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The effect of dietary sulfur on performance, mineral status, rumen hydrogen sulfide, and rumen microbial populations in yearling beef steers

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The effect of dietary sulfur on performance, mineral status, rumen hydrogen sulfide, and rumen microbial populations in yearling beef steers

by

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A thesis submitted to the graduate faculty
In partial fulfillment of the requirements for the degree of

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Major: Nutritional Sciences

Program of Study Committee:
Stephanie L. Hansen, Major Professor
James Russell
Daniel Loy
Steve Ensley

Iowa State University
Ames, Iowa
2011
DEDICATION

First and foremost this work is dedicated to my husband, Nate, for his ceaseless love and support. It is because of his dedication to me, his praise and belief in me, even when I did not believe in myself that this work has finally become a reality.

I also dedicate this to my parents, Mike and Lisa and my siblings, Carmen and Austin. Who have stood by me through it all with unending encouragement and love.

And above all to my Lord and Savior, Jesus Christ, without the strength graciously given me by faith, none of this would be possible.

For nothing is impossible with God. (Luke 1:37, NIV)
BIOGRAPHY

Erin Lisa Richter was born Erin Lisa Brasche on June 24, 1987 in Ames, Iowa to parents, Mike and Lisa Brasche. She was raised in Nevada, Iowa along with a younger brother, Austin and sister Carmen, and attended Nevada High School, graduating in 2005. Erin then attended Iowa State University in pursuit of a Bachelor’s degree in microbiology with double minors in horticulture and general biology. In 2007, she changed her last name to Richter after marrying Nathaniel Lawrence Richter on July 7th at Nevada First United Methodist Church. Upon graduation with her Bachelor’s cum laude in the spring of 2009, she began a Master’s degree in nutritional sciences the fall of the same year. She chose the home department of Animal Science the following spring of 2010. Upon completion of her Master’s she will be moving to not only a different time zone but a different culture in Mansfield, Connecticut to rejoin her husband and possibly pursue a call to ministry.
ACKNOWLEDGEMENT

The person to which the most acknowledgement is due for making this work a reality is Dr. Stephanie L. Hansen. Without her guidance, advice, patience, and encouragement this never would have been completed. The author would also like to thank Dr. Hansen for the opportunity to be the first ‘guinea pig’ in her lab, I know we have both learned a lot and I am so grateful for the lessons I have learned and the time I have spent here.

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List of Abbreviations:

A:P : acetate to propionate
Ca: calcium
Cu: copper
DDGS: dried distillers grains with solubles
DM: dry matter
DNA: deoxyribonucleic acid
Fe: Iron
H: hydrogen
H₂S: hydrogen sulfide
Mg: magnesium
Mn: manganese
N: nitrogen
NPN: non-protein nitrogen
N:S : nitrogen to sulfur
P: phosphorus
PEM: polioencephalomalacia
rRNA: ribosomal ribonucleic acid
rtPCR: real-time polymerase chain reaction
S: sulfur
SO₄: sulfate
SRB: sulfate reducing bacteria
VFA: volatile fatty acid
Zn: zinc
ABSTRACT

Sulfur (S) is a requisite part of the ruminant diet, but may sometimes be present in excess, leading to decreased animal performance and illness. In this experiment, 96 crossbred yearling steers (321 ± 29 kg BW) were used to determine the effects of dietary S concentration of a supplement fed to cattle on pasture and of the total diet in the finishing period. Steers were blocked by weight and allocated to smooth bromegrass-based pastures and supplemented with either a low S dried distillers grains with solubles (DDGS; 0.3% total dietary S; LS; n = 4 plots) or LS DDGS with additional S (0.45% total dietary S; HS; n = 4 plots). After 36 d on pasture, steers moved to the feedlot where half remained on the previous S treatment while half switched treatments, resulting in 4 treatments (LS-LS; LS-HS; HS-LS; and HS-HS; n = 6 feedlot pens). Steers were transitioned to the final finishing diet using a series of step up diets. Once fully transitioned, steers were receiving either 0.3% dietary S (LS) or 0.6% dietary S (HS). Plasma and liver mineral concentrations of half of the steers were determined at beginning of the trial (d 0), the end of the pasture period (d 35), and near the end of study (d 155). Plasma Mg was decreased (P = 0.05) in response to increased dietary S during the pasture period, however steers were not deficient. Copper concentrations in the plasma and liver were decreased (P < 0.05) in steers which had received increased dietary S when measured at the end of study (d 155). Average daily gain of steers did not differ (P = 0.50) between LS and HS during the pasture period, but was lower (P = 0.01) in HS vs. LS steers during the finishing period. Decreased gains translated to a tendency (P = 0.06) for hot carcass weight to be decreased in HS steers vs. LS steers. Fatty acid analysis of longissimus dorsi showed increased stearic and heptadecanoic acid (P = 0.04 and 0.01, respectively) in steers receiving increased dietary S. Cattle exposed to higher S diets during the pasture period did not show differences (P = 0.40) in performance based on the previous pasture treatment. However, cattle fed high dietary S on pasture had greater carcass fat cover (P = 0.01), suggesting S may have influenced lipid metabolism. Rumen H₂S concentration was determined 6 hr post-feeding at multiple time points throughout transition and finishing (d 36, 45, 52, 59, 66, 91,125, and 155). Concentrations of rumen H₂S did not differ while cattle were consuming a high forage diet (1733 and 1400 ppm for HS and LS, respectively; P = 0.54). During the feedlot period, rumen H₂S concentration
was affected by day of sampling ($P < 0.01$). Rumen $\text{H}_2\text{S}$ began to differ due to treatment when forage in the diet decreased to 15% of diet DM ($P = 0.04$). This increase in $\text{H}_2\text{S}$ in HS cattle was sustained throughout the finishing period ($P < 0.05$) when forage was present at 8% of DM. Peak $\text{H}_2\text{S}$ concentrations in HS steers were observed on d 91 when steers had been on the full finishing diet with 8% forage for 23 days (HS: 4813 ppm; LS: 1317 ppm; $P < 0.01$) and again near the end of trial (d 155; HS: 5275 ppm; LS 1248 ppm; $P < 0.01$).

Rumen fluid was collected esophageally for determination of VFA and lactate proportions at the end of the pasture period (d 36), during transition (d 56) and near the end of study (d 155). Lactate proportion was not affected ($P > 0.50$) by dietary S concentration and minimal effects on VFA profiles were observed. Isovalerate was decreased ($P = 0.002$) in cattle receiving elevated dietary S on d 155. Rumen fluid samples collected near the end of study (d 155) from steers which represented treatments maintained on the same S treatment throughout the study (LS-LS and HS-HS, n = 6 pens per treatment) were analyzed for sulfate reducing bacteria (SRB) populations and methanogens using quantitative real time PCR. Steers receiving the HS diet demonstrated an increase ($P = 0.03$) in total SRB and an increase ($P = 0.03$) in a SRB group which represents the SRB most commonly found in the rumen, specifically *Desulfovibrio desulfuricans*. The simple correlation between SRB populations and $\text{H}_2\text{S}$ concentrations ($R = 0.60$, $P = 0.05$) illustrates that a considerable portion of the variation in $\text{H}_2\text{S}$ concentrations among cattle may be accounted for by the differences in SRB populations. These combined effects of dietary S concentration on rumen $\text{H}_2\text{S}$ concentration, VFA proportions, and microbial populations illustrate that rumen metabolism is altered by increased dietary S. Furthermore, these effects on rumen metabolism may partially explain the decreases in performance, evidenced by reduced gains, and HCW, while part of the decrease in performance may also be explained by effects of increased dietary S on mineral status, especially Cu.
CHAPTER 1.
INTRODUCTION

Ruminants like all living organisms, require sulfur (S). This element is a critical part
of some B-vitamins, amino acids, and other cellular components. As with most minerals,
more is not always better. Sulfur toxicity has become a significant problem for cattle
producers across the country. In western states, high levels of sulfate in the water supply
create the potential for S toxicity in cattle, while in the Midwest increased utilization of
ethanol co-products in cattle diets has raised dietary S concentrations. Whether increased S
intake is due to water or feed sources, the effects on the animal are the same, as increases in
total dietary S can result in polioencephalomalacia (PEM), and decreased animal
performance (Gould, 1998).

Ruminants are susceptible to S toxicity because of the metabolism of S in the rumen.
Within the rumen are a myriad of microorganisms which digest and ferment dietary
components. One group of organisms, the sulfate reducing bacteria (SRB), compose a very
small portion of the population, usually representing less than 1% of the population
(Callaway et al., 2010); however, metabolism of S by SRB create a significant threat to
animal health. When diets are high in S, specifically in the form of sulfate, these organisms
utilize the sulfate and produce sulfide. This increased utilization of dietary S leads to
increased hydrogen sulfide in the rumen, and at high enough levels this toxic gas causes
PEM, which if not treated quickly may lead to animal death (Gould, 1998). Loneragan et al.
(2001) found that rumen H2S concentration peaks between 15 and 35 days on-feed, when
animals are fed a high concentrate diet, with additional S in the drinking water. Sulfur may
also negatively impact the ruminant’s mineral status, in particular copper (Cu). The
formation of thiomolybdates leads to a reduction of both Cu absorption and systemic Cu status when thiomolybdates enter circulation and scavenge Cu from biological processes (Suttle, 1991; Kandylis, 1984).

Considering that cattle are not as susceptible to increased dietary S when consuming diets higher in forage (NRC, 1996), this research sought to determine what effects exposure to increased dietary S would have on yearling steers when they first consume increased S while consuming a high forage diet. To this end, steers were backgrounded on pasture with a supplement of dried distillers grains with solubles where treatments only differed in the amount of S (added from sodium sulfate). This backgrounding was done to determine if adjusting the rumen to high S on a high forage diet would prevent some of the problems associated with high dietary S in the feedlot, such as the sharp increase in rumen H$_2$S during the early part of the feeding period. Data collected during this study included analysis of microbial populations, both the SRB populations and methanogens, at the end of study to determine what effects dietary S concentration had on these important rumen populations. It was also of interest to determine if the increases in H$_2$S gas concentrations are due to a change in SRB population numbers or due to a change in the metabolism of these organisms that allows them to increase their reduction of sulfate to sulfide without a change in population numbers.

**Thesis organization**

Immediately following this introduction will be Chapter 2, a detailed review of the literature pertaining to S in cattle, S toxicity, and SRB. Chapter 3 will present original research on the effects of dietary S concentration on yearling steer performance, mineral
status, and carcass characteristics during a pasture backgrounding period and during finishing. Chapter 4 contains analysis from the same study pertaining to the rumen, specifically, rumen hydrogen sulfide concentrations, volatile fatty acid proportions, and rumen microbial populations, including, SRB and methanogens. Finally, Chapter 5 will provide an overview of the findings of the research.
CHAPTER 2.

REVIEW OF LITERATURE

The role of sulfur in biological systems

Sulfur plays a key role in biological systems. Structurally, S is a part of the amino acids cysteine, cystine, and the essential amino acid methionine. Sulfur is also a precursor for the other S-containing amino acids (NRC, 1996). Biotin and thiamine, both B-vitamins and integral players in energy metabolism, also contain S as a part of their structure. Sulfur serves a structural role in mucins, specifically sulfomucins. Mucins are composed of a mixture of proteins, glycoproteins, and lipids which protect the alimentary tract from the contents in the lumen (Tabak, 1995). Microbes can utilize the S in sulfomucins resulting in recycling and utilization of the S (Deplancke et al., 2000). Sulfur is important to wool and hair production in small ruminants due to the high amounts of both cystine and methionine found in hair (Kandylis, 1984; Qi et al., 1994; NRC, 1996). These S-containing amino acids and B-vitamins are noted for their importance to milk and tissue protein production, collagen and connective tissue development, blood clotting, enzyme synthesis, and endocrine function (Kandylis, 1984).

The formation and biological action of iron-sulfur ([Fe-S]) clusters represent another way S is critical for the maintenance of life. These structures are critical to electron transfer in biological processes including photosynthesis and respiration, and comprise the active sites of many enzymes including nitrogenase, hydrogenase, radical S-adenosyl methionine enzymes, ferridoxin, and biotin synthase (Rees and Howard, 2003; Johnson et al., 2005;
Fontecave, 2006). The transfer of a single electron is usually facilitated by [2Fe-2S] or [3Fe-4S] clusters, whereas the more complex [4Fe-4S] clusters are more varied in their mechanisms (Fontecave, 2006). The diverse array of [Fe-S] clusters from the simple [2Fe-2S] clusters to complicated [4Fe-4S] clusters is believed to indicate the evolution of these complexes as one of the integral and original motifs of life on earth (Johnson et al., 2005). In an attempt to determine how many [Fe-S] cluster containing proteins could be found in cells, Fontecave (2006) reviewed the literature and determined that *E. coli* contains at least 110 different proteins that contain these clusters serving a multitude of functions.

**Ruminant requirements for sulfur**

Ruminants are unique in their need for S as the rumen microbiome provides the animal with adequate supplies of S-containing compounds once the needs of the rumen microbes are met (Kandylis, 1984). The beef NRC (1996) recommendation for S concentration in the diet of growing cattle is 0.15%, while a maximal level of 0.4% of the diet is suggested. In small ruminants, especially fiber producing animals like sheep and hair goats, the amounts of S required are higher to allow for adequate fiber production (Qi et al., 1994). The sheep NRC (1985) recommends that growing sheep receive 0.18 to 0.26% S in the diet, in part because of the high S content of wool that Qi et al. (1994) estimated to be 2.7% S.

When diets simply do not contain adequate concentrations of S, it should be supplemented for optimal animal health and production. This is in part to meet the S requirements of the rumen microbes to digest the contents of the rumen and in turn produce protein (NRC, 1996). In decreasing order of bioavailability, the following sources of S may
be used to supplement the diets of beef cattle: calcium sulfate, ammonium sulfate, sodium sulfate, sodium sulfide, and elemental S (NRC, 1996).

**Sulfur deficiency in cattle**

When dietary S concentrations are inadequate significant negative effects on animal performance have been observed. Sulfur deficiency in the ruminant may be characterized by anorexia and weight loss, weakness, dullness, excessive salivation, and eventual animal death if not corrected (Kincaid, 1988; NRC, 1996). In ruminants used for fiber production it has been noted that both quality and quantity of fiber are affected by S deficiency (Qi et al., 1994), which can result in the loss of wool (Kincaid, 1988). Some blood parameters can be measured to indicate S deficiencies, including increases in lactate, alanine, citrulline, cysteine, serine, and non-essential amino acids, and decreases in glycine and tyrosine (Kincaid, 1988). Though S is required for hair growth in ruminants, negative effects to hair growth are also observed as an effect of S toxicity, presumably due to the associated deficiency of Cu (Gooneratne et al., 1989). Thus, rough hair coat could indicate either S deficiency due to a low S-amino acid supply, or S toxicity because of the associated negative impacts on hair related to Cu deficiency.

**Factors affecting sulfur requirements of beef cattle**

Looking at S solely as a dietary constituent neglects the importance of the balance between nitrogen (N) and S in the ruminant diet. Kandylis (1994) reviewed works on nitrogen to sulfur (N:S) ratio and determined that the ideal ratio for cattle is in the range of 13.5:1 to 15:1. This ratio represents a balance for the rumen microbes, between providing
adequate levels of both S and N for microbial growth and incorporation of both elements into proteins and vitamins. These microbial products are later utilized by the ruminant for maintenance and growth. The importance of the N:S ratio was determined when researchers studying the effects of dietary urea concentration on animal performance found that by replacing true protein in the diet with non-protein N they inadvertently created S deficiencies. Lassiter et al. (1958) fed dairy heifers supplements containing urea at 3.0%, 5.0%, or 7.0% of the supplement, which accounted for 25% of diet DM, and found that DM intake was not different among cattle fed the different urea levels. However, efficiency of gain was linearly decreased as cattle average daily gains of 0.51, 0.38, and 0.29 kg/d for the 3.0, 5.0, and 7.0% urea treatments, respectively, were reported. Ignoring dietary S concentrations resulted in dietary S percentages as low as 0.11% of the diet with the highest urea concentration (7% urea in the supplement). This low S concentration likely resulted in the observed poor performance even though animals were receiving an easily digestible source of N. Because urea is not a protein, but rather a source of non-protein N, it does not contain the other elements needed for adequate microbial protein synthesis, like S, and these additional elements must be supplemented for optimal growth when a non-protein N source is utilized (Lassiter et al., 1958; Garrigus, 1970).

Limiting dietary S negatively affects multiple aspects of animal performance. While an imbalanced N:S ratio creates an inability to generate adequate S-containing amino acids and B-vitamins in the rumen, S deficiency also impacts lactate and volatile fatty acid (VFA) production in the rumen (Whanger and Matrone, 1970; Qi et al., 1994). Lactate accumulates in situations where S is limited, and this may be due to the preference of the SRB populations
to utilize lactate as a carbon source (Campbell and Postgate, 1965). Volatile fatty acid production is shown to improve when S reaches concentrations sufficient for the production of acetate by the sulfate reducers as a waste product of their lactate metabolism (Campbell and Postgate, 1965).

**Sulfur concentration in forages**

Feedstuffs vary in S content based on the feedstuff and the soil in which it was grown. Two common feedstuffs that often do not contain adequate S (S less than 0.15% of DM) are mature forages and corn silage, though sorghum forages are also noted to be consistently low in S (NRC, 1996). Other common forages which contain adequate S include alfalfa hays in all stages of bloom with decreases as the alfalfa matures and crude protein decreases (all stages at or above 0.25% S of DM) and early bloom orchardgrass hay (0.26% S of DM; NRC, 1996). Forages can also vary in S content based on geographic location. Feedstuffs grown in areas with S deficient soils will produce forages insufficient in S for animal growth (Qi et al., 1994). A specific study on the effects of S fertilization in the form of gypsum (calcium sulfate), at either 0 or 132 kg S/ha, found that amending the soil with S resulted in increased S content of the forage. This increase also improved digestibility as measured by apparent digestibility of NDF and ADF, and fiber fractions including cellulose, hemicellulose, and lignin in the steers fed S-fertilized forage (Spears et al., 1985). The authors suggested that many types of forage have lower S requirements than the animals to which the forages are fed. Therefore, adding S to the soil may not increase the yield of the forage, but the improvement in nutrient digestibility and animal performance may be worth the additional input (Spears et al., 1985). Sulfur-deficient forages seem to be more prevalent.
in the western United States than in other areas. This low forage sulfur content is interesting given the high concentrations of sulfate found in water in parts of the western United States, though the sulfate is typically found in well water, rather than the rainfall and snowmelt that supply the topsoil with water responsible for forage growth (Qi et al., 1994; Loneragan et al., 2001).

**Sulfur concentration in coproducts of the ethanol industry**

With the emergence of the biofuels industry, and the utilization of corn for ethanol, numerous coproducts of this process have entered the market and become common components of cattle diets. The process to generate ethanol from corn utilizes the starch portion of the corn grain for the production of the ethanol, while the fiber, fat, and protein components of the grain are concentrated in the coproducts (Schingoethe, 2006). According to Schingoethe (2006), for every 100 kg of corn that is fermented using a dry grinding process, 40.2 L of ethanol, 32.3 kg of CO$_2$, and 32.3 kg of dried distillers grains with solubles (DDGS) are produced. The DDGS are not the only coproduct produced, the initial stillage can also be centrifuged to produce thin stillage and coarse solids (Lardy, 2007). Thin stillage can then be evaporated to produce condensed distillers solubles (CDS). The coarse solids, also known as wet distillers grains (WDG), can be dried resulting in dried distillers grains (DDG). The WDG can be combined with the CDS to make wet distillers grains plus solubles (WDGS), which then can be dried to produce DDGS (Lardy, 2007). All these products differ in their nutritive value and ability to be stored long-term; however, in general they are all higher in fiber, fat, and protein than cracked dent corn (NRC, 1996; Lardy, 2007).
Regardless of form, distillers grains are often used to provide supplemental protein in finishing cattle diets due to their high levels of ruminally undegradable protein (Lardy, 2007). The energy content of distillers grains is often difficult to estimate, but it is generally equal to or greater than dry rolled corn. Therefore distillers grains are also useful as an energy source when used in the diet to replace corn or other grains (Lardy, 2007).

Coproduts cannot simply replace corn in the ration without considerations for the nutrient profile differences between coproduts and corn. When grains are utilized for ethanol production, the concentrations of multiple minerals including phosphorus (P), potassium, and S are all increased. Depending on the total composition of the diet this may not pose a problem, but the P level may result in a suboptimal calcium (Ca) to P ratio, requiring supplementation of additional Ca to adjust the ratio (Schingoethe, 2006; Lardy, 2007). The S concentration is also something of great concern as it can be both elevated and highly variable between batches of coprodut (Crawford, 2007; Lardy, 2007). This is as a result of the S inherent in the corn as well as additional S from sulfuric acid used in the fermentation process to control pH and bacterial contamination as well as clean distillation equipment (Crawford, 2007). While products of the ethanol industry contribute to increased dietary S concentrations, water concentrations of sulfate must also be taken into account when determining total S intake by cattle (NRC, 1996; Crawford, 2007).

**Sulfate in water**

The concentration of dissolved sulfate in drinking water varies widely depending on the geographical area. The western United States has experienced problems due to high sulfate water which have led to economic losses by cattle producers (Qi et al., 1994;
Loneragan et al., 2001; Crawford, 2007). Gould et al. (2002) conducted a survey assessing forage and water S concentrations at beef ranches across the United States and found that estimated total S intake (% DM) was highest at 1.0 to 1.91% in parts of Nebraska, North Dakota, and Texas. Similar surveys of water S concentration have been conducted for parts of the state of Iowa as well (Figure 1). Northwest Iowa is an example of an area which is highly populated with feedlots and water there can vary in sulfate content such that producers in this area must account for S intake from water in addition to dietary sources.

**Figure 1.** Northwest Iowa is one area of the Midwest which has high concentrations of dissolved sulfate in the water.
Cases of sulfate toxicity and S-related polioencephalomalacia (PEM) are increased during warmer parts of the year in regions such as Colorado and South Dakota as animals ingest higher amounts of sulfate from the water as they drink to combat the heat (Crawford, 2007).

Loneragan et al. (2001) saw negative effects on performance of feedlot steers due to high sulfate water when total S (water and diet combined) accounted for 0.22% of the overall diet DM. The effects of high sulfate water were similar to the effects of a high S diet, with decreased gains, intake, and feed efficiency in steers resulting from increasing sulfate concentration in the diet. Specifically, they found that for every 100 mg sulfate/L added to the water (equating to 33 mg S/L of water), ADG of cattle was decreased by 0.03 ± 0.006 kg.

In some areas well water results in high levels of sulfate, this increase in dietary S could significantly damage the profitability of an operation due to the decreases in performance. Another observation by the authors was that high sulfate water decreased water consumption by cattle, indicating that the animals were forming an aversion to the high sulfate treatment and thus may have been limiting their water intake during heat stressed conditions (Loneragan et al., 2001). Increased concentrations of sulfate in the water linearly decreased dressing percentage of cattle, but had no impact on marbling. The authors theorized that this decreased dressing percentage may have been a result of increased thoracic and abdominal viscera, and that during the beginning of the study, the intake of S may have exceeded the detoxifying abilities of the liver and resulted in systemic cellular damage, while respiratory distress may also account for increases in thoracic weight (Loneragan et al., 2001).
**Thiamin and thiaminase**

Thiamine is a S-containing B-vitamin, and ruminants may meet their need for thiamine through their diet or through production by rumen microbes (Rammell and Hill, 1986). Thiamine plays a role as a prosthetic group for many enzymes including transketolase, pyruvate decarboxylase, pyruvate dehydrogenase, and others (Rammell and Hill, 1986). Thiamine is also degraded in the rumen by microbial thiaminases (Rammell and Hill, 1986; Harmeyer and Kollenkirchen, 1989). There are two types of thiaminases present in the rumen and these two types utilize different mechanisms by which they degrade thiamine. The thiaminase I exoenzyme requires cosubstrates for the cleavage of thiamine to a free thiazole and the pyrimidin ring to which the cosubstrate is attached. Its activity is not only dependent on these cosubstrates but also inhibited by thiamine. Thiaminase II hydrolyzes thiamine into the pyrimidin and thiazole components without the addition of any cosubstrate (Harmeyer and Kollenkirchen, 1989). The amount of thiamine that actually reaches the intestine for absorption is dependent on the balance in the rumen between production of thiamine and destruction of thiamine by the various microbes. Ionophores and antibiotics have been shown to depress the apparent thiamine synthesized in the rumen, with this decreased thiamine synthesis being credited to the changes in microbial populations that occur. High concentrate diets have also been shown to reduce the amount of thiamine escaping the rumen, which is believed to be due to the decrease in pH that is not harmful to thiamine directly, but increases the activity of thiaminases. Increased thiaminase activity is credited as the most important cause of thiamine deficiency (Harmeyer and Kollenkirchen, 1989, Rammell and Hill, 1986).
The negative effects of high S diets on thiamine status of animals were evident in one Canadian study. Gooneratne et al. (1989) described a case study where one calf contracted PEM. The diets of the animals were subsequently found to be both deficient in Cu (7.1 mg/kg DM) and elevated in S (0.38%). Blood samples confirmed deficiency, defined by the authors as whole blood thiamine concentration below 40 µg/L, in 30% of the 105 calves and an average for all animals of 42.9 µg/L. To combat a related Cu deficiency, calves were supplemented with Cu (either 37.0 or 57.5 mg/kg DM) as part of the diet and dietary S was not changed. Upon completion of the trial, supplementation with either level of Cu was found to improve both the Cu status and the thiamine status of the animals (Gooneratne et al., 1989). This improved micronutrient status would indicate that there is an interaction between Cu status and thiamine production in the rumen even when S is more than adequate for the production of thiamine.

de Oliveira et al. (1997) support the detrimental effects of S on thiamine in an in vitro semi-continuous culture study with a factorial design utilizing a combination of acidogenic conditions, generated by using a high starch diet and infusion of reduced strength artificial saliva, S levels (0.2 or 0.5% dietary DM, as sodium sulfate) and supplemental thiamine (0 or 296 mmol). A 13 day fermentation was utilized, which allowed for modeling of thiamine metabolism in the rumen as sufficient time was given to address both the more immediate thiaminase activity and the thiamine production capabilities of the rumen microflora. The additional S resulted in decreased net thiamine production, which was not prevented with thiamine supplementation (de Oliveira et al., 1997). The authors themselves noted, however, that this decreased production of thiamine when extrapolated to the whole animal would not
be sufficient to impair thiamine status. In a previous study, de Oliveira et al. (1996) assessed thiamine status in sheep fed a thiamine-free, semi-synthetic diet. No effects of the thiamine-free diet on thiamine status were found, indicating that the findings in their later study may have been an artifact of the in vitro methods and not reflective of the rumen environment.

**Figure 2.** 1. Sulfate enters the rumen as part of the diet and is reduced by SRB. 2. Sulfide produced by the SRB combines with molybdenum to form thiomolybdates, this sequential process may results in multiple forms of thiomolybdate. 3. The trithiomolybdate and tetrathiomolybdate forms interact with Cu. 4. These highly substituted forms of thiomolybdate complex with Cu in the rumen and intestinal tract, resulting in systemic Cu deficiency. 5. Thiomolybdates cross into the bloodstream from the intestines. 6. In the bloodstream and tissues thiomolybdates are able to bind to Cu bound in proteins resulting in a functional decline in Cu status. (adapted from Suttle, 1991)
Mineral interactions associated with high sulfur diets

Chemically, S may interact with multiple minerals during the process of ruminant digestion and absorption, which may account for some of the changes in performance when animals consume high S diets. The most common and well documented interaction is with Cu (Suttle, 1975; El-Gallad et al., 1983). The interaction with Cu is a result of the formation of complexes of molybdenum (Mo) and S, called thiomolybdates (Suttle, 1991). Thiomolybdates possess two mechanisms which decrease the Cu status of the ruminant. First, these complexes can irreversibly bind Cu in the rumen, preventing it from being absorbed. Second, thiomolybdates can cross from the gastrointestinal tract into the bloodstream where they scavenge Cu from Cu-containing enzymes and irreversibly bind Cu to albumin, limiting the ability of Cu to be used by other processes (Figure 2; Suttle, 1991). As a result, these complexes decrease the Cu status of the animal by preventing the Cu from being absorbed in the gastrointestinal tract and by scavenging it from tissues throughout the body (Suttle, 1991).

Copper is a critical micronutrient for ruminants as well as other animals. In growing and finishing cattle, it is required at 10 mg/kg of DM intake, with a maximum tolerable concentration of 100 mg/kg (NRC, 1996). Signs of Cu deficiency include anemia, reduced growth, and altered hair growth and pigmentation (NRC, 1996). Metabolically, Cu is critical to the function of multiple enzymes including ceruloplasmin, superoxide dismutase, and cytochrome oxidase (NRC, 1996). Ceruloplasmin activity in fact has been used in multiple studies as a marker of Cu availability and function (Gengelbach et al., 1994; Ward and Spears, 1997; Hansen et al., 2008). Superoxide dismutase, which combats oxidative stress,
and cytochrome oxidase, a key protein in the electron transport chain, are Cu-dependent enzymes with impaired function as a result of thiomolybdate formation, this decreased enzyme function can result in damage to the mitochondria (Suttle, 1991). In a study previously discussed, the negative effect of elevated dietary S on Cu status is evident (Gooneratne et al., 1989). In this report, one calf contracted PEM, after which the diets of the cattle were found to be both deficient in Cu (7.1 mg/kg DM) and relatively high in S (0.38%). Blood samples confirmed deficiency of plasma Cu (defined by the authors as plasma Cu less than 0.3 mg/L) in 45% of the cattle. To combat this deficiency, calves were provided with additional Cu (either 37.0 or 57.5 mg/kg DM) as part of the diet and dietary S was not changed. After 3 weeks of supplementation with either concentration of Cu, the cattle were found to have improved Cu status with only 3% of calves still measured as deficient (Gooneratne et al., 1989).

Interactions between S and other minerals have also been observed. Spears et al. (1985) found decreased apparent absorption of both Ca and magnesium (Mg) in steers fed S-fertilized forages. The authors postulated that this interaction might be due to formation of insoluble complexes between these macrominerals and S in the rumen. Alternatively, exposure to H$_2$S resulting from S metabolism may affect Mg status. Rabbits exposed to one hr of inhalation of H$_2$S (72 mg/L) daily exhibited decreased serum Mg after 14 days of this exposure regimen (Beauchamp et al., 1984). Other research data suggest that cations such as zinc and iron may be susceptible to the formation of similar insoluble salts with the sulfide ion (Gould, 1998).
Sulfur toxicity

As stated previously, the NRC (1996) sets the maximum tolerable level of S in the diet at 0.4% of the total. When cattle ingest diets with high concentrations of S, toxicity can result. The major symptoms of acute toxicity described by the NRC (1996) include: muscular twitching, diarrhea, restlessness, blindness, breathlessness, and eventual death in some cases. This severe acute manifestation is generally referred to as PEM and will be discussed in more depth in later sections.

The concentration at which S becomes toxic is still unclear as various studies show great differences in the response to dietary S concentration. Part of this discrepancy is due to differences between forms of S used in these trials. In one study, weanling bull calves were fed 1.72% elemental S for 77 days as part of a purified diet without observed illness, although increases in liver, spleen, and testes weight at harvest were noted in calves fed diets with increased S concentration (Chalupa et al., 1971). Further research by the same group utilizing a similar purified diet of urea, starch, and glucose found that sodium sulfate at 0.62% of the diet (providing 0.21% S in the total diet) for 84 days also did not induce poor health in steers (Chalupa et al., 1973). Negative impacts on intake in dairy cattle were observed with S concentrations at or above 0.35% (Bouchard and Conrad, 1974). Overall, the NRC concentration of 0.4% S is used as the benchmark to represent the maximum concentration where animals can be expected not to suffer from disease (Kandylis, 1984).
The mechanism behind S toxicity is currently credited to the production of H$_2$S gas in the rumen (Figure 3). The process of H$_2$S gas production begins with the utilization primarily of lactate, although other carbon sources can be utilized, along with sulfate, by sulfate reducing bacteria (SRB) in the rumen. This process generates acetate and sulfide (Campbell and Postgate, 1965). The formation of H$_2$S from the sulfide ion is a pH-dependent process, with pKa’s for dissociation of the first ion at 11.96 and the second at 7.04, resulting in the majority of sulfide being reduced to H$_2$S in the rumen of an animal on a high concentrate feedlot diet (Beauchamp et al., 1984). The rumen gas is then eructated and between 70 - 85% of the eructed gases are inhaled (Dougherty and Cook, 1962). These inhaled rumen gases may cross into the bloodstream in the lungs (Dougherty and Cook, 1962).
Dougherty and Cook (1962) performed experiments where various gases, including \( \text{H}_2\text{S} \), were intentionally insufflated into the rumen to determine whether toxicity resulted from eructation and subsequent inhalation or was due to absorption of \( \text{H}_2\text{S} \) through the rumen wall. They discovered that in the case of \( \text{H}_2\text{S} \), the route is likely pulmonary. In their experiments, they paired sheep such that one had a patent trachea and the other had the trachea occluded. The sheep were then subjected to 300 mL of \( \text{H}_2\text{S} \) gas into the rumen. If a sheep is estimated to have 12 L of rumen gas headspace, this would equate to approximately 25,000 mg/L of \( \text{H}_2\text{S} \). The animal with the patent trachea in all replicates eventually collapsed, and the time it took to collapse depended on the frequency of eructation and time postprandial, indicating further that the effects were due to eructation. They also found that 900 mL of \( \text{H}_2\text{S} \) gas into the rumen of sheep with occluded tracheae resulted in symptoms similar to \( \text{S} \) toxicity and neurological damage in as little at 85 seconds (Dougherty and Cook, 1962). The onset of symptoms so soon after administration of \( \text{H}_2\text{S} \) gas would indicate that there was insufficient time for the eructation and inhalation route to account for disease development. Possibly, the toxic effects at the higher exposure developed as a result of \( \text{H}_2\text{S} \) crossing the rumen epithelia. The results of these two levels of \( \text{H}_2\text{S} \) exposure, 300 mL and 900 mL, provide an interesting look at the potential mechanism of \( \text{S} \) toxicity. At lower concentrations, ruminants may experience toxicity as a result of eructation. But if \( \text{H}_2\text{S} \) concentrations become high enough, the amount absorbed through the rumen epithelia may result in toxicity. This toxicity by the epithelial route may be because the amount of \( \text{H}_2\text{S} \) crossing the rumen epithelia exceeds the ability of the liver to detoxify \( \text{H}_2\text{S} \) prior to entering the blood supply to the rest of the body.
Historically, PEM was considered a disease of acute thiamine deficiency, with treatment consisting of administration of thiamine, often intravenous, intramuscular, or subcutaneous injections (Gould, 1998). Rammell and Hill (1986) went so far as to surmise that PEM develops because of progressive thiamine deficiency caused by thiaminases of bacterial or plant origin. The belief that PEM is solely due to thiamine deficiency is a misnomer as PEM can be caused by a variety of conditions. The word polioencephalomalacia when broken down into the word parts means polio-gray matter, as in brain and spinal cord; encephalo- brain or head; and malacia-tissue death or softening (Gould, 1998). Thus, the word polioencephalomalacia indicates that the gray matter of the brain is dying in response to the disease. It is important to make a distinction between PEM, which only indicates that the disease involves lesions in the gray matter and neurological dysfunction, and thiamine-deficiency PEM, where the disease is specifically caused by poor thiamine status in the animal. In fact, Mueller and Asplund (1981) showed in sheep that more than just a deficiency in thiamine was required for the nervous system symptoms to be present.

Polioencephalomalacia may be caused by many dietary disorders such as thiamine deficiency, water deprivation, sodium toxicity, lead poisoning, and S toxicity (Gould, 1998). Regardless of the cause, thiamine administration continues to be the recommended treatment and animals usually respond positively to treatment (Gooneratne et al., 1989; Crawford, 2007; Knight et al., 2008).

The symptoms of PEM include diarrhea, stargazing, blindness, head pressing, incoordination, seizures, recumbency, teeth grinding, and circling (Kandylis, 1984; Gooneratne et al., 1989; Gould, 1998; Crawford, 2007; Knight et al., 2008; Cammack et al.,
2010). These symptoms largely point to a neurological dysfunction and clinical diagnosis is dependent on the presentation of cerebrocortical necrosis (Gould, 1998). One clinical finding that a case of PEM may be S-related would be the observance of a H$_2$S or ‘rotten egg’ smell on the breath of the animal (Kandylis, 1984).

**Sulfate reducing bacteria in other environments**

As previously stated, the production of H$_2$S gas is due to the presence of SRB in the rumen. However, these organisms are present in many other habitats and discoveries from these other environments may hold insight into dealing with the problems associated with high dietary S in cattle and the resultant increase in H$_2$S production by SRB.

Sulfate reducing bacteria are not new bacteria. They are considered by some to be one of the first respiring microorganisms (Barton and Fauque, 2009). The effects of SRB have warranted research in areas as diverse as biocorrosion of metals (Barton and Faque, 2009), estuarine environments (Cappenberg, 1975; Oremland and Polcin, 1982), and biofilms of wastewater (Okabe et al., 1999; Baumgartner et al., 2006). Significant gains in knowledge have been achieved in these areas of research and much of this information may be applicable to the rumen.

One key observation in wastewater ecology research is the seemingly counter paradigm observation that SRB are able to withstand an oxic environment, and some genera have even been observed to utilize oxygen (Baumgartner et al., 2006), although consensus has not been reached on this new paradigm (Dolla et al., 2006). Previously, it was believed that SRB could not survive in an oxygenated environment, with two reasons for this belief.
First, the metabolism of sulfate reduction was calculated to be non-competitive in an oxygenated environment. That is to say, the SRB would be out competed for substrates needed for growth and would not reach a steady state in the community as a significant member of the population (Baumgartner et al., 2006). Secondly, since they evolved before free oxygen constituted a significant portion of the atmosphere, it is believed SRB would not have innate mechanisms to deal with oxidative stress and reactive oxygen species produced in an oxygen rich environment (Baumgartner et al., 2006; Dolla et al., 2006).

However, upon further exploration, it appears that SRB have a variety of mechanisms for dealing with oxygen. Baumgartner et al. (2006) suggested that Desulfovibrio vulgaris contains 4 times the chemical-sensing membrane proteins as the model organism E. coli, indicating they would be better suited to handle oxidative stress. Many of the oxygen sensing proteins are integral to the organism’s metabolism including L (+) lactate dehydrogenase and aldehyde dehydrogenase, while others are key to cell division and nucleic acid synthesis (Dolla et al., 2006). The stress from surrounding oxygen is also transient. Often in these environments, oxygen is produced during the day and dissipates at night. Similarly, in the rumen, oxygen can vary during times of ingestion and rumination while also differing in oxygen content near the rumen wall (Baumgartner et al., 2006; Dolla et al., 2006). Many of these mechanisms for dealing with oxygen can become overwhelmed and cease to function after 24 hours, while others are negatively affected after only one hour. The deleterious effects of oxygen are evidenced by absence of cell replication, and loss of long term cell viability with exposure longer than 24 hours (Dolla et al., 2006). Evidence also points to the production of anoxic microenvironments within what appears to be an oxygenated
environment allowing sulfate reduction to compete with the more energetically favorable alternatives simultaneously (Baumgartner et al., 2006).

Ability to withstand oxygen varies by phylogeny within the different groups of SRB. Since SRB are such an ancient group that they diverged before oxygen was prevalent and do not contain a common ability to deal with environmental oxygen (Baumgartner et al., 2006), different genera have been found to have different abilities to thrive in varying levels of oxygen (Okabe et al., 1999). Interestingly, the genus most prevalent in the rumen, *Desulfovibrio*, is one of the genera known to contain mechanisms for combating oxidative stress. As stated previously, some groups of SRB have been seen to utilize oxygen, whether *Desulfovibrio* have this ability is not clear. But their ability to withstand oxygen rich environments would indicate that they may be found in some unique areas of the rumen, namely the rumen wall, where there may be some diffused oxygen (Barton and Faque, 2009). If rumen SRB are found to withstand or utilize oxygen, it could indicate a metabolic advantage over other rumen organisms that are negatively affected by transient oxygen.

**Sulfate reducing bacteria in the rumen**

Initial discovery of SRB in the rumen depended upon culture techniques in the 1950’s. Lewis (1953) used mixed cultures to determine some of the energy sources utilized by sulfate reducers. The first scientist to isolate an SRB from the rumen was Coleman in 1960, when he isolated *Desulfotomaculum ruminis* from the rumen of a sheep. Another key group of rumen SRB, the *Desulfovibrio*, were discovered later by Howard and Hungate (1976), also using various culture-based techniques, to determine the metabolism and growth requirements of these unique cultures. These solely culture-based techniques were critical to
the discovery of these organisms, but they also have significant drawbacks. Sulfate reducing bacteria are very fastidious organisms and require specific conditions for adequate growth. Because of this, only the organisms most suitable for in vitro growth are recognized when culture techniques are used, biasing the scientists view to the exclusion of uncultured relatives. This bias can be overcome by utilization of modern molecular techniques.

The reduction of sulfate in the rumen was first discussed in the literature by Lewis (1953). Lewis (1953) investigated whether sulfate was reduced to H$_2$S in a manner comparable to that of N in the production of ammonia from nitrite and nitrate. He found that in sheep, this was indeed true, that sulfate was being reduced to H$_2$S. This reaction was confirmed both in vivo and in vitro using mixed cultures. With these mixed culture models Lewis (1953) determined that cultures containing sulfate reducers utilized a variety of hydrogen (H) sources including glucose, lactate, ethanol, citrate, and malate, among others.

One of the SRB was later isolated and characterized in greater detail by Coleman (1960). Coleman isolated an SRB from the rumen of a sheep, this organism was originally referred to as ‘Coleman’s organism’, and later was classified as *Desulfotomaculum ruminis* type strain: Coleman (Campbell and Postgate, 1965). Coleman (1960), through various culture-based experiments, determined some of the growth requirements of this SRB. It was determined to be an obligate anaerobe with a requirement for the B-vitamin biotin and for sulfate (Coleman, 1960). Its metabolic requirements for H were provided by pyruvate or formate and it was found to utilize lactate with the production of acetate and formate (Coleman, 1960). It was determined that sulfate could be provided at levels that were inhibitory even to these sulfate requiring organisms if included at concentrations greater than
576 mg/L (Coleman, 1960). Lewis’ (1953) previous work reached ruminal concentrations of sulfide as high as 471.2 mg/L without toxic effects. These concentrations together would indicate that between 471.2 mg/L and 576 mg/L the rumen and specifically the SRB reach a tipping point where S is toxic even to the organisms which have an integral requirement for S. However, compared to calculations for sulfate in cattle diets at 0.6% S at 10 kg of DM intake and an average rumen volume in a finishing steer of 45 L, resulting in a 4,000 mg/L sulfate, it would appear that something other than the sulfate concentration was contributing to the demise of these in vitro cultures. Both Lewis (1953) and Coleman (1960) found that under certain conditions sulfite and thiosulfate could be utilized by the SRB to produce H\textsubscript{2}S and they also both were able to produce growth with gaseous H as the H source.

Coleman’s organism marked a key discovery in rumen S metabolism in that it was the first organism isolated and shown to have sulfate reducing properties (Campbell and Postgate, 1965). As research progressed, other organisms were isolated and found to carry out even more of the sulfate reduction, accounting for many of the discrepancies seen in the early research using the *Desulfotomaculum*. *Desulfovibrio* species are generally considered to be of greater importance to rumen sulfate metabolism due to the differences in population density between the two groups. *Desulfovibrio* was estimated at 10\textsuperscript{8} organisms/mL of rumen fluid and *Desulfotomaculum* was estimated at 100 individual cells/mL of rumen fluid (Howard and Hungate, 1976). For reference, the total bacterial population of the rumen is estimated between 10\textsuperscript{10} and 10\textsuperscript{11} cells/g of rumen contents (Yokoyama and Johnson, 1988). A key difference between these two groups is their gram staining characteristics, with *Desulfovibrio* being gram negative and *Desulfotomaculum* being gram positive (Campbell
The work by Howard and Hungate (1976) was extremely similar to that of Coleman (1960), only the organism in question was *Desulfovibrio desulfuricans*. However, the purpose of the work was the same in both cases, to determine the metabolic requirements of SRB found in the rumen.

*Desulfovibrio* was similar to *Desulfotomaculum* in that both can utilize H gas for sulfate reduction, as well as lactate, ethanol, pyruvate, and formate (Coleman, 1960; Howard and Hungate, 1976). It did not utilize alanine or glucose, and was able to grow without sulfate when the H source was either pyruvate or choline. The *Desulfovibrio* also showed a need for provision of B-vitamins, through the lack of growth without the inclusion of either yeast extract or trypticase as B-vitamin sources in the growth media, although this does not specify which B-vitamins are required (Howard and Hungate, 1976).

**Sulfate-reducing bacteria as a member of the rumen microflora/ecosystem**

Sulfate-reducing bacteria of the rumen utilize lactate as a substrate for production of H to generate H$_2$S (Lewis, 1953; Coleman, 1960; and Howard and Hungate, 1976). Early research indicated that this could possibly be carried out with a co-culture of the *Desulfovibrio* and an unidentified *Bacteroides*. This combined culture showed growth with glucose, whereby the *Bacteroides* utilized the glucose and the waste product, lactate, was used by the SRB (Howard and Hungate, 1976). Other organisms are known to produce lactate in the rumen including *Streptococcus bovis*, and *Lactobacillus* species (Owens et al., 1998). This interaction between SRB and rumen lactate metabolism is a key interaction between organisms of the rumen, and it also is recognized as a major target for prevention of S toxicity as well as lactic acidosis. The SRB must compete with other rumen
microorganisms for the available lactate, so if other lactate-utilizing bacteria were increased, such as with a direct-fed microbial or SRB inhibitor, problems generated by the SRB would be expected to decrease. Other lactate utilizers include *Megasphaera elsdenii*, and the lactate-utilizing *Lactobacillus* species *acidophilus* (Owens et al., 1998).

Another important interaction between organisms in the rumen is the interaction of SRB with methanogens. Research in this area of interaction has centered in bioreactors and analysis of abnormal states of the reactors (Mizuno et al., 1997), and estuarine sediments (Cappenberg, 1975; Oremland and Polcin, 1982). Research in bioreactors indicates that methanogens and sulfate reducers compete for the H needed for their respective metabolisms (Mizuno et al., 1997). In bioreactors, methane production is a part of the process. However, when SRB and their wastes, namely H$_2$S, accumulate, methanogenesis is inhibited and the reactor may fail. This example may be extrapolated to the rumen and could indicate that when sulfate reduction and its product, H$_2$S, reaches a critical point, methanogens will be negatively affected. This decreased methane production could be beneficial if the carbons usually lost through methane were retained in the rumen and utilized by the ruminant, but it also may cause fermentation to be negatively affected and lead to decreased performance or illness in the animals if bacteria other than methanogens are similarly affected.

In research in estuarine environments, Cappenberg (1975) noted that there is actually a symbiosis at times between SRB and methanogens. In his experiments, SRB, specifically *Desulfovibrio desulfuricans*, and a *Methanobacterium* species, were co-cultured together in a continuous culture system. A waste product of SRB metabolism is acetate, which is utilized by methanogens for its metabolism and production of methane. Simultaneously, SRB can
prove to be detrimental to methanogens. Cappenberg (1975) found that if sulfide accumulated, methanogens were washed out of the continuous culture apparatus and were not recovered at the end of the experiment (Cappenberg, 1975). The SRB did not experience any benefits from methanogens making this a true commensalism (Cappenberg, 1975).

Another aspect of Cappenberg’s (1975) research analyzed the redox environment preferred by the two populations. It was found that the two populations were located at different depths of the estuary sediment being studied. Sulfate-reducing bacteria were more prevalent at lesser depths of the muddy sediment, where the redox potential was -100 to -150 mV. The methanogens were more common at greater depths where the redox potential was in the -250 to -300 mV range, but SRB and methanogens obviously overlap in their redox ranges if they can be grown together as Cappenberg (1975) did. This stratification was accredited to the toxic effects of H$_2$S to the methanogens and a survival mechanism by which the methanogens avoided the high sulfide levels (Cappenberg, 1975). For reference, the redox potential, measured by a combination pH probe which utilized a saturated calomel glass and silver probe, in the rumen of a Holstein cow fed a high grain diet was found to average just over -520 mV, and was just under -500 mV when fed a high forage diet (Mishra et al., 1970). When adjusted for the type of probe become -320 mV and -300 mV for the high concentrate and high forage diets, respectively.

Oremland and Polcin (1982) delved further into this issue and determined some other aspects of the competition between these groups for substrates. They found that SRB outcompete the methanogens for the substrates they share in common, namely H and acetate. When the methanogens were supplied with an adequate supply of substrates for
methanogenesis that are not utilized by the SRB the two could coexist, these substrates included methionine and methanol. When these were supplied at sufficient levels, or an abundance of H and/or acetate was provided, a toxic effect of sulfate ions was not found (Oremland and Polcin, 1982).

The combined results of these two studies are hard to resolve. Cappenberg (1975) found a toxic effect of SRB end products on methanogenesis, namely the $\text{H}_2\text{S}$ and sulfide ions produced, while Oremland and Polcin (1982) did not. But Oremland and Polcin (1982) did not measure the $\text{H}_2\text{S}$ potential, which may account for the lack of toxic effects they observed. Analysis of the methanogen specific substrates in a co-culture situation similar to the Cappenberg (1975) study would provide more insight into the complex interactions between these groups.

Stoichiometrically, methane is a loss of energy for ruminants. Research has been carried out to determine if efficiency of feed conversion to gain by cattle and ruminal methanogen populations are related. Zhou et al. (2009) recently completed work to help answer this question. In their study, cattle were blocked by feed efficiency (high and low) and analyzed rumen gas samples for methane production and pooled rumen fluid was used to construct methanogen 16S gene libraries. These libraries were then analyzed to determine methanogen taxonomies, to determine if there were population differences that could account for the differences in methane production and feed efficiency (Zhou et al., 2009). They found that all three items were correlated, and that certain population differences of methanogens were found to result in less methane and higher feed efficiency. Based on the interrelation between methanogens and SRB if growth of SRB could be encouraged and methane
production decreased, it might be possible that efficiency would be improved. However, this would have to be weighed against the possible problems with increased growth of SRB and H₂S production.

The application of new technology to assess sulfate-reducing bacterial populations

Much of the literature on SRB in ruminants has been culture based. Culturing generates great bias by only allowing analysis of organisms whose growth requirements are or can be met in vitro. It is estimated that only 1 to 10% of all bacterial species can be grown using modern culture techniques (Handelsman, 2004). In the case of SRB, recent studies have applied new molecular technologies to their study in different animal environments and ecosystems. Some of the earliest scientists to apply these new technologies to the study of SRB were in the study of SRB activities in wastewater ecosystems (Minz et al., 1999; Okabe et al., 1999).

Both of these wastewater studies used rRNA probes that were specific to certain genes of the SRB. Okabe’s (1999) research group used a 16S rRNA probe and fluorescent in situ hybridization to determine where within the wastewater biofilm SRB were located and their distribution in relation to oxygen. Minz’s (1999) group used a single probe specific to the conserved dissimilatory sulfite reductase (dsr) gene found in the SRB as an integral part of their sulfate respiration. Minz et al. (1999) were also hoping to determine which groups of SRB would be found in oxic environments. Both studies concluded that SRB could be found in oxic portions of the biofilm and found that species within the genus *Desulfonema* were some of the most likely to be found in the oxic layers (Minz et al., 1999, and Okabe et al.,
1999). Minz et al. (1999) also found that *Desulfotomaculum ruminis* was one of the species found only in the anoxic areas of the biofilm.

Recently, these technological developments and primers have been applied to other areas of research including animal science. Spence et al. (2008) conducted studies of SRB in swine slurry using molecular techniques to analyze the presence of the SRB in confinement lagoons and underground storage pits, they also analyzed these bacteria such that the relative quantity of SRB could be estimated. They used quantitative real-time polymerase chain reaction with specific primers for the *dsr* gene of three different phylogenetic groupings of SRB to determine which were present and to what extent they were present within each sample. They also used a degenerate primer set that was more encompassing of strains not found in the three groups, giving a more complete look at which populations were present. They discovered that SRB populations changed in relation to the season that swine manure had been sampled in the lagoon. Interestingly, the seasonal differences were mostly related to the SRB groups, with the group 1 SRB, most like *Desulfobulbus propionicus* organisms most prevalent during the winter, and group 3 SRB, the *Desulfovibrio desulfuricans*-like organisms most prevalent during the summer sampling. They also analyzed manure slurry from a study that utilized diets with two S concentrations, a high (0.23%) and low S diet (0.19%) and discovered that the diet with increased S resulted in more SRB detected in the manure slurry (Spence et al., 2008).

Recent investigations have shown that *Desulfovibrio* can comprise up to 1% of the bacteria in the feces of cattle fed a high concentrate diet that did not contain ethanol coproducts (Callaway et al., 2010). This percentage became undetectable with inclusion of
DDGS at up to 50% of the concentrate component. To determine these population changes, bacterial tag-encoded FLX pyrosequenceing was utilized with DNA isolated from the feces or rumen fluid samples (Callaway et al., 2010). This research indicated that DDGS resulted in a numerical decrease in SRB. But, this study neglected to determine the S concentrations of these various diets, and these changes may or may not be a result of dietary S differences. Thus, the influence of S on changes in SRB populations in ruminants has not been extensively analyzed using these new molecular tools. Using PCR and other emerging molecular methods to analyze rumen SRB population changes due to dietary concentration of S is an area of great interest.
LITERATURE CITED


http://www.extension.umn.edu/beef/components/pdfs/ManagingSulfur_Crawford.pdf


CHAPTER 3.

Elevated dietary sulfur concentration affects mineral status, performance, and carcass characteristics of beef steers

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ABSTRACT

Ninety-six crossbred yearling steers (321 ± 29 kg BW) were used to determine the effects of feeding cattle a high sulfur (S) diet on pasture prior to receiving a high S diet in the feedlot. Steers were blocked by weight, allocated to 2.4 ha bromegrass pastures (n = 4 plots per treatment), and supplemented with either low S dried distillers grains with solubles (DDGS; 0.3% total diet S; LS) or LS DDGS with additional S (0.45% total diet S; HS; additional from NaSO₄) for 36 days. On d 37, steers moved into the feedlot where half remained on the previous S treatment while the other half switched treatments, resulting in 4 treatments (LS-LS, LS-HS, HS-LS, HS-HS; LS: 0.3% total diet S, HS: 0.6% total diet S; n = 6 feedlot pens per treatment). During the pasture period, forage mass offered, grazing residual mass, and IVDMD of forage samples were not different among treatments (P > 0.40), and ADG did not differ (LS: 1.6 kg/d, HS: 1.7 kg/d, P = 0.54). Plasma Mg measured on d 35 was decreased in
response to increased dietary S during the pasture period ($P = 0.05$), though no effect was seen during finishing on d 155 ($P > 0.15$). Plasma and liver Cu concentrations measured on d 155 were decreased ($P < 0.05$) by HS diets. Increased dietary S during the feedlot period decreased ADG ($P = 0.01$) and tended to decrease HCW ($P = 0.06$). Steers receiving the HS diet had increased stearic acid and heptadecanoic acid ($P = 0.04$ and 0.01, respectively) percentages from rib facings collected at harvest. Exposing cattle to higher S diets while on a forage-based diet did not influence later performance on high S feedlot diets; however, cattle fed high dietary S on pasture had greater fat cover at harvest ($P = 0.01$) suggesting S may have influenced lipid metabolism.

**Key words:** cattle, co-products, mineral status, pasture, sulfur

**INTRODUCTION**

Sulfur (S) is a critical component of amino acids and B-vitamins (NRC, 1996). In cattle diets, concentrations above 0.4% S are of concern, as this exceeds the maximum tolerable level set by the NRC (1996). Coproducts of the ethanol industry are high in S because of the use of sulfuric acid to maintain pH, reduce bacterial growth and flush distillation equipment during ethanol production (Crawford, 1997). Not only are these feedstuffs often high in S but they are also variable in their S content (Crawford, 1997). High dietary S concentrations can result in decreased animal performance, polioencephalomalacia (PEM), and in some cases animal death (Gould, 1998). Cattle mineral status may also be influenced by dietary S concentration. Copper (Cu) is known to be negatively affected by S through the formation of thiomolybdates in the rumen (Suttle, 1991). Thiomolybdates may decrease the Cu status of the ruminant in two ways. First, these complexes can irreversibly
bind Cu in the rumen, preventing absorption later in the gastrointestinal tract. Second, thiomolybdates may also cross from the gastrointestinal tract into the bloodstream, scavenging Cu from cuproenzymes and causing albumin to irreversibly bind Cu, limiting its use by tissues (Suttle, 1991).

As a possible management method to reduce negative effects of high S diets fed to feedlot cattle, this study utilized a pasture backgrounding period where low or high dietary S supplements were fed. Because cattle tolerate more dietary S on a high forage diet (NRC, 1996), it was our hypothesis that exposing the rumen to high dietary S while animals were consuming a high forage diet would allow microbial populations to adjust to dietary S with less risk of toxicity to the cattle. As a result, cattle may experience less dramatic effects of high S diets on performance in the feedlot, where cattle consuming concentrate-based diets are more susceptible to S toxicity. It was also of interest to determine how this pasture backgrounding and subsequent S concentrations in the finishing diet would affect carcass quality and mineral status.

**MATERIALS AND METHODS**

Procedures and use of animals for this experiment were approved by the Iowa State University Animal Care and Use Committee.

**Experimental design**

Crossbred yearling steers (n = 96, 321 ± 29 kg initial BW) were used to determine the effects of high dietary S on mineral status, cattle performance, and carcass characteristics when cattle are fed dried distillers grains with solubles (DDGS). The study utilized a nested
design to analyze the effects of high S as part of a DDGS supplement provided to grazing cattle and a high S feedlot diet. Steers were blocked by weight and randomly assigned to treatment with either a low S DDGS (0.5% S DM, LS, n = 48) or the LS DDGS with an additional 0.3% S provided from sodium sulfate (0.8% S DM, HS, n = 48), resulting in an estimated total dietary S concentration of 0.30% for LS and 0.45% for HS during the pasture period of the study. Composition of the DDGS supplement is shown in Table 1. The study began in October of 2009 utilizing pastures that had been harvested during June or July of that year, after harvest forage was allowed to stockpile until the beginning of the study. Prior to being assigned to treatments all steers were grazed in a single bromegrass-based pasture for 7 d prior to the start of the study while receiving the LS DDGS supplement at 1% BW. On d 6 and 7 of this pre-study period, steers were weighed at 0800 before feeding of the supplement, to determine initial pasture-based BW. On d 0 of study, steers were assigned to 2.4 ha smooth bromegrass-based pastures (4 pastures per treatment, n = 12 steers per pasture) and were front stripgrazed weekly over a 36 d backgrounding period. Steers received the DDGS supplement at 1% of BW at 0900 daily. Consecutive day BW were taken again on d 35 and 36 to determine performance during the pasture period.

Measurements for pre-grazing and post-grazing forage masses were collected every two weeks during the pasture period to determine forage mass offered and grazing residual mass. Indirect estimates of forage mass were taken using a 0.25-m² falling plate meter (4.8 kg/m²) at 20 random sites within the area to be grazed in the next two week time period. During each sampling, the falling plate meter was calibrated by harvesting at least 9 sites (0.25-m²) to ground level using battery operated grass shears (Black and Decker, Baltimore,
Maryland). Forage clipped from these sites was dried at 60°C for 72 h and weighed hot to determine DM mass per hectare. Postgrazing masses were determined similarly with falling plate meter readings and harvested sites (0.25-m²) taken within the area that was grazed in the previous 7 d. Regression was used to develop an equation relating the harvested forage with falling plate meter readings taken at each of the sites on d 12, 21, 27, and 37 (Appendix; Macoon et al., 2003). Grazed area was determined by a rolling measure wheel (Meter-Man, Vernon Center, MN) to determine the dimensions of the rectangular plots, which were then used to determine area. Dry matter intake was estimated by addition of supplement intake and calculated forage intake, estimated from the forage disappearance.

Quality samples of forage were collected prior to the start of study (d -14), at midpoint of the pasture period (d 14), and after the end of pasture (d 37). Sward samples were collected by random selection of 10 sites within the area to be grazed of individual plots and were clipped using battery operated grass shears (Black and Decker, Baltimore, MD) to a height of 5 cm. The clippings from the 10 sites within each plot were then thoroughly mixed and a subsample of each separate plot was sent to a commercial lab (CEPS, Poultry Science Center L-209, University of Arkansas, Fayetteville, AR) for analysis of DM, ADF, NDF, CP and mineral concentrations (Table 2). In vitro digestible dry matter (IVDMD) of sward quality samples collected throughout the pasture period (d -14, d 14, and d 37) from all plots were determined via a modification of the Tilley and Terry (1963) method. Samples were incubated for 48 h in rumen fluid collected from a fistulated steer fed a grass hay diet, with NC-64 buffer, followed by an additional 24 h incubation with addition of an HCl-pepsin solution (Barnes and Marten, 1979).
On d 36, steers were moved from the pastures to feedlot pens, with half the steers remaining on the same treatment and half switching to the other treatment, resulting in 4 treatments total (LS-LS, HS-LS, HS-LS, HS-HS; n = 4 steers per pen, 6 replicate pens per treatment). For the first 10 d in the feedlot pens, steers were provided grass hay ad libitum along with 1% BW of their respective DDGS supplement. Steers were then transitioned to the feedlot diet from d 47 to d 67, utilizing 3 step-up diets where grass hay was replaced with an equal amount of cracked corn. Once fully transitioned, steers were finished on the final diet (Table 3) for 95 days, resulting in a total trial length of 164 d at harvest. Diets for the feedlot period were formulated to contain 0.3% total S in the LS treatment, and 0.6% for HS steers, with the additional S provided by NaSO₄. Actual S content varied between 0.2 and 0.3% for LS cattle and between 0.5% and 0.6% for HS cattle throughout the study as a result of differing S content of DDGS batches. The DDGS supplements, individual feed components and pen orts were sampled monthly and dried at 60°C for 48 h to determine DM. During the feedlot period, steers were weighed every 28 d and consecutive day weights were taken near the end of the trial (d 154 and 155) to determine final BW.

Animal sampling and mineral analysis

Forty-eight steers were randomly selected for sampling of blood and liver for determination of mineral status through the backgrounding and finishing periods (n = 2 per feedlot pen). Blood samples were taken on d 0, 35, 125, and 155 via jugular venipuncture into 7 mL trace mineral potassium-EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and placed on ice for transport to the laboratory. Samples were centrifuged at 1000 x g for 20 min at 4°C and plasma was aliquoted and stored at -20°C prior to analysis.
Liver biopsies were conducted on d 0, 35, and 155 using the method of Engle and Spears (2000). Both liver and plasma samples were sent to a commercial laboratory (CEPS, Fayetteville, AR) for analysis of Mg, Ca, P, S, Fe, Zn, Cu, and Mn.

**Carcass characteristics and fatty acid analysis**

Steers were shipped 151 km to a commercial abattoir in Denison, IA for harvest on d 164. Carcass data were collected by trained personnel including hot carcass weight, and after a 24 h chill carcass measures of ribeye area, kidney pelvic and heart fat (KPH), marbling score, and backfat thickness. Yield grades were calculated and quality grades were determined by a USDA grader.

Rib facings were collected from 46 steers (n = 12, 12, 12, and 10 for treatments LS-LS, LS-HS, HS-LS, and HS-HS, respectively) 24 h post harvest from the 12th - 13th rib split for determination of fatty acid composition. Lipids were extracted using a modified Folch wet tissue lipid extraction method (Folch et al., 1957). The total lipid extracted was calculated as a percentage of the original meat sample. Then the extracted lipid was esterified using the acetyl chloride/methanol method of Christie et al. (1972). Fatty acid methyl esters were analyzed by GC (model 3900; Varian Analytical Instruments, Walnut Creek, CA) using a 100m x 0.25mm x 0.2 µm fused silica capillary column (Supelco, Bellefonte, PA). Helium was used as the carrier gas. A temperature-programmed procedure was used. Initial column temperature was 70°C for 4 minutes, temperature was then increased 13°C per minute until column temperature reached 175°C. The temperature was maintained at 175°C for 27 minutes, after which it was increased 4°C per minute until the column temperature was 215°C, where it was maintained for 28 minutes. The detector temperature remained constant.
at 220°C throughout. The fatty acid composition was calculated using the peak areas on a percentage basis.

**Statistical analysis**

The mixed procedure of SAS version 9.2 (SAS Inst. Inc., Cary, NC) was used for analysis of all data. For data collected during the pasture period the model included the fixed effect of pasture treatment with the experimental unit of pasture plot (n = 4 plots per treatment). The model for analysis of the feedlot period data included the fixed effects of feedlot treatment, pasture treatment, and the interaction, and no random effects were used. Experimental unit for the feedlot period was the feedlot pen (n = 6 per treatment). Interactions with a $P$-value greater than 0.2 were removed from the model. The interaction of pasture and feedlot treatments were only significant for plasma P, and as a result treatment means were pooled within feedlot diet (n = 12 pens per treatment). Day 0 plasma and liver mineral values were used in a covariate analysis when appropriate ($P < 0.20$).

**RESULTS**

**Steer performance and carcass quality**

There were two incidences of PEM during the study. On d 88 (d 20 of full finishing diet), one steer receiving the LS-HS treatment exhibited blindness, ataxia, and head pressing and was removed from the trial and treated initially with intramuscular (IM) dexamethasone and intravenous thiamine. On d 89 (d 21 of full finishing diet), a steer on the HS-HS treatment also exhibited ataxia, blindness and circling, and was removed from the trial for one week and treated initially with IM dexamethasone and intravenous thiamine followed by
daily IM thiamine until fully recovered, and then returned to the pen prior to feeding on d 97. This steer was later removed from the trial permanently on d 125 due to a persistently high H₂S rumen gas concentration (data not shown). Intakes, ADG, and G:F data were adjusted for these changes in the number of cattle per pen.

For the first 35 days of study, steers were front stripgrazed weekly on smooth bromegrass-based pastures with a DDGS supplement fed at 1% of body weight. Forage disappearance (3.5 and 3.0 kg·hd⁻¹·d⁻¹, SEM ± 1.4, for HS and LS respectively), as calculated based on the forage mass offered (8.5 and 8.2 kg·hd⁻¹·d⁻¹, SEM ± 2.2, for HS and LS respectively) and residual grazing masses (1576 and 1648 kg/ha, SEM ± 72, for HS and LS respectively) did not differ due to treatment ($P > 0.40$). Intake of the DDGS supplement (3.6 and 3.6 kg·hd⁻¹·d⁻¹, SEM ± 0.13, for HS and LS respectively) also did not differ ($P = 0.40$). Quality of the pastures, as determined by IVDMD was not different between treatments ($P = 0.90$), but did decrease over time ($P = 0.01$). Mineral concentrations and protein content of the forages differed over time, but were not different by treatment and no interactions were observed ($P > 0.16$; Table 2). Specifically, Fe increased ($P < 0.01$) as did DM ($P = 0.05$) and Ca ($P = 0.05$). Conversely, there were decreases in CP ($P = 0.03$), P ($P < 0.01$), Cu ($P < 0.01$) over time, and a tendency for Mg ($P = 0.06$) to be decreased. Measurement of ADF varied over time ($P < 0.01$). Average daily gain did not differ ($P = 0.54$) due to inclusion of additional S in the DDGS supplement (1.6 and 1.7 kg·hd⁻¹·d⁻¹, SEM ± 0.08, for HS and LS respectively).
Prior pasture treatment did not affect performance of cattle during the finishing period \((P > 0.4)\). Inclusion of high S in a finishing diet tended to decrease DMI \((P = 0.15)\), but did not impact G:F \((P = 0.39; \text{Table 4})\). However, ADG was lower \((P = 0.01)\) when steers received the HS diet than the LS diet during the finishing period. Reduced ADG due to the HS diet translated to a tendency \((P = 0.06)\) for lower HCW in HS than LS cattle. Other measures of carcass quality including marbling, KPH, and ribeye area were not affected by dietary S concentration during the feedlot period. Interestingly, steers receiving the HS diet while on pasture, regardless of dietary treatment during the feedlot period, exhibited greater backfat thickness \((1.14 \text{ and } 0.93 \text{ cm, } \text{SEM} \pm 0.05, \text{ for HS and LS respectively}; P = 0.01)\) and higher USDA yield grade \((2.95 \text{ and } 2.68, \text{ SEM} \pm 0.10, \text{ for HS and LS respectively}; P = 0.05)\) than steers receiving the LS diet during the pasture period.

**Fatty acid composition**

Fatty acid composition and total lipid content of samples collected from the 12th - 13th rib split are shown in Table 5. Previous pasture treatment with high dietary S tended \((P = 0.07)\) to decrease cis-9 trans-11 conjugated linoleic acid (CLA; 0.34 and 0.48 g/100g of fatty acids, for HS and LS respectively, SEM \pm 0.052). No other effects of pasture dietary S concentration on fatty acid composition were observed. The ratio of saturated fatty acids (SFA) to polyunsaturated fatty acids (PUFA) was not affected by dietary S level during the feedlot period \((P = 0.59)\). Long chain fatty acids (> 16:0) tended \((P = 0.07)\) to be higher in HS cattle than LS cattle. Steers fed HS diets in the feedlot had increased \((P = 0.04)\) stearic acid (C18:0) and a tendency for increased \((P = 0.06)\) docosanoic acid (C 22:0). Steers fed HS diets also had decreased heptadecanoic acid (C17:0; \(P = 0.01)\) and tended to have lower
palmitic acid (C16:0; \( P = 0.08 \)), myristic acid (C 14:0; \( P = 0.06 \)) and \( \text{cis}-12 \ C18:1 \) \( (P = 0.08) \) percentage in meat.

**Mineral status of cattle**

After 35 d of receiving a HS supplement on pasture, plasma Mg concentrations were decreased \( (P = 0.05) \) although animals did not become deficient in Mg, while plasma P concentrations were increased \( (P = 0.05) \) in comparison to cattle receiving the LS supplement (Table 6). No differences \( (P > 0.20) \) due to dietary S were detected in the other minerals measured after 35 d on pasture (Table 7). During the finishing period, plasma P concentration was lower \( (P = 0.01) \) in HS steers than LS steers (Table 8). The plasma P response was opposite of that observed during the backgrounding period, resulting in a pasture treatment by feedlot treatment interaction \( (P = 0.03) \). Only a weak tendency \( (P < 0.15) \) for plasma Mg to be decreased in HS than LS steers was observed on d 155. Plasma and liver Cu concentrations were decreased in steers which received increased S in the feedlot diet \( (P < 0.05; \text{Table 9}). \)

**DISCUSSION**

In this study, 96 crossbred yearling steers were supplemented on pasture with 1% body weight daily of DDGS that was either HS \( (0.8\% \text{ S}; 0.45\% \text{ total dietary S}) \) or LS \( (0.5\% \text{ S}; 0.3\% \text{ total dietary S}) \). The hypothesis was that exposing cattle to high dietary S concentrations on a high forage diet would adjust the rumen populations to elevated S prior to the challenge provided by the switch to a high concentrate diet. We theorized that this
adjustment may decrease the $\text{H}_2\text{S}$ concentration during the peak time seen in other research to be between 15 and 30 d on a finishing diet (Loneragan et al., 2001).

During the time on pasture, DMI and BW gains did not differ between the treatments, but plasma Mg was decreased by high dietary S. Spears et al. (1985) observed a similar effect on circulating Mg concentrations in steers fed cool-season grass forages, tall fescue or orchardgrass, either supplemented with S (132 kg S/ha as gypsum) or not supplemented with S fertilizer. Sulfur fertilization of orchardgrass resulted in a 14% decrease in serum Mg in the cattle receiving the fertilized orchardgrass compared to those receiving the non-S fertilized orchardgrass. Spears et al. (1985) postulated that the negative effect of S on serum Mg might have been the result of the formation of insoluble complexes in the rumen preventing absorption of Mg from the digesta. In the present study, the relationship between S and Mg did not continue into the feedlot period of the study. Because the observed effects on plasma Mg were observed while cattle consumed a high forage diet, it is possible that the rumen conditions during this period were more favorable for the formation of the insoluble complex between S and Mg postulated by Spears et al. (1985). Magnesium serves an important role in the nervous system as an electrolyte (Katzman, 1966) and the importance of this electrolyte function in relation to S toxicity and the development of the neurological disorder PEM has not been documented.

Many studies have supported the antagonistic relationship between S, Cu, and molybdenum (Suttle, 1975; Spears et al. 1985; Gengelbach et al., 1994; Hansen et al., 2008). This interaction is caused by the formation of thiomolybdates in the rumen which prevent Cu absorption in the intestine and may also scavenge Cu from metabolic processes and enzymes.
in the body, further decreasing Cu status of the animal (Suttle, 1991). In this study, high dietary S resulted in a decrease in not only liver Cu concentration but also plasma Cu concentration; however, cattle did not reach a state of deficiency (NRC, 1996). This decreased Cu status may account for some of the decreased weight gain that was observed in the HS treatment. Copper is also critical to the function of the immune system as decreased Cu status has been shown to negatively affect both humoral and cellular immune responses (McDowell, 1992). Furthermore, Cu is important for cellular respiration, and is an integral part of several metalloenzymes, some of which play roles in antioxidant defense (McDowell, 1992).

Dietary S negatively impacted performance during the feedlot period resulting in decreased ADG and HCW during finishing. Other studies have found similar deleterious effects of high dietary S on performance of beef cattle (Zinn et al., 1999; Loneragan et al., 2001; Cammack et al., 2010). Feed conversion was not affected by diet S concentration, indicating that the decreased weight gain was at least partly due to the tendency for decreased DMI by steers receiving the HS diet. Zinn et al. (1999) found a linear decrease in efficiency with increasing dietary S concentration in steers, and Loneragan et al. (2001) reported similar effects of high S water on performance in steers. Regardless of whether high S diets are decreasing intake, efficiency or both, diets high in S appear to decrease animal gain, resulting in increased days on feed to reach the same level of finish as steers on lower S diets.

Determination of fatty acid composition when the diets only differ in S content allows this study to more definitively ascertain changes in lipid composition due to S, without the confounding differences in diet composition observed in other studies utilizing different
sources or levels of DDGS inclusion in the diet. Previous studies have found conflicting effects of distillers grains on marbling and fat deposition. Several studies have reported that inclusion of distillers grains negatively influence marbling scores of cattle (Depenbusch et al., 2008; Gunn et al., 2009) while others have not observed differences (Al-Suwaiegh et al., 2002; Depenbusch et al., 2009; Leibovich et al., 2009). This study found an apparent increase in fatness of steers receiving HS during the backgrounding period, regardless of dietary S concentration during the finishing period. This result may indicate that receiving HS during the pasture period altered nutrient partitioning in the steers and resulted in a fatter carcass, though it remains unclear how S may have caused this effect.

High dietary S during the finishing period decreased the percentage of several saturated fatty acids. Biohydrogenation is a microbial process in the rumen which reduces the unsaturated lipids from the diet to more saturated fats (Nafikov and Beitz, 2007). These differences may indicate that hydrogens that could be used for biohydrogenation were being diverted to a different aspect of rumen metabolism, likely the reduction of sulfate to sulfide by SRB and the subsequent production of H₂S from sulfide. Hydrogen competition between SRB and other ruminal processes has been of interest as multiple studies have utilized various substances as hydrogen sinks to prevent the production of H₂S when cattle are receiving high S diets (Knight et al., 2008; Cammack et al., 2010).

In conclusion, our hypothesis that exposing cattle to high dietary S concentrations while consuming a forage-based diet would improve future performance when cattle were fed diets containing high dietary S concentrations in the feedlot was not substantiated. But high dietary S during the pasture decreased plasma Mg concentrations compared to steers
receiving a low S diet, though the influence of S on Mg did not persist in the feedlot period. Indices of Cu status were decreased due to elevated dietary S during the feedlot period. Increased dietary S during the finishing period negatively influenced steers gains and retail product yield and also resulted in alterations to meat fatty acid profile.
LITERATURE CITED


Table 1. Ingredient composition of supplement fed to steers grazing stockpiled bromegrass

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% of diet, DM basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDGS ¹</td>
<td>98.04</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.90</td>
</tr>
<tr>
<td>Bovatec 91²</td>
<td>0.03</td>
</tr>
<tr>
<td>Trace mineral mix³</td>
<td>0.03</td>
</tr>
</tbody>
</table>

¹ 1.3% of DDGS was replaced with sodium sulfate to add 0.3% S to the supplement for HS steers.

² Provided approximately 200 mg Lasolacid·hd⁻¹·d⁻¹.

³ Provided per kg of diet: 0.1 mg Co, 10 mg Cu, 0.5 mg I, 20 mg Mn, 0.1 mg Se, 30 mg Zn.
Table 2. Analysis of forage offered to grazing yearling steers supplemented with distillers grains

<table>
<thead>
<tr>
<th>Item</th>
<th>Day -14</th>
<th>Day 14</th>
<th>Day 37</th>
<th>SEM</th>
<th>P value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, % DM</td>
<td>37.9</td>
<td>43.4</td>
<td>43.6</td>
<td>1.74</td>
<td>0.05</td>
</tr>
<tr>
<td>CP, % DM</td>
<td>14.1</td>
<td>13.0</td>
<td>11.4</td>
<td>0.64</td>
<td>0.03</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td>60.1</td>
<td>59.1</td>
<td>60.5</td>
<td>1.61</td>
<td>0.82</td>
</tr>
<tr>
<td>ADF, % DM</td>
<td>36.0</td>
<td>31.5</td>
<td>33.9</td>
<td>0.86</td>
<td>0.005</td>
</tr>
<tr>
<td>IVDMD, % DM</td>
<td>46.7</td>
<td>46.2</td>
<td>41.9</td>
<td>1.13</td>
<td>0.01</td>
</tr>
</tbody>
</table>
| Macrominerals, % DM
  Phosphorus       | 0.36    | 0.27   | 0.25   | 0.02| 0.001       |
  Calcium          | 0.54    | 0.60   | 0.66   | 0.03| 0.05        |
  Magnesium        | 0.20    | 0.17   | 0.18   | 0.008| 0.06       |
  Sulfur           | 0.17    | 0.16   | 0.16   | 0.007| 0.80       |
| Microminerals, mg/kg
  Iron             | 84.9    | 92.8   | 139.6  | 8.22| 0.001       |
  Manganese        | 49.5    | 60.7   | 61.1   | 10.1| 0.66        |
  Zinc             | 30.0    | 28.9   | 25.2   | 2.08| 0.25        |
  Copper           | 6.68    | 6.51   | 4.28   | 0.35| 0.001       |

$^1$ All treatment and treatment by day interactions, $P > 0.15$. 
Table 3. Ingredient composition of finishing diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% total diet, DM basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>48.00</td>
</tr>
<tr>
<td>DDGS</td>
<td>40.00</td>
</tr>
<tr>
<td>Chopped hay</td>
<td>8.00</td>
</tr>
<tr>
<td>Treatment corn(^1)</td>
<td>2.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.50</td>
</tr>
<tr>
<td>Bovatec 91(^2)</td>
<td>0.01</td>
</tr>
<tr>
<td>Salt</td>
<td>0.40</td>
</tr>
<tr>
<td>Vitamin A premix</td>
<td>0.06</td>
</tr>
<tr>
<td>Trace mineral mix(^3)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^1\) Vehicle for inclusion of the sodium sulfate.

\(^2\) Provided approximately 200 mg Lasolacid·hd\(^{-1}\)·d\(^{-1}\).

\(^3\) Provided per kg of diet: 0.1 mg Co, 10 mg Cu, 0.5 mg I, 20 mg Mn, 0.1 mg Se, 30 mg Zn.
Table 4. Dietary sulfur affects intake, feedlot gain, feed efficiency and carcass characteristics

<table>
<thead>
<tr>
<th>Item</th>
<th>Feedlot</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Sulfur</td>
<td>High Sulfur</td>
<td>SEM</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>Live performance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial BW, kg</td>
<td>321</td>
<td>321</td>
<td>29</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>DMI, kg DM/d</td>
<td>10.73</td>
<td>10.03</td>
<td>0.33</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>ADG, kg/d</td>
<td>1.64</td>
<td>1.47</td>
<td>0.03</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Gain:Feed</td>
<td>0.150</td>
<td>0.145</td>
<td>0.003</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Final BW, kg</td>
<td>585</td>
<td>566</td>
<td>11</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Carcass data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCW, kg</td>
<td>359</td>
<td>342</td>
<td>6.12</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Fat cover, cm</td>
<td>1.07</td>
<td>1.00</td>
<td>0.07</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Ribeye Area, cm²</td>
<td>83.01</td>
<td>80.66</td>
<td>1.26</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Yield Grade</td>
<td>2.85</td>
<td>2.77</td>
<td>0.12</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>KPH, %</td>
<td>1.94</td>
<td>2.00</td>
<td>0.08</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Marbling Score²</td>
<td>429</td>
<td>436</td>
<td>14.51</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

¹ n = 12 pens per treatment.

² Marbling score: 400 = small, 500 = modest.
Table 5. Effects of dietary sulfur on fatty acid composition of meat sample from 12\textsuperscript{th} -13\textsuperscript{th} rib split (g/100g of fatty acids)

<table>
<thead>
<tr>
<th>Item\textsuperscript{1}</th>
<th>Low Sulfur</th>
<th>High Sulfur</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 14:0</td>
<td>1.80</td>
<td>1.38</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>C14:1</td>
<td>0.37</td>
<td>0.31</td>
<td>0.045</td>
<td>0.31</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.36</td>
<td>0.41</td>
<td>0.058</td>
<td>0.55</td>
</tr>
<tr>
<td>C 16:0</td>
<td>24.52</td>
<td>22.86</td>
<td>0.64</td>
<td>0.08</td>
</tr>
<tr>
<td>C 16:1</td>
<td>2.48</td>
<td>2.28</td>
<td>0.19</td>
<td>0.47</td>
</tr>
<tr>
<td>C 17:0</td>
<td>0.95</td>
<td>0.78</td>
<td>0.043</td>
<td>0.01</td>
</tr>
<tr>
<td>C 18:0</td>
<td>16.31</td>
<td>17.79</td>
<td>0.49</td>
<td>0.04</td>
</tr>
<tr>
<td>C 18:1 cis-9</td>
<td>38.39</td>
<td>39.17</td>
<td>0.76</td>
<td>0.47</td>
</tr>
<tr>
<td>C 18:1 cis-11</td>
<td>0.15</td>
<td>0.16</td>
<td>0.018</td>
<td>0.84</td>
</tr>
<tr>
<td>C 18:1 cis-12</td>
<td>0.31</td>
<td>0.25</td>
<td>0.022</td>
<td>0.08</td>
</tr>
<tr>
<td>C 18:1 cis-13</td>
<td>0.17</td>
<td>0.18</td>
<td>0.010</td>
<td>0.43</td>
</tr>
<tr>
<td>C 18:1 trans-10 and 11</td>
<td>3.29</td>
<td>3.32</td>
<td>0.21</td>
<td>0.90</td>
</tr>
<tr>
<td>C 18:1 trans-12</td>
<td>0.27</td>
<td>0.36</td>
<td>0.054</td>
<td>0.28</td>
</tr>
<tr>
<td>C 18:1 trans-15</td>
<td>1.17</td>
<td>1.16</td>
<td>0.043</td>
<td>0.91</td>
</tr>
<tr>
<td>CLA cis-9 trans-11</td>
<td>0.41</td>
<td>0.42</td>
<td>0.052</td>
<td>0.96</td>
</tr>
<tr>
<td>C 18:2</td>
<td>7.68</td>
<td>7.82</td>
<td>0.36</td>
<td>0.78</td>
</tr>
<tr>
<td>C 18:3n3</td>
<td>0.20</td>
<td>0.21</td>
<td>0.030</td>
<td>0.85</td>
</tr>
<tr>
<td>C 22:0</td>
<td>0.31</td>
<td>0.36</td>
<td>0.017</td>
<td>0.06</td>
</tr>
<tr>
<td>C 22:1</td>
<td>1.00</td>
<td>1.14</td>
<td>0.11</td>
<td>0.39</td>
</tr>
<tr>
<td>SFA\textsuperscript{2}</td>
<td>44.26</td>
<td>43.58</td>
<td>0.74</td>
<td>0.53</td>
</tr>
<tr>
<td>MUFA\textsuperscript{3}</td>
<td>47.60</td>
<td>48.28</td>
<td>0.65</td>
<td>0.47</td>
</tr>
<tr>
<td>PUFA\textsuperscript{4}</td>
<td>8.29</td>
<td>8.44</td>
<td>0.35</td>
<td>0.76</td>
</tr>
<tr>
<td>PUFA:SFA</td>
<td>0.19</td>
<td>0.20</td>
<td>0.0098</td>
<td>0.59</td>
</tr>
<tr>
<td>MCFA\textsuperscript{5} ( &lt; 15:1)</td>
<td>2.54</td>
<td>2.10</td>
<td>0.18</td>
<td>0.010</td>
</tr>
<tr>
<td>LCFA\textsuperscript{6} ( &gt; 16:0)</td>
<td>97.62</td>
<td>98.21</td>
<td>0.22</td>
<td>0.07</td>
</tr>
<tr>
<td>Total lipid (g lipid/100g meat)</td>
<td>4.49</td>
<td>5.08</td>
<td>0.32</td>
<td>0.22</td>
</tr>
</tbody>
</table>

\textsuperscript{1} n = 12 per treatment.
\textsuperscript{2} Saturated fatty acid.
\textsuperscript{3} Monounsaturated fatty acid.
\textsuperscript{4} Polyunsaturated fatty acid.
\textsuperscript{5} Medium chain fatty acid.
\textsuperscript{6} Long chain fatty acid.
Table 6. Influence of dietary sulfur concentration while grazing bromegrass pastures on macromineral status of yearling steers

<table>
<thead>
<tr>
<th>Day 35(^1)</th>
<th>DDGS Supplement</th>
<th>SEM</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Sulfur</td>
<td>High Sulfur</td>
<td></td>
</tr>
<tr>
<td>Plasma, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.93</td>
<td>1.84</td>
<td>0.035</td>
</tr>
<tr>
<td>Calcium</td>
<td>10.6</td>
<td>10.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Phosphorus(^2)</td>
<td>15.5</td>
<td>17.4</td>
<td>0.34</td>
</tr>
<tr>
<td>Sulfur</td>
<td>113.8</td>
<td>113.3</td>
<td>1.33</td>
</tr>
<tr>
<td>Liver, g/kg DM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.59</td>
<td>0.58</td>
<td>0.14</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.32</td>
<td>0.29</td>
<td>0.14</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>11.84</td>
<td>11.48</td>
<td>2.06</td>
</tr>
<tr>
<td>Sulfur</td>
<td>6.92</td>
<td>7.00</td>
<td>2.32</td>
</tr>
</tbody>
</table>

\(^1\) \(n = 4\) plots per treatment.

\(^2\) Used d0 in covariate analysis.
Table 7. Influence of dietary sulfur concentration while grazing bromegrass pastures on micromineral status of yearling steers

<table>
<thead>
<tr>
<th>Day 35(^1)</th>
<th>DDGS Supplement</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Sulfur</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High Sulfur</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma, mg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>2.1</td>
<td>0.09</td>
<td>0.73</td>
</tr>
<tr>
<td>Zinc</td>
<td>1.2</td>
<td>0.04</td>
<td>0.53</td>
</tr>
<tr>
<td>Copper</td>
<td>1.1</td>
<td>0.04</td>
<td>0.24</td>
</tr>
<tr>
<td>Liver, mg/kg DM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>245.3</td>
<td>13.49</td>
<td>0.77</td>
</tr>
<tr>
<td>Manganese</td>
<td>10.1</td>
<td>0.72</td>
<td>0.94</td>
</tr>
<tr>
<td>Zinc</td>
<td>115.3</td>
<td>5.21</td>
<td>0.66</td>
</tr>
<tr>
<td>Copper (^2)</td>
<td>182.2</td>
<td>18.56</td>
<td>0.76</td>
</tr>
</tbody>
</table>

\(^1\) n = 4 plots per treatment.

\(^2\) Used d0 values in a covariate analysis.
Table 8. Influence of dietary sulfur concentration during the finishing period on macromineral status of yearling steers

<table>
<thead>
<tr>
<th>Item</th>
<th>Day 125</th>
<th></th>
<th>Day 155</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Sulfur</td>
<td>High Sulfur</td>
<td>SEM</td>
<td>P value</td>
</tr>
<tr>
<td>Plasma, mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.93</td>
<td>1.90</td>
<td>0.03</td>
<td>0.44</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.97</td>
<td>9.78</td>
<td>0.12</td>
<td>0.28</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>15.7</td>
<td>14.9</td>
<td>0.33</td>
<td>0.05</td>
</tr>
<tr>
<td>Sulfur</td>
<td>92.2</td>
<td>92.7</td>
<td>2.35</td>
<td>0.87</td>
</tr>
<tr>
<td>Liver, g/kg DM</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Magnesium</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Calcium</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

1 n = 12 pens per treatment.
2 d 125 represents 57 d on full finishing diet.
3 d 155 represents 87 d on full finishing diet.
4 d 0 used as covariate for d 125.
5 d 155 pasture treatment by feedlot treatment interaction (P = 0.04).
6 Liver was not sampled on d 125.
Table 9. Influence of dietary S concentration during the finishing period on micromineral status of yearling steers

<table>
<thead>
<tr>
<th>Item</th>
<th>Day 125</th>
<th>Day 155</th>
<th>SEM</th>
<th>P value</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Sulfur</td>
<td>High Sulfur</td>
<td></td>
<td></td>
<td>Low Sulfur</td>
<td>High Sulfur</td>
</tr>
<tr>
<td><strong>Plasma, mg/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>2.08</td>
<td>1.92</td>
<td>0.10</td>
<td>0.30</td>
<td>2.11</td>
<td>2.21</td>
</tr>
<tr>
<td>Zinc</td>
<td>1.18</td>
<td>1.16</td>
<td>0.03</td>
<td>0.71</td>
<td>1.30</td>
<td>1.27</td>
</tr>
<tr>
<td>Copper</td>
<td>0.85</td>
<td>0.82</td>
<td>0.03</td>
<td>0.42</td>
<td>0.77</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Liver, mg/kg DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>254.7</td>
<td>246.4</td>
</tr>
<tr>
<td>Manganese</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>9.5</td>
<td>9.6</td>
</tr>
<tr>
<td>Zinc</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>104.4</td>
<td>103.1</td>
</tr>
<tr>
<td>Copper</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>280.3</td>
<td>196.9</td>
</tr>
</tbody>
</table>

1. n = 12 pens per treatment.
2. d 0 covariate for d 125.
3. Liver not sampled on d 125.
4. d 0 covariate for d 155.
CHAPTER 4.

Effects of elevated dietary sulfur concentration on rumen hydrogen sulfide concentration and bacterial populations

A paper to be submitted to Journal of Animal Science

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ABSTRACT

Crossbred yearling steers (n = 96; 321 ± 29 kg BW) were used to determine the effects of dietary S concentration of a grain supplement fed to cattle on pasture and in the feedlot on microbial populations and rumen parameters. Steers were blocked by weight, allocated to bromegrass pastures, and supplemented with either low S DDGS (0.3% total dietary S; LS; n = 4 plots) or LS DDGS with additional S from NaSO₄ (0.45% total dietary S, HS; n = 4 plots). After 35 d on pasture, steers moved to feedlot where half remained on the previous S treatment while half switched treatments, resulting in 4 treatments (LS-LS, LS-HS, HS-LS, and HS-HS; LS = 0.3% S; HS = 0.6% S; n = 6 feedlot pens). No interactions for any measures between pasture treatment and feedlot treatment were observed, so data were pooled by feedlot treatment (n = 12 per treatment). Rumen fluid was collected esophageally on d 36, during transition to the full feedlot diet (d 56) and near the end of study (d 155) for
analysis of VFA and lactate proportions. Lactate was not affected \((P > 0.50)\) by dietary S concentration. Rumen H\(_2\)S concentrations were measured on d 36, 45, 52, 59, 66, 91, 125, and 155. Rumen H\(_2\)S concentrations did not differ among treatments until forage concentration decreased to 15\% of diet DM \((d 66; P = 0.04)\). Concentrations of H\(_2\)S in HS steers increased as forage percentage of the diet decreased during transition, even though S intake of HS steers remained consistent throughout this time. Two incidences of polioencephalomalacia were observed on d 88 and d 89 and coincided with elevated H\(_2\)S concentrations on d 91, 23 d on the full finishing diet. Sulfate reducing bacteria (SRB) populations from rumen fluid collected on d 155 were measured using real-time quantitative PCR (LS-LS and HS-HS only, \(n = 6\) per treatment). Copy numbers of total SRB and a SRB group representing *Desulfovibrio desulfuricans* were increased \((P = 0.03)\) in HS steers than LS. Rumen H\(_2\)S concentrations on d 155 were correlated with total SRB populations \((R = 0.6; P = 0.05)\). In conclusion, dietary roughage concentration influenced rumen H\(_2\)S concentration in steers fed high S diets. Further, differences in rumen SRB populations partially account for the variation in H\(_2\)S concentrations observed in this study.

**Key words:** cattle, hydrogen sulfide, methanogens, sulfate reducing bacteria, sulfur

**INTRODUCTION**

Modern cattle diets often contain high concentrations of sulfur (S) because of the inclusion of coproducts of the ethanol industry (Crawford, 2007). The mechanism behind S toxicity and polioencephalomalacia (PEM) development is currently credited to the production of hydrogen sulfide (H\(_2\)S) gas in the rumen (Gould, 1998). This gas is produced as a waste product of the sulfate reducing bacteria (SRB) populations present in the rumen.
Sulfate ($\text{SO}_4$) is utilized, along with lactate or other carbon donors, by the SRB to produce sulfide ions, which then combine with free hydrogen to form $\text{H}_2\text{S}$ (Campbell and Postgate, 1965; Beauchamp et al., 1984). A recent study suggested that SRB populations make up as much as 0.75% of the rumen population in cattle fed a high concentrate diet that did not contain dried distillers grains with solubles (DDGS; Callaway et al., 2010). Susceptibility of individual animals to PEM is highly variable and may be due to differences in ruminal production of $\text{H}_2\text{S}$ (Gould, 1998). This lead our research to the hypothesis that animal variation in SRB populations contribute to the differences in $\text{H}_2\text{S}$ concentration and PEM susceptibility. Previous work on SRB in the rumen has been largely culture based and has been limited to estimates of SRB as a proportion of the general population of the rumen (Howard and Hungate, 1976; Cummings et al., 1995). Recent work investigating SRB in swine slurry have determined populations using modern molecular techniques, specifically real-time quantitative PCR (Spence et al., 2008). Application of these techniques to identify SRB in the rumen has not previously been reported.

Therefore, the objectives of this study were: 1) to determine how concentration of dietary $\text{S}$ and roughage level of the diet may affect rumen $\text{H}_2\text{S}$ concentrations of yearling steers, 2) to determine how dietary $\text{S}$ influences microbial populations of the rumen, including SRB and methanogens, and how these changes in microbial populations may explain animal variation in rumen $\text{H}_2\text{S}$ concentrations, and 3) to determine if exposing cattle to high dietary $\text{S}$ while consuming a forage-based diet would allow the rumen to adjust to high $\text{S}$ in a lower risk situation preventing the high rumen $\text{H}_2\text{S}$ concentrations usually associated with high $\text{S}$ finishing diets.
MATERIALS AND METHODS

Procedures and use of animals for this experiment were approved by the Iowa State University Animal Care and Use Committee.

**Dietary treatments**

Crossbred yearling steers (n = 96, 321 ± 29 kg initial BW) were utilized to study the effects of dietary S concentration on rumen H\textsubscript{2}S concentrations, microbial SRB and methanogen populations, and lactate and VFA proportions. Steers were blocked by weight and randomly assigned to one of eight 2.4 ha smooth bromegrass-based pastures (n = 12 steers per plot). Two dietary treatments were utilized, the first being a supplement of low S DDGS (0.5% S in supplement; 0.3% total dietary S; LS; n = 4 plots) and the second being the LS DDGS with an additional 0.3% S provided from sodium sulfate (0.8% S in supplement; 0.45% total dietary S; HS; n = 4 plots; Table 1). Steers were front stripgrazed weekly over a 36 d period and received the DDGS supplement at 1% of BW at 0900 daily. On d 36 steers were moved from the pastures to feedlot pens, with half the steers remaining on the same treatment (HS or LS) and half switching to the other treatment, resulting in 4 treatments total (n = 4 steers per pen, 6 replicate pens per treatment). For the first 10 d in the feedlot steers consumed ad libitum hay and 1% BW of the DDGS supplement representing their feedlot treatment. Steers were transitioned to the feedlot diet from d 47 to d 67 utilizing 3 step-up diets where grass hay was replaced with an equal amount of cracked corn (Table 2). On d 67 steers began consuming the final feedlot diet (Table 3) and remained on this final diet for 97 days, resulting in a total trial length of 164 d at harvest. During the feedlot period, steers were fed low (LS) and high S (HS) diets. Initially dietary S was 0.2% and 0.5% for LS
and HS respectively. On d 126, a new batch of DDGS was used which increased the S concentrations to 0.3% and 0.6% for LS and HS respectively. Individual diet components and pen orts were analyzed for S concentration (ICP; Optima 7000, Perkin Elmer, Waltham, MA) for the calculation of S intake. Data for S intake were separated over time into sections which represent intake between each ort sampling.

*Rumen VFA and lactate measures*

One steer from each pen was randomly selected for measurement of rumen parameters. Rumen fluid was collected 6 h after feeding at the end of the backgrounding period, during transition, and end of sampling (d 36, d 56, d 155, respectively) by inserting plastic tubing into the rumen via the esophagus, and applying slight suction to assist in collection of fluid. The fluid was then strained through four layers of cheesecloth and frozen at -20°C for later analysis. Prior to VFA analysis strained rumen fluid samples were thawed and inverted, briefly vortexed (5-10 s), and a 5 mL subsample was centrifuged at 2,400 x g for 30 min at 4°C. Following centrifugation, a 3 mL subsample of the centrifuged rumen fluid was thoroughly mixed with 600 µL of 25% metaphosphoric acid (g/mL), and refrozen at -20°C prior to final analysis. The mixture was thawed and centrifuged at 790 x g for 10 min, and 1 mL of the clarified supernatant was combined in a GC vial with 100 µL of 0.4% 2-ethylbutyric acid (vol/vol) internal standard. Samples were analyzed for VFA concentrations by GC (Varian Inc., Colombia, MD), using a 30x0.25x0.25 DB-FFAP column (Agilent, Santa Clara, CA).
Lactate concentration in rumen fluid was analyzed using a commercially available kit (L-lactate assay kit; Biomedical Research Service, Buffalo, NY). Briefly, rumen fluid was initially centrifuged at 2,400 x g for 30 min at 4°C, and an aliquot was removed and refrozen. The rumen fluid was thawed and centrifuged a second time at 790 x g for 10 min, and an aliquot analyzed for lactate concentration. A standard line was generated using a lactate stock solution provided as part of the commercial kit, samples were analyzed at 492 nm as recommended by the kit manufacturer. Software associated with the spectrophotometer was used to generate the standard line (SPECTRA Max PLUS, Sunnyvale, Ca). A sample of doubly centrifuged rumen fluid was used as the blank for the spectrophotometric analysis of each separate day analyzed to account for differences in rumen fluid color due to sampling time.

**Rumen hydrogen sulfide gas concentration measurement**

Rumen H$_2$S measures were determined on the same 24 steers chosen for rumen fluid assessment and were collected on d 36, 45, 52, 59, 66, 91, 125, and 155 of the overall study at 6 h postprandial. Hydrogen sulfide concentrations of rumen gas were measured using commercially available gas detector tubes (Kitagawa, Japan). The sample area was prepared by clipping the area of the left side paralumbar fossa, and thoroughly scrubbing with betadine and isopropyl alcohol. Samples were collected by insertion of a sterile 10 cm, 16 gauge needle into the paralumbar fossa area. The needle was then fitted to a 1 mL syringe connected with a short piece of tubing to the gas detector tube and volumetric gas sampling pump (Matheson-Kitigawa 8014-400B, Kitigawa, Japan). A 50 mL or 100 mL sample of rumen gas was drawn into the detector tube depending on the time point and treatment the
animal was receiving, and H$_2$S concentration was determined based on the amount of color change of silver nitrate or copper sulfate in the tube again depending on the treatment and timepoint.

**DNA isolation from rumen fluid**

Microbial DNA was isolated from strained rumen fluid collected on d 155 from steers which remained on the same dietary S treatments for the whole study (LS-LS, and HS-HS; n = 6 steers per treatment) using the methods of Yu and Morrison (2004). Briefly, 1 mL of rumen fluid was subjected to two rounds of bead-beating in the presence of NaCl and SDS, followed by precipitation with ammonium acetate, then isopropanol. The precipitate was then resuspended and treated with RNase and proteinase K, followed by DNA purification using a QIAgen DNA mini stool kit (QIAGEN, Valencia, CA). The quality and concentration of DNA were measured at A$_{260}$ and A$_{280}$ using a ND-1000 spectrophotometer (Nandrop Technologies, Wilmington, DE).

**Real-time PCR analysis and standard curves**

Real-time PCR assays were conducted on a MJ Research PTC-200 peltier thermal cycler (MJ Research Inc., Waltham, MA) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Sample DNA (2 µl) was used in a reaction mixture containing 12.5 µl of 2 X iQ SYBR Green Supermix, 500 nmol l$^{-1}$ of the specific forward and reverse primers (Table 4), and molecular grade H$_2$O to a final volume of 25 µl. Three specific SRB groups were targeted using primers developed for swine slurry SRB population quantification by Spence et al. (2008). Group 1 primers were generated to provide a match to
the amino acid sequences of *Desulfobulbus* related organisms and matches most closely with *Desulfobulbus propionicus*. Group 2 primers targeted SRB which closely resembles other *Desulfovibrio*, however, the specific bacterial populations expected to be identified using this primer set remain largely unknown. Finally, the group 3 SRB primers match with *Desulfovibrio desulfuricans*, a species expected to be found in the rumen based on previous culture work (Howard and Hungate, 1976). Gene copy numbers for the highly conserved dsrA gene copies for each of the 3 SRB groups were determined using a separate plasmid for each group (kindly provided by C. Spence and colleagues, ARS, Peoria, IL). Each plasmid contained one copy of the dsrA gene and thus did not require a correction for copy number (Klein et al., 2001). Genomic DNA from *Methanosarcina acetivorans* was used to generate the standard line for methanogen quantification, as this organism has been reported to possess a single copy of the conserved mcrA gene (Nunuora et al., 2006). The universal bacterial ribosomal 16S gene was used to determine the total bacterial population (Harms et al., 2003). Quantification of bacterial numbers were determined using a standard from purified *Escherichia coli* genomic DNA, and data were divided by a ratio of the average 16S copy number per bacteria (3.6 copies) and the known copy number of 7 in the genomic *E. coli* DNA used (Klappenberg et al., 2001). Temperature gradient curves were analyzed to determine the optimal annealing temperature for each set of primers (Table 4). Standards and blanks (nanopure water) were analyzed in duplicate, while samples were analyzed in triplicate. Reaction conditions were as follows: 95°C for 5 min (1X), then 95°C for 30 s, then optimal annealing temperature for 30 s (40X). For the 16S and mcrA quantification an elongation step of 72°C for 30 s was included after annealing for the 40 cycles. Data from the real-time PCR reactions were analyzed using Opticon Monitor 3.1 software (Bio-Rad
Laboratories). These values were then used to determine the percentage of the total rumen population which each SRB group, the total SRB (determined by adding the 3 separate SRB groups together), and methanogens represented by dividing each respective group by the calculated total bacterial population (16S) and converting this value to a percentage.

**Statistical analysis**

The Mixed procedure of SAS version 9.2 was used to analyze all data (SAS Inst. Inc., Cary, NC). The model included the fixed effects of feedlot and pasture treatment and the interaction, no random effects were used. Experimental units were pasture plot during the backgrounding period (n = 4 per treatment) and feedlot pen during the finishing period (n = 6 per treatment). Interactions with P values greater than 0.20 were removed from the model. Rumen H$_2$S concentrations and dietary S intake measures were analyzed as repeated measures. Rumen fluid VFA proportions determined using GC were combined with lactate proportions determined colorimetrically and quantified as proportions to account for any discrepancies caused by saliva contamination during esophageal collection. Lactate and VFA data were analyzed as separate days (d 36, d 56, d 155) as each day represents a different dietary condition (high, medium, and low dietary forage, respectively). Bacterial population data were analyzed based on the S treatment that steers received throughout the study. Data were log transformed to allow the bacterial population data to meet statistical assumptions of equal variance. Means and standard error of the mean are reported from a Univariate analysis on SAS version 9.2 (SAS Inst. Inc.). The Corr procedure of SAS was used to determine correlations between SRB group copy numbers and rumen H$_2$S concentrations across treatments (n = 6 steers per treatment).
RESULTS

Sulfur intake

During the pasture period, daily S intake was greater ($P < 0.01$) in for HS steers compared with LS steers (23.7 g and 14.2 g, respectively). Dietary S intake in HS steers was greater ($P < 0.01$; Figure 1) than in LS steers throughout the transition and finishing period. Concentration of S in the diet also varied by time ($P < 0.01$), and a treatment by time interaction ($P < 0.01$) was observed. Sulfur intake in both treatment groups decreased slightly as steers transitioned to a full finishing diet. This was because the corn replacing the hay in the diet was lower in S (0.20% S and 0.11% S in hay and corn, respectively). Early in the finishing period, diets contained either 0.2% total dietary S (LS) or 0.5% total dietary S (HS). Dietary S intake within each treatment did not differ between days 42 – 125 ($P > 0.20$); however, S intake was increased ($P < 0.01$) in both treatments from d 126 to the end of study because a new load of DDGS was fed, resulting in total dietary S of 0.3% and 0.6% for LS and HS respectively for the remainder of the study.

Rumen hydrogen sulfide concentrations

Concentrations of rumen H$_2$S did not differ due to dietary S concentration when cattle were consuming a pasture-based diet (1,733 and 1,400 ppm for HS and LS, respectively; $P = 0.54$). A treatment by day interaction was observed for concentration of H$_2$S during the finishing period ($P < 0.01$). Rumen concentrations of H$_2$S in HS steers were not different ($P > 0.15$) from LS steers until d 66 (1,818 ppm and 643 ppm in HS and LS, respectively; $P = 0.04$), when forage content of the diet was decreased to 15% (Figure 2). Rumen H$_2$S
remained greater ($P < 0.01$) in HS than LS steers for the remainder of the trial (d 91, 125, 155) during which time steers consumed the final finishing diet containing 8% hay.

**Bacterial populations**

When bacterial population data were analyzed by raw copy number, group 1 SRB did not differ ($P = 0.74$) due to dietary S concentration, and group 2 SRB tended ($P = 0.11$, Figure 3) to be increased due to elevated dietary S concentration. Group 3 SRB showed the greatest increase in population numbers with a full-fold increase in HS steers ($P = 0.03$) relative to LS steers. The calculated total SRB population demonstrated a similar increase ($P = 0.03$) in the HS steers, and is likely due to the large portion of the total SRB population made up by the group 3 SRB (Figure 3). Methanogens were not affected by dietary S concentration ($P = 0.21$).

Sulfate-reducing bacteria and methanogen population data were also analyzed as a percentage of the total bacterial population using the universal 16S gene (Figure 4). No significant differences in percentage of the population were observed, though total SRB populations were found to comprise a very small proportion of the total bacteria analyzed, accounting for 0.007% in HS and 0.004% in LS ($P = 0.34$). Simple correlations between d 155 rumen H$_2$S concentrations and d 155 SRB populations measured on the same steers were determined using raw gene copy numbers for the SRB (Table 5). Concentrations of H$_2$S and copy numbers of SRB group 2 (R = 0.59; $P = 0.05$), group 3 (R = 0.60; $P = 0.05$) and the total SRB population (R = 0.60; $P = 0.05$) were correlated, suggesting that increases in these SRB populations explain at least some of the increase and variation observed in ruminal H$_2$S in this study.
**Volatile fatty acid and lactate proportions**

Proportions of VFA reflect the decrease in forage content of the diet from d 36 to 155, with acetate decreasing as a proportion of the VFA from near 70% at the end of the pasture period (d 36) to less than 50% at the last sampling point (d 155; Table 6). Steers receiving the HS diet had lower ($P = 0.02$) valerate proportion compared with LS steers on d 36. On d 56, HS-fed steers had greater propionate ($P = 0.03$) and tended to have lower butyrate and isovalerate ($P = 0.06$ and $P = 0.09$, respectively) compared to LS steers. By d 155, isovalerate was significantly lower ($P = 0.002$) in HS steers than LS steers. Elevated dietary S in d 155 tended to decrease acetate and increase propionate ($P = 0.08$ and $P = 0.10$, respectively) shifting the acetate: propionate ratio closer to 1 in the HS steers ($P = 0.09$). Dietary S did not affect lactate percentage ($P > 0.50$ at any timepoint).

**DISCUSSION**

The purpose of this study was to determine the effects of exposure to high dietary S concentrations to growing cattle consuming a high forage diet on subsequent performance in the feedlot when dietary S was also elevated. It was our hypothesis that exposing the rumen microbes, specifically the SRB, to increased concentrations of S while cattle consumed a high forage diet would allow the SRB population to adjust to the increased dietary S during a time when the animal is at less risk of toxicity due to the type of diet. Previous research by Loneragan et al. (2001) found that H$_2$S concentrations in the rumen peak between 15 and 30 d on a high concentrate, high S diet, this research sought to determine if exposing the rumen to increased dietary S prior to a high concentrate finishing diet would decrease this peak ruminal H$_2$S concentration.
In this study, H$_2$S concentration in the rumen gas was shown to increase in response to increased dietary S, but not until after the amount of forage in the diet dropped below 30%. Data from this study suggest that the increase in H$_2$S concentration does not occur until after the roughage content of the diet goes below a certain threshold, in this case the between 30% and 15% hay in the diet. During this time, S intake remained steady in the respective treatments, indicating that this increase in H$_2$S production was not in response to an increase in dietary S intake by the steers. It is possible that decreasing rumen pH as corn was substituted for hay in the diet provided an environment in which more sulfide was converted to H$_2$S since this conversion is a pH-dependent process with pKa’s of 11.94 and 7.04 for dissociation of the first and second ions, respectively (Beauchamp et al., 1984). This increase may also be related to the preference of SRB for waste products of starch metabolism, most notably lactate (Lewis, 1953; Coleman, 1960; Howard and Hungate, 1976); however, lactate proportions measured on d 36, 56, and 155 were not different due to dietary S in this study.

In the present study, 2 incidences of PEM in steers receiving the HS diet coincided with a peak in H$_2$S concentrations (d 91; 23 d on full finishing diet). The timing of PEM observations is similar to that described by others, with peak incidences being reported between 17 and 35 d on feed in studies utilizing high S water (Loneragan et al., 2001). The 2 cases in the present study were identified by farm personnel in the afternoon of d 88 and the early morning of d 89, respectively. Interestingly, rumen H$_2$S concentration measured upon intitial diagnosis and treatment of the d 88 case (9 h post feeding) was 25,000 ppm, a value approximately 5 times the average rumen H$_2$S concentration measured in the HS steers on d 91 (4813 ppm). Rumen H$_2$S concentration of the second steer was measured at 0800 on d 89.
and measured 1,000 ppm, which is likely related to the nearly 24 h that had passed since the steer had been fed the HS diet on d 88.

The suspected cause behind PEM development is inhalation of H$_2$S during the process of eructation of rumen gases (Gould, 1998). Dougherty and Cook (1962) suggested that 70-85% of eructated rumen gas is inhaled by the animal. When they specifically focused their attention on H$_2$S, these authors reported that insufflation of 300 mL of H$_2$S gas into the rumen of sheep with an open trachea resulted in eventual collapse, while sheep with the trachea occluded did not exhibit such symptoms of acute toxicity (Dougherty and Cook, 1962). They also noted that the time to collapse depended on the time post-feeding as well as frequency of eructation, giving further indication that the effects of rumen H$_2$S are related to inhalation of eructated rumen gas and variations in animal susceptibility to S toxicity may relate to eructation habits.

Hydrogen sulfide concentrations measured on d 125 were lower for both treatments, although HS remained elevated in comparison to LS. Sulfur intake had not changed over this time period, indicating that the rumen populations may have adjusted to the diet type and amount of S present in the diet. After this point, a new load of DDGS was used, which increased dietary S. Rumen H$_2$S concentrations measures at the last sampling (d 155) were elevated in comparison to d 125 measurements, possibly indicating that the rumen populations still have a propensity for increased H$_2$S in response to increased dietary S content after the rumen has adapted.
This increase in H$_2$S concentration may have multiple possible causes from a microbiological perspective. Either the SRB population is increasing in numbers resulting in increased gas production, or the metabolism of the SRB is shifted to increase the production of H$_2$S without a change in population. Sulfate-reducing bacteria represent a very small proportion of the overall population in the rumen; however, they create a significant risk to animal health through the production of H$_2$S and its subsequent toxic effects on cellular metabolism and animal performance (Gould, 1998). The *Desulfovibrio* in particular are considered to be the most prominent SRB in the rumen (Howard and Hungate, 1976). In our study, *Desulfovibrio* were found to be numerically increased by increased dietary S, with a full fold increase in HS steers than LS steers. This numerical increase did not translate into differences in the percentage of the total rumen bacterial population comprised of SRB (0.007% and 0.004% for HS and LS, respectively). The small number of samples (n = 6 per treatment) may have been a limitation in this study.

Sulfide is a waste product of SRB sulfate metabolism and contributes to increased H$_2$S in the rumen of cattle on high S diets causing PEM and animal death (Gould, 1998). In the present study, *Desulfovibrio* populations were correlated with H$_2$S concentrations, indicating that the increase in this population is likely responsible for at least a portion of the increased H$_2$S observed, rather than a shift in metabolic capacity of the SRB. Based on the increases in SRB populations that were observed at the end of this study, this study supports the implication that the SRB are increasing in population rather than some alteration in their metabolic capacity to utilize the increased S. This correlation also may explain the variation observed between individual animals in response to exposure to elevated dietary S. In the
future, researchers may investigate SRB populations of cattle prior to exposure to high
dietary S to determine if animals possess this propensity for H$_2$S production before
encountering a diet high in S. It also may be of interest to develop primers which target other
known SRB populations of the rumen, including *Desulfotomaculum*, to determine the effects
of other SRB populations on H$_2$S concentration.

In this study S had no effect on methanogen populations, although numerically
methanogens decreased to nearly half in the HS treatment animals, 0.06% of the rumen
population in HS compared to 0.11% in LS. Methane production has been shown in other
studies to be negatively affected by dietary S concentration (Qi et al., 1994; Weimer, 1998).
Mizuno et al. (1997) found that in bioreactors methanogens and SRB compete for H and that
H$_2$S inhibited methanogenesis (Mizuno et al., 1997).

Protozoa may also be impacted as these populations may be altered by high
concentrate diets leading to a decrease in their populations early in feeding a high concentrate
diet though populations may recover later in the feeding period (Towne et al., 1990).
Protozoa have also been found to influence protein metabolism in the rumen such that
defaunation decreases N recycling and ammonia concentrations (Yanez-Ruiz et al., 2007;
Kiran and Mutsvangwa, 2010). The effects of defaunation on microbial protein metabolism
may be related to the decreases seen in isovalerate proportion in rumen fluid of HS steers.
The effect of S on acetate and propionate result in an A:P ratio closer to 1 for the high S
treatment on d 155. Thompson et al. (1972) also found decreases in A:P ratio when dietary S
is increased. The reason for this is not clear because SRB are known to produce acetate as a
waste product of their S metabolism (Coleman, 1960), although their small percentage of the
rumen population makes their contribution to the total acetate likely negligible. Protozoa are also known to produce acetate (Yokoyama and Johnson, 1988), and defaunation has been shown to affect VFA profiles in a manner similar to those seen here in response to dietary S (Kiran and Mutsvangwa, 2010). Thus, it may be possible that S is negatively affecting protozoal populations leading to the changes observed in VFA.

In conclusion, S is interrelated with multiple aspects of rumen metabolism and animal health. Rumen H₂S concentrations were elevated when cattle had been consuming the finishing diet for 23 d. At this point, S intake had been constant, illustrating that this increase in H₂S was likely due to changes in the rumen populations in response to the dietary change from a high forage diet to a high concentrate finishing diet. The increase in rumen H₂S concentrations correlated well with the increases in the population numbers of *Desulfovibrio* SRB in rumen fluid determined using real-time PCR on d 155. This correlation would indicate that increased populations of SRB rather than some shift in their metabolism may be responsible for a considerable portion of the increased H₂S on this day. Furthermore, the H₂S concentrations were not affected by dietary S concentrations until forage decreased below 30%, possibly reflecting ruminal pH decreases and increased SRB energy substrate availability during this time. Future research may investigate other rumen populations and time points to determine how populations and conditions are changing during dietary transition and at times of observed peak H₂S concentrations.
LITERATURE CITED

http://www.extension.umn.edu/beef/components/pdfs/ManagingSulfur_Crawford.pdf


Table 1. Ingredient composition of supplement fed to steers grazing stockpiled bromegrass

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% of diet, DM basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDGS(^1)</td>
<td>98.04</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.90</td>
</tr>
<tr>
<td>Bovatec 91(^2)</td>
<td>0.03</td>
</tr>
<tr>
<td>Trace mineral mix(^3)</td>
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</tbody>
</table>

\(^1\) 1.3% of distillers dried grains with solubles (DDGS) was replaced with sodium sulfate to add 0.3% S to the supplement for HS steers.

\(^2\) Provided approximately 200 mg Lasolacid·hd\(^{-1}\)·d\(^{-1}\).

\(^3\) Provided per kg of diet: 0.1 mg Co, 10 mg Cu, 0.5 mg I, 20 mg Mn, 0.1 mg Se, 30 mg Zn.
Table 2. Ingredient composition of transitions diets

<table>
<thead>
<tr>
<th>Item</th>
<th>Ad Libitum</th>
<th>Transition diet 1</th>
<th>Transition diet 2</th>
<th>Transition diet 3</th>
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<tr>
<td>Days of study</td>
<td>36-46</td>
<td>47-54</td>
<td>55-61</td>
<td>62-68</td>
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<tr>
<td>Ingredients, %</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Chopped Hay</td>
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<td>45</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>DDGS Supp$^1$</td>
<td>1% BW</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DDGS$^2$</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Basal Diet$^3$</td>
<td>3</td>
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<td>3</td>
<td></td>
</tr>
<tr>
<td>Cracked Corn</td>
<td>10</td>
<td>25</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Treatment Corn$^4$</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Distillers dried grains with solubles supplemented as fed to cattle while on pasture.

$^2$ Distillers dried grains with solubles.

$^3$ Provided per kg of diet: 0.1 mg Co, 10 mg Cu, 0.5 mg I, 20 mg Mn, 0.1 mg Se, 30 mg Zn, 4 g Salt, 15 g Limestone, 0.6 g vitamin A premix, and approximately 200 mg Lasolacid·hd$^{-1}$·d$^{-1}$.

$^4$ Vehicle for inclusion of additional sulfur as sodium sulfate.
Table 3. Ingredient composition of finishing diet

<table>
<thead>
<tr>
<th>Ingredient</th>
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<tbody>
<tr>
<td>Corn</td>
<td>48.00</td>
</tr>
<tr>
<td>DDGS&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Chopped hay</td>
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<tr>
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</tr>
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<td>Bovatec 91&lt;sup&gt;3&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Salt</td>
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<tr>
<td>Vitamin A premix</td>
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<tr>
<td>Trace mineral mix&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<sup>1</sup> Distillers dried grains with solubles.

<sup>2</sup> Vehicle for inclusion of sodium sulfate.

<sup>3</sup> Provided approximately 200 mg Lasolacid·hd<sup>-1</sup>·d<sup>-1</sup>.

<sup>4</sup> Provided per kg of diet: 0.1 mg Co, 10 mg Cu, 0.5 mg I, 20 mg Mn, 0.1 mg Se, 30 mg Zn.
Table 4. Oligonucleotide primers and annealing temperatures used for rtPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Bacteria population target</th>
<th>Sequence (5’-3’)</th>
<th>Length (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grp1fd</td>
<td>Group 1 <em>Desulfobulbus propionicus</em> like SRB</td>
<td>GYGAGTGGKCTGCTAYGA</td>
<td>19</td>
<td>64</td>
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<tr>
<td>Grp1rd</td>
<td></td>
<td>CCAGGTGCCGATAACRGC</td>
<td>18</td>
<td></td>
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<tr>
<td>Grp2fd</td>
<td>Group 2 <em>Desulfovibrio</em> like SRB</td>
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</tr>
<tr>
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<td>GCWGCTACGCAACCGGTTGGG</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Grp3fw</td>
<td>Group 3 <em>Desulfovibrio desulfuricans</em> like SRB</td>
<td>CTGCQAATATGCTGCTACA</td>
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<td>64</td>
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<tr>
<td>Grp3rd</td>
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<td>GGGGCAARCCGTCGAACCTTG</td>
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<tr>
<td>mcrA f+</td>
<td>Methanogens</td>
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<tr>
<td>1055F</td>
<td>Universal bacterial 16S</td>
<td>ATGGGTCGTGTCAGCT</td>
<td>16</td>
<td>58</td>
</tr>
<tr>
<td>1392R</td>
<td></td>
<td>ACGGGGCGGTTGTGAC</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

1 Primers obtained from the ISU DNA Facility Oligo Synthesis Service, Iowa State University, Ames, IA, USA.

2 Primer sequences: all SRB primers: Spence et al., 2008; methanogens: Luton et al., 2002; Universal 16S: Harms et al., 2003.
Table 5. Simple correlations for sulfate reducing bacteria (SRB) populations and rumen H$_2$S gas concentration

<table>
<thead>
<tr>
<th>Microbial population$^\dagger$</th>
<th>Day 155 Rumen H$_2$S, R</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 SRB</td>
<td>0.39</td>
<td>0.33</td>
</tr>
<tr>
<td>Group 2 SRB</td>
<td>0.59</td>
<td>0.05</td>
</tr>
<tr>
<td>Group 3 SRB</td>
<td>0.60</td>
<td>0.05</td>
</tr>
<tr>
<td>Total SRB</td>
<td>0.60</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$^\dagger$ n = 6 per treatment.
Table 6. Rumen VFA proportions are affected by high dietary sulfur concentrations

<table>
<thead>
<tr>
<th>VFA, % of total</th>
<th>Low Sulfur</th>
<th>High Sulfur</th>
<th>SEM</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 36</strong>$^1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>68.57</td>
<td>68.39</td>
<td>1.05</td>
<td>0.90</td>
</tr>
<tr>
<td>Propionate</td>
<td>21.01</td>
<td>20.61</td>
<td>0.53</td>
<td>0.58</td>
</tr>
<tr>
<td>Butyrate</td>
<td>10.66</td>
<td>9.61</td>
<td>0.67</td>
<td>0.26</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>0.37</td>
<td>0.33</td>
<td>0.06</td>
<td>0.63</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>0.39</td>
<td>0.33</td>
<td>0.67</td>
<td>0.52</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.89</td>
<td>0.70</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.39</td>
<td>0.34</td>
<td>0.09</td>
<td>0.67</td>
</tr>
<tr>
<td>A:P ratio</td>
<td>3.28</td>
<td>3.33</td>
<td>0.08</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>Day 56</strong>$^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>51.85</td>
<td>52.41</td>
<td>1.56</td>
<td>0.80</td>
</tr>
<tr>
<td>Propionate</td>
<td>28.12</td>
<td>32.22</td>
<td>1.22</td>
<td>0.03</td>
</tr>
<tr>
<td>Butyrate</td>
<td>15.05</td>
<td>12.11</td>
<td>1.00</td>
<td>0.06</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>1.82</td>
<td>1.32</td>
<td>0.20</td>
<td>0.09</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>1.88</td>
<td>1.72</td>
<td>0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.68</td>
<td>0.71</td>
<td>0.23</td>
<td>0.91</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.94</td>
<td>1.63</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Day 155</strong>$^3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>47.48</td>
<td>44.41</td>
<td>1.20</td>
<td>0.08</td>
</tr>
<tr>
<td>Propionate</td>
<td>36.04</td>
<td>41.37</td>
<td>2.30</td>
<td>0.10</td>
</tr>
<tr>
<td>Butyrate</td>
<td>11.99</td>
<td>10.21</td>
<td>1.27</td>
<td>0.32</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>0.58</td>
<td>0.46</td>
<td>0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>1.45</td>
<td>0.58</td>
<td>0.18</td>
<td>0.002</td>
</tr>
<tr>
<td>Valerate</td>
<td>1.76</td>
<td>2.10</td>
<td>0.28</td>
<td>0.42</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.46</td>
<td>0.39</td>
<td>0.09</td>
<td>0.53</td>
</tr>
<tr>
<td>A:P ratio</td>
<td>1.38</td>
<td>1.12</td>
<td>0.10</td>
<td>0.09</td>
</tr>
</tbody>
</table>

$^1$ d 36 data represent the forage treatment that steers had received up to d 35, where diets contained either 0.3% or 0.45% S for low and high S, respectively (n = 4 plots per treatment).

$^2$ d 56 samples were collected during transition when cattle were eating transition diet 2, where diets contained either 0.2% or 0.5% S for low and high S, respectively (n = 12 pens per treatment).

$^3$ d 155 samples were collected near the end of the trial, where diets contained either 0.3 or 0.6% S for low and high S respectively (n = 12 pens per treatment).
Figure 1. Dietary sulfur (S) intakes are affected by dietary S treatment ($P < 0.01$) either low S (0.2% S d 47 – 125, 0.3% S d 126-155) or high S (0.5% S d 47-125, 0.6% d 126-155) and day of sampling ($P < 0.01$).
Figure 2. Rumen hydrogen sulfide concentrations are affected by dietary S concentration (low S 0.2% S d 45 - 125 and 0.3% S for d 155, high S 0.5% S d 45 – 125 and 0.6% S for d 155, \( P < 0.01 \)), day of sampling \(( P < 0.01 \), and the interaction \(( P < 0.01 \). Days when high sulfur steers exhibited rumen hydrogen sulfide concentrations greater than low sulfur steers \(( P < 0.05 \) are denoted by \(*\).
Figure 3. Dietary sulfur concentration (0.3% S for low sulfur, 0.6% S for high sulfur) quantitatively affects microbe populations in rumen fluid ($\dagger P = 0.11$, $* P = 0.03$).
Figure 4. Sulfate reducing bacteria and methanogen population changes in response to increased dietary sulfur concentration (0.3% S for low sulfur, 0.6% S for high sulfur) as a percentage of the total bacterial population ($P > 0.20$).
CHAPTER 5. GENERAL CONCLUSION

Sulfur, although required by ruminants can, in excess, result in significant health problems for the animal. The S content of finishing cattle diets is increasing and will likely continue to increase as coproducts of the ethanol industry, which are higher in sulfur, out compete more traditional feedstuffs like corn and soy. As a result management methods to reduce the risk of S-related performance problems and incidence of disease, including polioencephalomalacia (PEM), are of interest to the industry.

The purpose of this research was to determine the effects of high dietary sulfur in yearling beef steers on measures of animal performance and rumen environment. Furthermore, this research sought to determine if backgrounding cattle with a DDGS supplement containing elevated dietary S would adjust the rumen to the increased dietary S content in a lower risk environment and reduce the risk for toxicity when cattle began finishing in the feedlot.

The combined results of this research related to increased dietary S on animal performance and the rumen environment illuminate some of the mechanisms behind the negative impacts of increased dietary S on these parameters. This research indicates that prior exposure to increased dietary S did not reduce S toxicity later in the feedlot, nor did it increase S toxicity.

One aspect of the decrease in performance observed in cattle fed diets high in S is due to the interactions of S with other minerals especially Cu. In this study effects on copper were observed, specifically both plasma and liver Cu concentrations were decreased in cattle
receiving high dietary S when measured on d 155, but not at any other timepoint, nor were there effects due to the previous pasture treatment. Interestingly, plasma Mg was negatively affected when measured after 35 d of a high forage diet during the pasture period, but this effect was not sustained during the finishing period. Possibly this interaction is related to the differences in the rumen environment when a high forage diet is consumed in comparison to a high concentrate finishing diet.

Hydrogen sulfide concentrations in the rumen measured highest on d 91, 23 d on a full finishing diet, which contained 8% roughage as chopped hay. This peak in H₂S concentration coincided with many aspects of the research. The peak is 23 d into a full finishing diet, which coincides with the estimated peak concentration of H₂S between 14 and 30 d on a high concentrate diet. Furthermore, the peak was also observed shortly after 2 cases of PEM during this study. It is also interesting to note that H₂S concentrations did not differ between the S treatments until d 66 when cattle were fed a diet with 15% roughage, indicating that roughage level indeed may play a role in H₂S concentrations in the rumen gas.

The production of H₂S gas in the rumen is credited as the cause of S-related PEM. Sulfide is generated by sulfate-reducing bacteria (SRB) in the rumen as an integral part of sulfate respiration, and in the reducing environment of the rumen this sulfide becomes H₂S. The analysis of rumen microbial populations in this study, specifically SRB, indicate that part of the variation and difference observed between individual animals in production of H₂S gas in the rumen may be related to the differences in SRB populations. This variation between animals in both SRB population and H₂S concentration may partially explain why
individual cattle fed the same high S diet will respond differently in performance and propensity for S-related illness.

Future researchers may wish to analyze other microbial populations in the rumen to determine how they are affected by dietary S concentrations. Lactate is a choice substrate for SRB and in this study proportions of lactate in the rumen fluid were not altered by S concentration in the animal diet. Analysis of populations integral to lactate production and utilization in the rumen may help explain how these populations respond to the increased competition for lactate. This study found no differences between treatments in the methanogen population. Methanogens are known to compete with sulfate-reducing bacteria for hydrogens. Future researchers may analyze this population to determine if this population is indeed not affected by dietary sulfur concentrations or if the small number of samples used in this study limited the ability to observe the differences. Furthermore, analysis of methane concentrations and methanogen populations in high and low S diets may be of interest to determine how S and SRB populations affect their populations and metabolism. Protozoa are known to generate molecular hydrogen in the rumen and are also decreased when cattle receiving a high concentrate diet. The interrelationship between protozoa, methanogens, and sulfate-reducing bacteria over time in the rumen of cattle consuming high S diets may help explain changes in H$_2$S concentrations as cattle transition from high forage to high concentrate finishing diets, as this is the time period where the greatest risk of S toxicity is observed.
## APPENDIX

### Supplemental Table

Regression Analysis of Forage Masses

<table>
<thead>
<tr>
<th>Day of experiment</th>
<th>Regression Equation</th>
<th>$R^2$</th>
<th>Experimental Units (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 12 pregrazing</td>
<td>$Y = 285.96x - 1118.7$</td>
<td>0.76</td>
<td>8</td>
</tr>
<tr>
<td>d 21 postgrazing</td>
<td>$Y = 295.18x - 86.84$</td>
<td>0.76</td>
<td>5</td>
</tr>
<tr>
<td>d 27 pregrazing</td>
<td>$Y = 193.69x + 234.33$</td>
<td>0.71</td>
<td>11</td>
</tr>
<tr>
<td>d 37 postgrazing</td>
<td>$Y = 255.33x + 658.11$</td>
<td>0.73</td>
<td>11</td>
</tr>
</tbody>
</table>