Chapter I

Introduction

Weaning exposes calves to many stressors. Calves weaned on the farm are subjected to the stressors of feed change, separation from the dam, and often a change in environment. Calves that are purchased and brought to a new place for additional growth are subjected to further stressors of transportation, co-mingling with new animals, muscle trauma, and feed and water restriction. The additive effects of these stressors can result in immune system compromise and illness such as respiratory infection when combined with mineral deficiencies, which can be exacerbated by stress-induced anorexia.

Newly weaned cattle are susceptible to Bovine Respiratory Disease (BRD), a broad term that includes both viral and bacterial respiratory infections. Also called "shipping fever," this disease complex is caused by a variety of pathogens including *Mannheimia (Pasteurella) hemolytica* and Parainfluenza virus 3 (PI-3) (Briggs et al, 1988). Signs of BRD include nasal and (or) ocular discharge, fever (rectal temperature >39.5°C), cough, lethargy, depression and inappetance. This illness causes economic loss to producers due to calves' decreased weight gains, mortality, and treatment costs. A survey of small feedlots (100-1,000 animals marketed annually) in the United States reported death losses ranging from 1.5 to 2.7%, with 70% of losses attributed to respiratory disease (USDA-APHIS, 1994). Stress, exposure to pathogens, and energy/mineral imbalances associated with decreased feed intake decrease immune function and increase the risk of developing BRD.

Microminerals such as selenium (Se), copper (Cu), and zinc (Zn) have important roles in maintaining an optimally functioning immune system. These minerals are part of the antioxidant enzymes glutathione peroxidase (Se) and superoxide dismutase (SOD) (Cu and Zn). Superoxide dismutase catalyzes the reduction of superoxide radicals to

hydrogen peroxide, while GSH-Px catalyzes the reduction of peroxides to alcohols and water. Together these enzymes protect cellular membranes from highly destructive oxygen free radicals produced during oxygen-consuming activities such as cellular respiration and oxidative burst. A calf deficient in these minerals has decreased enzymatic activity, and increased cellular damage (Boyne and Arthur, 1979; Arthur et al., 1981; and Gyang et al., 1984). Damaged immune cells compromise immune function, leading to decreased ability to eliminate pathogens and increased disease incidence.

Stress causes the release of glucocorticoids and neuropeptides such as endorphins that have suppressive effects on cellular immune function (Anderson et al., 1999; Murata and Hirose, 1991; and Tizard, 2000). Some effects include decreased blastogenic response of lymphocytes after exposure to mitogen (Blecha et al., 1984), and decreased interleukin-2 (IL-2) production by lymphocytes (Gillis et al., 1979). Although these effects usually diminish within 7 days, this is sufficient time for invading pathogens to proliferate and cause illness. In addition, initial viral infection can suppress immune function, so that calves with viral infections have potential to develop secondary bacterial infections (Briggs et al. 1988; Carter, 1973; and Yates, 1982).

Producers must focus on methods to decrease stressors associated with weaning while simultaneously correcting any mineral deficiencies in newly weaned calves. Supplementation of calves pre-weaning with one or more minerals can help overcome deficiencies and improve immune function.

The present experiments were designed to evaluate 1) effects of weaning stressors on GSH-Px activity in erythrocytes vs. leukocytes; and 2) effects of single-mineral (Se) vs. multi-mineral (Se, Cu, Zn, and Mn) supplementation on health and immune responses of weaned calves.

Chapter II

Literature Review

The Bovine Immune System

Innate versus acquired immunity

Abbas et al. (1991) describes two classes of immunity: innate and acquired. Innate immunity consists of physical and chemical barriers to foreign antigens, the complement system, phagocytes, and macrophage-derived cytokines. Physical and chemical barriers include skin, tears, mucus, and "self-cleaning" processes such as sneezing, coughing, vomiting and diarrhea. The complement system is a cascade of events where a series of complement proteins bind to foreign cell walls, which ultimately results in lysis of these cells. Phagocytes, such as myeloid cells (neutrophils, basophils, and eosinophils) and monocytes, circulate in the bloodstream until signaled chemically to migrate out of the blood and into infected tissues (diapedesis)(Tizard, 2000). Foreign cells are opsonized and engulfed. There are two methods of destroying engulfed cells. Myeloid cells have granules that contain lytic enzymes, or they can release highly reactive oxygen species via a process called "oxidative burst." These reactive species include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$), which damage cell membranes (Boyne and Arthur, 1979). Macrophage-derived cytokines, including interleukin-1 (IL-1), IL-12, and tumor necrosis factor, stimulate production of lymphocytes and other cytokines. Innate defenses do not require exposure to antigen to elicit their effects; therefore, their effects are not targeted to any particular antigen but are involved in broad-scale defense (Tizard, 2000).

Acquired immunity consists of antibodies, lymphocytes, and lymphocyte-derived cytokines (IL-2 and IL-4)(Abbas et al., 1991). Antibodies are immunoglobulin molecules

produced by lymphocytes upon exposure to antigen. Antigens are any substance that can induce an immune response, such as bacterial proteins. Antibodies bind to antigen and mark the antigen as "non-host," enabling immune cells to identify and destroy foreign particles.

There are two classes of lymphocytes: B lymphocytes (B cells) are constituents of antibody-mediated (humoral) immunity, while T lymphocytes (T cells) are responsible for cell-mediated immunity (CMI), which targets intracellular infections. Lymphocytes live longer than phagocytic cells, with lifetimes ranging months to years (in the case of memory B cells), versus the two to three day lifespan of neutrophils (Tizard, 2000).

Acquired defenses require exposure to antigen in order to function, and target specific antigens for which antibodies have been produced. Vaccines are a method of exposing a host to foreign antigen and triggering antibody production against that antigen. The first time an animal is exposed to a foreign antigen, it takes up to 2 weeks for an immune reaction to be mounted, have lymphocytes produce specific antibodies for that antigen, and finally clear the antigen from the host. However, response time upon the second or third exposure is decreased to only a few days, because memory B cells recognize the antigen from previous exposure (Tizard, 2000). Decreased host response time means host immune cells can target and eliminate foreign cells more quickly, preventing extensive invasion of foreign cells and hence disease.

Effects of stress on mineral status and immune function

Stressors such as muscle trauma, movement restriction, thermal stress, infection, and change in feed alter a calf's mineral status. Stress is induced in a variety of situations including transport, handling, medical treatment, confinement, mixing with new animals, and other factors associated with weaning. An animal that is marginally mineral-deficient may become more deficient during stress due to decreased feed intake,

increased urinary/fecal excretion of minerals, or increased utilization/mobilization of minerals by various organ systems. Mineral-deficient animals have impaired immune function, which increases the occurrence of disease (Nockels, 1993). Bovine respiratory disease (BRD) is a broad term that encompasses illness caused by any of several pathogens such as *Mannheimia (Pasteurella) haemolytica* and Parainfluenza virus. Newly weaned calves are highly susceptible to BRD due to immune-suppressive stresses associated with weaning and transport, and increased exposure to pathogens (Galyean et al., 1999). Bovine Respiratory Disease causes enormous monetary loss to producers through calf death, treatment costs, and decreased weight gains.

Stressors cause release of adrenocorticotropic hormone (ACTH), which stimulates release of cortisol, glucocorticoids, and aldosterone from the adrenal cortex, and epinephrine and norepinephrine from the adrenal medulla. These substances can affect immune cell activity and mineral balance. Cousins (1985) reported that stress and trauma cause lymphocytes to produce IL-1, which causes increased adrenal glucocorticoid production and increased pancreatic glucagon production. These substances, along with epinephrine, trigger increased hepatic and renal production of metallothionein (MT), which binds metal ions such as Cu and Zn in the blood and removes them from circulation. This is a protective function, as it makes metal ions unavailable for bacterial use, but it also makes metal ions unavailable for host cell functions.

Anderson et al. (1999) mimicked the effects of stress on the hypothalamic-pituitary-adrenocortical axis by injecting Hereford steers with dexamethasone. Two doses of dexamethasone were administered to 6 yearling steers: 0.08 mg/kg IM (short-acting), and then 0.25 mg/kg IM 37 hours later (long-acting); 6 steers were injected with saline (controls). Calves were bled at the following times: 36.5 hours post-(first) injection, and d -2, 2, 3, 4, 6, 9, and 12 post-(second) injection. Treatment with

dexamethasone resulted in increased total white blood cell counts (leukocytosis), increased neutrophil counts (neutrophilia), and decreased lymphocyte counts (lymphopenia) throughout d 9 of the experiment.

Murata et al. (1987) examined the effects of transportation stress on lymphocyte subpopulations, lymphocyte blastogenesis, and neutrophil function in calves. Four castrated Holstein calves were transported by truck for 4 h. Blood samples before and after transport were examined for total and differential leukocyte counts, lymphocyte blastogenesis, neutrophil Nitroblue tetrazolium (NBT) reduction (a measure of oxidative burst), and plasma cortisol concentrations. Transportation caused leukocytosis and neutrophilia at 0 and 4 h post-transport, and decreased lymphocyte blastogenic response. Altered responses were back to baseline by d 4 post-transport. Neutrophils had increased NBT reduction at 0 and 4 h post-transport, which returned to baseline by 20 h post-transport. Cortisol concentrations were increased at 0 h, then returned to baseline by 4 h.

Orr et al. (1990) studied the effects of transportation and disease on serum Cu and Zn. Two trials were performed to examine effects of market-transit stress on serum Cu and Zn concentrations. Two additional trials were performed to determine effects of inoculation with infectious bovine rhinotracheitis virus (IBRV) on serum Cu and Zn concentrations. In Trials 1 (n=80) and 2 (n=100), English crossbred male calves were purchased and transported 1, 967 km to the Texas Agricultural Experiment Station (TAES, Amarillo, TX). All calves were castrated, vaccinated with four-way clostridial vaccine, administered $1x10^6$ units vitamin A (IM), and dewormed with Thiabenzole. Calves were visually evaluated for BRD and records of temperature and morbidity scores were kept. Calves were bled on arrival and d 7, 8, 9, and 10 post-arrival. Peak morbidity occurred on d 7, and serum Zn decreased in morbid and healthy calves. Morbid calves had lower Zn concentrations than healthy calves, with lowest concentrations coinciding

with peak morbidity. Copper concentrations were higher in morbid vs. healthy calves on d 9. In Trials 3 (n=37) and 4 (n=8), calves sero-negative for IBRV were inoculated intranasally with $2.7x10^5$ plaque-forming units (pfu) of IBRV. Blood was obtained via jugular indwelling catheters before and after inoculation, and serum Cu and Zn were measured. Serum Zn decreased on d 4 post-inoculation, while serum Cu increased 1-2 days after inoculation.

Increases in Cu concentration could be caused by increased absorption from the gut and/or increased ceruloplasmin production. Ninety percent of serum Cu is bound to ceruloplasmin, and stress has been shown to increase ceruloplasmin production (Nockels, 1993). Decreases in Zn concentration could be caused by increased urinary and fecal losses, binding by acute-phase proteins such as metallothionien (MT), or reallocation from serum into tissues. Increased MT production can be triggered by muscle catabolism. Fifty to sixty percent of total body Zn is in muscle. Stress, feed deprivation and trauma cause breakdown of muscle proteins for energy, which releases Zn. Increased serum Zn concentrations trigger increased production of MT, which binds the released Zn and causes serum Zn concentrations to decrease (Nockels, 1993).

Supplementing livestock with key micronutrients, such as Se, Zn, and Cu is an important step towards maintaining health. Compensation for mineral losses in stressed animals is critical, especially if their mineral status pre-stress is marginal or deficient.

Selenium

Discovery and significance

Swedish chemist Jons Jacob Berzelius first discovered Se deposits in a sulfuric acid factory. Selenium was linked with workers' illnesses at the factory and was viewed as a poisonous nuisance (Oldfield, 1999). In the 1950s, German biochemist Klaus Schwarz discovered Se's importance as an essential nutrient in rats (Schwarz and Foltz,

1957), and this discovery spurred further research of diseases caused by Se deficiency: exudative diathesis in chicks (Patterson, et al., 1957), white muscle disease in calves and lambs (Muth et al., 1958), and hepatosis dietetica in pigs (Mori and Masters, 1970). Micro-quantities of Se were found to have positive effects on animal health (Oldfield, 1999).

Determination of Se status in cattle

Selenium status can be determined from serum, whole blood, or liver tissue. Liver and serum Se reflect short-term changes in intake. Whole blood or erythrocyte Se concentrations change slowly and do not accurately reflect short-term changes in intake because of the long life span of erythrocytes (135-160 days) (Thompson et al., 1981). If Se intake is constant, any of the above methods can be used to determine Se status in a calf. The following table shows blood and serum Se concentrations for an adequate, marginal, or deficient calf:

Table 1. Bovine serum and whole blood Se concentrations

	Deficient	Marginal	Adequate
Whole Blood	0.01-.04 ppm	0.05-0.06 ppm	0.07- > 0.10 ppm
Serum	< 0.05 ppm		0.05-0.40 ppm

*adapted from Castellan et al., 1999 and Olson, 1978.

Methods of supplementation

Forages supply adequate Se to grazing animals if the forages contain > 0.06 mg Se/kg DM. Selenium in soils exists in many forms: elemental Se, selenide, selenite, selenate, and organic Se compounds such as selenocysteine. Selenate and organic Se compounds are the most available for plant uptake (Oldfield, 1999). Bioavailability of Se to plants depends on soil pH, oxidation-reduction potential, soil texture, organic matter, competitive ions, and plant species. Soil content of Se is not always a good indicator of

Se concentrations in plants, as some plants, such as *Medicago sativa,* accumulate Se more readily than others (Witte and Will, 1993). Selenium is incorporated into plants by substitution of Se for sulfur (Su) in amino acids such as methionine and cystine, creating seleno-amino acids, which provide animals with available Se (Neuhierl et al., 1999).

Forages grown on soils with low Se concentrations cannot provide adequate amounts of Se to maintain an animal's optimum health. Many supplementation methods are available to increase or maintain a calf's Se status including injections, drenches, Se-containing salt blocks or mineral mixes, Se pellets, adding Se to drinking water, and slow-release intra-ruminal glass boluses.

Injections are a short-term correction for animals that are deficient or have increased need of Se, such as during weaning or other times of stress. In a study by Swecker et al. (1989) a Se injection containing 0.1mg Se/kg body weight (BW) boosted whole blood Se concentrations quickly, but the concentration decreased to baseline within two weeks. A single injection may not be sufficient to increase the status of a severely deficient animal to adequate status (Maas et al., 1993).

Currently, the Food and Drug Administration allows 120 mg of Se/kg mineral mix (120ppm), with consumption not to exceed 3 mg/head/day. However, increased amounts may be needed in times of high stress, such as weaning, pregnancy, and lactation. Some researchers conclude that higher levels of Se are beneficial during these times (Eversole et al., 1988; Swecker et al., 1989).

Eversole et al. (1988) examined the efficacy of various supplements in increasing the Se status of severely deficient weaned beef calves. The six treatments were: 1) single IM injection of sodium selenite (0.1mg Se/kg BW) plus 20 mg Se/kg trace mineral supplement; 2) 20 mg Se/kg trace mineral supplement (control);
3) 40 mg Se/kg trace mineral supplement; 4) 80 mg Se/kg trace mineral supplement;

5) 160 mg Se/kg trace mineral supplement; 6) 1.1 kg/head/day dried brewer's grain (an organic source of Se). Treatments 1, 3, 4, 5, and 6 increased and maintained whole blood Se concentrations > 70 μg/L, while treatment 2 resulted in marginal Se status in the calves in that group. These results indicate that although all methods of supplementation increased whole blood Se concentrations, treatment 2 (the maximum amount of Se supplement allowed by the FDA at that time) was insufficient to improve calves' Se status to adequate.

Some forms of Se are more effective than others in increasing the Se status of cattle. Pehrson et al. (1999) supplemented 103 pregnant Hereford dams with either organic or inorganic Se to determine the effects of Se source on the Se status of their calves. Both groups had access to a mineral supplement containing 30 mg Se/kg supplement. The source of Se was either sodium selenite (inorganic) or Se-yeast (organic). Two blood samples were taken one month apart from cows and calves to compare whole blood and plasma Se between treatments. Cows and calves in the Se-yeast group had higher whole blood and plasma Se concentrations compared to the sodium selenite group. Dams in the Se-yeast group also had higher Se concentrations in their milk compared to the selenite group, and there were significant correlations (r=.59 to .68) between Se concentrations in milk or whole blood and the calves' whole blood and plasma Se concentrations. Calves in the Se- yeast group had adequate Se status, while calves in the selenite group had marginal status (130 μg/L and 84 μg/L for whole blood, 48 μg/L and 34 μg/L for plasma, respectively).

Effects of Se deficiency on calf health and immune function

There are many stages in progressive Se deficiency. If intake of Se is inadequate for a calf's needs, the first sign of deficiency is decreased Se concentrations in storage tissues such as the liver. After stores have been depleted, blood concentrations and

urinary excretion decrease. If Se intake continues to be inadequate, Se-dependent enzyme activity is compromised. Stages progress through sub-clinical and clinical signs (such as white muscle disease) until the effects of Se deficiency are irreversible, where no amount of supplementation can reverse the effects of deficiency. It is important to note that enzymatic activity, and hence immune function, are compromised long before any overt signs of deficiency are noticeable.

Boyne and Arthur (1979) examined the effects of Se deficiency on neutrophil function in cattle. Eight 3-month-old Fresian steers were assessed as Se-deficient by the absence of whole blood GSH-Px activity and whole blood Se concentrations <0.01 ppm. Four calves were fed a basal Se-deficient diet, while the other 4 calves were fed a basal diet supplemented with 0.1 mg Se/kg DM as sodium selenite. Steers were fed these diets for 26 weeks before blood was sampled. Neutrophils were isolated from whole blood at 26, 29, 33, 38, 44, and 45 weeks. Several assays were performed to assess neutrophils' ability to phagocytose and kill *Candida albicans*. There was no difference between groups in neutrophils' ability to phagocytose *C. albicans*, but neutrophils' ability to kill ingested bacteria was 3 times greater in supplemented animals versus non-supplemented. Neutrophils from deficient calves had decreased peroxidase activity, evidenced by decreased Nitroblue Tetrazolium (NBT) reduction, compared to supplemented calves. At weeks 38 and 45, whole blood GSH-Px was undetectable in Se-deficient steers, while supplemented steers had mean GSH-Px activities of 7.0 ± 2.6 and 3.7 ± 0.9 m units per 10^6 neutrophils, respectively.

In a follow-up study, Arthur et al. (1981) examined the effects of Se deficiency on neutrophil O_2 consumption and oxygen radical production. Neutrophils were isolated from Se-deficient and Se- supplemented calves. Oxygen uptake, cytochrome *c* reduction, and 2-(p-iodophenol)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction

were measured in neutrophils before and after zymosan stimulation. Oxygen consumption, cytochrome *c* and INT reduction were not different between Se-deficient and Se-supplemented calves before stimulation. After stimulation, neutrophils from supplemented calves consumed O_2 and reduced cytochrome *c* and INT at much greater rates than neutrophils from deficient calves.

Selenium deficiency also affects lymphocyte function. Droke and Loerch (1989) examined the effects of vitamin E and Se supplementation on performance, health status, and serum antibody response to *Pasteurella haemolytica* (a bacteria associated with Bovine Respiratory Disease) in steers new to the feedlot. Treatments included 1) no supplementation; 2) IM injection of 25 mg Se as sodium selenite (Na_2SeO_3); 3) IM injection of 340 IU Vit E as [d]-α-tocopherol acetate; 4) IM injection of 25 mg Se plus 340 IU vit E. Although average daily gain (ADG) and days sick were not different among treatment groups, calves receiving Se and/or vit E had higher IgG titers to *P. haemolytica* at d 7. Calves supplemented with both vitamin E and Se had the highest IgG titres, compared to the other 3 groups. Researchers noted that increased antibody titers did not convey increased health status, as evidenced by lack of difference among groups in average days sick per steer. This may be caused by morbidity due to pathogens other than *P. haemolytica*.

Effects of Se supplementation in cattle

Supplementing cattle with Se has many desirable effects. Castellan et al. (1999) reported increased average daily gain (ADG) in Hereford-Angus calves given multiple injections (0.05 mg/kg body weight, SC) on d 2, 70, 114, and 149 after birth, while ADG in calves given a single injection on d 2 after birth did not differ from control calves that received no injection.

Selenium supplementation also enhances dairy cow's resistance to mastitis.

Ndiweni and Finch (1996) investigated the effects of in vitro Se and vitamin E supplementation of first lactation dairy cows. Polymorphonuclear leukocytes (PMN) are the major cellular defense mechanism within the mammary gland. Both vitamin E and Se enhanced the chemotactic and random migration of PMN and increased production of superoxide following stimulation with phorbol myristate acetate (PMA). These effects result in PMN that more effectively target and kill organisms such as *Staphylococcus aureus* that cause mastitis. The authors of this study noted no synergistic effects between vitamin E and Se, contrary to other studies (Spears et al., 1986; Pollock et al., 1994).

Based on results presented by Eversole et al. (1988), calves' Se status at time of supplementation affects the rate and overall increase in concentration of blood Se, with deficient animals having greater increases in blood Se concentrations over a shorter amount of time versus marginal or adequate animals. Wright et al. (1997) concluded that vitamin E and/or Se supplementation did not influence post-weaning performance, stress responses, or vaccination responses in beef calves with adequate vitamin E status.

Interactions of Se with other micronutrients

The immune system is a complex network of cell types and cellular signals. Other micronutrients, such as vitamin E, Zn, and Cu, interact with Se both synergistically and antagonistically and affect immune cell function.

Spears et al. (1986) observed that injecting Hereford-Simmental cows with 408 IU vitamin E and 30 mg Se (as sodium selenite) 3-4 months pre-partum and at 60-d intervals thereafter reduced calf death losses from 15.3% to 4.2%, and slightly increased weaning weights ($P<0.10$). Debilitated calves given a single injection of 5.5 mg Se and 75 IU vit E/100 kg BW showed rapid recovery from diarrhea and unthriftiness.

Pollock et al. (1994) examined the effects of Se, vitamin E (in the form of α-tocopherol), and both nutrients together on lymphocyte proliferative responses in Se- and

vitamin E-deficient calves. Lymphocytes from calves supplemented with Se alone showed enhanced responses to keyhole limpet haemocyanin (KLH) in the presence of serum from calves supplemented with α-tocopherol. The percentages of B cells were highest in calves receiving both supplements.

Maas et al. (1994) studied the effects of sustained-release Se boluses on plasma and hepatic Cu concentrations in 32 Se-deficient heifers; 16 heifers received intra-reticular boluses and 16 were untreated (controls). There were no differences between control and treatment groups in mean hepatic or plasma Cu concentrations at d 68, 112, and 188 post-treatment, while treated heifers had higher mean blood Se concentrations. Other studies reported conflicting results on the effects of Se supplementation on tissue Cu concentrations in cattle. Amer et al. (1973) reported increased hepatic Cu concentrations in Se-supplemented calves. Thirty-six newborn male Holstein calves were assigned 1 of 6 treatments in a 3x2 factorial design. There were 3 levels of Cu supplementation (0, 100, or 200 ppm CuSO4) and 2 levels of Se supplementation (0 or 0.7ppm sodium selenate). Blood was collected via jugular venipuncture bi-weekly, and Cu and ceruloplasmin were measured. Selenium alone or with Cu caused a significant decrease in plasma ceruloplasmin activity, especially in the first 4 weeks. Selenium and Cu supplemented together increased hepatic Cu compared to Cu supplementation alone. They concluded there was a negative effect of Se on plasma Cu concentrations but a positive effect of Se on hepatic Cu concentrations. Fehrs et al. (1981) fed 0 or 100 ppm supplemental Cu as copper carbonate and 0 or 1.0 ppm Se as sodium selenite to 16 male Holstein calves for 15 days in a 2x2 factorial design. On d 13, calves were administered an oral dose of Se-^{75} and euthanized 48 h later. Copper concentrations in the pancreas, spleen, kidney, muscle, and spinal cord were unaffected by Se treatments, but heart and lung Cu concentrations were increased in groups fed 1.0 ppm Se. Gleed et al. (1983)

examined the effects of Se and Cu supplements on weight gain, serum Cu, and whole blood Se and GSH-Px activity in 6-week old beef steers. One hundred twenty-six Hereford x Friesian and 25 Charolais x Friesian calves were assigned to 1 of 4 treatment groups: 1) subcutaneous (SC) injection of 200 mg Cu as copper calcium edetate; 2) SC injection of 0.15 mg Se/kg BW as sodium selenite; 3) both Cu and Se injections; 4) no injection (control group). Statistical analysis showed no interaction between the supplements on any of the responses measured, although each mineral supplement increased the serum or blood concentrations of that mineral. Koh and Judson (1987) reported antagonism between hepatic Cu concentrations and Se supplementation. Fifteen Hereford steers and 20 Hereford heifers 4-8 months old were allocated to 1 of 5 orally-administered treatments: 1) control; 2) 10g copper oxide (CuO) capsule; 3) two 30g Se bullets; 4) 10 g CuO +2 Se bullets; 5) two 100g glass bullets (134 g/kg of Cu as CuO and 3 g/kg elemental Se). The Se bullets exerted an antagonistic effect on availability of Cu from CuO, evidenced by reduced Cu concentrations in plasma, blood, blood cells and liver, when compared to controls. Selenium supplementation alone did not affect liver Cu concentration. The mechanisms for this interaction are unknown.

Reffett et al. (1986) evaluated the effects of Se and Zn supplementation on performance, immune response, and blood characteristics of 80 stressed weanling beef calves. Treatments were: 1) control; 2) Se injection 15 mg Se/head; 3) zinc diet = 25 mg Zn/head/day as zinc oxide (ZnO); 4) zinc diet + Se injection. Calves were purchased at a sale and transported 80 km. Calves were blocked by GSH-Px activity into Se-deficient and Se-adequate groups to account for differences in Se status (nutritional background) before administration of treatments. The increase in whole blood GSH-Px activity was greater for calves in the Se-deficient group (vs. Se-adequate) that received Se alone. Zinc-supplemented calves had decreased GSH-Px activities on d19 ($P<0.006$) compared

to calves in the Se and Se + Zn groups. GSH-Px activity of calves in the Zn diet + Se injection group increased 35%, compared to calves in the Se injection group, which had a 78% increase, indicating a negative interaction between zinc and Se. Treatment did not affect total leukocyte counts, neutrophil/lymphocyte counts, nor levels of antibody titers to infectious bovine rhinotracheitis virus (IBRV) and para-influenza 3 (PI_3).

Glutathione peroxidase

Origin and importance in immune function

Selenium is an integral part of the enzyme glutathione peroxidase (GSH-Px), which contains selenocysteine in the catalytic portion of the enzyme. Glutathione peroxidase catalyzes the reduction of peroxides produced by phagocytic cells as they attack foreign antigens, as well as peroxides produced as by-products of cellular oxygen consumption. Without this enzyme, the host's own cells are subject to oxidative damage from these reactive products. Almost all cells, including erythrocytes and leukocytes, contain GSH-Px as protection against cellular oxidation.

Deficiency of Se, and hence GSH-Px, results in accumulation of H_2O_2, which would, in turn, cause increased lipid peroxide formation. These peroxides may inhibit the hexosemonophosphate shunt, thereby limiting the amount of $NADP^+$ available for the production of O_2 and H_2O_2. A lack of these reactive oxygen species would cause decreased ability of PMN to kill ingested cells as seen in Se-deficient animals (Gyang et al., 1984).

Distribution of GSH-Px in tissues and blood components

There are five selenocysteine-containing GSH-Px enzymes identified at this time: cytosolic (classical) GSH-Px (cGSH-Px), gastrointestinal GSH-Px (GI-GSH-Px), plasma GSH-Px (pGSH-Px), phospholipid hydroperoxide GSH-Px (PHGSH-Px), and sperm

nuclei GSH-Px (snGSH-Px). Cytosolic GSH-Px is the enzyme most commonly measured for immune function determination, and is present in nearly all tissues, although it is unevenly distributed (Behne and Kyriakopoulos, 2001). Bovine tissues with high GSH-Px activity include the spleen, myocardium, erythrocytes, brain, thymus, adipose tissue, and striated muscle (Scholz et al., 1981). In adult bovine blood, 98% of GSH-Px and 73% of Se is associated with erythrocytes (Scholz et al., 1979).

A non-Se-dependent GSH-Px also exists, which does not contain Se as its structural component and is not decreased by Se deficiency (Scholz et al., 1981). This enzyme is found in the liver, lungs, adrenal glands, testes, kidney medulla and kidney cortex, with the liver having the highest percentage of non-Se-dependent GSH-Px activity.

Mature erythrocytes are incapable of protein synthesis. GSH-Px is incorporated into erythrocytes at erythropoiesis, therefore erythrocyte GSH-Px activity is only an indicator of a calf's Se status over the past 120 d, and does not reflect short-term changes in Se intake (Thompson et al., 1981).

Determination of GSH-Px activity

Glutathione peroxidase activity reflects a calf's Se and oxidative status. Thompson et al. (1981) reported a high correlation (r= .97) between blood Se concentrations and erythrocyte GSH-Px activity in samples from 50 mixed age cattle. The following table summarizes values for deficient, marginal, and adequate Se concentrations and GSH-Px activities in whole blood:

Se Status	Blood Se (ppm)	Whole blood GSH-Px activity (mU/mg heme/min)
Deficient	< 0.04	< 15
Marginal	0.05 - 0.06	15-25
Adequate	> 0.07	25-100

*Adapted from Scholz et al., 1979.

Glutathione peroxidase activity can be used as an indicator of a calf's oxidative status, or its ability to produce reactive oxygen species during oxidative burst. Whole blood (erythrocyte), serum, and liver GSH-Px activities are commonly measured. There are advantages and disadvantages to using plasma versus liver versus erythrocyte GSH-Px to determine oxidative status. Glutathione peroxidase is stable in erythrocytes, has activity highly correlated with blood Se, and Se contamination of tubes is avoided. However, erythrocyte and whole blood GSH-Px are useful only as long-term indicators of Se intake (Thompson et al., 1981). Plasma GSH-Px is a good indicator of short-term changes in Se intake, but GSH-Px activity in plasma is much lower than in erythrocytes, and not as stable. Damaged blood cells can leak GSH-Px into plasma, resulting in falsely high values (Wilson and Judson, 1976). Liver GSH-Px assays require liver biopsies, which are time-consuming and invasive (Thompson et al., 1981).

Effect of Se deficiency and supplementation on GSH-Px activity and immune function

Selenium status and GSH-Px activity are highly correlated. An animal that has deficient or marginal Se status will also have decreased GSH-Px activity compared to an animal that is Se-adequate. Supplementing a deficient or marginal calf with Se increases GSH-Px activity both in serum and whole blood, although there is a 4-12 wk lag in the increase in whole blood or RBC GSH-Px from time of supplementation (Maas et al.; 1993; Spears et al., 1986; and Stabel et al., 1989).

Enjalbert et al. (1999) examined the efficacy of pre-partum versus post-partum supplementation of Se in improving Se status, and the effects of supplementation on GSH-Px activity in Se-deficient cows and calves. In the pre-partum group, 36 beef cows were supplemented orally for 15 d late in gestation with 13.0, 32.5, or 45.5 mg Se/head/day as sodium selenite. Glutathione peroxidase activity in whole blood and serum were measured for cows and their calves. In the post-partum group, cows were

supplemented orally with 0, 13.0, or 32.5 mg of Se/cow/day for 15 d post-partum, and calves were injected with 1.38 mg Se on d 2 and 45 after birth. Although both methods of supplementation resulted in adequate Se status in the dams, calves from dams supplemented post-partum did not maintain adequate Se status. Glutathione peroxidase activity increased most rapidly in cows fed 45.5 mg Se supplement, and GSH-Px activities in this group remained high 98 d after supplementation ended.

Gyang et al. (1984) examined the effects of supplementation of Se-deficient Holstein dairy cows with vitamin E and Se. The treatments were: 1) injection of 35 mg sodium selenite and 350 mg vit E; 2) injection of 20 mg sodium selenite and 1000 mg vit E; 3) no treament (control). Polymorphonuclear lymphocytes from supplemented cows had increased ability to kill ingested *Staphylococcus aureus* (a bacterium that causes mastitis), as well as increased GSH-Px activity when compared to deficient cows.

Swecker et al. (1989) supplemented weaned beef calves with 20, 80, 120, 160, or 200 mg Se/kg salt-mineral mixture (fed ad libitum) or 0.1mg parenteral Se and 0.22 IU of vitamin E/kg body weight in addition to 20 mg Se/kg mineral mix (fed ad libitum). Calves supplemented with 80-200 mg Se/kg salt-mineral mixture or 0.1mg Se plus 20 mg Se/kg mineral mix had higher IgG titers to hen egg lysozyme (HEL) than calves receiving 20 mg Se/kg mineral mix alone.

Other studies have shown variable results in antibody titer levels between Se-deficient and adequate calves. Stabel et al. (1989) reported no difference in antibody titers against *P. haemolytica* between Se-deficient and adequate calves, although GSH-Px activities were higher in supplemented calves. Reffett et al. (1988) evaluated effects of dietary Se supplementation on primary and secondary immune response in calves challenged with infectious bovine rhinotracheitis virus (IBRV). Eight male Holstein calves were intranasally inoculated with IBRV on d 0 and 35 of the 70 d study. Calves

were allotted to either Se-deficient (0.03 mg/kg diet) or Se-supplemented (0.2 mg/kg diet) treatments. Whole blood and plasma GSH-Px activities increased after IBRV inoculation in Se-supplemented calves but not Se-deficient calves. Selenium-supplemented calves also had higher IBRV antibody titers and total IgM titers, although total IgG titers were not affected by Se status.

Sordillo et al. (1993) examined the effects of Se supplementation on lymphocyte proliferation, interleukin-2 (IL-2) production, and GSH-Px activity in 13 Holstein cows. Selenium-deficient cows had decreased peripheral blood mononuclear cell (PBMC) GSH-Px activity, but PBMC proliferative responses when stimulated with concanavalin A (Con A), pokeweed mitogen (PWM), or polyhemaglutinin (PHA) were no different between deficient and adequate groups. Although monocyte numbers were decreased in Se-deficient cows, the production of IL-2 and the number of IL-2 receptors on lymphocytes were no different between groups.

In summary, calves that are stressed during weaning have increased need for minerals such as Se, Zn, and Cu, because they have decreased feed intake, increased immune system challenges, and changes in mineral metabolism. Supplementation is especially critical in animals that are deficient in these nutrients. The Se status of a calf affects GSH-Px activity, which is a measure of a calf's oxidative status, that is, its ability to fight off infection and stay healthy. Increased health means decreased economic losses to the producer.

Chapter III

Experiment 1. Effect of weaning stressors on erythrocyte vs. leukocyte glutathione peroxidase activity

Abstract

The purpose of this experiment was to evaluate the effects of weaning stressors on serum Se and leukocyte and erythrocyte glutathione peroxidase (GSH-Px) activity in calves. Sixty-four Angus and Angus-cross calves from 2 sources (Bland and Kentland) were bled pre-weaning (d –7), after transportation at weaning (d 0), and post-weaning (d 7, 14, 21). Serum Se, and leukocyte and erythrocyte GSH-Px activity from each calf at each date were measured. Neutrophil and lymphocyte counts were used as an indicator of stress in the calves. The effects of cell type, time, and cell type x time interaction on GSH-Px activity were analyzed using mixed model repeated measures analysis of variance (ANOVA). The effects of source and time on serum Se and neutrophil and lymphocyte counts were also analyzed. Erythrocyte GSH-Px activity remained constant over time, while leukocyte GSH-Px and serum Se increased on d 7 ($P<0.0001$). Serum Se in Kentland calves increased from deficient to adequate concentrations from d 0 to 7. Neutrophil counts increased between d –7 and 0 and decreased between d 0 and 7, while lymphocyte counts decreased between d –7 and 0 and increased d 0 to 7. Predicting Se status in stressed calves using leukocyte GSH-Px or serum Se concentration may overestimate pre-stress status. Leukocyte GSH-Px could be used as an indicator of a calf's oxidative status in short-term experiments, where erythrocyte GSH-Px is not useful.

Introduction

The enzyme glutathione peroxidase (GSH-Px) protects cells from oxidative damage. Cells produce reactive oxygen species as a by-product of oxygen metabolism and oxidative burst. Cellular GSH-Px is a tetramer, excluding phospholipid-hydroperoxide GSH-Px, which a monomer. Each subunit contains selenocysteine, which has a catalytic function. GSH-Px catalyzes the reduction of organic peroxides (R-O-O-H) into alcohols (R-O-H) and water, using glutathione (GSH) as a source of reducing equivalents (GSSG = oxidized glutathione)(Paglia and Valentine, 1967):

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{\text{GSH-Px}} R\text{-}O\text{-}H + GSSG + 2H_2O$$

The reduction of peroxides prevents lipid peroxidation and cellular membrane damage.

In previous studies, GSH-Px activities of whole blood or erythrocytes were used to assess Se status and oxidative function in calves (Maas et al., 1993; Spears et al., 1986; Stabel et al., 1989; and Thompson et al., 1981). GSH-Px is incorporated into erythrocytes at erythropoeisis, and the long life span and slow turnover of this population makes erythrocyte GSH-Px unsuitable for studying short-term changes in Se intake or GSH-Px activity. Serum GSH-Px reflects short-term changes, but serum GSH-Px activity is lower than in whole blood, can be falsely elevated by cell lysate, and is not as stable as cellular GSH-Px.

Leukocytes also contain GSH-Px, and the lifespan of certain cells such as neutrophils is shorter than erythrocytes (3-4 d vs. 135-160 d). The advantage of measuring leukocyte GSH-Px is ability to assess short-term changes in oxidative or Se status brought on by stressors or dietary treatments.

The objective of this portion of the study was to compare GSH-Px activities in leukocytes and erythrocytes from calves at weaning. Glutathione peroxidase activities were compared across 5 times (pre-weaning (d –7), weaning (d 0), and d 7, 14, and 21

post-weaning) to identify changes across time and between cell types. Serum Se concentrations were also measured to determine whether this response is affected by weaning stressors. Neutrophil and lymphocyte counts were used as an indicator of stress in the weanling calves.

Materials and Methods

Experimental Design: Sixty-four beef calves were blocked by source according to their pre-weaning origin (Kentland Research Facility or Bland Correctional Facility). Glutathione peroxidase activity in red and white cell samples was measured for each calf at each bleeding date for repeated measures: d-7 (pre-weaning), d 0 (weaning), d 7, 14, and 21 (post-weaning). Results were standardized using amount of protein per sample to yield GSH-Px activity in mU/mg protein per sample. Serum Se concentrations, and neutrophil and lymphocyte counts were measured for each calf at each date.

Animals, facilities and feed: Two groups of weanling Angus/Angus cross calves (steers and heifers) were used for this part of the experiment. The first group of 34 calves was born at the Bland Correctional Center in Bland, VA, and then transported 110 km to the VPI Beef Center (Blacksburg, VA) at weaning (d 0). Calves had access to a trace mineral mix pre-weaning. The mix contained 30 ppm Se. The second group consisted of 30 Angus calves that were raised pre-weaning at the Kentland research facility and transported 15 km to the VPI Beef Center at weaning (d 0). These calves were creep-fed pre-weaning and also had access to a Se-containing trace mineral mix. The heifers and steers from each group were pastured separately on four different pastures post-weaning.

Blood collection: Blood was collected from each calf via jugular venipuncture into 7mL lithium-heparin and 7mL EDTA vacutainer tubes at d–7 (pre-weaning), 0 (weaning), 7, 14, and 21 (post-weaning). The d 0 bleeding occurred after transportation from the farm of origin to the VPI Beef Center. A blood film was prepared from each sample within 5 hours of collection for cell differentials using blood from the EDTA vacutainer tubes. Blood in the lithium-heparin tubes was stored at 4°C until processing the next day. Glutathione peroxidase activity was measured in both a red and white cell pellet isolated from each sample at each bleeding date. Serum from each sample (lithium-heparin) was

separated and saved for serum Se determination. Serum was stored at -75°C until analysis.

Cell differentials: Blood films were prepared from each sample for differentials. The first 100 cells were counted in the monolayer and numbers of individual cells within a type were noted.

Isolation of cells: Red and white cell pellets were isolated from each sample at each date (see Appendix A for isolation technique). Briefly, lithium heparin tubes were mixed on a rocker apparatus until samples reached room temperature.

Red cell pellet: 1 mL of whole blood was pipetted from each sample and placed in a 1.5mL microcentrifuge tube. The microcentrifuge tubes were centrifuged 5 min @ 1200xG (4ºC). The serum and white cell layer from each tube was pipetted off and discarded, leaving the red cell pellet.

White cell pellet: the lithium heparin tubes were centrifuged 5 min at 1200xG (4ºC). After centrifugation, the serum layer from each sample was pipetted off and transferred to a separate 12x75mm polypropylene tube. Serum was saved for serum Se analysis (stored at -75°C). Next, the white cell layer was pipetted off and added to a conical tube containing 35mL of lysis buffer (see Appendix A for preparation). After 3 washing steps, each purified white cell pellet was re-suspended in 1mL Hank's Balanced Salt Solution (HBSS)(Gibco, Inc.) and transferred to a 1.5mL microcentrifuge tube.

All samples were stored at –75°C until analysis.

Determination of Serum Se: Serum was thawed to room temperature and diluted 10:1 with deionized water. Serum Se concentration for each sample at each date was determined using graphite furnace atomic absorption spectrometry absorbing at 196nm (PerkinElmer LLC, Norwalk, CN). Standard Se dilutions (5, 10 and 20 ppb) were used to create a standard curve from which the concentration of Se (in ppm) for each unknown

sample was calculated.

Preparing samples for analysis via Oxscan:

Each sample was thawed at room temperature and vortexed to mix.

White blood cell samples: 20µL of white cell sample was pipetted into each of 2 cuvettes for duplicate analysis.

Red blood cell samples: Each pellet was diluted with 1mL deionized water, then further diluted by transferring 200µL diluent plus 20µL diluted red cells to a separate microcentrifuge tube. 20µL of the final dilution was pipetted into each of 2 cuvettes for duplicate analysis.

Analysis of samples: Glutathione peroxidase activity for each sample was measured via the Oxyscan GPx-340^{TM} assay. This assay is an indirect measure of GSH-Px activity. Oxidixed glutathione (GSSG) is produced as GSH-Px reduces organic peroxides. Oxidized glutathione is transformed back to a reduced state (GSH) by glutathione reductase (GR), using NADPH as a source of hydrogen molecules. NADPH is reduced to $NADP^+$. Production of $NADP^+$ is accompanied by a decrease in absorbance that is measured at 340nm (A_{340}). The rate of decrease in A_{340} is directly proportional to the GSH-Px activity in the sample.

For this assay, the Oxyscan^{TM} instrument (OXIS Health products, Inc.) is first calibrated using 5 known concentrations of calibrators (0.0 (blank), 5.0, 10.0, 20.0, 30.0, 40.0 mU activity). 50µL of each concentration is pipetted into 2 cuvette wells for duplicate analysis and a standard curve is generated at 340nm. To run samples, 200µL of deionized water is added to the first 2 wells of the cuvette (blanks). Then 20µL of each diluted sample is added to 2 successive wells for duplicate analysis. The instrument aspirates samples one at a time and adds each to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction is initiated by addition of tert-

butyl hydroperoxide and A_{340} is measured. GPx-340^{TM} assay and calibrator kits were purchased from OXIS Health products, Inc., and contain all the reagents necessary for the assay.

Protein content for each sample was analyzed in duplicate using the Biorad protein analysis (Biorad Laboratories)(Appendix B). The Bio-Rad protein assay is a dye-binding assay in which a differential color change of dye occurs in response to various concentrations of protein. Absorbance at 595nm for unknown samples is compared to a standard curve prepared from 7 known protein concentrations of bovine serum albumin (BSA)(0.400, 0.300, 0.200, 0.100, 0.05, 0.025, 0.000 mg/mL). The mean protein amount per sample (mg/mL) was used to standardize GSH-Px activity (mU) per mg protein per sample (mU/mg).

Statistical Analysis: The effects of cell type, time, and cell type x time interaction on GSH-Px activity were analyzed using mixed model repeated measures analysis of variance (ANOVA), with source (Kentland vs. Bland) as a blocking factor and autoregressive first order as the covariance structure. The effects of time and source on serum Se, neutrophil counts, and lymphocyte counts were also analyzed. Cell type x time interaction effects were analyzed using test of effect slices by time, which compares the mean GSH-Px activity from each cell type at each date. Source x time interaction effects were analyzed using contrasts, which compares the change in slope between two dates between sources.

Results

Figure 1 depicts GSH-Px activity across time for leukocytes and erythrocytes. Erythrocyte GSH-Px activity did not change over time, while leukocyte GSH-Px activity increased on d 7($P<0.0001$).

Serum Se across time for each source is shown in Table 1. Serum Se concentrations in the Kentland calves increased from 0.044 to 0.075 ppm from d 0-7, then decreased after d 7. Serum Se in the Bland calves increased from d-7 through d 7 (0.016-0.034 ppm) but concentrations were lower than Kentland calves on every bleeding date.

Neutrophil counts increased from d-7 to 0, decreased from d 0 to 7, and then started to increase again through d 14-21. Lymphocyte counts decreased from d-7 to 0, increased from d 0 to 7, and remained constant through d 7 to 21 (Figure 2). Bland calves' lymphocyte counts increased more rapidly between d 0 and 7 than the Kentland calves' (Figure 3).

Discussion

The average life span of a bovine erythrocyte is 135-160 days, and GSH-Px is incorporated only at erythropoesis. The lag time for changes to appear in erythrocyte GSH-Px is 60-90 days following stress or diet change (Maas, 1993; Thompson et al., 1991). Erythrocyte GSH-Px activity of calves in this study remained constant over 5 sampling dates, despite stressors (transportation, separation from the dam, feed and water restrictions, and change in environment). This result is similar compared to other researchers (Maas et al., 1993; Spears et al., 1986; Stabel et al., 1989; and Thompson et al., 1981).

Immune functions are affected by stress. Leukocyte GSH-Px activity increased the first week post-weaning, then decreased to normal pre-weaning levels as calves adjusted to their new environment. Leukocytes are nucleated and certain populations such as neutrophils have short life spans, so GSH-Px activity in these cells is more dynamic compared to erythrocytes.

Olson (1978) stated that serum Se concentrations between 0.05-0.40 ppm are adequate. Source of calves (Bland vs. Kentland) was a large source of variation in serum Se concentrations. Bland calves had lower serum Se concentrations compared to Kentland calves at all bleeding dates. Bland calves were Se-deficient pre-weaning, with mean serum concentrations of 0.016 ppm. Kentland calves were marginally Se-deficient pre-weaning (serum Se=0.044 ppm on d -7). From d 0 to 7, Kentland calves' serum Se increased to adequate concentrations (0.075 ppm on d 7), but decreased to marginal concentrations by d 14. Bland calves' serum Se concentrations increased from d-7 to 0 (0.016-0.030 ppm), but concentrations were always deficient. This post-transportation increase (not seen in Kentland calves) could be a result of the longer distance traveled by the Bland calves.

Serum Se variations between sources could be explained by differences in mineral supplementation pre-weaning and/or environment and husbandry techniques. Both groups of calves had access to Se-containing trace mineral mix pre-weaning. However, only Kentland calves were creep-fed, which allowed these calves to adjust to a different diet and method of feeding. These calves would be more likely to consume trace mineral mix from a mineral feeder, whereas calves that were not creep-fed would be less likely to eat mineral mix. Although Bland calves had access to Se-containing trace mineral mix, their deficient Se status indicates that these calves did not consume enough to maintain adequate Se status.

The peak in Kentland calves' serum Se concentrations on d 7 could be due to release of liver stores of Se in response to stress. Bland calves, which had lower serum Se concentrations compared to Kentland calves, may not have had adequate Se stores in the liver to induce as great an increase. Short-term changes in leukocyte GSH-Px seem to mirror changes in serum Se levels. Both leukocyte GSH-Px and serum Se increased in response to weaning stressors.

Source was used as a blocking factor in the analysis of leukocyte versus erythrocyte GSH-Px activity, so effect of source on GSH-Px activity was not ascertained. Bland calves were Se-deficient, therefore probably had decreased GSH-Px activities compared to Kentland calves. Further research should be conducted to study the leukocyte GSH-Px activity in deficient versus adequate calves in response to stress.

Neutrophil and lymphocyte counts were used in this study to ascertain whether or not calves were actually stressed. Neutrophil and lymphocyte counts were affected by stress with results similar to other studies (Anderson et al., 1999; Blecha et al., 1984; and Murata et al., 1987). Neutrophil counts increased and lymphocyte counts decreased from d-7 to 0, which is consistent with results from past research where transportation of

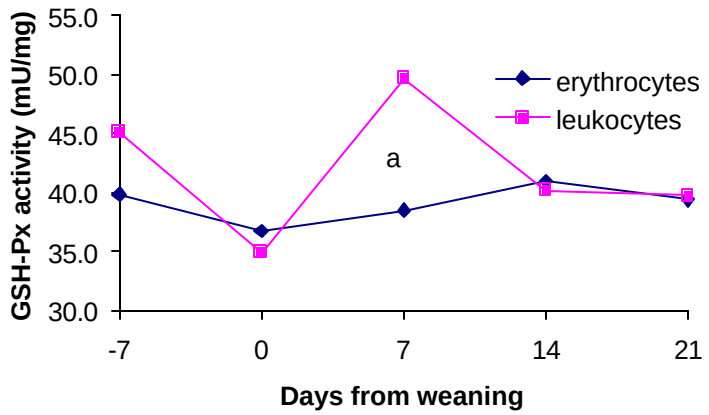
calves caused neutrophilia and lymphopenia compared to pre-transport levels (Murata et al., 1987) or non-shipped controls (Blecha et al., 1984). The greater increase in lymphocyte counts seen in the Bland calves between d 0 and 7 may have been a result of the longer distance traveled by these calves, or other transportation-linked stressors such as higher ambient temperature.

Several factors could cause the increase in leukocyte GSH-Px activity seen in this experiment. One factor could be increased production of GSH-Px protein by leukocytes. Erythrocytes, which lack nuclei, are incapable of producing more GSH-Px protein, but leukocytes could. A decrease in the amount of protein per sample could cause an increase in GSH-Px activities, because the activities for all samples are standardized by dividing GSH-Px activity by the amount of protein per sample, yielding GSH-Px activity in mU/mg protein. An increase in GSH-Px activity could also be caused by decreased degradation of GSH-Px protein, resulting in a longer half-life. It is not known whether there is any difference in GSH-Px expression between lymphocytes, neutrophils, or other types of leukocytes.

Further research needs to be conducted to explore the causes of changes in GSH-Px activities in leukocytes in response to stress. Future studies could include effects of Se supplementation on leukocyte GSH-Px activity, leukocyte activity in Se-deficient vs. Se-adequate calves, and differences in GSH-Px activity of different populations of leukocytes (neutrophils vs. lymphocytes, for example).

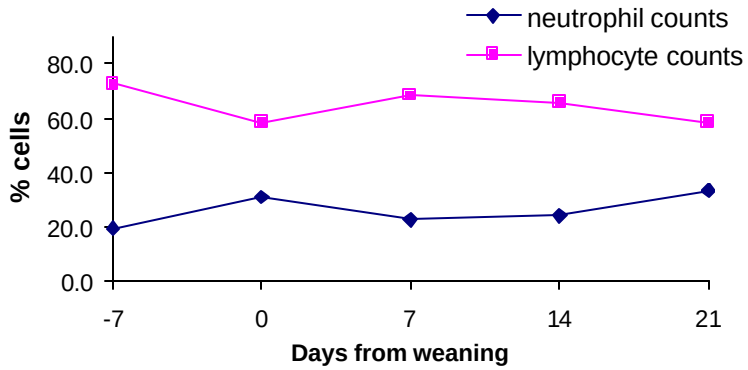
Experiment 1. Tables and Figures

Figure 1. Leukocyte and erythrocyte glutathione peroxidase activity in weaned calves †‡



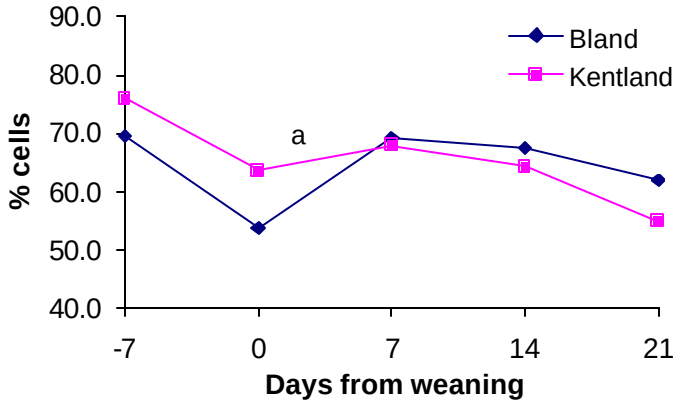
† a - indicates significant difference (P<0.0001) between means within a day
‡ each value is the mean of n=64 observations

Figure 2. Neutrophil and lymphocyte counts of calves during the weaning period †



† each value is the mean of n=64 observations

Figure 3. Kentland and Bland calves' lymphocyte counts during the weaning period [†‡]



[†] a - indicates date interval where significant interaction occurred (P=0.02)
[‡] each value is the mean of n=34 observations (Bland) and n=30 observations (Kentland)

Table 1. Mean serum Se concentrations (ppm) for Bland and Kentland calves [†]

	Days from weaning				
Source	-7	0	7	14	21
Kentland	0.044 ± .003	0.044 ± .002	0.075 ± .002	0.047 ± .002	0.048 ± .002
Bland	0.016 ± .002	0.030 ± .002	0.034 ± .002	0.034 ± .002	0.023 ± .002

[†] each value is the mean of n=34 (Bland) and n=30 (Kentland) ± SEM

Chapter IV

Experiment 2. Effects of single mineral versus multi-mineral supplementation on immune and health responses of weanling calves.

Abstract

Two experiments were performed on the effects of single mineral (Se) versus multi-mineral (Se, Cu, Zn, Mn) supplementation on health and immune responses of weanling calves. The first trial (Trial 1) consisted of 36 Angus heifers born and weaned on-site at SVAREC. Heifers were randomly allotted to 1 of 2 dietary treatments: 1) 15% CP at 0.5% BW; 2) no supplement; and 1 of 3 injection treatments: 1) Multi-min on d 0; 2) Mu-Se on d 0; 3) no injection. The main effects of diet, injection treatment, and date as well as their interactions were analyzed using mixed model repeated measures ANOVA for the following immune and health responses: phagocytosis, oxidative burst, neutrophil/lymphocyte/total leukocyte counts, weight, temperature, whole blood Se, and serum Cu and Zn concentrations. Whole blood Se and serum Cu increased post-weaning and serum Zn decreased post-weaning. Mu-Se-supplemented heifers gained weight faster between d 14-28 vs. Multi-Min-supplemented or control heifers. This experiment was repeated (Trial 2) using 48 steers purchased at auction and transported to SVAREC. Steers were randomly assigned to 1 of 4 pasture management systems and 1 of 3 injection treatments (the same injection treatments as Trial 1). The main effects of pasture management, injection treatment, and date, as well as pasture management x date and injection treatment x date interactions on 10 responses (same as in Trial 1) were analyzed. Whole blood Se and serum Cu increased post-stress and serum Zn decreased post-stress. Oxidative burst activity decreased in Mu-Se and Multi-min supplemented steers between d 0-4 vs. control steers. Multi-min-supplemented steers had higher phagocytic activity vs. steers in either Mu-Se or control groups.

Introduction

Calves with mineral deficiencies have decreased immune function (Boyne and Arthur, 1979; Eversole et al., 1988; and Swecker et al., 1989). Calves that are marginally mineral-deficient can become more deficient when stressed due to decreased feed intake, increased sequestration by tissues and proteins, increased fecal/urinary losses, and increased demands on the immune system (Cousins, 1985 and Nockels et al., 1993). Decreased immune function increases the likelihood of disease occurrence, with Bovine Respiratory Disease (BRD) as the most common illness in weanling calves. Calf illness results in treatment costs, decreased weight gains, death losses, and decreased profits for producers. There may be benefits to treating potentially mineral-deficient calves with mineral supplements at weaning to correct existing deficiencies and boost immune function. Minerals such as Se, Cu, and Zn are part of the antioxidant enzymes GSH-Px (Se) and SOD (Cu and Zn). These enzymes protect cells from oxidative damage by catalyzing the reduction of toxic oxygen species into less reactive species.

The objective of this study was to investigate whether multi-mineral supplementation (Se, Cu, Zn, and Mn) is more beneficial to calf health versus single mineral (Se) supplementation. The effects of three treatments 1) no treatment (control); 2) Mu- Se injection at weaning; 3) Multi-Min injection at weaning; on immune and health responses were examined. Two trials were performed on two groups of calves. Trial 1 examined the effects of mineral supplementation on heifers born and weaned on-site at the Shenandoah Valley Agricultural Research and Extension Center (SVAREC)(Raphine, VA) and supplemented pre-weaning with a Se-containing trace mineral mix. Trial 2 repeated the same 3 injection treatments on weanling steers (which in previous years were mineral-deficient) purchased from a livestock sale and transported to SVAREC.

Experiment 2. Trial 1: SVAREC heifers

Materials and Methods

Animals, housing and feeding: Thirty-six spring-born Angus crossbred heifers born and weaned on-site at SVAREC were used in this portion of the experiment. At each bleeding, heifers were weighed and rectal temperatures taken. Daily morbidity scores (Perino and Apley, 1998) and treatment records were kept (see Table 1 for morbidity score criteria). Any heifer with a temperature over 105.0°F on a bleeding date was treated with Micotil and Banamine (dosage determined by weight); between bleeding dates, heifers with a morbidity score >1 were treated with Micotil.

Four groups of 9 heifers (average weight 230 kg) were allotted by weight to 4 pastures. Two groups were fed a 15% CP supplement (see experimental design). All groups were supplemented with 25 lbs trace mineral mix (see Table 2 for Guaranteed Analysis of the mineral mixture used) every 2 weeks before and after the experiment. Every 2 weeks, groups were rotated among the 4 pastures to minimize the effects of differences in pasture quality. Four weeks after the start of the experiment hay was fed to all heifers due to the lack of forage in the pastures. Heifers were vaccinated 3 weeks prior to weaning against bovine respiratory disease (BRD), infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PI-3), and bovine viral diarrhea (BVD) with Pyramid 4® (Fort Dodge Animal Health, Fort Dodge, IA), against clostridial disease with Vision 7® (Intervet, Boxeer, Netherlands) and *Brucella abortus*. Heifers were also treated on d 0 (weaning) for parasites with Cydectin® pour-on anthelmintic.

Experimental Treatments and Design: Thirty-six heifers were assigned to dietary supplement and injection treatments using a split-plot design with diet supplement in the whole plot and injection treatment in the subplot. Heifers were blocked by weight and allotted within blocks to 1 of 4 pastures so that an even representation of light, medium

and heavy heifers was in each pasture. Each pasture group was randomly assigned 1 of 2 dietary treatments 1) no supplement; 2) 15% crude protein (CP) at 0.5% body weight. Within each pasture group, heifers were blocked by weight into 3 weight blocks, with the 3 lightest in block 1, the next 3 lightest in weight block 2, and the next 3 lightest in weight block 3. Within weight blocks, 1 of 3 injection treatments was randomly assigned to each heifer: 1) no injection (control); 2) Mu-Se injection (Schering-Plough); or 3) Multi-Min injection (RXV Products). Dosage was determined by the heifer's weight at weaning (d 0) so that each heifer was injected with 0.5cc supplement per 45kg BW; injections were administered subcutaneously (SC). Mu-Se contains 5mg/mL Se and 50mg/mL vitamin E; Multi-Min contains 20mg/mL Zn, 10mg/mL Cu, 5 mg/mL Se, and 20 mg/mL Mn. Responses of interest were phagocytosis, oxidative burst, neutrophil, lymphocyte and total white cell counts, weight, temperature, whole blood Se, serum Cu, and serum Zn concentrations.

Blood collection: Blood was collected from each heifer via jugular venipuncture into: 7mL lithium heparin vacutainer tubes (for cell assays), 10mL EDTA vacutainer tubes (for blood films and whole blood Se analysis), and two 7mL no-additive trace mineral vacutainer tubes (for serum Cu and Zn analysis). Samples were collected at d 0 (weaning), d 7, 14, and 28 (post-weaning). Samples were stored at 4°C until processing.

Cell differentials: Blood films were prepared from each sample at each date and submitted to the Clinical Pathology lab for staining and differential counts.

Mineral analysis: Whole blood Se concentration was measured using graphite furnace atomic absorption spectrometry. Serum Cu and Zn concentrations were measured using flame atomic absorption spectrometry (AAnalyst 800, PerkinElmer Instruments, Norwalk, CN)(Welz, 1985).

Forage analysis: Forage and hay samples were ground to pass a 1mm-mesh screen of a

Wiley mill (Thomas Wiley, Laboratory Mill Model 4, Arthur H. Thomas Co., Philadelphia, PA). Hay samples were ground individually, sub-sampled, and composited by week for chemical analysis. Forage, hay, and supplement samples were analyzed for dry matter (DM), neutral detergent fiber (NDF)(Van Soest and Wine, 1967), acid detergent fiber (ADF)(Van Soest, 1963), and nitrogen, using Kjeldahl analysis (AOAC, 1990). All forage and supplement samples were wet-ashed with 2:1 (V/V) HNO_3:$HClO_4$ for mineral analysis (Muchovej et al., 1986). Digested samples were analyzed for Zn, Cu, and Se using an atomic absorption spectrometer (AAnalyst 800, PerkinElmer Instruments, Norwalk, CN).

Preparation of leukocytes for assays: Leukocytes were isolated from 1 mL whole blood (see Appendix C for isolation technique). Briefly, samples were mixed on a rocking apparatus at room temperature for 20 minutes. One mL whole blood was added to 45 mL lysis buffer (same as used in the GSH-Px experiment) for each sample. White cells were isolated and purified using successive centrifugation and washing steps. The final leukocyte pellet was resuspended in 2 mL Hank's Balanced Salt Solution (HBSS)(JRH Biosciences). One drop of leukocyte suspension was placed on a hemacytometer to ascertain cell counts. Leukocytes in 4 of 16 squares of the hemacytometer grid were counted and the following formula used to calculate total number of leukocytes/2mL sample:

$$4 \times \#\text{ cells counted} \times 2 \times 10^4$$

This calculation yielded the number of cells per 2mL of suspension and also the # cells per mL whole blood (cells were originally isolated from 1 mL whole blood). The volume of the 2mL cell suspension was adjusted by adding HBSS to result in a final cell concentration of 1^6 cells/mL (the concentration needed for the phagocytosis and oxidative burst assays). Four mLs total were needed to run both assays, each requiring a 1mL

control and a 1mL test sample.

Phagocytosis assay: (See Appendix C for cell preparation)(See Appendix D for detailed assay instructions). The purpose of this assay is to measure the phagocytic capability of neutrophils represented by their ability to phagocytize fluorescent polystyrene beads. For each sample, 1 mL leukocyte suspension containing 10^6 cells/mL was pipetted into each of 2 polypropylene test tubes, one for the "cold" (control) sample and one for the "warm" sample. Following addition of fluorescent beads to each sample and incubation for 60 minutes at either 0°C (control sample) or in a 37°C water bath (warm sample), reactions were stopped by addition of 3mL PBS-gelatin EDTA. Each sample was analyzed on a flow cytometer (Coulter Epics XL-MCL) emitting at 525 nm. This instrument measures the mean channel fluorescence, representative of neutrophils' uptake of fluorescent beads. The control samples were baseline, representing random binding of beads to neutrophils. This amount was subtracted from the % fluorescence by the "warm" samples, giving net % fluorescence.

Oxidative burst assay: (See Appendix C for cell preparation)(See Appendix E for detailed assay instructions). This assay indirectly measures hydrogen peroxide (H_2O_2) production by cellular oxidative burst by measuring the percent fluorescence of activated leukocytes. Dichlorofluorescin diacetate (DCF-DA) (non-fluorescent) is added to each sample. DCF-DA diffuses into cells and is hydrolyzed by intracellular esterases to 2'7'-Dichlorofluoroscin (DCFH). Leukocytes are activated by (phorbol 12-myristate 12-acetate (PMA). DCF is oxidized intracellularly by hydrogen peroxide to 2'7'-dichlorofluorescein, which is highly fluorescent (Bass et al., 1983). For each sample, 1 mL leukocyte suspension containing 10^6 cells/mL was pipetted into each of 2 polypropylene test tubes, one for the control sample and one for the "test" sample. One µL/mL of 5mM DCF-DA was added to each test tube for a final concentration of 5µM

DCFH-DH. All test tubes were incubated for 15 minutes in a 37°C water bath. Following incubation, PMA was added to the "test" samples while HBSS was added to the control samples. All samples were incubated 10 min in a 37°C water bath. Mean fluorescence was detected by a flow cytometer emitting at 525nm. Net % fluorescence was calculated by subtracting the % fluorescence emitted by the control sample (baseline) from the % fluorescence emitted by the test sample.

Statistical Analysis: The main effects of diet supplement, injection treatment, and date, as well as their interactions were analyzed using repeated measures mixed model ANOVA with spatial power as a covariance structure. A Tukey-Kramer Differences of LS Means test was performed on the date effect for all responses. Contrasts were used to test diet x date and injection treatment x date interactions, and *P*-values were compared to a=.05/3=0.0167 (Bonferoni correction) for differences between the 3 injection treatments, and a=.05/2=0.025 for the 2 diet treatments.

Results

2-way Interaction Effects:

Table 4 lists the *P*-values for the test of contrasts for the dietary supplement x date interaction effects on weight, serum Cu and serum Zn. Calves supplemented with 15% CP at 0.5% BW gained weight more rapidly between d 14-28 than unsupplemented calves ($P<0.0001$)(Figure 1).

Serum Cu concentrations in the unsupplemented group increased more rapidly from d 0-7 than in the supplemented group ($P=0.008$). After d 7, serum Cu concentrations were similar for both groups (Figure 2).

Serum Zn concentrations decreased more rapidly in the supplemented group from d 0-7, compared to the non-supplemented group ($P=0.016$). After d 7 serum Zn concentrations were similar for both groups (Figure 3).

Calves in the Mu-Se injection treatment group gained weight faster between d 14-28 than calves in either the control ($P=0.02$) or Multi-Min ($P=0.01$) groups (Figure 4).

Main Effects:

There was a date effect for all responses of interest (see Table 5- LS means for all responses across date). A Tukey-Kramer Differences of Least Square Means test was performed on the LS means of all responses (see Table 6 for adjusted *P*-values).

Phagocytosis and oxidative burst: Phagocytic activity of neutrophils decreased from d 0-7, increased from d 7-14, and then decreased sharply on d 28 to below d 0 values. Hydrogen peroxide production (measured by the oxidative burst assay) increased from d 0 through d 7 and 14, and then decreased on d 28.

Neutrophil, lymphocyte, and total white cell counts: Neutrophil counts decreased from d 0 to d 7, then increased back to d 0 levels by d 14-28. Lymphocyte counts changed opposite to neutrophil counts, increasing on d 7 and then decreasing back to d 0 levels on

d 14-28. Total white cell counts decreased from d 0 through d 28.

Body weight and temperature: Weight increased across all dates. Heifers gained 19 kg from d 0 to d 28. Body temperature decreased between d 0 and 7 (105.2 to 102.3°F) and then increased through d 14-28.

Whole blood Se, serum Cu and Zn: Selenium concentrations increased on d 7, decreased at d 14, and then increased to greater-than d 0 concentrations by d 28. Serum Cu and Zn concentrations changed in opposite ways, with serum Cu increasing through d 14 and then decreasing to d 0 concentrations, and serum Zn decreasing on d 7 and then increasing to d 0 concentrations by d 28.

Other effects: A table of average morbidity scores and number of heifers treated with Banamine and Micotil is presented in Table 7. Scores are grouped by week and injection treatment administered.

Forage and hay nutritive analysis results are shown in Table 8.

Discussion

The heifers raised at SVAREC were fed a trace mineral salt containing Se, Cu, and Zn both pre-weaning and throughout the experiment. These heifers had adequate whole blood Se status at weaning (>0.10 ppm). Based on research performed by Galyean et al. (1999) and Wright et al. (1997), no effect of injection treatment was expected on the responses measured in this experiment. Heifers supplemented with Mu-Se had greater weight gain than unsupplemented calves or calves supplemented with Multi-min. Negative interaction between minerals in Multi-min may account for the difference in weight gain between Mu-Se- and Multi-min-supplemented heifers. All other responses measured did not appear to benefit from parenteral mineral supplementation at weaning. Excess minerals are secreted from the body via urine or feces, or stored in tissues (Nockels, 1993).

The responses studied changed over time due to the effects of the weaning stressors placed on the calves (separation from the dam, change in feed and environment, etc.), with peak changes occurring between d 0 (weaning) and d 14. By d 28 most responses had returned to pre-weaning concentrations.

Changes in immune responses: Stress seemed to have a negative effect on the phagocytic activity of neutrophils, evidenced by the decrease in phagocytosis (a measure of neutrophils' ability to engulf foreign particles) on d 7. This ability rebounded to pre-stress levels by d 14, but then decreased precipitously on d 28. Mishandling of the cells during preparation for the assays could cause cell death, and account for the decline in phagocytic activity on d 28. However, each diluted white cell sample was divided into 4 aliquots each containing 1^6 cells to provide cells for both the phagocytosis and oxidative burst assays. If cell death occurred, it was not in the original cell preparation but during assay preparation.

Oxidative burst activity increased from d 0 to d 7, indicating increased hydrogen peroxide-producing capacity of neutrophils. Murata et al. (1987) reported similar results, where transportation stress triggered increased NBT reduction (another assay that measures oxidative burst).

Borderline neutropenia was observed on d 7 (mean=16.1%)(see Table 3 for normal bovine hematology values), but counts increased by d 14 to pre-weaning counts. Lymphocyte counts increased on d 7 and then decreased to pre-weaning counts. These results conflict with results published by others (Anderson et al., 1999; Blecha et al.1984; and Murata et al. 1987), where stress induced neutrophilia and lymphopenia. Total white cell counts decreased from d 0-7, which conflicts with previously published results, where stress increased total white cell counts (Anderson et al., 1999; Blecha et al.,1984; and Murata et al. 1987). Total cell counts on d 0 were low-normal ($4.73x10^6$ cells/mL), and decreased to below normal by d 28 ($2.80x10^6$ cells/mL).

Changes in health responses: Heifers gained weight throughout the experiment, gaining an average of 19 kg between d 0 and d 28. Heifers supplemented with 15% CP at 0.5%BW did not have any difference in rate of weight gain until d 28. These results are consistent with a study by Austin (unpublished data) where heifers supplemented with either 15% crude protein (CP) or 30% CP at 0.5% or 1.0% BW had similar weight gains compared to unsupplemented heifers until d 28, when heifers fed the 15% CP supplement at 0.5% BW had higher gains than those supplemented with either 30% CP at 0.5% BW or 15%CP at 1% BW. By d 42 all heifers in supplemented groups had higher average daily weight gains compared to heifers in the unsupplemented group.

Changes in mineral concentrations: Forages and hay supplement contained insufficient Se concentrations (<0.06 mg Se/kg diet) to maintain adequate selenium status, which is common in forages grown in the Southeast United States (Kubota, 1972). There was no

benefit of additional supplementation of either Se alone or Se in conjunction with Cu and Zn on mineral status. Previous experiments reported conflicting results of effects of Cu or Zn and Se supplemented together. Koh and Judson (1987) reported that when supplemented together, Se exerted an antagonistic effect on Cu concentrations in plasma, blood, blood cells and liver, compared to controls and Cu and Se supplemented individually. Gleed et al. (1983) found no interaction between Cu and Se, while Amer et al. (1973) reported increased hepatic Cu concentrations in Se-supplemented calves. Reffett et al. (1986) reported that calves supplemented with Zn and Se together had decreased whole blood GSH-Px activities compared to calves receiving the Se injection alone, indicating a negative interaction between Zn and Se. The difference in weight gain between injection treatment groups could be attributable to negative interactions between Cu, Zn, and Se, since heifers in the Mu-Se-supplemented group had increased weight gain compared to control or Multi-Min groups.

Weaning stressors had different effects on Se, Cu, and Zn. While whole blood Se and serum Cu concentrations increased, serum Zn concentrations decreased. Other researchers have noted similar effects of stress on Cu and Zn concentrations (Nockels et al., 1993, Orr et al., 1990, Stabel et al., 1993).

The increase in whole blood Se was probably due to increases in serum Se, since erythrocyte selenium is unlikely to change in such a short time (Maas, 1993 and Thompson et al., 1981). Increased Se concentration could result from release of stored Se from the liver and other tissues into the bloodstream, since even unsupplemented control steers' Se concentrations increased (nonsignificantly). In Experiment 1, weaning stress caused increased serum Se concentrations. Kentland calves' serum Se concentrations increased from marginal concentrations pre-weaning to adequate concentrations by d 7 post-weaning.

Changes in serum Cu and Zn can be affected by liver metabolism of these minerals. Hormones and metal binding proteins such as metallothionine (MT) and ceruloplasmin produced as a result of stress can affect release or sequestration of minerals such as Cu and Zn, causing increased or decreased serum concentrations. Stress can also cause increased excretion of minerals via feces and urine. In this experiment, Se and Cu were secreted into serum between d 0 and d 7, while Zn was removed from serum.

These different effects of stress on mineral concentrations mean that the mineral status of a stressed calf does not accurately predict its pre-stress blood mineral status. If minerals are stored in the liver, stress could cause release of these minerals into blood, and the calf's status would seem better than it truly was. Conversely, stress could increase sequestration of minerals by binding proteins or increased losses via urine or feces, so an animal that is marginal or severely deficient in that mineral becomes more deficient.

Tables and Figures
Experiment 2, Trial 1. SVAREC heifers

Table 1. Criteria for morbidity scoring

Morbidity scale

1 = normal, no signs of disease
2 = noticeable depression, signs of weakness are usually not apparent
3 = marked depression, moderate signs of weakness may be apparent but without significant alteration of gait
4 = severe depression accompanied by signs of weakness such as altered gait or lowered head
5 = moribund, unable to rise

- adapted from Perino and Apley, 1998

Table 2. Guaranteed analysis of the mineral supplement given to the SVAREC heifers

Mineral	**Min amount**	**Max amount**
Calcium	10.7 %	12.8 %
Phosphorus		6.5 %
Salt (NaCl)	16.2 %	19.5 %
Potassium		1.1 %
Magnesium		11.2 %
Sulfur		1.9 %
Zinc		510 ppm
Manganese		3,970 ppm
Copper		2,500 ppm
Iodine		136 ppm
Cobalt		97 ppm
Selenium		120 ppm
Vitamin A		345,800 IU/lb
Vitamin D		85,600 IU/lb
Vitamin E		500 IU/lb

Manufactured for King Ag Products, Inc. (Pulaski, VA)

Table 3. Normal bovine hematology values

	Cell Type					
WBC	Segs.	Bands	Lymph.	Mono.	Eos.	Baso.
4.0-12.0 x 106/L	15-45%	0-2%	45-75%	2-7%	2-20%	0-2%

* Reference intervals, Clinical Pathology Lab, Veterinary Teaching Hospital, Virginia-Maryland Regional College of Veterinary Medicine.

Table 4. *P*-values for test of contrasts for dietary supplement x date interaction effect on weight, serum Cu, and serum Zn for SVAREC heifers

		Date Interval		
Response	Dietary supplement*	0-7	7-14	14-21
weight	no supp v supp	0.5208	0.4816	<0.0001
[Cu] µg/dL	no supp v supp	0.0080	0.5170	0.3864
[Zn] µg/dL	no supp v supp	0.0158	0.9459	0.4270

* supplement = 15% CP fed at 0.5% BW

Table 5. Least square means, standard errors, and *P*-values for the date effect for all responses [†]

	Days post-weaning				
Response	0	7	14	28	*P*-value
Phagocytosis (% fluorescence)	37.3 ± 3.2	19.6 ± 2.9	53.8 ± 3.0	3.7 ± 3.1	<0.0001
Oxidative burst (% fluorescence)	9.70 ± 1.4	15.8 ± 1.45	17.1 ± 1.40	8.90 ± 1.4	<0.0001
Neutrophil count (% cells)	22.1 ± 1.7	15.8 ± 1.7	19.8 ± 1.8	21.2 ± 1.7	0.0052
Lymphocyte count (% cells)	74.2 ± 1.7	79.6 ± 1.7	72.6 ± 1.7	73.5 ± 1.6	0.0054
Total WBC/mL ($x10^6$)	4.7 ± 0.21	3.8 ± 0.21	4.1 ± 0.21	2.8 ± 0.21	<0.0001
Weight (kg)	218.4 ± 4.3	227.5 ± 4.3	231.8 ± 4.4	237.4 ± 4.5	<0.0001
Temperature (°F)	105.2 ± 0.16	102.3 ± 0.16	103.3 ± 0.16	103.7 ± 0.16	<0.0001
[Se] ppm	0.150 ± 0.007	0.175 ± 0.007	0.158 ± 0.007	0.210 ± 0.007	<0.0001
[Cu] µg/dL	52.9 ± 4.5	69.0 ± 4.5	81.7 ± 4.5	63.7 ± 4.6	<0.0001
[Zn] µg /dL	74.6 ± 3.0	54.1 ± 3.0	69.2 ± 3.0	66.0 ± 3.0	<0.0001

[†] each value is the LS mean of n=36 observations ± SEM

Table 6. Adjusted *P*-values for the Tukey-Kramer differences of LS means test of date effect for all responses

	Date Interval		
Response	0-7	7-14	14-28
Phagocytosis (% fluorescence)	<0.0001	<0.0001	<0.0001
Oxidative burst (% fluorescence)	0.0008	0.8139	<0.0001
Neutrophil count (% cells)	0.006	0.1523	0.8721
Lymphocyte count (% cells)	0.0546	0.008	0.9692
Total WBC count/mL (x 10^6)	0.0015	0.6794	<0.0001
Weight (kg)	<0.0001	0.0003	0.0001
Temperature (°F)	<0.0001	<0.0001	0.3838
[Se] ppm	0.001	0.05	<0.0001
[Cu] µg/dL	<0.0001	<0.0001	<0.0001
[Zn] µg /dL	<0.0001	<0.0001	0.7323

Table 7. Average morbidity scores and number of animals treated for SVAREC heifers, grouped by days post-stress and injection treatment

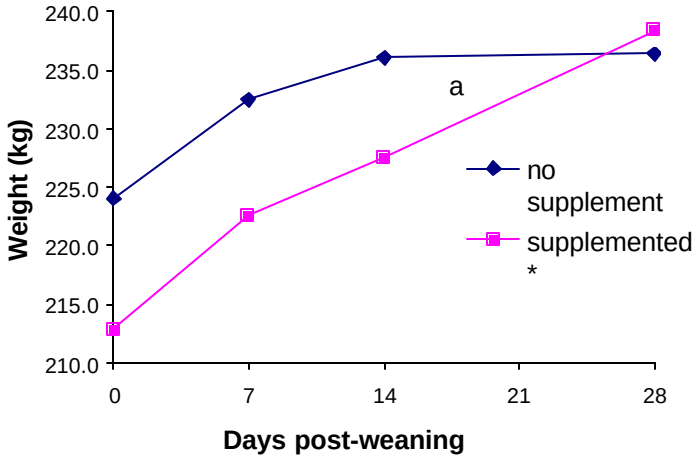
Morbidity	Control	Mu-Se	Multi-Min
d 0	1	1	1
d 7	1.17	1.5	1.42
d 14	1	1	1
d 28	1	1	1
Treated*			
d 0	0	0	0
d 7	2	5	3
d 14	0	0	0
d 28	0	0	0

* "Treated"=w/ Micotil and Banamine for illness/fever

Table 8. Results of forage analysis for the SVAREC heifers (averaged across 4 pastures)

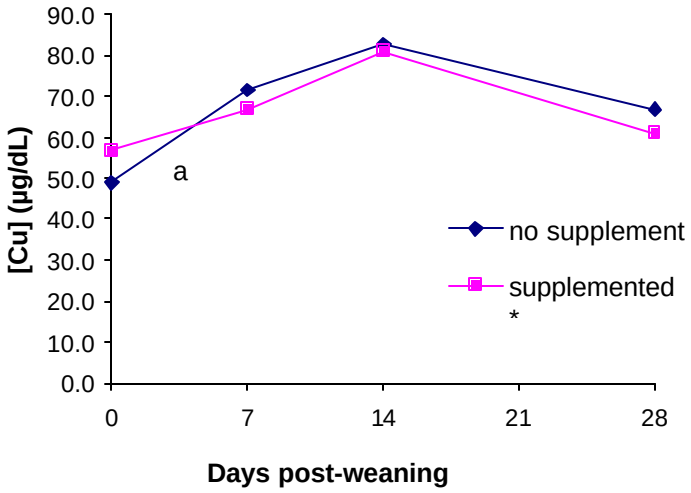
Date	DM	% CP	%NDF	% ADF	Se (ppm)	Cu (ppm)	Zn (ppm)
0	92.77	17.4	53.9	34.2	0.019	5.3	20.5
7	93.10	14.5	55.1	27.9	0.011	5.3	17.5
14	93.92	12.7	58.1	29.8	0.016	5.1	20.0
28	92.51	13.4	59.9	30.1	0.027	3.4	16.9
average	93.1	14.5	56.8	30.5	0.018	4.8	18.7
Hay analysis (overall)	92.2	14.7	34.7	17.4	0.004	2.7	18.8

Figure 1. Body weight of supplemented and unsupplemented heifers by days post-weaning [†‡]



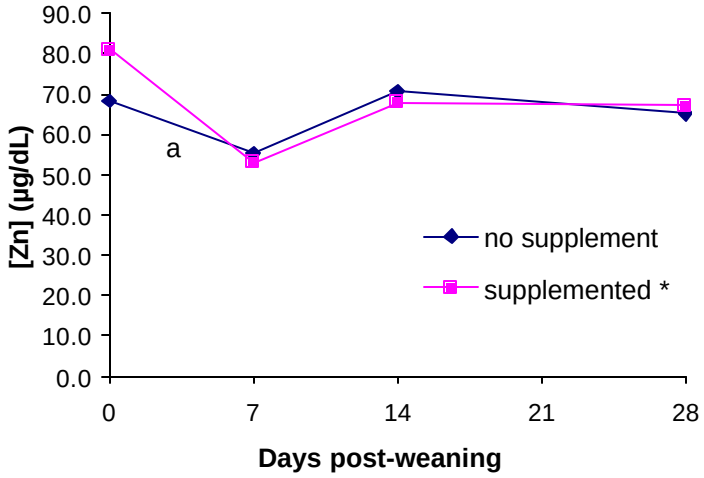
[†] a - indicates date interval where significant interaction occurred ($P<0.0001$)
[‡] each value is the mean of n=18 observations
* supplement = 15% CP fed at 0.5% BW

Figure 2. Serum Cu concentration in supplemented and unsupplemented weanling heifers †‡



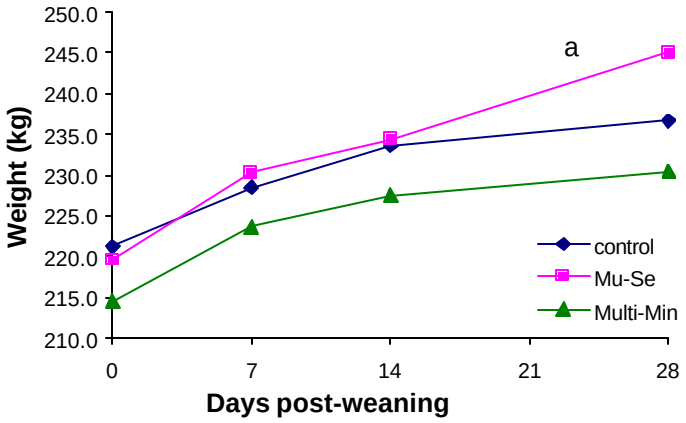
† a - indicates date interval where significant interaction occurred (*P*=0.008)
‡ each value is the mean of n=18 observations
* supplement = 15% CP fed at 0.5% BW

Figure 3. Serum Zn concentration in supplemented and unsupplemented weanling heifers †‡



† a - indicates date interval where significant interaction occurred (*P*=0.02) according to a test of contrasts in SAS
‡ each value is the mean of n=18 observations
* supplement = 15% CP fed at 0.5% BW

Figure 4. Weight gain in weanling heifers, grouped by injection treatment †‡



† a - indicates date interval where significant interaction occurred*;* slopes between Mu-Se and Multi-Min /control are significantly different ($P<.05$)

‡ each value is the mean of n=12 observations

Trial 2: SVAREC nutrient management steers

Materials and Methods

Animals, housing and feeding: Forty-eight weanling steers purchased at auction from 2 sources were used in this trial. Steers were allotted into 12 groups of 4 steers each and put onto 12 pastures. The 12 pastures contained 3 replications of 4 pasture management systems (see experimental design). Steers were also fed ground corn, starting with 0.23 kg /head/day at arrival (d 0) and increasing by 0.23 kg/head/day over 7 days to achieve a final supplementation of 1.8 kg/head/day. Steers were vaccinated upon arrival with Pyramid 4® and Vision 7®. Steers were weighed and temperatures taken at each bleeding date. Daily morbidity scores and treatment records were kept (morbidity score criteria same as for heifers in Trial 1). Any steer with a temperature over 105.0°F on a bleeding date was treated with Micotil and Banamine (dosage determined by weight). Between bleeding dates, steers with a morbidity score >1were treated with Micotil.

Experimental Design: Forty-eight steers were randomly allotted among 4 pasture management systems and 3 injection treatments using a 2 way factorial design. The 4 pasture management systems were: 1) control (no treatment); 2) litter fed; 3) litter applied; and 4) inorganic fertilizer; each pasture management system was replicated 3 times for a total of 12 pastures. Before arrival of the steers, the 12 pastures were randomly allotted into 1 of 3 treatment blocks with an equal representation of the 4 pasture management systems in each block. Three injection treatments (same as or the heifers in Trial 1) were randomly assigned to 1 of the 3 treatment blocks. Steers were allotted to 1 of 4 pasture blocks as they were unloaded off the truck. The first 12 steers unloaded were assigned to pasture block 1, the next 12 to pasture block 2, and so on for blocks 3 and 4. Within each pasture block, the 12 steers were assigned to 1 of 12 pastures. The injection treatment administered to each steer was determined by which

pasture it was assigned (see Table 1 for treatment/pasture assignments). The dosage for the mineral supplements was determined by weight on d 0 (the day of the steers' arrival) and administered on d 0. The dosage and mineral content of the injections was the same as in Trial 1.

Blood collection: Blood was collected from each steer via jugular venipuncture into 7mL lithium heparin vacutainer tubes (for cell assays), 10mL EDTA vacutainer tubes (for blood films and whole blood selenium analysis), and two 7mL no-additive trace mineral vacutainer tubes (for mineral analysis). Samples were collected at d 0 (arrival), d 4, 14, and 28 (post-arrival). Whole blood samples were stored at 4°C until processing. The responses of interest for the nutrient management steers were the same as for the heifers in Trial 1.

Preparation of leukocytes for assays: see heifers, Trial 1

Cell differentials: see heifers, Trial 1

Mineral analysis: see heifers, Trial 1

Forage analysis: see heifers, Trial 1

Phagocytosis assay: see heifers, Trial 1

Oxidative burst assay: see heifers, Trial 1

Statistical Analysis: The main effects of pasture management, injection treatment, time, as well as the time x management and time x treatment interactions were analyzed using mixed model repeated measures ANOVA. A Tukey-Kramer Differences of LS Means test was performed for treatment or date main effects on responses. Contrasts were used to test time x management and time x treatment interactions, and p-values were compared to a=.05/3=.0167 (Bonferoni correction) for differences between the 3 injection treatments and a=.05/6=.008 for the 4 pasture management systems.

Results

2-Way Interaction Effects:

Oxidative burst decreased between d 0 and 4 in steers supplemented with Mu-Se or Multi-min, while oxidative burst did not change between d 0 and 4 in control steers ($P<0.01$). Serum Zn concentrations decreased between d 0 and 4 in control and Multi-min supplemented steers, but increased in Mu-Se supplemented steers ($P<0.005$). After d 4 there was no difference between injection treatment groups in changes in serum Zn concentrations across time.

Serum Zn concentrations of steers on the litter applied pasture management systems decreased between d 0 and 4, while serum Zn of steers on the inorganic pasture management systems increased between d 0 and 4 ($P=0.004$) (Figure 3). There was no difference in changes in serum Zn concentrations between pasture management systems after d 4.

Main Effects:

Table 2 contains *P*-values for main effects of time, pasture management, and injection treatment. There was a date effect for all responses, and a treatment effect on phagocytosis ($P=0.03$) and whole blood Se concentration ($P=0.02$). Least square means of each response by date are shown in Table 3, and LS means for phagocytosis and Se concentration for each injection treatment are in Table 5. Table 4 contains adjusted P-values for the date effects on all responses measured, and Table 6 contains the P-values for the injection treatment effects on phagocytosis and Se concentration.

Phagocytosis and oxidative burst: Both phagocytosis and oxidative burst decreased from d 0-4, then increased to pre-stress levels (oxidative burst) or slightly lower (phagocytosis) through d 28. Steers in the Multi-Min group had higher phagocytic activity ($P=0.04$) than steers in either the control group or the Mu-Se groups.

Neutrophil, lymphocyte and total white cell counts: Neutrophil counts decreased on d 4 ($P<0.0001$), then increased through d 14-28. Lymphocyte counts increased on d 4 ($P<0.0002$), then remained constant from d 14-28. Total white cell counts decreased on d 4 ($P<0.01$), then increased to d 0 levels by d 14.

Weight and temperature: Weight increased from d 0-4 ($P<0.0001$), and from d 14-28 ($P<0.0001$), and temperature decreased between d 0 and d 14 ($P=0.002$).

Blood Se, serum Cu and serum Zn concentrations: Blood Se concentrations decreased from d 0-d 14 ($P=0.002$). Steers in the Mu-Se and Multi-Min groups had higher whole blood Se concentrations than steers in the control group. Serum Cu concentrations increased from d 0-14 ($P<0.001$), while serum Zn concentrations decreased from d 0-14 ($P<0.0001$). Both serum Cu and Zn concentrations returned to d 0 concentrations by d 28.

Other Effects: Average morbidity scores for steers by week and injection treatment are shown in Table 7. Forage analysis results are shown in Table 8.

Discussion

Steers in Trial 2 were exposed to more stressors than the heifers in Trial 1. In addition to separation from the dam and diet changes, the steers were transported, co-mingled with other steers, and had feed and water restrictions. Additional stressors may amplify the effects of weaning on immune and health responses.

Changes in immune responses: The effect of stress on phagocytic activity was not the same for the steers as it was for the heifers in Trial 1. Steers' phagocytic activity did not decrease drastically on d 28 as was seen in the heifer trial. Whether this was due to environmental factors or laboratory error is unknown. Oxidative burst decreased in steers on d 4, while it increased in the heifers. Steers had decreased neutrophilic phagocytic activity and decreased hydrogen peroxide production subsequent to stress. Steers supplemented with Multi-min had greater phagocytic activity than steers given either Se alone or no supplement. It appears that multi-mineral supplementation is more beneficial neutrophilic phagocytic activity than supplementation with Se alone or no supplement. Oxidative burst decreased in Mu-Se and Multi-min supplemented calves between d 0-4, while oxidative burst in the control group did not change. This seems to indicate that mineral supplementation is detrimental to oxidative burst activity in steers. Differences may also be due to differences in d 0 (baseline) oxidative burst values. Although not significant, oxidative burst between d 4-14 increased in control steers, and decreased in Mu-Se and Multi-min supplemented steers. Supplementation of either Se alone or with other minerals may have a negative effect on neutrophilic hydrogen peroxide production within a week post-stress.

As seen in the heifer trial, stress caused decreased neutrophil counts and increased lymphocyte counts from d 0-4. In Experiment 1 (GSH-Px experiment), weaning and transportation stressors caused increased neutrophil and decreased lymphocyte counts. If

heifers and steers could have been bled pre-stress, it is possible neutrophil counts actually increased from pre-stress to d 0, as occurred in calves in Experiment 1. Murata et al. (1994) reported neutrophilia in transported calves immediately post-transport, and Anderson et al. (1999) reported neutrophilia and lymphopenia 13.5 hours following dexamethasone injection. In these experiments, stress caused rapid secretion of neutrophils into the bloodstream, and removal of lymphocytes. Steers were bled post-transport, and heifers were stressed by separation from their dams and loading into the chute for processing. It is possible these stressors triggered increased neutrophil and decreased lymphocyte counts on d 0 that were not detected because there were no pre-stress counts with which to compare. The changes from d 0-4 (steers) or 7(heifers) could represent return to normal pre-stress levels.

Total white cell counts were higher across all dates in the steers versus the heifers. Although total white cell counts decreased from d 0-4, they never decreased below normal counts as occurred in the heifer experiment. The decrease in total white cell counts compared to increases reported by others (Anderson et al., 1999; Blecha et al.,1984; and Murata et al., 1987) could be explained by the same reasoning as for neutrophil/lymphocyte counts.

Changes in health responses: Overall, steers gained 26.3 kg over the 28 days of the experiment, or 0.94kg/day. Body temperature decreased steadily from d 0-28 as steers recovered from weaning and/or transportation stresses. Both steers' and heifers' temperatures were increased above normal on d 0. Increased temperatures were expected on d 0 as a result of stress-induced cytokine release. Gengelbach et al. (1997) states that cytokine-induced fever and decreased feed intake indicate increased immune response. Steers' and heifers' high temperatures on d 0 were probably indicative of cytokine production and increased immune function as a result of stressors and antigen exposure.

Increased body temperature can also be a by-product of ambient temperatures and movement.

Changes in mineral concentrations: Most of the nutrient management steers bought for a previous study were Se-deficient (Austin, unpublished data). Mineral supplementation was expected to improve health and immune responses measured. Steers in this experiment were marginally Se-deficient on d 0 (mean Se concentration=0.054 ppm). Steers in the Mu-Se and Multi-Min groups had higher whole blood Se concentrations than steers in the control group, but these steers still had whole blood Se concentrations considered marginally deficient. Whole blood Se concentration was the same for steers supplemented with either Mu-Se or Multi-Min. This was expected since both supplements contain the same amount of Se (5.0 mg/mL). As in a previous experiment (Maas et al., 1993), a single injection of Se was insufficient to increase Se concentrations to adequate status. Forages grazed by steers had insufficient Se concentrations to maintain adequate Se status. Supplementation with a Se-containing trace mineral ration in addition to the injection would likely increase the steers' Se status to adequate and maintain their status over time.

Injection treatment x date interaction effects were due to differences in mean d 0 values between injection treatment groups. Significant interaction effects did not exist beyond d 4. These results also applied the pasture management x date interaction; after d 4, serum Zn concentrations changed at the same rate across all times for all pasture management systems.

As seen the heifer trial, whole blood Se and serum Cu increased post-stress, while serum Zn decreased.

Tables and Figures

Experiment 2, Trial 2. SVAREC nutrient management steers

Table 1. Nutrient management steers-assignment of pasture and injection treatments

Treatment	Paddock	Steer ID	Trt 2
Fertilizer	1/2	31	
		32	
		33	
		34	
Litter applied	3/4	35	
		36	
		37	
		38	
Litter fed	5/6	39	
		40	
		41	
		42	
Control	7/8	43	
		44	
		45	
		46	

Treatment	Paddock	Steer ID	Trt 3
Fertilizer	9/10	47	
		48	
		49	
		50	
Litter applied	11/12	51	
		52	
		53	
		54	
Litter fed	13/14	55	
		56	
		57	
		58	
Control	15/16	59	
		60	
		61	
		62	

Treatment	Paddock	Steer ID	Trt 1
Fertilizer	17/18	63	
		64	
		65	
		66	
Litter applied	19/20	67	
		68	
		69	
		70	
Litter fed	21/22	71	
		72	
		73	
		74	
Control	23/24	75	
		76	
		77	
		78	

trt1=no inj
trt2=MuSe
trt3=Multi-min

Table 2. *P*-values for main effects on health and immune responses measured in the SVAREC nutrient management steers

	Date effect *P*-value	Inj. Trt. effect *P*-value	Past. Mang. effect *P*-value
Response			
Phagocytosis (% fluorescence)	<0.0001	0.0334	0.2498
Oxidative burst % fluorescence)	0.0008	0.3448	0.8266
Neutrophil Count (% cells)	<0.0001	0.6554	0.8022
Lymphocyte Count (% cells)	<0.0001	0.6775	0.4965
Total WBC/mL ($x10^6$)	0.0149	0.0798	0.2083
Weight (kg)	<0.0001	0.5791	0.5342
Temperature (F)	<0.0001	0.7373	0.1642
[Se] ppm	<0.0001	0.0220	0.2137
[Cu] µg/dL	0.0003	0.1358	0.5320
[Zn] µg /dL	<0.0001	0.1357	0.1663

Table 3. Least square means and SE for date effect on health and immune responses measured in the SVAREC nutrient management steers †

	Date			
Response	0	4	14	28
Phagocytosis (% fluorescence)	27.0 ± 1.4	12.8 ± 1.3	20.8 ± 1.4	20.0 ± 1.3
Oxidative burst % fluorescence)	35.7 ± 3.3	18.3 ± 3.3	24.9 ± 3.3	39.1 ± 3.3
Neutrophil Count (% cells)	37.8 ± 1.7	22.3 ± 1.7	24.2 ± 1.7	28.9 ± 1.7
Lymphocyte Count (% cells)	55.6 ± 2.0	70.1 ± 2.0	70.2 ± 2.0	65.1 ± 2.0
Total WBC/mL ($x10^6$)	6.4 ± 0.23	5.2 ± 0.23	6.1 ± 0.23	5.8 ± 0.23
Weight (kg)	193.9 ± 2.0	203.7 ± 1.6	207.5 ± 1.8	220.2 ± 2.0
Temperature (°F)	104.1 ± 0.13	103.9 ± 0.13	103.5 ± 0.13	103.2 ± 0.13
[Se] ppm	0.054 ± 0.004	0.058 ± 0.004	0.039±0.004	0.038 ± 0.004
[Cu] µg/dL	49.0 ± 2.3	53.9 ± 2.3	61.6 ± 2.3	47.7 ± 2.3
[Zn] µg /dL	54.4 ± 1.9	47.6 ± 1.9	30.3 ± 1.9	58.4 ± 1.9

† each value is the mean of n=48 observations

Table 4. Adjusted *P*-values for the Tukey-Kramer differences of LS means test for date effect on health and immune responses in the SVAREC nutrient management steers

	Date Interval		
Response	0-4	4-14	4-28
Phagocytosis (% fluorescence)	<0.0001	0.009	0.01
Oxidative burst % fluorescence)	0.006	0.4809	0.001
Neutrophil Count (% cells)	<0.0001	0.8238	0.04
Lymphocyte Count (% cells)	0.0002	0.9999	0.2795
Total WBC ($x10^6$)	0.01	0.0941	0.4092
Weight (kg)	<0.0001	0.0894	<0.0001
Temperature (°F)	0.3420	0.0738	0.0003
[Se] ppm	0.6176	0.0001	<0.0001
[Cu] µg/dL	0.3018	0.05	0.1325
[Zn] µg /dL	0.1188	<0.0001	0.007

Table 5. Least square means of phagocytosis and whole blood Se concentration in the SVAREC nutrient management steers, grouped by injection treatment †

	Injection treatment		
Response	Control	Mu-Se [1]	Multi-min [2]
Phagocytosis (% fluorescence)	18.9 ± 0.815	19.0 ± 0.693	22.5 ± 0.693
[Se] ppm	0.031 ± 0.0052	0.058 ± 0.0052	0.053 ± 0.0052

† each value is the mean of n=48 observations
[1] Mu-Se contains 5 mg/ml Se and 50 mg/mL vitamin E
[2] Multi-min contains 20 mg/mL Zn, 10 mg/mL Cu, 5 mg/mL Se, and 20 mg/mL Mn;

Steers were injected with 0.5cc supplement per 45 kg BW

Table 6. Adjusted *P*-values for the Tukey-Kramer differences of LS means test for injection treatment effect on phagocytosis and whole blood Se concentration in the SVAREC nutrient management steers

	Injection Treatment †		
Response	1 v 2	1 v 3	2 v 3
Phagocytosis (% fluorescence)	0.9976	0.0548	0.044
[Se] ppm	0.0241	0.0523	0.8012

† where 1=control, 2=Mu-Se, 3=Multi-min

Table 7. Average morbidity scores and number of animals treated for the SVAREC nutrient management steers, grouped by days post-stress and injection treatment

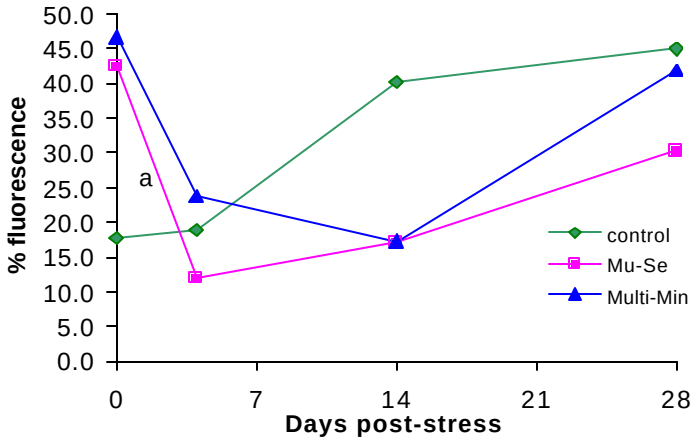
Morbidity	Control	Mu-Se	Multi-Min
d 0	1.01	1.01	1.00
d 4	1.02	1.00	1.00
d 14	1.02	1.00	1.00
d 28	1.01	1.00	1.00
Treated*			
d 0	1	1	5
d 4	0	0	2
d 14	1	1	1
d 28	1	0	0

* "Treated"=w/ Micotil and Banamine for illness/fever

Table 8. Forage analysis for the SVAREC nutrient management steers

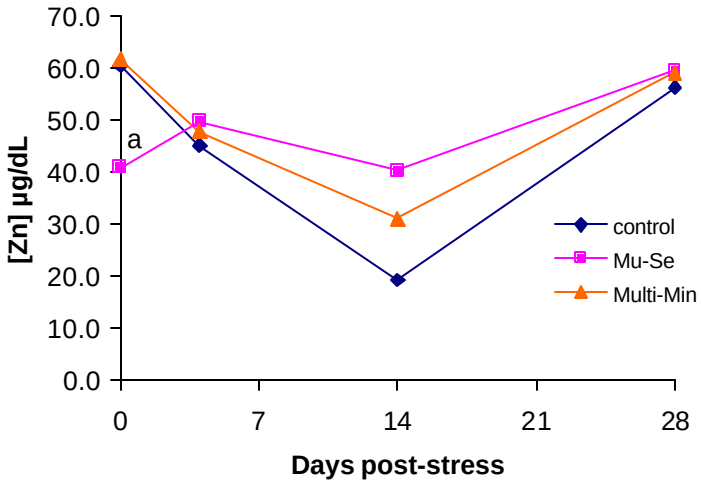
d 0	%DM	%CP	%NDF	%ADF	[Se] ppm	[Cu] ppm	[Zn] ppm
Inorganic Fertilizer	92.7	12.8	55.5	27.2	0.022	2.28	18.64
Litter Applied	92.4	13.6	54.9	27.1	0.006	1.84	18.64
Litter Fed	92.9	12.6	54.9	26.8	0.012	1.90	34.90
Control	93.7	12.3	55.2	27.1	0.012	1.24	19.95
d 4							
Inorganic Fertilizer	95.4	10.4	59.9	30.3	0.032	2.1	17.4
Litter Applied	95.3	11.8	59.4	29.9	0.017	2.3	17.3
Litter Fed	95.1	11.5	59.2	29.2	0.015	2.3	17.5
Control	95.1	11.4	57.6	29.0	0.012	2.0	19.6
d 14							
Inorganic Fertilizer	95.5	10.8	63.5	31.5	0.003	2.2	12.8
Litter Applied	95.6	11.4	64.7	32.3	0.008	2.4	14.2
Litter Fed	95.0	11.1	63.3	31.7	0.005	2.8	15.8
Control	95.6	11.9	63.0	31.6	0.007	2.4	13.6
d 28							
Inorganic Fertilizer	96.8	13.1	62.4	30.4	0.010	2.6	17.8
Litter Applied	95.9	12.9	65.5	32.3	0.016	2.6	19.7
Litter Fed	94.7	13.1	63.9	31.6	0.012	2.7	19.4
Control	97.5	13.3	63.1	31.2	0.013	2.6	17.5

Figure 1. Oxidative burst in weanling steers, grouped by injection treatment †‡



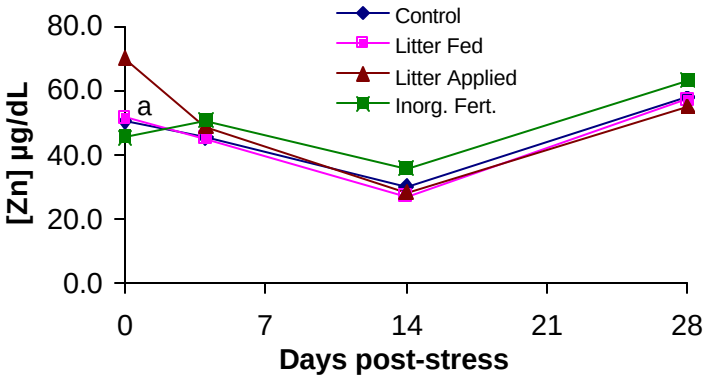
† a - indicates date interval where significant interaction occurred ($P<0.01$) according to a test of contrasts in SAS
‡ each value is the mean of n=16 observations

Figure 2. Serum Zn concentrations in weanling steers, grouped by injection treatment †‡



† a - indicates date interval where significant interaction occurred ($P<0.005$) according to a test of contrasts in SAS
‡ each value is the mean of n=16 observations

Figure 3. Serum Zn concentrations in weanling steers, grouped by pasture management system [†] [‡]



[†] a - indicates date interval where significant interaction occurred ($P<0.004$) according to a test of contrasts in SAS

[‡] each value is the mean of n=12 observations

Chapter V.

Conclusions

Stress affects mineral status and various health and immune responses in different ways. In the first experiment, weaning stressors seemed to have positive effects on the immune system, evidenced by increased leukocyte GSH-Px activity. In the heifer trial in experiment 2, oxidative burst (hydrogen peroxide production) increased in response to weaning stressors. It makes sense that GSH-Px activity and oxidative burst would increase together, because increased antioxidant activity would be needed to neutralize the hydrogen peroxide produced by neutrophils. Serum Se also increased, and it is possible that leukocytes utilized Se from serum to make more GSH-Px. Decreased oxidative burst in the steer trial was most likely due to their Se deficiency. Additional stressors experienced by the steers could also have depressed neutrophilic hydrogen peroxide production.

Phagocytic activity decreased in both the heifer and steer trials. Stress may cause release of hormones or other substances that negatively affect neutrophil phagocytic activity. Although neutrophil counts seemed to decrease from d 0 to 4 or 7 in Experiment 2, this might represent a return to normal counts after an increase immediately following stress. Conclusions cannot be drawn without pre-stress counts for comparison.

Stress has different effects on different minerals. While stress may trigger increases in concentrations of some mineral such as Se and Cu, it decreases others, like Zn. Mineral balance is affected by numerous factors such as absorption rates, binding to proteins, excretion via urine and feces, and utilization by tissues. If a producer wants a true measure of calves' mineral status, measurements should be taken either before stress or 3-4 weeks after stress to avoid stress-induced elevations or depressions.

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Appendices

Appendix A: Glutathione peroxidase assay - Cell isolation and preparation.

Lysis Buffer: (makes 1L)

8.03 g NH_4Cl
0.84 g NaHCO3
2.92 g EDTA free acid

Mix and add deionized water to a final volume of 1L. Add sodium hydroxide (NaOH) to increase pH to 7.4.

Isolation of red cell pellet

1. Mix samples for 20 minutes at room temperature on a rocking apparatus.
2. Pipette 1mL whole blood from each sample and place in a 1.5 mL microcentrifuge tube.
3. Centrifuge microcentrifuge tubes for 5 min @ 1200xg.
4. Following centrifugation, remove and discard the serum and buffy coat from each sample, leaving a red cell pellet.
5. Stored at -75°C until analysis.

Isolation of white cell pellet

1. Centrifuge samples for 10 min @ 500xg after 20 min mixing at room temperature.
2. Pipette off and discard plasma layer using Pasteur pipette.
3. Carefully pipette buffy coat (containing white cells) and transfer to a 50mL conical tube containing 35mL lysis buffer.
4. Centrifuge samples for 10 min @ 500xg.
5. Following centrifugation, pour off supernatant, leaving the white cell pellet at the bottom of the tube.
6. Add 2 mL lysis buffer and 10mL Hank's Balanced Salt Solution (HBSS)(Gibco) to each sample. Thoroughly mix cell pellet, HBSS, and lysis buffer via pipetting.
7. Incubate samples for 10 minutes at room temperature and then centrifuge 10 min @ 500xg.
8. Pour off supernatant and add 10mL HBSS to each sample.
9. Immediately centrifuge samples 10 min @ 500xg.
10. Pour off supernatant and add 1 mL HBSS added to each sample.
11. Thoroughly mix each sample via pipetting and then transfer to a 1.5mL microcentrifuge tube.
12. Store samples at -75°C until analysis.

Appendix B: Protein analysis via Bio-Rad Protein Assay

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized water (DDI). Filter through Whatman #1 filter (or equivalent) to remove particulates. Store diluted solution at room temperature in a brown bottle for up to 12 days.
2. Prepare 7 dilutions of bovine serum albumin to prepare a standard curve (0.4, 0.3, 0.2, 0.1, 0.05, 0.025, 0.00 mg/mL(blank), diluted in deionized water).
3. Add 125µL dye reagent to each well and mix gently using microplate mixer.
4. Pipette 10µL of each standard and sample solution into separate microtiter plate wells. Sample solutions should be run in duplicate (2 wells per sample).
5. Add 125µL additional dye reagent to each well and mix thoroughly.
6. Incubate 5 minutes at room temperature. Absorbance increases over time, so samples should not incubate longer than 1 hour.
7. Measure absorbance at 595nm.

Appendix C: Phagocytosis and oxidative burst assay cell preparation

KRH-gelatin

Mix together the following:
97 mL deionized water
10 mL 9% NaCl solution
616 µL 1M KCl
600 µL .5M $MgSO_4$
20 mL .1M Hepes-TEA (pH 7.4)
0.108 g Knox unflavored gelatin

PBS-gelatin-EDTA

Mix together the following:
100 mL PBS
0.1 g EDTA disodium salt
0.1 g Knox unflavored gelatin

Isolation of white blood cells

1. Thoroughly mix samples at room temperature on a rocking apparatus for 20 min.
2. Pipette 1mL whole blood from each sample and place in a 50mL conical tube containing 45mL lysis buffer.
3. Incubate samples for 10 min at room temperature then centrifuge for 10 min @ 500xg, 20°C.
4. Poured off supernatant, leaving the white cell pellet at the bottom of the tube.
5. Add 2mL lysis buffer and 10mL HBSS to each sample and mix thoroughly.
6. Incubate samples for 10 min, then centrifuge for 10 min @ 500xg, 20°C.
7. Pour off supernatant, add 10mL HBSS to each sample and mix thoroughly.
8. Immediately centrifuge samples 10 min @ 500xg, 20°C.
9. Pour of supernatant and re-suspended pellet in 2mL HBSS.

Appendix D: Phagocytosis assay

1. Pipette 1mL of cell suspension containing 1^6 cells/mL into round bottom 12X75mm plastic test tubes, 2 for each sample; one represents the control (labeled C), the other represents the heat-incubated sample (labeled H).
2. Centrifuge samples 10min @ 500xg, 20°C.
3. Pour off supernatant. Add 1mL KRH-gelatin, 100µL fetal bovine serum (FBS), and 20µL diluted fluorescent polystyrene beads (Fluoresbrite Beads, 1 micron/FITC conjugated, Polysciences) to each tube.
4. Incubate control samples in an ice bucket (0°C) for 1 hour. Incubate test samples in a 37°C hot water bath for 1 hour.
5. After incubation stop the reaction by pipetting 3mL PBS-gelatin-EDTA into each tube.
6. Centrifuge samples 10min @ 500xg, 20°C.
7. Pour off supernatant and resuspend the cells in 1mL HBSS in preparation for analysis with the flow cytometer. Net % fluorescence is calculated by subtracting the value for the control sample from the value of the corresponding warm (test) sample.

Note: if phagocytosis assay is not to be run immediately, substitute 300µL paraformaldehyde for 1mL HBSS to fix cells. The assay can be run up to 3 days later.

Appendix E: Oxidative burst assay

1. Add 1mL white cell suspension containing 10^6 cells/mL to round bottom plastic 12x75mm tubes, 2 for each sample; one is labled "C" for the control sample and the other is labeled "H" for the test sample.
2. Add 1µL/mL DCF-DA to each sample for a final concentration of 5.0 µM DCFH-DA.
3. Incubate samples 15 minutes in a 37°C hot water bath.
4. Add 12.5 µL PMA (mitogen) to the "test" tubes and 12.5 µL HBSS to the control tubes.
5. Incubate samples 10 minutes in a 37°C hot water bath.
6. Analyze samples on a flow cytometer. Net % fluorescence is calculated by subtracting the value for the control sample from that of the corresponding test sample.

Vita

Alba Maria Montana Shank, daughter of Albert Frank and Alba Vittoria Shank, was born on October 25, 1976 in Charlottesville, Virginia. She graduated from Charlottesville High School in June 1994. She received a Bachelor of Science in Animal and Poultry Sciences in May 1998, and a Bachelor of Science in Biology in May1999 from Virginia Polytechnic Institute and State University. She is a member of Alpha Zeta Honor Fraternity. She will begin work on her DVM degree at Virginia-Maryland Regional College of Veterinary Medicine starting in Fall 2002.

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Alba Maria Montana Shank