Data were analyzed using PROC MIXED with the Tukey-Kramer adjustment applied post-hoc. No treatment effects were detected for blood variables or calf weights. Glutathione peroxidase activity was not altered by treatment ($P = 0.38$). Glutathione

reductase activity declined ($P < 0.004$) with each subsequent sampling from d -7 to 7. Likewise, plasma Se decreased ($P < 0.001$) throughout the trial, with the exception of the period between d 0 and 1, where no transport effects were detected. Plasma MDA was highest on d -7 ($P = 0.01$) and lowest on d 7 ($P < 0.001$), with no transport effects detected between d 0 and 1. Treatment x date interactions were detected ($P < 0.05$), with NC calves having lower MDA than all other calves on d 0, and lower MDA than C calves on d 1. Control and FL steer BW increased with each measurement day ($P < 0.05$), whereas BW of NC steers were not different between d -7 and 0 ($P > 0.05$), but increased on d 7 ($P < 0.05$). Final calf BW was not affected by treatment ($P = 0.81$). Results suggest that two-stage weaning has little effect on examined markers of oxidative stress. Stressors in the current trial may have been less severe than those sometimes encountered by weaning calves.

Key words: calves, glutathione peroxidase, glutathione reductase, malondialdehyde, oxidative stress, shipping, weaning

INTRODUCTION

Routine management strategies of the beef industry unavoidably induce stress in cattle. Stressors may be physical or psychological, and are often associated with exposure to novel experiences and environmental conditions (Grandin, 1997). In calves, processes involving weaning and transport are generally necessary, and serve as appropriate models of commonly encountered stressors. Decreased resistance to infectious disease and elevated risk of injury result from these practices (Tarrant and Grandin, 2000). Though

reasons for calf susceptibility during these times are multifactorial, vulnerability may be traced back to biochemical processes within the body.

Oxidative stress has been implicated in the pathophysiology of transport-related maladies (Chirase et al., 2001; McBride et al., 2001; Chirase et al., 2004; Pregel et al., 2005; Urban-Chmiel, 2006; Wernicki et al., 2006). Although oxidative stress is simply interpreted as an imbalance between prooxidants and antioxidants in favor of the former, a more functional definition incorporates the potential of oxidant-induced damage (Sies, 1991). Thus, diminished antioxidant defenses or excess oxidative insults resulting from transport and weaning stressors may be deleterious to tissues, and may be linked to manifestation of disease.

Concentration of reactive oxygen species is greater in white blood cells isolated from calves after shipping, likely as a result of enhanced respiratory burst (Urban-Chmiel, 2006). Antioxidant status may be lowered by stress. Chirase et al. (2004) observed decreased total antioxidant capacity in serum of transported calves, and noted a continual decline of this capacity for 28 d post-transport. Similarly, Pregel et al. (2005) concluded that total antioxidant status, assessed as the ability of serum to reduce cupric cations, was a useful tool for measuring stress in transported dairy calves. They observed an increase in antioxidant capacity of serum after calves were allowed a 2 mo recovery period following transport. Serum concentrations of the antioxidant, vitamin E, are reduced in shipped steers, and exposure to simulated dust storm further exacerbates the decline (Chirase et al., 2001). Vitamin E supplementation in receiving rations increases ADG and decreases morbidity in received cattle (Gill et al., 1986; Hays et al., 1987), highlighting the importance of antioxidants to health. The antioxidant supplement,

AgradoTM, is also beneficial in improving the health of received cattle, as it decreases morbidity and the average number of medical treatments required for calves received from auction (Stovall et al., 1999). Despite having lower pre-transit concentrations of serum vitamin E, steers fed AgradoTM have greater post-transit vitamin E and A status than non-supplemented steers (McBride et al., 2001). Steers fed AgradoTM also consume more feed and have greater post-shipment weight gain. Chirase et al. (2001) observed decreased ADG and increased bovine respiratory disease in conjunction with decreased post-transport concentrations of serum vitamins A and E.

Investigations of oxidative stress during the period surrounding weaning are lacking. Previous investigators have indicated that weaning methods employing two-stage methodologies are preferable to traditional abrupt weaning techniques in terms of alleviating physiological and psychological stress in calves. Both anti-suckle nose clips and single fence-line separations have been effectively used to gradually wean beef calves. Calves weaned in two-stages via nose clip vocalize less, exhibit less pacing behavior, and spend more time eating and lying down after terminal separation from dams (Haley et al., 2005). Compared to calves completely separated in one step, fence-line weaned calves also settle down sooner after weaning (Nicol, 1977). Fence-line weaned calves walk less, vocalize fewer times, and lay down more during the absolute separation phase (Price et al., 2003).

A trial was conducted to test the hypothesis that both weaning and shipping exacerbate biomarkers of oxidative stress, but that two-stage weaning treatments alleviate this oxidant-induced stress. Lipid oxidation products in the plasma were measured due to their widespread consideration as markers of oxidative damage. Activities of the

antioxidant enzymes, glutathione peroxidase (**GSH-Px**) and glutathione reductase (**GR**) were assessed in leukocytes because of antioxidant enzyme importance in immune cell function.

EXPERIMENTAL PROCEDURES

Experimental Design

Thirty-six crossbred Angus steers (initial weight and age: 243 ± 20.8 kg; 221 ± 19.7 d) reared at Shenandoah Valley Agriculture Research and Extension Center (Steeles Tavern, VA, $37^{\circ}92' N$, $79^{\circ}20' W$) were blocked by weight and allotted to control (**C**), fenceline (**FL**), and nose-clip (**NC**) weaning treatments. Control and treatment groups were replicated twice, and each replicate was assigned its own paddock so that each replicate within treatment consisted of 4 steers per paddock. On d -7, steers were weighed and bled by jugular venipuncture into evacuated tubes containing heparin and EDTA (Becton Dickinson, Franklin Lakes, NJ). At this time, anti-suckle nose-clips (Nasco Farm and Ranch, Fort Atkinson, WI) were attached to the muzzles of steers in the NC group, while steers in the FL treatment were separated from their dams by a single fence. Control and NC steers remained with their dams on pasture for the next 6 d.

On d 0, steers were sampled as previously described, and then completely separated from their dams. Anti-suckle clips were removed from the NC group and all calves were transported 172 km (approximately 2 h) to Virginia Tech's Kentland Farm (Blacksburg, VA, $37^{\circ}11' N$, $80^{\circ}35' W$). Here, all steers were commingled on a dry-lot overnight with *ad libitum* access to hay and water. The next morning, d 1 of the trial, steers were again sampled (24 h post-separation), and regrouped by initial weaning replicate onto nine 1.1 ha tall fescue pastures. A final sampling was conducted on d 7.

Calf health was monitored throughout the trial and all animal handling procedures were approved by the Virginia Tech Animal Care and Use Committee. One FL steer had a piece of residual testicle (left over from banding) removed on d 0. All variables for this steer were similar to those of contemporaries, and it remained in the statistical analysis.

Sample Processing and Analysis

Upon arrival to the lab, 2 mL of whole blood was placed into 12 mL of lysis buffer (pH=7.2, 150 mM NH₄Cl, 10 mM NaHCO₃, 10 mM EDTA) to remove residual erythrocytes. After 10 min of incubation at room temperature, the buffer containing cells was centrifuged at 470 x g for 10 min to pellet the leukocytes. Cells were resuspended in 12 mL of lysis buffer diluted 1:5 (v/v) with Hank's Balanced Salt Solution (Gibco Industries Inc., Langley, OK), incubated an additional 10 min at room temperature, and re-pelleted by centrifugation. The resulting leukocyte pellet was reconstituted with 1 mL of Hank's Balanced Salt Solution, and frozen at -70 °C until analysis for GR and GSH-Px activity.

Heparinized blood was centrifuged for 5 min at 3000 x g and 4 °C. Plasma was removed and stored at -70 °C until malondialdehyde (MDA) analysis. Assessment of GR and GSH-Px in leukocyte lysates, as well as MDA in plasma, was carried out with colorimetric assay kits (OXIS International, Portland, OR). Glutathione peroxidase activity was measured using a coupled test procedure (Flohe, 1989) in which the action of GSH-Px is coupled to that of GR, and the change in absorbance at 340 nm is measured as NADPH is oxidized in the presence of substrate *tert*-butyl hydroperoxide. Glutathione reductase activity was determined spectrophotometrically at 340 nm after the oxidation of NADPH in the presence of excess disulfide glutathione (Carlberg and Mannervik, 1985).

Enzyme activity was expressed in milliunits and defined as the amount of enzyme catalyzing the reduction of one nmol of *tert*-butyl hydroperoxide per minute at pH 7.6 and 25 °C for GSH-Px, and as the amount of enzyme catalyzing the reduction of one nmol of disulfide glutathione per minute at pH 7.6 and 25 °C for GR. All enzyme measurements were balanced for the total protein content of the leukocyte isolate (Bio-Rad Laboratories, Hercules, CA), which was quantified using the Coomassie blue method of Bradford (1976). Malondialdehyde was evaluated as a marker of lipid peroxidation by recording the formation of a spectrophotometrically detectable carbocyanine dye that results from the reaction of MDA with N-methyl-2-phenylindole at 45 °C (Gerard-Monnier et al., 1998). The concentration of Se in plasma was determined with atomic absorption spectrophotometry using a graphite furnace on a Perkin Elmer AAnalyst 800 (PerkinElmer Life and Analytical Science Inc., Wellesley, MA). Plasma was diluted 10-fold in distilled water and, after addition of a Pd/Mg nitrate matrix modifier (Van Cauwenbergh et al., 1990), spectra absorption was measured at 196 nm.

Statistical Analysis

Data were analyzed using repeated measures PROC MIXED with pasture block as the experimental unit (SAS Inst. Inc., Cary, NC). Weaning treatment, day, and weaning treatment x day were main effects, with day as the repeated term. The ar = 1 autoregressive heterogeneous structure was utilized for estimating covariance. For calf weight, post-shipping (d 1) weight was excluded from the model. Tukey-Kramer adjustment was applied *post hoc* to test significance of detected weaning treatment x date interactions. Significance was determined at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Leukocyte Glutathione Peroxidase

Neither treatment ($P = 0.38$), nor day ($P = 0.16$) effects were detected for leukocyte GSH-Px activity in weanling steers (Table 4.1). Prior work in our lab indicated that GSH-Px activity of leukocytes was elevated in calves at 7 d post-weaning (Shank, 2002). In humans, GSH-Px activity increases after long-term exercise training regimens, a response that may be an adaptive mechanism against physical stressors (Tessier et al., 1995; Tauler et al., 2006a). Lymphocyte GSH-Px either increases (Tauler et al., 2006b) or decreases (Tauler et al., 2004) in reaction to short-term physical stress in well-trained athletes; an inconsistency that may be related to the intensity of the stress. Alternatively, GSH-Px activity in neutrophils consistently declines after short-term physical strain (Tauler et al., 2002; Tauler et al., 2004). Total leukocyte GSH-Px activity was evaluated in steers for the present study, and fluctuations in white cell populations around weaning were not assessed.

Stabel et al. (1989) reported that signs of morbidity coincided with elevated plasma GSH-Px in weaned and transported calves challenged with *Manheimia* (formerly *Pasturella*) *hemolytica*. These authors found no evidence of whole blood GSH-Px alteration in the face of *Manhemia* exposure, but noted that both plasma and whole blood GSH-Px activity were higher in calves that had been supplemented with Se. Glutathione peroxidase activity in post-weaned calves is highly associated with pre-weaning Se availability (Beck et al., 2005), a finding not surprising given the essentiality of Se to the

Table 4.1 Least squares means with pooled standard errors for plasma Se and malondialdehyde (MDA), along with leukocyte glutathione peroxidase (GSH-Px) and glutathione reductase (GR) in all steers

Variable	Day ¹				SE	<i>P</i> -value ²		
	-7	0	1	7		T	D	TxD
GSH-Px, ³ mU/mg	146	133	138	150	6.7	0.38	0.16	0.79
Se, ⁴ ppb	65.5 ^a	54.4 ^b	55.2 ^b	47.4 ^c	2.05	0.08	<0.001	0.20
GR, ⁵ mU/mg	35.5 ^a	25.0 ^b	16.9 ^c	13.2 ^d	0.87	0.35	<0.001	0.41
MDA, ⁶ μM/L	22.1 ^a	20.0 ^b	19.2 ^b	11.1 ^c	1.32	0.062	<0.001	0.001

^{a,b,c,d} Within a variable, means with an uncommon superscript differ by day.

¹ October 6, 13, 14, and 20, 2007 for days -7, 0, 1, and 7, respectively.

² *P*-value is probability of significance for treatment (T), day (D), and treatment x day (T x D).

³ Glutathione peroxidase activity of leukocytes expressed as milliunits per mg of lysate protein.

⁴ Plasma Se in parts per billion.

⁵ Glutathione reductase activity of leukocytes expressed as milliunits per mg of lysate protein.

⁶ Plasma malondialdehyde in micromoles per liter.

structure and function of GSH-Px (Rotruck et al., 1973). Glutathione peroxidase activity, therefore, is dependent on Se status in addition to oxidative stress and adaptive responses.

Plasma Selenium

Because of the well established relationship between Se and GSH-Px, plasma Se status of calves was evaluated for each sampling date in the present trial. Control calves tended to have lower plasma Se than NC calves over the course of the trial (50.5 ± 2.87 and 59.7 ± 2.87 ppb, respectively; $P = 0.084$), but no other treatment effects were detected. Lower plasma Se in C calves during the study was likely a function of pre-trial Se status, as these calves had numerically lower plasma Se at trial onset (d -7). All calves had equal access to trace minerals, so these differences may be related to the high variation in individual animal intake when mineral supplements are offered *ad libitum*. In contrast to GSH-Px, which remained steady over time, plasma Se decreased during the course of the trial (Table 4.1). With the exception of the time from d 0 to 1, when no change in plasma Se was detected ($P = 0.63$), Se declined with each successive sampling ($P < 0.001$). Prior to d -7 all calves and dams had *ad libitum* access to a Se-containing mineral mixture (Ca 11.8%, P 6.5%, Mg 11.2%, Zn 0.51%, Cu 0.25%, I 0.014%, Mn 0.40%, Se 0.012%, and vitamins D₃ and E at 185600 and 1100 IU/kg, respectively; King AG Products, Inc., Pulaski, VA). The absence of supplemental Se during the trial may explain the gradual decline in plasma Se. Whole blood Se is expected to be more static over time due to the life span of erythrocytes, but plasma Se reflects short-term Se nutrition (Surai, 2006). Therefore, it is not surprising that Se would decline with the progression of time in the current trial. Alteration in plasma Se without a response of leukocyte GSH-Px may be explained by several factors. Although Se is tightly correlated

to blood GSH-Px activity in Se deprived animals, correlation between Se and GSH-Px is weakened in animals that are not Se deficient (Surai, 2006). In cattle, plasma Se concentrations in the range of 2 to 25 ppb are indicative of deficiency (Puls, 1988). The Se status of steers in the present study was higher than this range, even after some Se depletion occurred. Alternatively, whereas plasma Se is a dynamic marker, leukocyte GSH-Px status may be more static due to the life span of cells.

Leukocyte Glutathione Reductase

Leukocyte GR activity was not altered by weaning treatment ($P = 0.35$; Table 4.1). Activity of GR did, however, decline at each successive sampling ($P < 0.004$). Several authors have reported the possibility of an adaptive upregulation of GR with the onset of oxidant-related stress (Ohno et al., 1986; Schirmer et al., 1989; Townsend et al., 2003; Tauler et al., 2006b). Although we are unaware of any reports of immune cell GR activity in calves subjected to weaning and transport stress, our findings contrast the theory that GR is induced by stress. Our data supports that of previous researchers who have indicated that GR actually declines in response to physical stress. Erythrocyte GR activity is lowered in humans undergoing long-term training regimens (Tessier et al., 1995; Tauler et al., 2006a), whereas neutrophil GR activity is decreased in athletes after short bouts of exercise (Tauler et al., 2004).

Depleted GR could relate to decreased feed intake during the trial. Wohaieb and Godin (1987) reported declines in erythrocyte GR activity in rats suffering from starvation-induced weight loss. Steers in the present study could have been nutritionally limited in the days following the termination of suckling, as they adapted to solely meeting nutritional needs with forage. Likewise, feed intake is often sub-par immediately

following a period of transport, and weight loss is practically unavoidable during this time. Still, limiting nutrition would not explain the decline in GR activity seen in C steers from d -7 to d 0. Daily gains rebounded well prior to d 7 indicating a return of normal feed intake and adequate energy status. Another explanation for alterations in leukocyte GR might relate to the dynamic nature of the leukocytes examined.

Psychological stress in cattle induces a surge of glucocorticoids that not only enhances the release of neutrophils from bone marrow, but also may induce survival and persistence in already circulating cells (Burton et al., 2005). For this reason, neutrophil counts, along with neutrophil to lymphocyte ratios, are generally increased in calves subjected to stressors associated with weaning (Hickey et al., 2003), and shipping (Blecha et al., 1984; Cole et al., 1988; Phillips et al., 1989). White cell differentials were not evaluated in the current experiment, and leukocytes were not separated into respective populations for determination of GR activity. Neutrophilia may have altered the population of cells that we were evaluating, and been responsible for the observed decline in GR activity. Glutathione reductase activity in divergent populations of bovine leukocytes has not been described.

Plasma Malondialdehyde

Accumulation of lipid peroxidation products has previously been associated with mortality and morbidity in shipped cattle. Chirase et al. (2004) found that serum MDA concentrations in calves tripled after transportation. More importantly, prevalence of respiratory disease was found to be positively connected with MDA, and calves that died after transport had 44% higher MDA than surviving calves.

In the current study, C calves tended ($P = 0.062$) to have greater plasma MDA concentrations than NC steers (21.2 ± 2.0 and 14.3 ± 2.0 $\mu\text{M/L}$, respectively). As previously stated, C steers also tended to have lower plasma Se, a mineral important to synergistic functioning of antioxidant systems (Miller et al., 1993). Why this relationship was observed without detecting any treatment effects for the Se dependent enzyme, GSH-Px, is difficult to explain, but it may relate to the essentiality of Se in another antioxidant enzyme. Thioredoxin reductase is also a selenoprotein, which, like GSH-Px, has the ability to reduce lipid hydroperoxides to less reactive substrates (water and alcohols). Primary oxidation products, such as lipid hydroperoxides, are formed in pathways leading to the creation of secondary lipid oxidation products like MDA. Although thioredoxin reductase was not quantified in the current trial, O'Boyle et al. (2006) suggested that the enzyme might be a more accurate gauge of oxidant status in mid-lactation dairy cows than GSH-Px. Sordillo et al. (2007) described a simultaneous decrease in thioredoxin reductase activity and total antioxidant potential in PBMC of transitioning cows, and suggested that the enzyme was important to the antioxidant defense in this population of white cells.

A day effect, and treatment x day interaction was detected for plasma MDA (Table 4.2). Both C and FL steers had higher plasma MDA on d 0 than NC steers ($P < 0.05$). This elevation in lipid peroxidation of C steers persisted on d 1, as MDA remained greater than that in NC steers ($P = 0.015$). Malondialdehyde in FL and NC steers decreased from d -7 to d 0 ($P < 0.015$), whereas C steer MDA was greater on d 0 than d -7 ($P < 0.01$). In addition, MDA of NC and FL steers was unchanged from d 0 to d 1 ($P > 0.8$), while MDA in C steers decreased ($P < 0.05$). Plasma MDA of all steers was greatest

Table 4.2 Least squares means with pooled standard errors for plasma malondialdehyde¹ from control (C), fenceline (FL), and nose-clip (NC) weaned steers on days -7, 0, 1, and 7 surrounding weaning

	Day				SE
	-7	0	1	7	
C	21.9 ^{Aa}	25.8 ^{Ab}	22.9 ^{Aa}	14.0 ^{Ac}	2.28
FL	23.9 ^{Aa}	20.2 ^{Ab}	19.9 ^{ABb}	11.2 ^{Ac}	2.28
NC	20.6 ^{Aa}	13.8 ^{Bb}	14.9 ^{Bb}	8.0 ^{Ac}	2.28

¹Plasma malondialdehyde in micromoles per liter.

^{A,B,C}Means within day with no common superscript differ by treatment ($P < 0.05$).

^{a,b,c}Means within treatment with no common superscript differ by day ($P < 0.05$).

on d -7 ($P = 0.01$), intermediate on d 0 and d 1, and lowest on d 7 ($P < 0.001$; Table 4.1). The failure to detect any increase in plasma MDA ($P = 0.40$) in response to transport (d 0 to d 1) was not expected. Chirase et al. (2004) observed increased MDA in calves transported 1,930 km, a distance that is approximately 11 times greater than the distance of transport in the current study. In addition to the data presented by Chirase et al. (2004), amplification of lipid peroxidation in reaction to shipping stress has been described elsewhere. Wernicki et al. (2006) reported large increases in plasma TBARS on d 1 to 3 after transportation, before witnessing a gradual decline on the 6th d, and a return to baseline levels on the 9th d post-transport. Oxidative stress associated with cattle transport has also been evidenced by excessive accumulation of leukocyte lipid oxidation products (Urban-Chmiel, 2006). The lower MDA concentration detected on d 7 in steers from the current study is an effect that is similar to the post-shipping decline in lipid oxidation seen by Wernicki et al. (2006). Still, other day effects are not in agreement with what would be expected from the literature. Elevated plasma MDA on d -7 could be explained by cold and rainy environmental conditions on that day. Layout of facilities required that the weanlings be crowded into sorting alleys and exposed to these stressful conditions before pre-weaning treatments could be applied and blood could be collected. In addition, sample handling time may have masked day effects occurring between the first two samplings and the last two samplings of the study. All blood samples were placed on ice immediately after collection, but samples from Steele's Tavern (d -7 and 0) had to be transported for approximately 1.5 h before they could be processed and frozen at the lab. Some lipid oxidation within the sample could have occurred during this period. Transit time for samples collected at Kentland Farm (d 1 and 7) was only 20 min. Finally, it is

important to point out that the present study assessed MDA via a different methodology than those utilized in the cited trials. Wernicki et al. (2006) and Urban-Chmiel (2006) quantified MDA as TBARS, whereas Chirase et al. (2004) used high-pressure liquid chromatography. Thiobarbituric acid reactive substances detect a wide range of lipid peroxidation products, and are rather nonspecific for MDA (Griffiths et al., 2002). Conversely, high-pressure liquid chromatography would be expected to be highly specific, and perhaps, more accurate than the spectrophotometric procedures that were employed in the present trial.

Calf Weight Gain

Calf weights were influenced by day ($P < 0.001$) and treatment x day ($P < 0.02$). No treatment effects were detected for calf weights over the course of the study ($P = 0.81$). Body weight of C and FL steers increased on each measurement day, whereas body weights of NC steers were not different between d -7 and 0, but increased on d 7 (Table 4.3). Nicol (1977) suggested that fenceline weaning has no effect of calf weight at 2, 5, or 20 d after separation (Nicol, 1977). In that trial, fenceline separated calves only weighed more than control calves on the first day after weaning. Conversely, Price et al. (2003) reported greater cumulative weight gains in calves separated by a fenceline for 7 d, than in abruptly separated calves. Gradual weaning effects on weight were still evident at 2 and 10 wk post-weaning in that study, and were noted regardless of calf preconditioning (bunk-breaking or not), and environment (pasture or drylot). Haley et al. (2005) found that calves gradually weaned via the aid of nose clips gained more weight than traditionally weaned calves in the first 8 d after weaning. This effect was noted when nose clips were attached for 3, 5, or 14 d.

Table 4.3 Least squares means with pooled standard errors for body weight¹ of control (C), fenceline (FL), and nose-clip (NC) weaned steers on days -7, 0, and 7 surrounding weaning

Treatment ²	Day ³			SE
	-7	0	7	
C	244.5 ^a	253.2 ^b	264.8 ^c	6.15
FL	241.2 ^a	247.1 ^b	264.7 ^c	6.15
NC	243.1 ^a	245.4 ^a	262.3 ^b	6.15

¹Body weight in kilograms.

²Overall, no treatment effects were detected for steer weight ($P = 0.81$).

³Steer weight increased with time ($P < 0.02$).

^{a,b}Means within treatment with no common superscript differ by day ($P < 0.05$).

IMPLICATIONS

Negligible effects of gradual weaning by fenceline or nose clip were detected in the present study, suggesting that management strategies employing two-stage weaning have little influence over biomarkers of oxidative stress that were examined. Moreover, no benefit of gradual weaning on post-weaning weight gain was observed. Leukocyte GSH-Px activity during weaning and transport was more static than predicted, and was not reflective of short-term Se status. Conversely, GR activity in leukocytes was diminished during gradual weaning and shipping. Short-term Se status may play some role in alleviating lipid oxidation, regardless of GSH-Px activity. Lipid oxidation assessed as plasma MDA was difficult to predict under the conditions of the current experiment. Although plasma MDA was lowered after a rest period following calf transport, no effect of transport may be seen if prior stressors have already elevated this marker. No steers were treated for illness during the trial, suggesting that steers had adequate antioxidant defenses to deal with oxidative insults that they may have encountered. In addition, stressors encountered by calves in the present experiment may not have been as severe as those described in previous trials.

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CHAPTER V

EFFECT OF FORAGE OR GRAIN FINISHING ON PRE-HARVEST ANTIOXIDANT STATUS, AND RELATIONSHIP TO LIPID OXIDATION IN GROUND BEEF PRODUCED

ABSTRACT: Forages contain natural antioxidants, which may be beneficial to beef production systems in terms of improving both animal health and marketable product. Forty-eight crossbred Angus steers (323 ± 38 kg) were used to assess the influence of grain- and forage-based diets on redox balance pre- and post-harvest. Steers were fed a high concentrate diet (CON) or stocked on alfalfa (*Medicago sativa*; ALF), pearl millet (*Pennisetum glaucum*; PM), or naturalized (NAT) pastures (n = 12 per treatment). They were bled prior to harvest to determine serum β -carotene, α -tocopherol, and γ -tocopherol, along with plasma malondialdehyde and trolox equivalent antioxidant capacity (TEAC). After harvest, a sub-set of the steers (n = 4 per CON, ALF and NAT treatments) were used to determine β -carotene and α -tocopherol content of *longissimus* tissue, along with crude fat and d 1, 4, and 7 thiobarbituric acid reactive substances (TBARS) in ground beef. Prior to harvest, β -carotene and α -tocopherol were higher in forage-fed steers, but γ -tocopherol was higher in CON steers. Malondialdehyde was lower in steers grazing PM than in all other steers ($P < 0.05$), whereas TEAC in steers grazing PM was higher (CON and NAT; $P < 0.05$), or tended to be higher (ALF; $P < 0.06$) than in other treatments. In ground beef, CON steers had lower β -carotene and α -tocopherol ($P < 0.001$), but higher crude fat ($P < 0.001$) and TBARS ($P < 0.005$) than forage-finished steers. Lipid oxidation increased in all ground beef over time ($P < 0.005$). The β -carotene and α -tocopherol

concentrations of beef were also higher ($P < 0.01$) in steers grazing NAT compared to those grazing ALF, but no differences in TBARS were detected ($P = 0.52$). Serum α -tocopherol and β -carotene were correlated ($P < 0.05$) to beef α -tocopherol and β -carotene ($r = 0.66$ and 0.88 , respectively). Results indicated that forages promoting TEAC may decrease malondialdehyde and protect cattle against oxidative stress. Antioxidants like α -tocopherol and β -carotene protected forage-finished beef from lipid peroxidation.

Key words: antioxidants, cattle, finishing, forage, lipid, oxidation

INTRODUCTION

Forage-based production systems can alter the fat composition of cattle tissues, resulting in beef products that possess greater percentages of PUFA (Laborde et al., 2002; Steen et al., 2003; Martin and Rogers, 2004; Mir et al., 2004; Noci et al., 2005). Lower $n - 6:n - 3$ ratios and higher PUFA:saturated fatty acid ratios present in forage-produced products are considered healthier from a human nutrition standpoint. However, the unique double-bond character exhibited by unsaturated lipids make PUFA particularly susceptible to chain-propagated autoxidation reactions (Scollan et al., 2005; Scollan et al., 2006). Upon oxidation, lipids can create “warmed-over flavor”, “rancidity”, or, more generally, “meat flavor deterioration” (Gray and Crackel, 1992). Forage-finished beef has been cited as possessing off-flavors that are characteristic of lipid oxidation (Wood et al., 2003; Campo et al., 2006; Scollan et al., 2006), a finding that may relate to the accrual of lipid oxidation products over time (Reverte et al., 2003). In contrast, fat-soluble vitamins such as α -tocopherol and β -carotene are available in forages. These vitamins may accumulate in tissues (Simonne et al., 1996; Yang et al., 2002a; Gatellier et al., 2004;

Muramoto et al., 2005) and, because of their antioxidant capacity, play a role in counterbalancing oxidation of lipids in forage-fed beef (Mercier et al., 2004; Realini et al., 2004a; Descalzo et al., 2005; Campo et al., 2006).

Much of the current literature indicates that the antioxidant character of forage-finished beef may be able to compensate for the propensity of its lipids to oxidize. However, understanding the appropriate balance of antioxidants required for this effect is difficult, and is confounded by the synergistic nature in which antioxidants work. For this reason, assessment of total antioxidant capacity of biological samples is desired. Little information is available regarding the effect of forage finishing on pre-slaughter antioxidant status, an effect that may relate to both animal health and well-being, and to post-harvest oxidation potential of tissues. A two-phase trial was conducted to test the hypothesis that ground beef from cattle finished on forage-based diets has less lipid oxidation during display, and that this resistance to oxidation is related to greater pre-slaughter antioxidant status. We hypothesized that finishing diets promoting greater antioxidant status would be coupled with less plasma lipid oxidation pre-mortem. Objectives of phase 1 were to compare antioxidant status and plasma lipid oxidation of cattle finished on a traditional, high concentrate-based feedlot diet to that of cattle finished on forage diets based on three different types of pasture. Objectives of phase 2 were to associate antioxidant status with lipid oxidation of ground beef produced.

EXPERIMENTAL PROCEDURES

Experimental Design

General. Forty-eight Angus crossbred steers from the Shenandoah Valley Agriculture Research and Extension Center (Steele's Tavern, VA) were winter stockered

from early December 2005 through early April 2006 at West Virginia University's Reedsville Farm, (Arthurdale, WV) on forage systems designed to produce an ADG of 0.45 kg. Prior to the spring and summer finishing phase, steers (323 ± 38 kg) were blocked by weight and allotted to one of 4 treatment diets: traditional, grain-based concentrate (**CON**; Appendix 12), pearl millet (*Pennisetum glaucum*; **PM**), alfalfa (*Medicago sativa*; **ALF**), and naturalized pasture (**NAT**). Naturalized pasture was composed of Kentucky bluegrass (*Poa pratensis* L.), orchardgrass (*Dactylis glomerata* L.), tall fescue (*Festuca arundinacea* Schreb.) and white clover (*Trifolium repens* L.). Each treatment consisted of 3 replicates, with 4 steers per replicate and a total of 12 steers per treatment. The finishing phase ran from April 6, 2006 through September. The CON steers were finished at the Shenandoah Valley Agriculture Research and Extension Center on dry-lot, and fed individually using electronic gates (American Calan, Northwood, NH). Forage finished steers grazed naturalized pasture at Willow Bend Farm (Union, WV) from early April through mid August, prior to application of the forage finishing treatment. On August 15, 2006, forage-finished steers began grazing their respective finishing forage, and remained on these forages until harvest on October 2, 2006. Finishing forages were rotationally grazed with dry matter allotment to ensure ad libitum intake. Steers on pasture had free access to a commercial mineral mixture (Vigortone No. 35S, North American Nutrition Companies, Inc., P.O. Box 5002, Lewisburg, OH 45338-5002; Appendix 13) free choice at all times. While grazing alfalfa, cattle were also allowed a commercial bloat block (Bloat Guard, Sweetlix, P.O. Box 8500, Mankato, MN 56002; Appendix 14). Steers on pasture were de-wormed (Ivomec-Eprinex, Merial Limited, 2100 Ronson Road, Iselin, NJ 08830), and received fly-control

treatment via commercial pour-on products (Durasect II, Pfizer Animal Health, Exton, PA 19341; Elector, Elanco Animal Health, A Division of Eli Lilly and Company, Indianapolis, IN 46285) throughout the grazing season. All procedures involving animals during the study were approved by respective institutional Animal Care and Use Committees, and all medicinal slaughter regulations were followed.

Phase 1. In this phase, the effect of finishing diet on pre-slaughter antioxidant status and plasma lipid peroxidation was evaluated. Steers finished on forage at Willow Bend Farm (PM, ALF, and NAT) were bled by jugular venipuncture on August 15, 2006, and, 44 d later on September 28, 2006. At each sampling time, CON steers at the Shenandoah Valley Agriculture Research and Extension Center were bled similarly, but one day after the forage-fed steers (on August 16, and September 29, 2006, respectively). Blood samples were collected into evacuated tubes containing heparin, and additive-free evacuated tubes (Becton Dickinson, Franklin Lakes, NJ). Heparinized blood was placed immediately on ice for transport to the laboratory, whereas blood in additive-free tubes was wrapped in aluminum foil to exclude light, and then allowed to clot at ambient temperature. Upon arrival at the laboratory, heparinized blood was centrifuged for 5 min at 2500 x g and 4 °C. Plasma was removed and stored at -70 °C. Serum was separated from clotted samples by centrifugation for 10 min at 3000 x g, and was stored at -70 °C in amber, light-blocking vials.

Total antioxidant status of plasma was assessed as trolox equivalent antioxidant capacity (TEAC; Randox Laboratories Ltd., Oceanside, CA) on an Olympus AU-400 clinical chemistry analyzer (Olympus America Inc., Center Valley, PA) at the Virginia-Maryland Regional College of Veterinary Medicine Veterinary Teaching Hospital. The

TEAC assay evaluates the ability of a biological sample to reduce the radical cation, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Miller et al., 1993; Prior and Cao, 1999). This radical is generated via ferryl myoglobin derived from metmyoglobin reacting with hydrogen peroxide, and has absorption maxima at 650, 734 and 820 nm. The ability of antioxidants in plasma to inhibit spectra formation is compared to that of the water-soluble vitamin E analogue, trolox, and TEAC is expressed as mmol trolox equivalents per liter of plasma.

Lipid peroxidation in plasma was assessed as malondialdehyde (MDA), and was determined with colorimetric assay kits (OXIS International, Portland, OR). Briefly, MDA concentration was measured by recording the formation of a spectrophotometrically-detectable carbocyanine dye, which results from the reaction of MDA with N-methyl-2-phenylindole at 45 °C (Gerard-Monnier et al., 1998). Sample MDA was compared to a curve generated using 1,1,3,3-tetramethoxypropane as the standard.

Serum was pooled by replicate (3 per treatment) for analysis of the dietary antioxidants, β -carotene, α -tocopherol, and γ -tocopherol. Pooled serum (1.0 mL) was spiked with internal standards of α -tocopherol acetate and retinol acetate (Sigma-Aldrich, St. Louis, MO). Spiked serum was vortexed with the addition of 0.01% butylated hydroxytoluene in ethanol (800 μ L; Sigma-Aldrich, St. Louis, MO), extracted twice with hexane (1.0 mL; Honeywell Burdick & Jackson, Morristown, NJ), and evaporated under N flow. Samples were then resuspended in cyclohexane (200 μ L; EMD Chemicals Inc., San Diego, CA) and filtered through a 0.2 μ m micropore PVDF membrane (Pall Corporation, East Hills, NY) before HPLC injection. The α - and γ -tocopherol

concentrations of samples were determined by normal phase HPLC using an Agilent Zorbax-Si (150 x 4.6 mm) column and an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) with wavelength settings of 295 and 330 nm for excitation and emission, respectively. The injection volume was 10 μ L and the mobile phase was (99.7:0.3) hexane:isopropanol (EMD Chemicals Inc., San Diego, CA) at a flow rate of 1 mL/min. Concentration of β -carotene in samples was quantified using reverse-phase HPLC with an Agilent Zorbax XDB-C8 (150 x 4.6 mm) column and a Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) with UV-visible wavelength detection set at 450 nm. The injection volume was 10 μ L and the eluent was methanol (Honeywell Burdick & Jackson, Morristown, NJ) with a flow rate was 1.5 mL/min. Samples were run in duplicate, and antioxidant concentrations were calculated based on peak area of external standards (Sigma-Aldrich, St. Louis, MO) and percent recovery of the appropriate internal standard.

Phase 2. In this phase, antioxidant status was related to the oxidation of lipid in post-harvest tissue. On October 3, 2006 steers were transported to a commercial slaughterhouse (Cargill Meat Solutions Corporation, Wyalusing, PA) and harvested. Rib sections of carcasses were removed, vacuum-packaged, and shipped at 4 °C to the Clemson University Meat Laboratory. Rib sections from 4 steers each from CON, NAT, and ALF treatments were selected at random, and at 14 d post-harvest were unwrapped. The 9-10-11th rib section was removed and dissected into lean, fat and bone (Hankins and Howe, 1946). The lean was ground once and formed into patties. Sample patties were placed on styrofoam trays, overwrapped with oxygen permeable film, and stored at 4 °C in a lighted cooler. Oxidative stability of lipid in ground patties was determined by

measuring 2-thiobarbituric acid reactive substances (**TBARS**) at 1, 4, and 7 d post-grinding via the fluorometric method of Jo and Ahn (1998). Results were expressed as mg of MDA produced per kg of sample. Total lipid content of ground lean samples were determined after drying at 95°C for 24 h using the Ankom XT15 Extraction System (Ankom Technology, Macedon, NY) and hexane as the solvent. The α -tocopherol and β -carotene concentrations of *longissimus muscle* (**LM**) samples were determined according to Liu et al. (1996) using a Shimadzu Prominence HPLC with diode array detector (Shimadzu, Columbia, MD).

Statistical Analysis

Phase 1. Pre-harvest plasma MDA and plasma TEAC were analyzed using PROC MIXED with steer as the experimental unit, treatment as a main effect, and treatment replication as a random effect (SAS Inst. Inc., Cary, NC). August values for MDA and TEAC served as covariates and means were separated via the Tukey-Kramer adjustment. Significance was determined at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$. Concentration of β -carotene, α -tocopherol, and γ -tocopherol in pooled serum was not statistically analyzed, but is presented in Table 5.1 for comparison.

Phase 2. Post-harvest β -carotene and α -tocopherol concentration in LM, along with total lipid in ground beef were analyzed using PROC GLM with steer as the experimental unit, and treatment as a main effect (SAS Inst. Inc., Cary, NC). Ground beef TBARS were analyzed as repeated measures in PROC MIXED with steer as the experimental unit. Treatment and time were main effects, and the Tukey-Kramer adjustment was used to separate significant time effects. (SAS Inst. Inc., Cary, NC). Orthogonal contrasts (CON vs. forages, and ALF vs. NAT) were used to compare

differing ($P < 0.05$) treatment means for β -carotene, α -tocopherol, total lipid, and TBARS. Pearson correlation coefficients for pre-harvest serum antioxidants and respective post-harvest concentrations in LM were determined using PROC CORR (SAS Inst. Inc., Cary, NC).

RESULTS

Phase 1. Treatment effects were detected for both plasma MDA, and plasma TEAC. Steers finished on PM had lower plasma MDA than CON, ALF, and NAT finished steers ($P < 0.05$; Figure 5.1). Correspondingly, PM steers had greater plasma TEAC than CON or NAT steers ($P \leq 0.05$; Figure 5.2), but only tended to have greater plasma TEAC than ALF ($P = 0.057$) steers. Plasma MDA and plasma TEAC did not differ between CON, ALF, and NAT steers. Analysis of pooled serum samples revealed that steers finished on forages had serum β -carotene and α -tocopherol concentrations that were numerically greater than those in CON steers, yet had serum γ -tocopherol concentrations that were numerically lower than CON steers (Table 5.1). Serum γ -tocopherol appeared to numerically increase in the CON steers over time, whereas serum β -carotene numerically increased in steers being finished on forages.

Phase 2. As expected, finishing treatment altered redox balance of muscle tissue samples post-harvest. Concentrations of both β -carotene and α -tocopherol were greater ($P < 0.001$) in LM samples taken from steers finished on forages than in samples taken from CON steers (Table 5.2). Type of forage further affected meat antioxidants, as NAT steers had greater concentrations of LM β -carotene ($P < 0.001$) and α -tocopherol ($P = 0.008$)

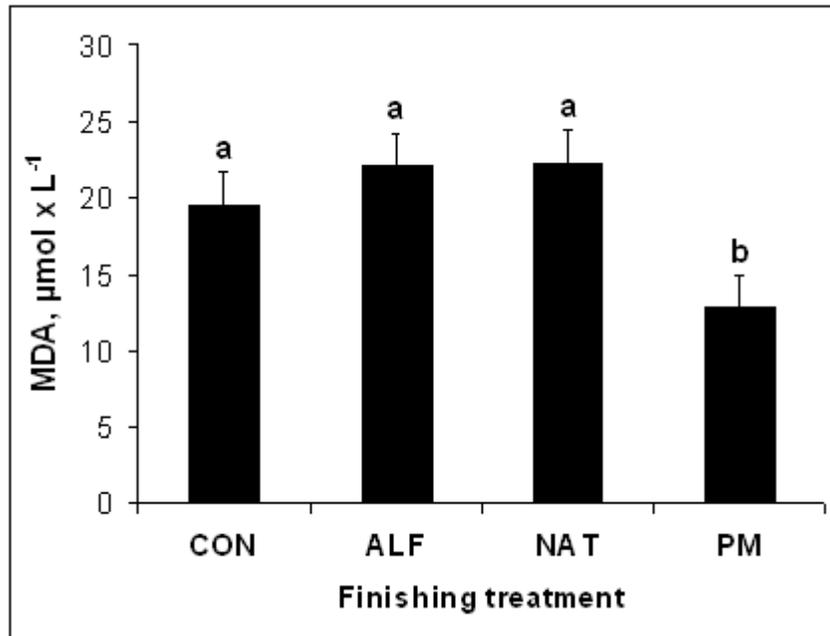
Table 5.1 Mean β -carotene, α -tocopherol, and γ -tocopherol in pooled¹ serum collected in August and September²

Antioxidant	Treatment			
	Concentrate	Alfalfa	Pearl millet	Naturalized
<u>beta-carotene, ppm</u>				
August	0.27	3.55	3.67	3.73
September	0.15	8.29	8.69	7.49
<u>alpha-tocopherol, ppm</u>				
August	3.66	5.59	8.25	6.28
September	3.52	7.14	5.86	6.23
<u>gamma-tocopherol, ppb</u>				
August	200	51.7	35.8	58.9
September	277.5	43.3	41.9	26.2

¹Serum was pooled by replicate (3 pools per treatment).

²Collected on August 15, 2006 and September 28, 2006 for steers fed forages, and on August 16, 2006 and September 29, 2006 for steers fed concentrate.

Figure 5.1 Least squares means (\pm SE) for pre-harvest plasma¹ malondialdehyde (MDA) in steers from different finishing treatments²

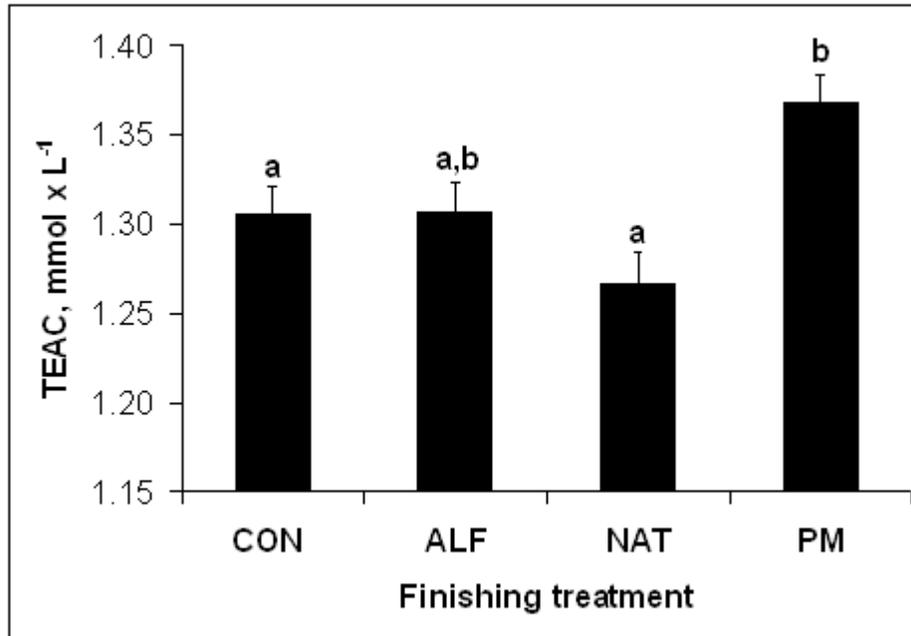


¹Plasma collected on September 28, 2006 from steers fed forage, and on September 29, 2006 from steers fed concentrate.

²($n = 12$) steers each from concentrate (CON), alfalfa (ALF), naturalized pasture (NAT), and pearl millet (PM) finishing treatments.

^{a,b}Least square means without common superscripts differ ($P < 0.05$).

Figure 5.2 Least squares means (\pm SE) for trolox equivalent antioxidant capacity (TEAC) of pre-harvest plasma¹ in steers from different finishing treatments²



¹Plasma collected on September 28, 2006 from steers fed forage, and on September 29, 2006 from steers fed concentrate.

²(n = 12) steers each from concentrate (CON), alfalfa (ALF), naturalized pasture (NAT), and pearl millet (PM) finishing treatments.

^{a,b,c}Least square means without common superscripts differ ($P < 0.05$).

than ALF steers. Crude fat was greater ($P < 0.001$) in ground beef from CON steers than from forage-finished steers, but was not different ($P = 0.37$) between ALF and NAT treatments (Table 5.2). Ground beef TBARS were greater in beef from CON steers than in beef from steers finished on forages ($P < 0.005$), but was not different between ALF and NAT treatments ($P = 0.52$; Figure 5.3). Compared to d 1 post-grinding, TBARS in beef from all treatments were greater on d 4 and 7 post-grinding ($P < 0.005$), but not different between d 4 and 7 ($P = 0.24$).

As expected, pre-harvest concentrations of antioxidants in serum were positively correlated with their respective concentrations in LM after harvest (Table 5.3). Serum β -carotene was correlated ($r = 0.88$; $P < 0.001$) to LM β -carotene, and serum α -tocopherol was correlated ($r = 0.66$; $P = 0.03$) to LM α -tocopherol.

DISCUSSION

Phase 1. Antioxidants such as α -tocopherol and β -carotene are present in fresh, vegetative forages (Hess, 1993; Noziere et al., 2006a). Because of the greater concentration of these fat-soluble vitamins in forages, grazing animals are generally expected to have greater antioxidant status than animals consuming preserved forages. Numerically greater concentrations of α -tocopherol and β -carotene in serum from steers finished on forages in the present experiment support this concept. Plasma β -carotene and α -tocopherol are greater in pasture-stocked cattle than in contemporaries fed grain-based diets (Yang et al., 2002a; Descalzo et al., 2005). Plasma α -tocopherol is depleted within 6 wk (Arnold et al., 1993), whereas serum β -carotene is depleted within 2 wk (Yang et al., 1993), when steers are transitioned from forage-based to concentrate-based diets. Less is known about the concentration of γ -tocopherol in the blood of grazing cattle,

Table 5.2 Least squares means and contrasts for post-harvest variables in beef of steers from different finishing treatments

Variable	Treatment ¹			SE ²	P -value ³	Contrasts ⁴	
	CON	ALF	NAT			CON vs. Forages	ALF vs. NAT
beta-carotene, µg/g	0.16	1.90	3.62	0.222	<0.001	<0.001	<0.001
alpha-tocopherol,	3.55	7.97	12.30	0.908	<0.001	<0.001	0.008
Crude fat, %DM	24.90	14.12	12.00	1.584	<0.001	<0.001	0.368

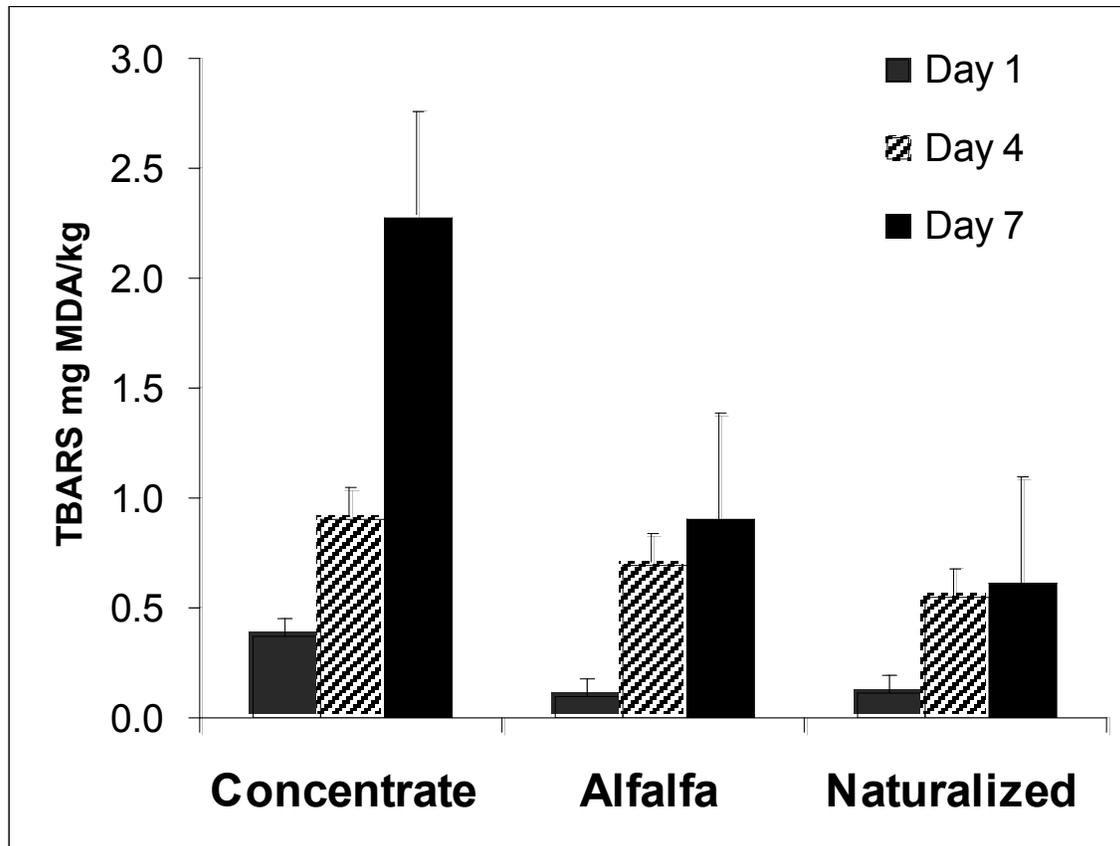
¹CON = concentrate; ALF = alfalfa; NAT = naturalized pasture.

²Pooled standard error of the mean.

³Probability of a treatment effect.

⁴P-values of respective contrasts.

Figure 5.3 Least squares means (\pm SE) for thiobarbituric acid reactive substances TBARS in ground beef on days¹ 1, 4, and 7 after grinding from steers fed concentrate, alfalfa, and naturalized pasture finishing treatments²



¹d 1 less than d 4 ($P < 0.001$) and d 7 ($P < 0.005$); d 4 versus d 7 ($P = 0.24$).

²Concentrate versus forages ($P < 0.005$); alfalfa versus naturalized pasture ($P = 0.52$).

Table 5.3 Pearson correlation coefficients for pre-harvest serum antioxidants¹ and post-harvest muscle antioxidants² from steers³ for which thiobarbituric acid reactive substances (TBARS) analysis was conducted

	serum alpha-tocopherol and <i>longissimus</i> alpha-tocopherol	serum beta-carotene and <i>longissimus</i> beta-carotene
<i>r</i>	0.66	0.88
<i>P</i> -value	0.03	<0.001

¹Serum collected on September 28, 2006 from steers on forage, and on September 29, 2006 from steers on dry-lot.

² α -tocopherol and β -carotene measured in tissue taken from the *longissimus dorsi*.

³(n = 4) steers each from concentrate, alfalfa, and naturalized pasture finishing treatments.

but Al-Senaidy (1996) found that plasma α -tocopherol concentration was 3.5 times greater than plasma γ -tocopherol in randomly selected bulls. Information regarding the diet being fed to these bulls was not available. Prior to harvest, the ratio of α -tocopherol to γ -tocopherol in pooled serum from the present study ranged from 13 in CON steers, to 173 in steers consuming forages. Although dietary intake of respective tocopherols was not assessed in the current experiment, dissimilar concentrations of α -tocopherol and γ -tocopherol in forages versus concentrates best explains the numerically higher γ -tocopherol content of serum from CON steers. Corn is higher in γ -tocopherol than in α -tocopherol (Hidioglou et al., 1992; Goffman and Bohme, 2001), whereas γ -tocopherol is often less than α -tocopherol in leaf tissue (Burrows and King, 1968; Hess, 1993).

To appreciate oxidant-induced damage prior to harvest, the present experiment compared pre-harvest plasma lipid oxidation in cattle finished on CON or various forages. Plasma MDA was not different in CON steers compared to ALF and NAT steers, so the lower α -tocopherol and β -carotene content of CON plasma was not reduced to the point of promoting oxidative stress. However, steers finished on PM had lower MDA than CON, ALF, and NAT steers prior to harvest, indicating a lower propensity for oxidative damage of cellular membranes *in vivo*. The lower concentration of MDA in PM steers was likely a function of their greater plasma TEAC. Plasma TEAC intends to reflect the contribution of all antioxidants, working synergistically, to reduce oxidative species in plasma. Analysis of α -tocopherol, β -carotene, and γ -tocopherol in serum from PM steers revealed no striking discrepancies of these antioxidants from those in serum of steers finished on other forages, so the greater antioxidant capacity of PM steers was likely due to the presence of other, unidentified low-molecular-weight antioxidants. A

wide range of natural phenolic and polyphenolic compounds are present, albeit in variable proportions, in leaf tissue of vascular plants (Bertram et al., 1980; Lewis, 1993). Pearl millet pastures may have supplied greater amounts of these natural antioxidants to PM steers, thereby elevating their overall antioxidant status and preventing lipid oxidation. O'Grady et al. (2006) included natural phenolic compounds from tea catechins and rosemary extract in a barley-based concentrate ration being fed to bullocks, but found that plasma TEAC was unaffected by antioxidant supplementation over a 90 d finishing period, possibly because of the relatively low level of antioxidant inclusion in the ration. Plasma TEAC in steers is also unaltered by inclusion of PUFA in the diet (Scislowski et al., 2005), and TEAC of LM extract is not changed when cattle are finished on pasture instead of concentrates (Gatellier et al., 2004). Castillo et al. (2003) observed cows in peak or late lactation and found that lipid hydroperoxides were more concentrated in plasma of cows in peak lactation, but that plasma TEAC was not changed. These authors suggested that lipid peroxidation was increased by oxidative stress associated with increased metabolic demand, and that hydroperoxides accumulated because of insufficient TEAC. Other researchers observed greater plasma TEAC in lactating cows versus dry cows, and proposed that the finding was due to the high levels of vitamin A and E included in the lactation diet (Mandebvu et al., 2003). Their work also noted seasonal effects on plasma TEAC that may have been associated with availability and antioxidant content of forages. The present study is the first report of a forage-based diet increasing plasma TEAC, and consequently decreasing plasma lipid oxidation in cattle. Reduction of oxidative damage may be beneficial to cattle health and welfare (Chirase et al., 2004).

Phase 2. The relationship between forage-based diets and antioxidant concentrations in beef muscle has been well established. The present study confirmed results of previous studies indicating greater amounts of β -carotene (Simonne et al., 1996; Sonon et al., 2005b), α -tocopherol (Yang et al., 2002b; Gatellier et al., 2004), or both (Yang et al., 2002a; Descalzo et al., 2005; Muramoto et al., 2005), in meat from forage-fed cattle, than in meat from concentrate fed cattle. Arnold et al. (1993) suggested that muscle tissue can be saturated with α -tocopherol in 12 to 18 wk, and that greater than 18 wk is required for depletion of muscle α -tocopherol. Yang et al. (2002a) reported that cattle grazing on pasture could achieve concentrations of α -tocopherol in their tissues that were at least as high as those produced by vitamin E supplementation in grain-fed animals. The greater amount of β -carotene and α -tocopherol in forages versus concentrates best explains the antioxidant accumulation in pasture-finished beef during the present study. Our data further indicated that LM from NAT steers have more β -carotene and α -tocopherol than LM from ALF steers. Antioxidant concentrations in forages were not determined, but results suggest that NAT pastures may have been richer than ALF pastures in these antioxidants. Alternatively, even though steers were rotated to ensure *ad libitum* DMI, DM availability of vegetative leaf may have been greater in NAT pastures, leading to greater overall leaf tissue intake.

Given the observed deviation of LM antioxidants, we are left to hypothesize why β -carotene and α -tocopherol concentrations were not numerically different in serum obtained from steers grazing NAT and ALF prior to harvest (Phase 1). Discrepancies may be related to the dynamic nature of these antioxidants in blood compared to muscle. In cattle, β -carotene in plasma or serum increases quickly in response to dietary inclusion

(Seren et al., 1971; Mora et al., 2000), whereas switching cows from high to low carotenoid diets diminishes plasma β -carotene within 2 wk (Noziere et al., 2006b). Likewise, plasma α -tocopherol content is either saturated or depleted faster than muscle α -tocopherol upon change in α -tocopherol content of the diet (Arnold et al., 1993). O'Grady et al. (2001) noted that plasma α -tocopherol was saturated within 2 wk in steers supplemented with 300 IU α -tocopheryl acetate/kg feed. Using multiple levels of supplementation, Arnold et al. (1993) found that cattle supplemented with low amounts of α -tocopherol had greater α -tocopherol in plasma and LM than non-supplemented cattle. However, when low levels of α -tocopherol supplementation were compared to high levels of supplementation, LM α -tocopherol was increased, although plasma α -tocopherol was not. Data from that trial compare favorably to data from the present study if NAT and ALF pastures are considered to be high and lower sources of α -tocopherol, respectively. Liu et al. (1995) suggested that plasma α -tocopherol, unless known to be at steady-state, is not a good indicator of muscle concentration. Despite this assertion, and the afore-mentioned discrepancies between ALF and NAT treatment groups, when all steers were considered, β -carotene and α -tocopherol concentrations in serum collected prior to harvest were positively correlated to β -carotene and α -tocopherol concentration in the LM ($r = 0.88$ and $r = 0.66$, respectively). These correlations confirm a link between plasma and LM antioxidants in the present study. Irie et al. (1999) also reported a correlation ($r = 0.78$) between plasma and muscle α -tocopherol.

Lipid oxidation may explain the negative relationship observed between forage-finished beef and sensory acceptability of beef in previous studies (Schroeder et al., 1980; Melton et al., 1982; Larick et al., 1987). Reverte et al. (2003) froze restructured steaks

from cattle that had been finished on either alfalfa, or a corn supplemented diet. They found that after 3 mo of freezer storage TBARS increased dramatically in steaks from forage-fed cattle, yet remained relatively unchanged in steaks from corn-fed cattle. In steers that were forage or grain-fed, Yang et al. (2002b) observed elevated concentrations of muscle TBARS when tissues from both groups of steers were exposed to aerobic conditions for 7 d. Realini et al. (2004a) noted greater initial TBARS in ground beef from concentrate-fed cattle, but found that TBARS in samples from pastured cattle surpassed TBARS of concentrate-fed beef after 8 d of display.

In the present study, TBARS accumulated in ground beef from all finishing treatments over time, but ground beef from forage-fed steers had less lipid oxidation than beef from CON steers. Oxidative stability of burger from forage-finished steers is likely due, in part, to increased antioxidant content of the meat. Numerous researchers have suggested that the greater α -tocopherol content of forage-finished beef is responsible for preventing TBARS accumulation in comparison to concentrate-finished beef (O'Sullivan et al., 2002; O'Sullivan et al., 2003; O'Sullivan et al., 2004; Realini et al., 2004a; Campo et al., 2006). Other antioxidants present in forage have also been suggested to offer protection against lipid peroxidation in meat. Descalzo et al. (2005) observed 3 times less oxidation in meat from forage-fed steers compared to grain-fed steers, and reasoned that greater amounts of β -carotene and α -tocopherol found in forage-produced beef had a cooperative effect in diminishing lipid oxidation. Additional plant compounds like phytic acid, and flavonoids derived from grass have been postulated to explain lower TBARS in beef when cows were finished on grass rather than cereal grain-based concentrates (Mercier et al., 2004). Inclusion of an antioxidant supplement in the finishing diet of

steers reduces TBARS in ground beef produced (Walenciak et al., 1999). Realini et al. (2004b) found the oxidative stability of ground beef from grain-fed cattle could be improved by vitamin C treatment. Conversely, ground beef from cattle finished on tall fescue could not be improved with vitamin C, possibly because of a greater prior antioxidant concentrations, which prevented oxidation in any of the ground beef, treated or not treated, from fescue-fed cattle (Realini et al., 2004b).

Even though β -carotene and α -tocopherol concentrations were greater in meat from NAT steers, lipid oxidation in ground beef from ALF and NAT steers was not different at any time during the current experiment. The statistical power of the present study was limited to a certain degree by the small sample size, and the magnitude of difference in antioxidant concentration may have been too small to greatly impact TBARS formation. Faustman et al. (1989) concluded that beef was protected against oxidative insult when it contained at least 3.0 μg α -tocopherol/g muscle, whereas Arnold et al. (1993) reported that it was maximally protected with 3.3 μg α -tocopherol/g muscle. Although α -tocopherol concentration in meat from CON steers (3.55 $\mu\text{g/g}$) was close to this critical value, steers from all finishing treatments in our study had tissue concentrations of α -tocopherol that were at least this high. Arnold et al. (1993) investigated the oxidative stability of steaks, whereas our study examined TBARS in ground beef. Grinding of muscle may disrupt the integrity of cellular membranes, which contain high percentages of unsaturated phospholipids. When this occurs, PUFA may be liberated and exposed to oxygen so that antioxidant components of tissues may not be able to override the susceptibility of lipids to oxidize. For satisfactory oxidative stability, ground beef may require greater antioxidant content than steaks. Ground beef from ALF

and NAT steers in our trial may have possessed sufficient antioxidant capacity, whereas ground beef from CON steers did not. Similar reasoning may explain TBARS accumulation in ground beef despite the α -tocopherol concentration achieved (3.91 $\mu\text{g/g}$) in other research (Realini et al., 2004a).

Alternatively, TBARS accumulation may have been greatly affected by overall lipid content of the ground beef. Concentrate-finished beef often possess a greater % fat (DM) compared to forage-finished beef (Miller et al., 1981; O'Sullivan et al., 2003; Realini et al., 2004a; Realini et al., 2004b; Descalzo et al., 2005; Muramoto et al., 2005; Sonon et al., 2005a), and intramuscular fat deposition in steers increases greatly after 3 mo on concentrate feed (Duckett et al., 1993). In the present trial, crude fat was approximately 2 times higher in ground beef from CON steers than ground beef from ALF and NAT steers. Considering autoxidation of lipids, a greater amount of fatty acid substrate could lead to branching of chain-propagated oxidation pathways, causing greater accumulation of peroxidation end products, in spite of the unsaturation index of the ground beef as a whole. Fatty acid composition was not determined in the current trial, but ground beef from CON steers may have had significant total amounts of peroxidisable (unsaturated) fatty acids despite the assumption that the percentage of peroxidisable lipid (as a percentage of total lipid) would be much higher in ground beef from forage-finished steers.

Finally, ground beef from ALF and NAT steers may have been protected by other antioxidants that were not quantified in meat. In trial 1, steers grazing PM had greater plasma TEAC than other steers prior to harvest, despite having numerically similar concentrations of serum α -tocopherol, β -carotene, and γ -tocopherol. Unfortunately, meat

analysis was conducted prior to plasma analysis, and due to limitation of resources, we chose not to include ribs from PM steers in our random sampling. It is uncertain whether the greater total antioxidant capacity of steers grazing PM would have been reflected in the oxidative stability of their muscle tissues.

IMPLICATIONS

Forage-based finishing schemes altered redox balance in cattle, increasing their body stores of dietary antioxidants like β -carotene and α -tocopherol. Pearl millet enhanced plasma total antioxidant status, an effect which reduced lipid peroxidation products in plasma, and may be beneficial to animal health. Pre-harvest β -carotene and α -tocopherol status was correlated with concentrations of the antioxidants in beef, and was linked to prevention of lipid oxidation. Oxidative potential of beef is a multifaceted concept that depends on the delicate balance between antioxidants like β -carotene and α -tocopherol, and prooxidants like unsaturated fats. Often overlooked is the role that total fat amount, rather than percent of unsaturated fatty acids plays on accrual of oxidation products. In line with much of the current literature, our data indicated that the antioxidant character of forage-finished beef made up for the propensity of its lipids to oxidize.

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CHAPTER VI

SUMMARY AND CONCLUSION

Oxidative phenomena play significant roles, affecting stability and function of physiological systems. The concept of oxidative stress is one that has been theorized in depth over the past two decades. Recognizing the pathogenicity of reactive oxygen species, investigators from the biomedical community have associated oxidative stress with a diverse assortment of disorders. Debates over the role of oxidative stress as a cause of pathogenesis, rather than a by-product compounding more fundamental aspects of disease, are often unresolved. A great deal of literature is available to veterinary and animal scientists interested in the impact of redox balance on bovine health and production management. The current thesis supplies a thorough compilation of such literature, while expanding the field of knowledge through investigations of redox status in production scenarios common to Virginia's beef industry.

Information was added to the knowledge-base surrounding endophyte-infected tall fescue consumption and heat stress in cattle. Fescue toxicosis was confirmed via established indicators like increased body temperatures and decreased serum prolactin. For the first time, GR activity, and the reduced to oxidized glutathione ratio (GSH:GSSG) were examined in immune cells from animals grazing endophyte-infected tall fescue under heat stress. Glutathione peroxidase activity and plasma MDA were determined as additional biomarkers of oxidative stress. It was hypothesized that cattle exhibit altered glutathione redox in immune cells as a by-product of heat stress, but that shifts in glutathione redox are not a cause of fescue toxicosis.

In calves weaned using different strategies, neither leukocyte glutathione peroxidase and glutathione reductase, nor plasma lipid oxidation were useful in predicting differences in oxidative stress. Previous research has implicated oxidative stress in the morbidity and mortality of transported cattle, and other biomarkers of oxidative stress might prove to be useful tools when cattle are monitored under more extreme conditions. Still, it should be remembered that there was no need to treat any calves in the present study for shipping fever. Antioxidant defenses of these calves may have been sufficient to prevent oxidative-insult and any stress-related maladies previously linked to oxidative stress.

Finally, work described herein confirms that natural antioxidants derived from forages are useful in pasture-based beef production. Measurement of individual antioxidant compounds in meat is helpful in determining resistance of beef to lipid oxidation. Although not specific, assay of TBARS is a valuable, widely-used tool in ascertaining lipid stability of beef. The trolox equivalent antioxidant capacity test was helpful in establishing that certain forage-based diets may improve overall antioxidant status. Research presented is the first to show that forages increasing total antioxidant status can prevent oxidative stress, decreasing lipid peroxidation *in vivo*. Future investigations may confirm the benefit of such effects from an animal health standpoint.

In conclusion, redox balance in living systems is difficult to quantify, and biomedical scientists must continue to strive for new techniques of assessment. Although the methods employed in the current research by no means represent all of the broad, existing resources, they demonstrate that some already available assays can be useful indicators of oxidative phenomena in cattle.

APPENDIX 1

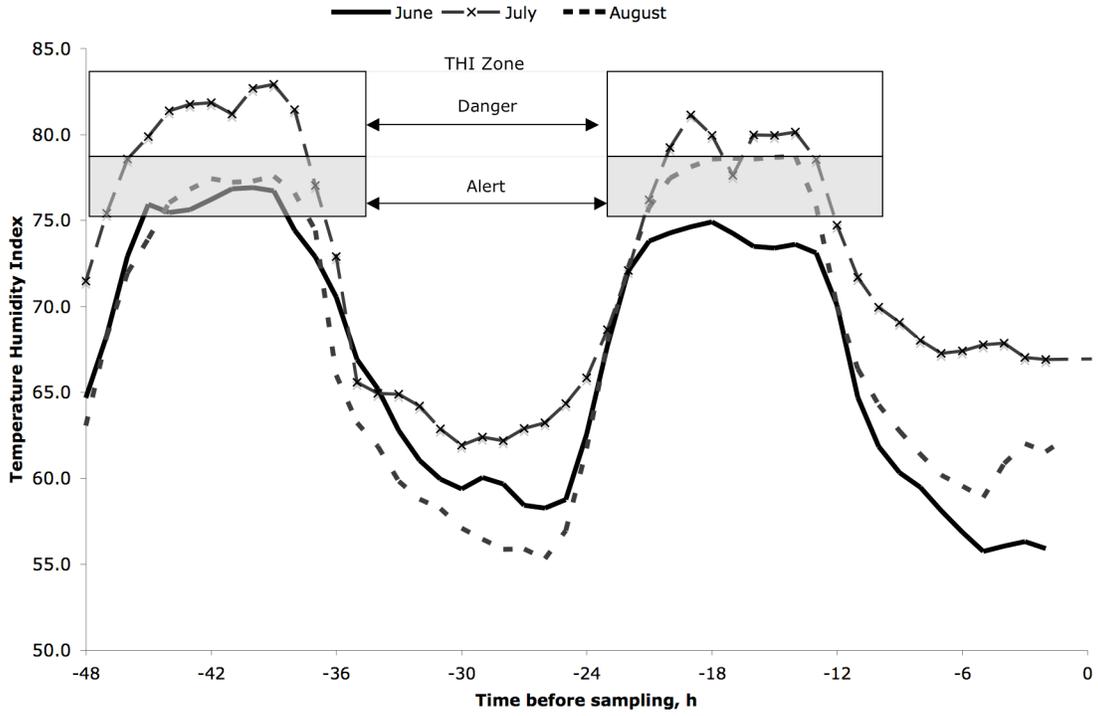
Means (\pm SD) for average temperatures, maximum temperatures, minimum temperatures, and temperature fluctuations recorded by intravaginal temperature loggers in heifers grazing Kentucky 31 wild type endophyte-infected (E+) and Kentucky 31 endophyte-free (E-) tall fescue

	Average, °C	Maximum, °C	Minimum, °C	Flux ^a , °C
June				
E+	38.3 \pm 0.4	39.5 \pm 0.5	37.5 \pm 0.3	2.0 \pm 0.4
E-	38.2 \pm 0.4	39.4 \pm 0.5	37.6 \pm 0.5	1.8 \pm 0.3
July				
E+	38.5 \pm 0.3	40.1 \pm 0.3	37.3 \pm 0.3	2.8 \pm 0.4
E-	38.4 \pm 0.3	40.1 \pm 0.3	37.4 \pm 0.4	2.7 \pm 0.4
August				
E+	38.6 \pm 0.4	39.8 \pm 0.4	37.6 \pm 0.3	2.2 \pm 0.4
E-	38.2 \pm 0.4	39.3 \pm 0.5	37.3 \pm 0.6	2.0 \pm 0.5

^aFlux defined for each heifer as the difference between the maximum temperature recorded and the minimum temperature recorded.

APPENDIX 2

Temperature Humidity Index (THI) during 48 h intravaginal temperature logging



APPENDIX 3

Mean environmental conditions¹ recorded at Kentland Farm over the entire data collection period in June, July and August, 2006

Climatic condition	Daytime			Nighttime		
	June	July	August	June	July	August
Temperature, °C	23.7	26.7	25.0	16.7	19.6	16.7
Relative humidity, %	81.2	83.5	74.8	100	100	100
Sunshine, kilowatts/m ²	0.556	0.604	0.573	n/a	n/a	n/a
THI ²	72.1	77.1	73.2	62.0	67.2	62.0

¹Average ambient conditions for the two days; i.e. the two-day average from 0800 to 1900 inclusive for daytime values, and the two-day average from 2000 to 0700 inclusive for night-time values.

²THI = (0.8 x temperature) + [(% relative humidity/100) x (temperature - 14.4)] + 46.4.

APPENDIX 4

Alkaloid content¹ of wild type endophyte-infected (E+) and endophyte-free (E-) Kentucky 31 tall fescue paddocks before the experiment (2005) and after the experiment (2006)

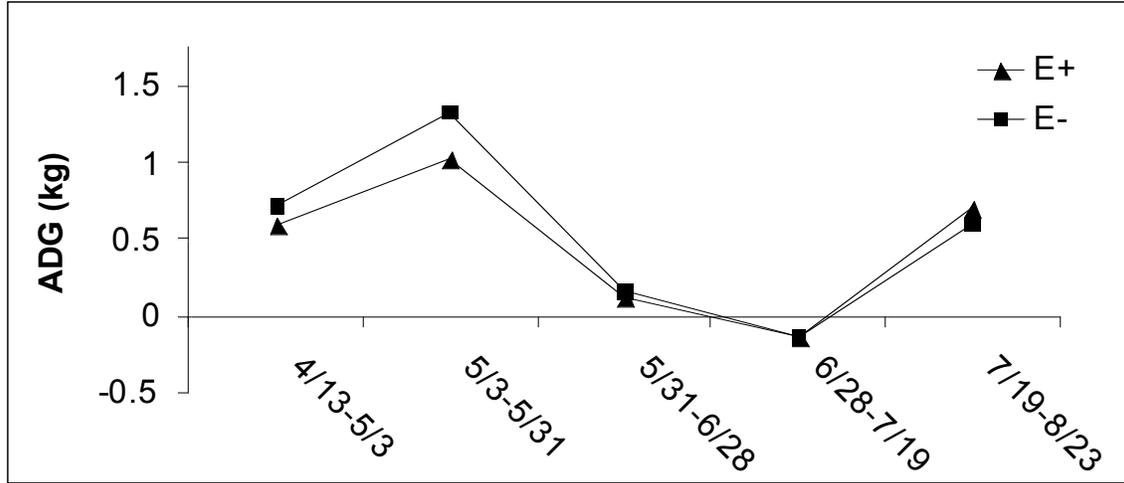
Year	E+ Paddocks				E- Paddocks			
	1	2	3	4	1	2	3	4
2005	1855	797	1075	1254	34	208	30	35
2006 ²	2177	2343	1135	-	181	198	199	-

¹Alkaloid content in nanograms per gram (dry matter basis).

²Alkaloid analysis not available for paddock 4 in 2006.

APPENDIX 5

Average daily gain¹ for heifers grazing wild type endophyte-infected (E+) and endophyte-free (E-) Kentucky 31 tall fescue pastures during the summer of 2006²



¹Average daily gain in kilograms.

²Heifers were stocked together on naturalized pasture on 4/13, and were allotted to treatment pastures on 5/3.

APPENDIX 6

Mean (\pm SD) hematocrit (%) for heifers grazing wild type endophyte-infected (E+) and endophyte-free (E-) Kentucky 31 tall fescue pastures in June, July, and August 2006

	June ¹	July ²	August ³
E+	42 \pm 0.04	39 \pm 3.7	40 \pm 3.2
E-	42 \pm 0.04	39 \pm 2.2	40 \pm 2.9

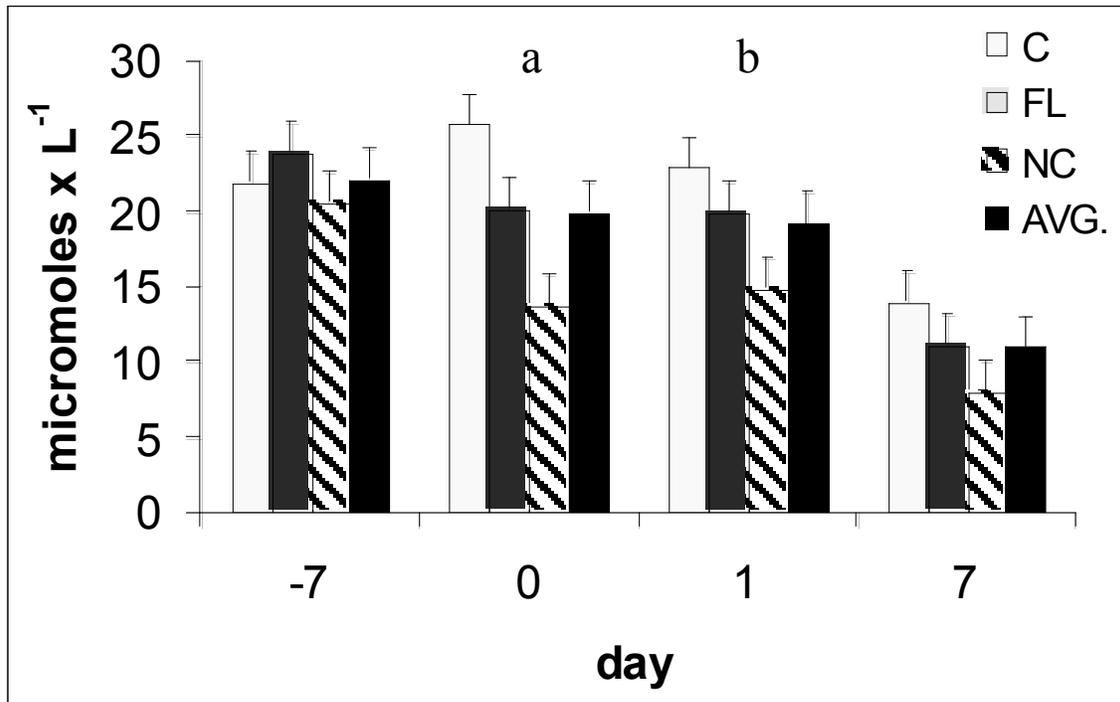
¹June 30, 2006.

²July 21, 2006.

³August 25, 2006.

APPENDIX 7

Average plasma malondialdehyde (MDA) of all treatments*, shown with plasma MDA of control, fenceline, and nose-clip treatments on days -7, 0, 1, and 7



^aControl and fenceline calves different from nose clip calves ($P < 0.05$).

^bControl calves different from nose clip calves ($P = 0.015$).

*Date effect ($P < 0.05$): all calves greatest on d -7, lowest on d 7, and intermediate on d 0 and 1.

APPENDIX 8

To assess the effect that suckling may have on the amount of potentially oxidizable lipid substrate in the blood, plasma samples from control (C) calves 7 d prior to (d -7), and 7 d after (d 7) weaning were assayed for total triglyceride (TG) concentration. Analysis was carried out on an Olympus AU400 (Center Valley, PA) in the clinical pathology lab at the Virginia-Maryland Regional College of Veterinary Medicine teaching hospital. Similarly, the concentration of non-esterified fatty acids (NEFA) in serum from the same calves was obtained from the data of Boland et al. (2007)¹. Serum NEFA, along with plasma TG and malondialdehyde (MDA) are listed in the following table. No dependent relationship of MDA on plasma TG or serum NEFA could be established.

Means (\pm SD) for plasma malondialdehyde, plasma triglycerides, and serum non-esterified fatty acids¹ in control calves seven days prior to, and seven days after weaning

	Day -7 (suckling)	Day +7 (no suckling)
MDA, ^a μ M	21.9 \pm 10.3	14.0 \pm 7.6
TG, ^b mg/dL	22.7 \pm 11.9	26.3 \pm 8.2
NEFA, ^c mEq/L	0.54 \pm 0.20	0.31 \pm 0.16

¹Data obtained from H. T. Boland, G. Scaglia, W. S. Swecker, Jr., and N. C. Burke. Effect of different pre-weaning treatments on behaviour, performance, and blood metabolites of beef calves. Applied Animal Behaviour Science. Submitted for publication: July 2007

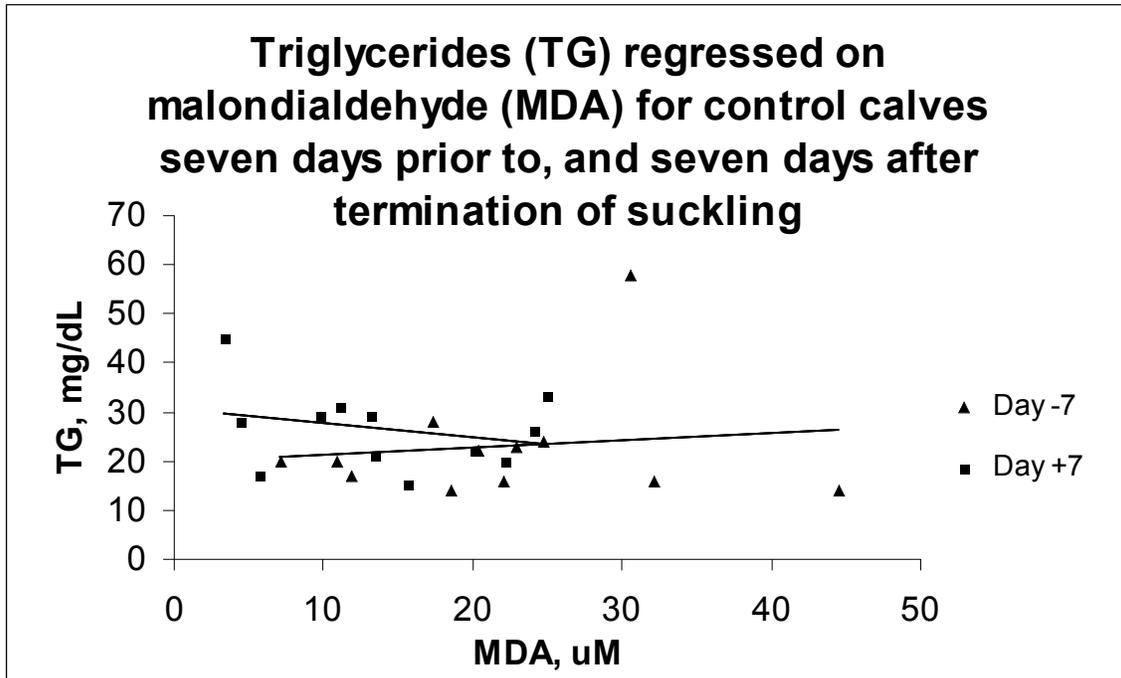
^aPlasma malondialdehyde in micromoles per liter.

^bPlasma triglycerides in milligrams per decilitre.

^cSerum non-esterified fatty acids in milliequivalents per liter.

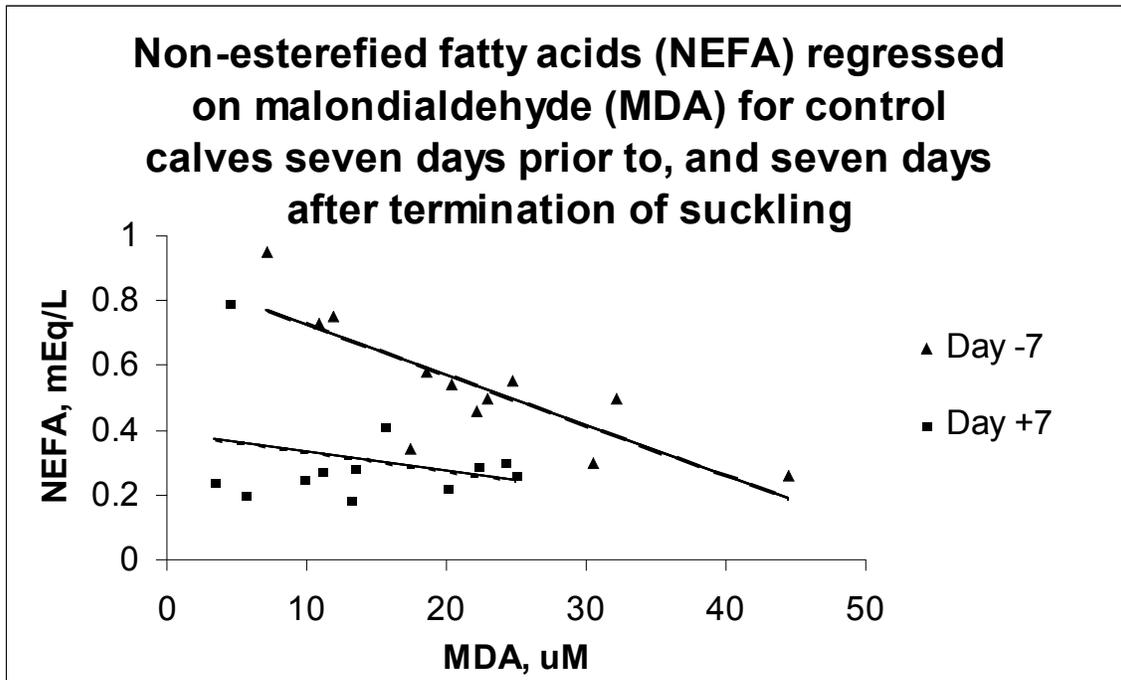
APPENDIX 9

Plasma malondialdehyde regressed upon plasma triglycerides and serum non-esterified fatty acids in control calves seven days prior to, and seven days after weaning



Day -7: $y = 0.15x + 19.4$; $r^2 = 0.017$

Day +7: $y = -0.30x + 30.5$; $r^2 = 0.08$



Day -7: $y = -0.016x + 0.88$; $r^2 = 0.66$

Day +7: $y = -0.006x + 0.39$; $r^2 = 0.07$

APPENDIX 10

Urinary nitrotyrosine

In addition to reactive oxygen species, reactive nitrogen species have been implicated in the pathogenesis of disease. Nitric oxide is a gas that is prevalent in biological systems. It plays important roles in cellular signaling and capillary vasodilation (it can act like a hormone or neurotransmitter), but it is also a radical. As such, it can diffuse through membranes and damage DNA, proteins, and lipids. It is utilized as part of the respiratory burst mechanism of phagocytes. In this system, it plays an important role in killing microbial invaders, and it is tightly linked to necessary natural inflammatory processes. At the same time, when concentrations are too high, the free radical properties of nitric oxide allow it to induce a wide array of tissue damage. Upon interaction with superoxide ($O_2^{\bullet-}$) it can form the oxidizing agent peroxynitrite. This molecule is responsible for the generation of 3-nitrotyrosine, and detection of 3-nitrotyrosine in the blood or urine is used as an indicator that peroxynitrite has been formed. Nitrotyrosine accumulation with concurrent tissue damage has been observed in ruminants (Pfister et al., 2002). Excretion of nitrotyrosine in the urine evidences the potential of oxidative stress (Schwemmer et al., 2000; Radak et al., 2003).

In the present weaning and transport study (Chapter IV), urine was collected from a sub-set of steers ($n = 6$) from each treatment (total $n = 18$) at every sampling date (d -7, 0, 1, and 7). Urine was frozen on dry ice and nitrotyrosine concentration was later assayed by enzyme linked immunosorbent assay (Radak et al., 2003). Nitrotyrosine concentration in the urine of steers at all samplings was below the limit of detection (2 nM) of the ELISA kits (OXIS International, Portland, OR) that were used. Results

suggested that nitrotyrosine excretion in urine of weaned calves was negligible, peroxynitrite formation was minimal, and oxidative stress associated with reactive nitrogen species was not noteworthy.

Pfister, H., K. A. Remer, M. Brcic, R. Fatzer, S. Christen, S. Leib, and T. W. Jungi. 2002.

Inducible nitric oxide synthase and nitrotyrosine in Listeric encephalitis: A cross-species study in ruminants. *Vet. Pathol.* 39:190-199.

Radak, Z., H. Ogonovszky, J. Dubecz, G. Pavlik, M. Sasvari, J. Pucsok, I. Berkes, T.

Csont, and P. Ferdinandy. 2003. Super-marathon race increases serum and urinary nitrotyrosine and carbonyl levels. *Eur. J. Clin. Invest.* 33:726-730.

Schwemmer, M., B. Fink, R. Kockerbauer, and E. Bassenge. 2000. How urine analysis

reflects oxidative stress – nitrotyrosine as a potential marker. *Clin. Chim. Acta.* 297:207-216.

APPENDIX 11

Mean (\pm SD) hematocrit (%) for control (C), fenceline (FL), and nose-clip (NC) steers on d -7, 0, 1, and 7 of the experiment

Treatment	Day -7	Day 0	Day 1	Day +7
C	45 \pm 7.9	42 \pm 4.3	44 \pm 5.0	43 \pm 3.9
FL	46 \pm 7.8	44 \pm 4.5	44 \pm 3.8	44 \pm 6.3
NC	45 \pm 4.2	43 \pm 4.1	45 \pm 4.1	43 \pm 4.3

APPENDIX 12

Average feed composition of concentrate diets¹ (CON) fed to steers on dry-lot at SVAREC, Steele's Tavern, VA

Date started	Feedstuffs (%DM)				
	Corn silage	Shelled corn	Soybean meal ²	Limestone	TM salt ³
4/6/06	69.55	21.57	8.33	0.20	0.35
4/20/06	50.23	42.21	7.09	0.17	0.29
5/9/06	34.98	58.50	6.11	0.15	0.25
5/23/06	23.44	70.84	5.37	0.13	0.22
6/9/06	14.40	80.50	4.79	0.12	0.20

¹Vitamin A added to the ration at an amount required to provide 20,000 IU•head⁻¹•day⁻¹.

²Without hulls (49% CP).

³Composed of 1.4 ppm Co, 141 ppm Cu, 281 ppm Mn, 4.2 ppm Se, 281 ppm Zn, 7.0 ppm I, 30,960 IU/kg vitamin A, 3,870 IU/kg vitamin D, and 140 IU/kg vitamin E.

APPENDIX 13

Composition of mineral block supplied¹ to grazing steers at Willow Bend Farm, WV

Item	As-fed basis
Calcium, minimum, %	12.00
Calcium, maximum, %	14.00
Phosphorus, minimum, %	8.00
NaCl, minimum, %	12.00
NaCl, maximum, %	14.00
Magnesium, minimum, %	0
Potassium, minimum, %	1.60
Sulfur, minimum, %	1.50
Fluorine, maximum, %	0.08
Cobalt, minimum, ppm	60
Iodine, minimum, ppm	50
Iron, minimum, ppm	6,000
Manganese, minimum, ppm	1,900
Selenium, minimum, ppm	20
Zinc, minimum, ppm	2,800
Vitamin A, minimum, IU/kg	661,387
Vitamin D3, minimum, IU/kg	220,462
Vitamin E, minimum, IU/kg	220

¹Supplied by North American Nutrition Companies, Inc., P.O. Box 5002, Lewisburg, OH 45338-5002.

APPENDIX 14

Composition of bloat block^{1,2,3} supplied to steers grazing alfalfa at Willow Bend Farm, WV

Guaranteed Analysis	As-fed basis
Crude protein, minimum, %	4.0
Crude fat, minimum, %	0.5
Crude fiber, maximum, %	12.5
Salt, minimum, %	19.5
Salt, maximum, %	23.0
Potassium, minimum, %	1.8
Iodine, minimum, ppm	43
Selenium, minimum, ppm	13

¹Active drug ingredient, Poloxalene (6.6%).

²Contains ethoxyquin (0.03%) and BHT (0.095%) as preservatives.

³Sweetlix, P.O. Box 8500, Mankato, MN 56002.

APPENDIX 15

Means (\pm SD) for plasma trolox equivalent antioxidant capacity (TEAC)¹ and malondialdehyde (MDA)² in steers allotted to various finishing treatments³

Variable	Treatment			
	Concentrate	Alfalfa	Naturalized pasture	Pearl millet
TEAC				
August	1.31 \pm 0.044	1.32 \pm 0.047	1.33 \pm 0.064	1.33 \pm 0.064
September	1.30 \pm 0.069	1.31 \pm 0.056	1.27 \pm 0.070	1.37 \pm 0.059
MDA				
August	14.8 \pm 5.64	17.4 \pm 6.18	17.3 \pm 5.73	19.9 \pm 9.07
September	17.3 \pm 7.66	22.1 \pm 10.56	22.2 \pm 8.63	15.0 \pm 6.45

¹Trolox equivalent antioxidant capacity expressed as mmol trolox equivalents per liter.

²Malondialdehyde in micromoles per liter.

³Collected on August 15, 2006 and September 28, 2006 for steers fed forage, and on August 16, 2006 and September 29, 2006 for steers fed concentrate.

APPENDIX 16

Mean values for carcass data¹ from steers finished on forages or concentrate

Treatment	Carcass weight (kg)	Maturity ²	Marbling score ³	Backfat (cm)	Ribeye area (cm ²)	KPH fat ⁴ (%)	Quality grade ⁵	Yield grade
Naturalized pasture	248.0	1.0	2.5	0.5	65.2	1.3	8.6	1.6
Alfalfa	247.6	1.0	2.7	0.5	63.2	1.2	9.2	1.8
Pearl millet	268.2	1.0	3.1	0.6	67.7	1.5	9.9	1.8
Concentrate	364.3	1.0	5.3	1.5	85.8	2.0	13.5	2.6

¹Steers were harvested on October 3, 2006 and carcasses were evaluated on October 4, 2006.

²A maturity = 1; B maturity = 2; C maturity = 3.

³Trace = 2; Slight = 3; Small = 4; Modest = 5; Moderate = 6; Slightly abundant = 7.

⁴Kindney, pelvic, and heart fat.

⁵Standard = 8,7,6; Select = 11,10,9; Choice⁻ = 12; Choice⁰ = 13; Choice⁺ = 14; Prime⁻ = 15.

APPENDIX 17

Carcass data¹ for steers from which thiobarbituric acid reactive substances (TBARS) analysis was conducted

Treatment	Carcass weight (kg)	Maturity ²	Marbling score ³	Backfat (cm)	Ribeye area (cm ²)	KPH fat ⁴ (%)	Quality grade ⁵	Yield grade
<u>Naturalized pasture</u>								
46	259.5	1	3	0.3	61.3	2.0	9	1.82
75	287.3	1	3	1.0	63.9	1.5	11	2.53
127	224.5	1	2	0.3	63.9	1.0	7	1.26
140 ⁶	245.9	1	1	0.3	69.7	1.0	6	0.92
<u>Alfalfa</u>								
95	277.3	1	3	0.5	72.3	1.5	9	1.55
106	242.7	1	3	0.8	64.5	1.5	11	1.95
116	242.0	1	3	0.5	61.9	1.0	9	1.73
31R	246.1	1	3	0.5	67.7	1.5	9	1.56
<u>Concentrate</u>								
73	305.2	1	4	0.8	83.9	2.0	12	1.50
80	351.8	1	5	1.3	87.1	2.0	14	2.15
92	404.5	1	6	1.8	92.9	1.5	14	2.61
125	387.0	1	7	2.0	78.1	2.5	15	3.68

¹Steers were harvested on October 3, 2006 and carcasses were evaluated on October 4, 2006.

²A maturity = 1; B maturity = 2; C maturity = 3.

³Trace = 2; Slight = 3; Small = 4; Modest = 5; Moderate = 6; Slightly abundant = 7.

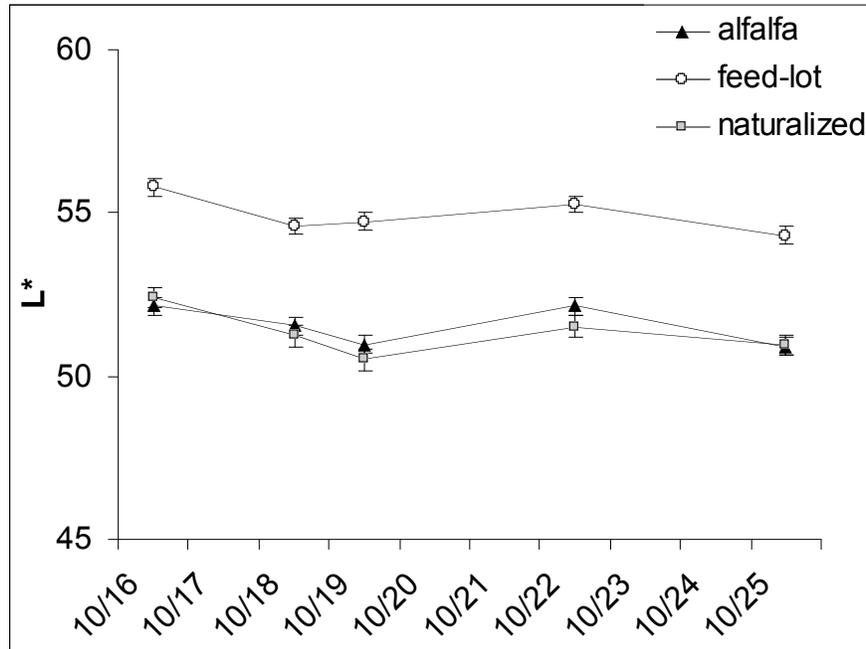
⁴Kidney, pelvic, and heart fat.

⁵Standard = 8,7,6; Select = 11,10,9; Choice⁻ = 12; Choice⁰ = 13; Choice⁺ = 14; Prime⁻ = 15.

⁶Graded a bullock.

APPENDIX 18

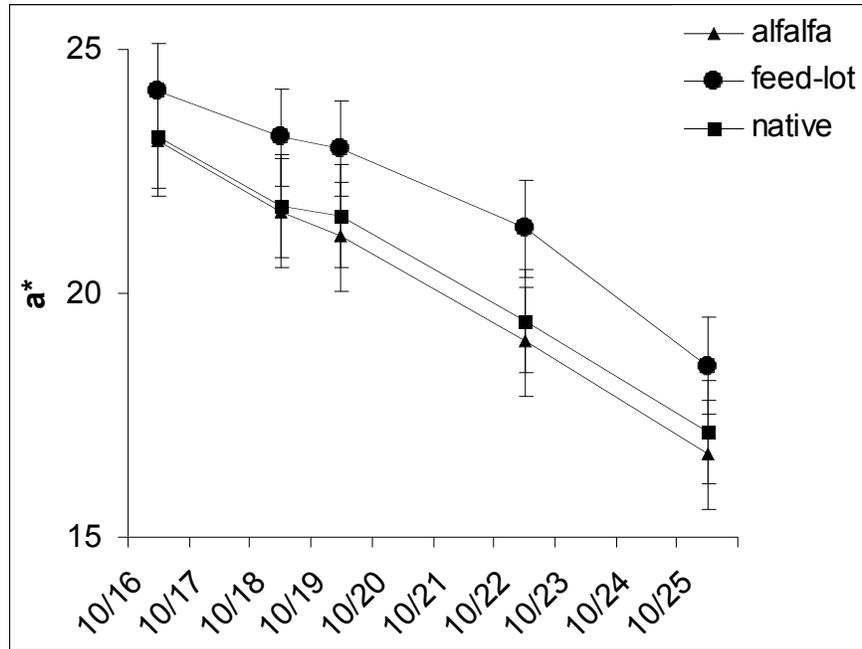
Lightness (\pm SD) of beef patties over time



Color measurements of ground beef used for TBARS analysis (Chapter V) were recorded for L^* (lightness) on d 1, 3, 4, 7, and 10 post-grinding using a Minolta chromameter (CR-410, Minolta Inc., Osaka, Japan).

APPENDIX 19

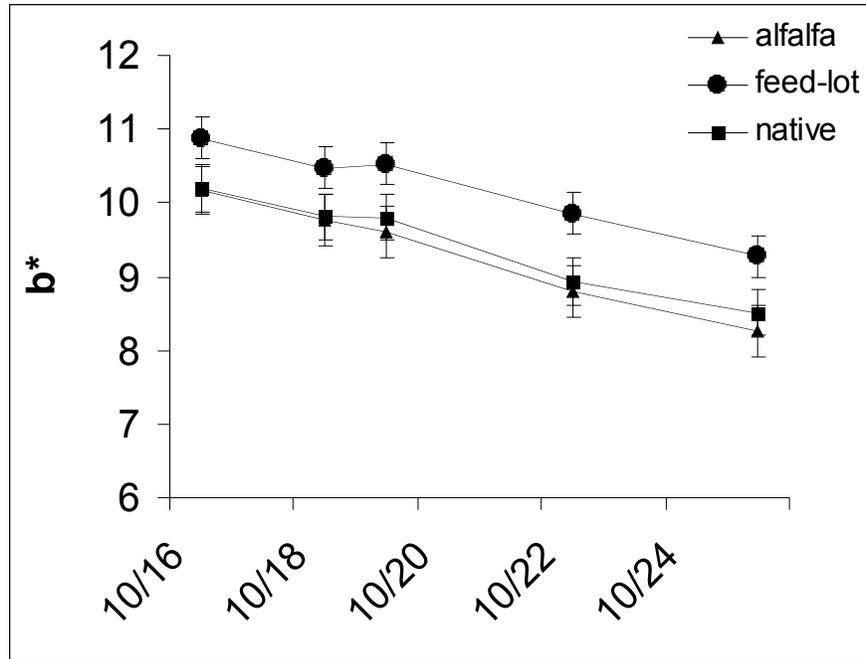
Redness (\pm SD) of beef patties over time



Color measurements of ground beef used for TBARS analysis (Chapter V) were recorded for a^* (redness) on d 1, 3, 4, 7, and 10 post-grinding using a Minolta chromameter (CR-410, Minolta Inc., Osaka, Japan).

APPENDIX 20

Yellowness (\pm SD) of beef patties over time



Color measurements of ground beef used for TBARS analysis (Chapter V) were recorded for b^* (yellowness) on d 1, 3, 4, 7, and 10 post-grinding using a Minolta chromameter (CR-410, Minolta Inc., Osaka, Japan).

APPENDIX 21

To examine the relationship between pre-harvest antioxidant status and lipid oxidation in ground beef, backward elimination was carried out using PROC REG (SAS Inst. Inc., Cary, NC). Pre-harvest β -carotene, α -tocopherol, MDA, and TEAC were compared to TBARS in beef on d 1, 4, and 7 after grinding. Plasma MDA and TEAC were not associated with TBARS at any time. Conversely, serum α -tocopherol was linked to lipid oxidation on d 1, and serum β -carotene was linked to lipid oxidation on d 7. As previously discussed, inherent antioxidant properties of beef may protect it against oxidative degradation. The relationship detected between these serum antioxidants and ground beef TBARS is probably a function of the positive correlation that was established between serum and LM antioxidants.

Summary of backward elimination for pre-harvest variables regressed upon thiobarbituric acid reactive substances (TBARS) concentration of ground beef on d 1, 4, and 7

Step ¹	TBARS					
	Day 1		Day 4		Day 7	
	Variable removed	<i>P</i> -value	Variable removed	<i>P</i> -value	Variable removed	<i>P</i> -value
1	beta-carotene	0.68	TEAC	0.85	TEAC	0.97
2	MDA	0.52	alpha-tocopherol	0.74	alpha-tocopherol	0.77
3	TEAC	0.36	beta-carotene	0.73	MDA	0.13
4	alpha-tocopherol*	0.08	MDA	0.41	beta-carotene**	0.03

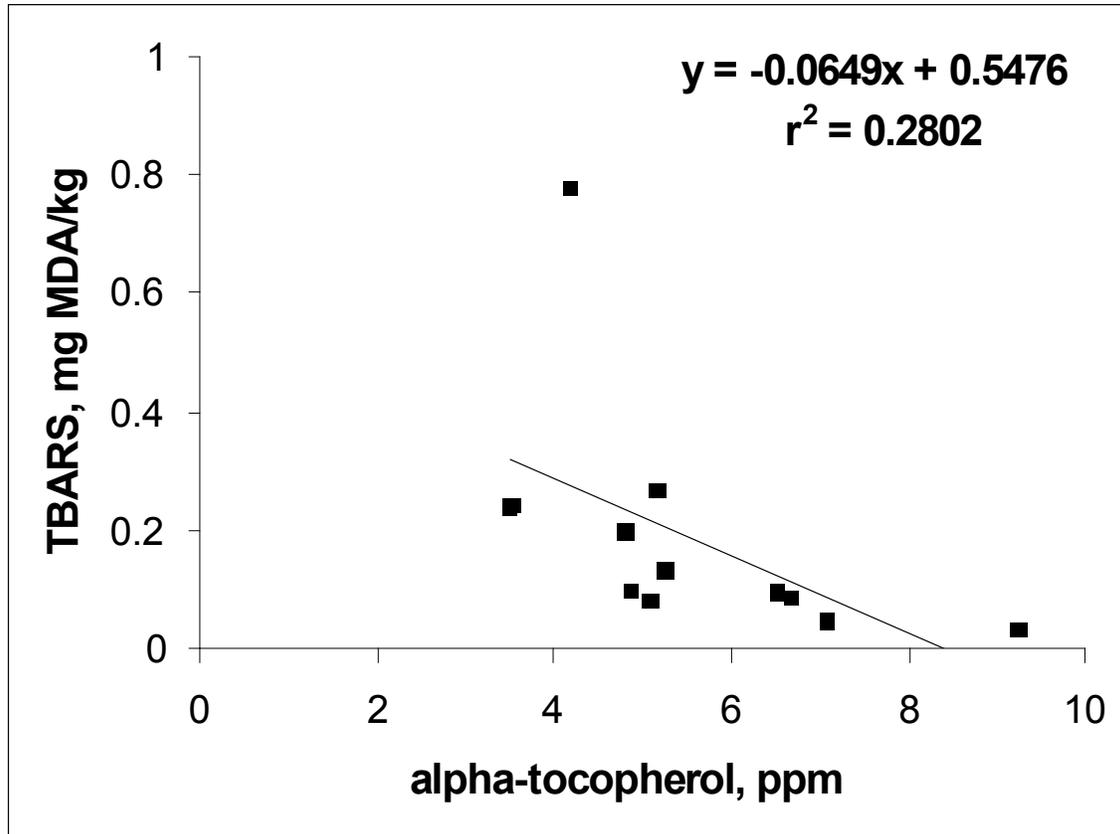
¹Backward elimination step.

*Pre-harvest serum α -tocopherol tended to affect TBARS in ground beef on d 1.

**Pre-harvest serum β -carotene affected TBARS in ground beef on d 7.

APPENDIX 22

Ground beef thiobarbituric acid reactive substances (TBARS)¹ regressed upon serum α -tocopherol collected² from steers³ prior to harvest



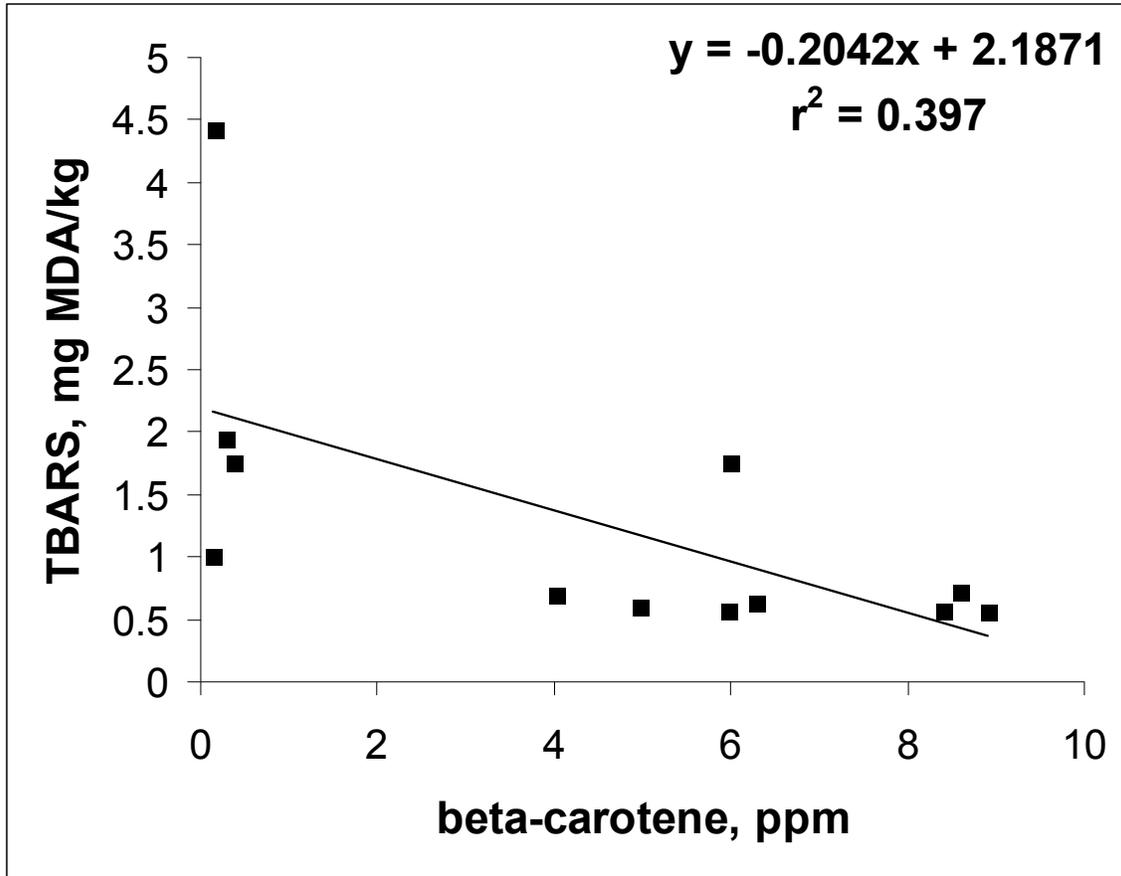
¹TBARS in beef on day 1 after grinding.

²Serum collected on September 28, 2006 from steers on forage, and on September 29, 2006 from steers on dry-lot.

³(n = 4) steers each from concentrate, alfalfa, and naturalized pasture finishing treatments.

APPENDIX 23

Ground beef thiobarbituric acid reactive substances (TBARS)¹ regressed upon serum β -carotene collected² from steers³ prior to harvest



¹TBARS in beef on day 7 after grinding.

²Serum collected on September 28, 2006 from steers on forage, and on September 29, 2006 from steers on dry-lot.

³(n = 4) steers each from concentrate, alfalfa, and naturalized pasture finishing treatments.

VITA

Nathaniel is the son of James and Carla Burke, born May 17, 1983. He grew up with his one sister, Casey, in Page County Virginia, where he graduated with honors from Page County High School in June, 2001. Nathaniel enrolled as an undergraduate student at Virginia Tech the following fall. In 2005 he received an honors scholar diploma, graduating summa cum laude with a Bachelor of Science in Human Nutrition, Foods and Exercise. He then joined VMRCVM, pursuing a Master of Science under the supervision of Dr. Terry Swecker. Nathaniel defended his M.S. thesis during the 2007 summer session, and in August he will matriculate as a member of the college's DVM class of 2011. In addition to production animal medicine and agriculture, Nathaniel's interests include Hokie athletics, bass fishing, deer hunting, classic country music, and bird hunting with his English Setters.