ABSTRACT

ARELARO ARTIOLI, LUIS FELIPE. Frequency of Energy Supplementation and Its Effects on Growth and Immunity of Recently Weaned Beef Calves.
(Under the direction of Philipe Moriel)

In the first study, the effects of frequency of energy supplementation on growth and measurements of innate and humoral immune responses of preconditioning beef steers following vaccination were evaluated. Angus steers (n = 24; 221 ± 6.3 kg; 177 ± 4 d of age) were weaned on d –7 and kept in a single drylot pen with free access to tall fescue hay and concentrate DMI at 0.5% of BW (50:50 mix of SH and CGF pellets; DM basis) from d –7 to 0. On d 0, steers were stratified by BW and age and randomly assigned to 1 of 8 feedlot pens (3 steers/pen). Treatments were randomly assigned to pens (4 pens/treatment) and consisted of steers provided daily free access to ground tall fescue hay and similar weekly concentrate DMI (1% of BW times 7 d), which was divided and offered either daily (S7) or 3 times weekly (S3; Monday, Wednesday, and Friday) from d 0 to 42. Steers were vaccinated against infectious bovine rhinotracheitis (IBR), bovine viral diarrhea virus (BVDV), *Mannheimia haemolytica,* and clostridium on d 7 and 21. Steers offered concentrate daily had greater ($P \leq$ 0.02) BW on d 42, overall ADG, and total DMI, but similar (P = 0.14) G:F, than S3 steers. On days that S7 and S3 steers were offered concentrate, total DMI was greater and hay DMI was less for S3 vs. S7 steers ($P \leq$ 0.05). On days that only S7 steers were supplemented, hay DMI was greater, but total DMI was less for S3 vs. S7 steers ($P \leq$ 0.05). Mean CP and NEg intake were greater ($P \leq$ 0.03) for S7 vs. S3 steers. Plasma cortisol concentrations on d 7 and 28, and mean plasma haptoglobin concentrations, but not liver mRNA expression of haptoglobin (P = 0.75), were greater for S3 vs. S7 steers ($P \leq$ 0.03). Plasma IGF-1 concentrations on d 0 and urea nitrogen on d 1 and 3, relative to vaccination, were greater for

S7 vs. S3 steers ($P \leq 0.008$). Positive seroconversion to BVDV-1b on d 42 and mean serum BVDV-1b titers were greater for S7 vs. S3 steers ($P \leq 0.05$). In summary, decreasing the frequency of concentrate supplementation from daily to three times weekly, during a 42-d preconditioning period, decreased growth performance, increased plasma concentrations of haptoglobin and cortisol, and decreased vaccine-induced antibody production against BVDV-1b of beef steers.

The second study aimed on decreasing frequency and rate of wet brewers grains (WBG) supplementation, as an alternative to allow decreased supplementation frequency during preconditioning, without having the negative effects aforementioned. At 14 d post-weaning (d 0), Angus heifers (n = 36; 213 ± 2 kg of BW; 254 ± 7 d of age) were stratified by BW and age, and randomly assigned to 1 of 12 drylot pens (3 heifers/pen). Treatments were randomly assigned to pens, in a 2 × 2 factorial design, and consisted of heifers provided ground tall fescue hay ad libitum (55% TDN, 12% CP of DM) and supplemented with WBG (75% TDN, 36% CP of DM) either daily (7X) or 3 times weekly (3X; Monday, Wednesday and Friday) at 0.5 or 1.0% of BW (DM basis) for 42 d. Heifers were vaccinated against IBR, BVDV, Mannheimia haemolytica and clostridium on d 14 and 28. Heifers fed WBG 3X weekly had less hay DMI (2.6 vs. 3.2 ± 0.16 kg/d; $P < 0.0001$), but greater total DMI (5.6 vs. 3.8 ± 0.16 kg/d; $P < 0.0001$) than 7X heifers on days that all heifers received WBG supplementation. However, overall hay and total DMI was not affected ($P \geq 0.40$) by supplementation frequency. Thus, ADG, BW and G:F from d 0 to 42 did not differ among treatments ($P \geq 0.29$). Plasma concentrations of haptoglobin on d 15 and cortisol on d 14 were greater for 3X vs. 7X heifers ($P \leq 0.04$), respectively. Heifers fed WBG at 0.5% of BW tended to have greater plasma cortisol concentrations on d 15, 17 and 35 ($P \leq 0.09$) than

heifers fed at 1.0% of BW. Serum BVDV-1a titers were greater (P = 0.04) for 7X vs. 3X heifers on d 42 (4.2 vs. 3.3 ± 0.28 log2), whereas serum titers against BVDV-2 and IBR were greater for heifers fed WBG at 1.0 vs. 0.5% of BW (7.6 vs. 6.7 and 3.3 vs. 2.8 ± 0.19 log2, respectively). In summary, decreasing WBG supplementation frequency (7 vs. 3 times weekly) or rate (1.0 vs. 0.5% of BW) for recently weaned beef heifers did not affect growth, but decreased vaccine-induced antibody production against pathogens associated with bovine respiratory disease, during a 42-d preconditioning period.

Frequency of energy supplementation and its effects on growth and immunity of recently weaned beef calves.

by
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DEDICATION

This thesis is dedicated to my family, teachers and friends. Most especially, though, Cenise Arelaro, the best friend, teacher and mother I could have asked for.

BIOGRAPHY

Luis Felipe Arelaro Artioli was born in Atibaia, Sao Paulo, Brazil, and lived in Socorro, Sao Paulo, Brazil for eighteen years before moving to Botucatu, Sao Paulo, Brazil to pursue his B.S degree in Animal Science at Sao Paulo State University (UNESP). In 2014, he had the opportunity of an internship for five months at the Mountain Research Station, Waynesville, NC as a North Carolina State University Livestock Specialist Assistant under Dr. Philipe Moriel guidance. Luis received his B.S in Animal Science in 2014 and was offered the opportunity to continue working with Dr. Moriel and obtain a M.S degree in Animal Science at North Carolina State University. As a master's student at NC State, Luis continued working with research and extension with Dr. Moriel. The research topic of interest was in beef cattle nutrition, with focus on methods of storage of wet brewers grains, supplementation of the dams and its effects in the offspring growth performance and immune system, and the principal one, the effects on growth performance and immune system of different supplementation strategies to beef cattle during stressful events, such as weaning, preconditioning and vaccination periods. Oral and poster presentations about Dr. Moriel's group research results were accomplished in five conferences and several smaller meetings. For the extension role, Luis helped on the organization of two workshops, four field days, and the Mountain Area Conference; Luis attended more than ten other conferences, workshops or field days. He also helped Dr. Moriel in other extension related fields, as the "regional livestock specialist assistant".

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CHAPTER 1

Literature Review: Frequency of concentrate supplementation for beef cattle

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Introduction

Economical losses associated with bovine respiratory disease (BRD), such as antibiotic treatment, mortality and decreased feed efficiency have reached between $800 to 900 million dollars in 2001 (Chirase and Greene, 2001). For instance, feedlot cattle that received 1, 2 or 3 treatments for BRD returned $40.64, 58.35 and 291.93 less, respectively, than untreated animals (Fulton et al. 2002). A great portion (79%) of this loss on return was associated with decreased carcass weight and quality grade (Gardner et al., 1999). Incidence of BRD has increased despite advances in vaccines and antibiotics available for prevention and treatment of the disease (Loneragan et al., 2001; Babcock et al., 2009; Fulton, 2009; Holland et al., 2010). It is estimated that about 15% of calves placed in the feedlot will develop BRD (USDA-APHIS, 2001) with the majority of BRD cases in beef cattle occurring within 30 d post-weaning or 14 d after feedlot entry (Kirkpatrick et al., 2008, Artioli et al., 2015). Preconditioning (PC) is one alternative calf management procedure that might reduce the incidence of BRD. Some PC protocols involve feeding high concentrate-based diets for 45 d or longer (Dhuyvetter, 2003), in order to acclimate calves to people and bunk feeding and to improve their growth and immunity during the feedlot receiving period. However, labor is one of the obstacles for producers not adopting a PC program. Hence, it was hypothesized that decreasing the frequency of concentrate supplementation would be one alternative to reduce costs associated with labor and feeding, and increase the PC implementation by beef cattle producers. There are not known published studies evaluating the impact of reducing the frequency of concentrate supplementation on calf growth performance and immune response. Therefore, the following research studies evaluated the

effects of decreasing the frequency of energy supplementation from daily to three times weekly, during a 42-d PC period, on growth performance and post-vaccination measurements of innate and humoral immune response of beef calves (Chapter 2), and the effects of decreasing frequency and rate of energy supplementation from daily to three times weekly, during a 42-d PC period, on growth performance and post-vaccination measurements of innate and humoral immune response of beef calves (Chapter 3).

Preconditioning

The concept of preconditioning calves was conceived in the mid-1960s at Iowa State (Patrick, 1967). Currently, there is no standardized definition or protocol for PC calves. However, calf PC generally refers to pre- or post-weaning management practices that are intended to optimize the animal's immune system, reduce the stress of weaning and the incidence of respiratory diseases (Lalman and Mourer, 2014), and prepare calves to enter a backgrounding program or to go directly into a feedlot system (Dhuyvetter et al., 2005). The benefits of PC include less morbidity and mortality, improved post-weaning growth performance (Lalman and Mourer, 2014), and greater carcass quality compared to non-preconditioned calves (Thrift and Thrift, 2011). However, the risks and costs associated with castrating, feeding, deworming and vaccinating calves around weaning time has slowed the adoption of PC as an operational practice by beef cattle producers.

Preconditioning has been shown to be beneficial for cow-calf farmers that adopt the program through selling a heavier calf, premium calf prices and calf reputation at sale. Based on Superior Livestock Auction data from 2014, PC calves (VAC 45 preconditioning

program, Merck® Animal Health) received a premium of approximately $12.06 cwt. ($0.27/kg) relative to non-preconditioned calves. For stocker/feedlot buyers, PC is beneficial through decreased mortality and morbidity rates, less expenses with BRD treatment and greater calf performance, carcass weight and quality grade. Returns associated with preconditioned calves in the feedlot are in the $40 to $60 range per hd ($0.16 to $0.24/kg; Dhuyvetter et al., 2005).

Roeber et al. (2001) compared carcass quality and number of hospital visits for feedlot cattle that was preconditioned or not, prior to sale. Preconditioned calves originated from two different PC programs: 1) Certified: Preconditioned for Health (CPH) and 2) Kentucky Cattlemen's Association (KCA) Gold Tag. A third group consisted of calves purchased from auction markets (AM) of unknown treatment and herd health processing history. Average hospital visits were greater for AM than for CPH and KCA (1.97, 0.55 and 0.70, respectively). Morbidity and mortality rates of beef calves that required at least one hospital visit were greater for AM (77.3 and 11.4%, respectively) compared to CPH (34.7 and 1.1%, respectively) and KCA calves (36.7 and 1.1%, respectively). Hot carcass weights were lower for cattle treated twice or more (344.6 kg) and greater for cattle treated only once or not treated (357.4 kg and 353.8 kg, respectively). In summary, non-preconditioned beef calves had greater morbidity rates, which increased costs associated with medical treatment and mortality rates, resulting in greater economic losses than preconditioned beef calves.

Supplementation of forage based diets

Forage for pasture is the cheapest and most available feed source for beef cattle. Tall fescue (*Lolium arundinaceum*) is the dominant forage found in production systems stretching from central Oklahoma to central North Carolina, and from northern Alabama to Kentucky, covering more than 15 million hectares (Bouton and Hopkins, 2003). Growing cattle require a high plane of nutrition to maintain adequate performance (NRC, 1996). The requirements of a 500lb growing steer gaining 2lb of BW daily are 69 and 12.8% for TDN and CP, respectively (DM basis; NRC, 1996). Depending on the season, tall fescue nutritional composition ranges from 49 to 80% TDN, and 9 to 20% CP of DM (Burns and Chamblee, 1979, 2000b; Poore et al., 2006). Therefore, supplementation of energy is necessary since tall fescue may not meet the energy requirements of growing steers during most part of the year. Supplementation programs need to be designed according to: (1) the nutritional requirements of the animal to be supplemented; (2) inadequacies of the forage consumed; and (3) economic viability (DelCurto et al., 2000; Kunkle et al., 2000).

Associative effects are the interaction between nutrients in different feed sources in a diet. It can result in greater (positive associative effects) or lower (negative associative effects) growth performance than expected from the individual ingredient. These associative effects often occur when both grain and forage are included in the diet, due primarily to changes in intake and/or digestibility of the fibrous components of forage (Dixon and Stockdale, 1999). Negative associative effect is related to a reduction in ruminal pH (Hoover, 1986) and activity of celluloytic enzymes (Martin et. al., 2001), decreased bacterial attachment to fibrous material (Hiltner and Dehority, 1983), and an increase in lag time for digestion (Mertens and Loften, 1980). Supplements often decrease forage DMI when

TDN:CP ratio is < 7, supplemental TDN greater than 0.7% of BW, or when forage intake when fed alone is greater than 1.75% of BW (Moore et al., 1999). Nevertheless, concentrate supplementation-induced depression in forage intake increases as forage quality increases (Horn and McCollum, 1987) and as supplement intake increases (Moore et al., 1999), particularly if the supplement source is high in non-structured carbohydrates (NSC).

Fibrolytic bacteria activity is diminished at pH < 6 because of the need to increase their energy requirement for maintenance. These microbes have to utilize more energy to maintain intercellular pH, which results in slower microbial population growth and can eventually lead to the wash out of specific microbial populations from the rumen (Strobel and Russell, 1986; Russell and Wilson, 1996). Decreasing the lag time for microbial colonization of fiber by supplementing soluble carbohydrates can increase forage digestion and intake (Hiltner and Dehority, 1983). The supply of nutrients that are deficient, such as protein, can also increase intake (Köster el al., 1996; Kunkle et al., 2000) and digestibility (Moore et al., 1999) of low-quality forages, but does not result in adequate energy intake by the animal (Bowman and Sanson, 1996). Therefore, supplementation programs providing energy-based supplements with adequate levels of protein may be a good alternative for cow-calf operations when lack of energy intake is observed.

Energy supplementation

As aforementioned, growing beef calves often require supplementation for maximum BW gains while grazing (Moore et al., 1999) and are supplemented daily at 1% of BW (as-fed basis) or greater (Drewnoski and Poore, 2012). An approach often followed to

supplement forage-fed cattle is to provide sufficient protein, minerals and vitamins to balance deficiencies in the forage, and then provide supplemental energy if it will provide a return over cost (Kunkle et al., 2000). Moore et al. (1999) stated that supplementation with rumen degradable protein (RDP) resulted in positive associative effects when the forage TDN:CP ratio was >7:1, whereas negative associative effects would be expected from RDP supplementation when the ratio was <7:1 in the forage. Cool season grasses, including tall fescue, generally have a TDN:CP <7, and therefore, energy supplementation should be more efficacious than protein supplementation on PC programs for animals fed this type of forage.

Energy supplements containing NSC, which make up a large portion (≥70%) of grains, can depress forage intake and digestibility (Sanson et al., 1990; Olson et al., 1999), because of the negative associative effect previously cited. Chase and Hibberd (1987) fed mature beef cows with low-quality native grass hay (4.3% CP, 72.1% NDF and 52.5 % ADF; DM basis) and offered iso-nitrogenous supplements containing 0, 1, 2 or 3 kg/d of ground corn (as-fed basis). Daily hay DMI decreased linearly as supplemental corn increased (8.8, 8.2, 6.4 and 5.1 kg of hay DMI for 0, 1, 2 and 3kg of supplemental corn, respectively). Ruminal NDF disappearance rate (3.9, 3.3, 2.1 and 1.4%/h for 0, 1, 2 and 3kg of supplemental corn, respectively) and ruminal ammonia concentrations (2.20, 1.12, 0.88 and 0.61 mg/d for 0, 1, 2 and 3kg of supplemental corn, respectively) also decreased linearly as amount of supplemental corn increased, and may have limited microbial growth and forage intake. Beck et al. (2006) reviewed that by feeding a large amount of corn based supplements to beef calves grazing on a high-quality tall fescue pasture, the asynchrony of available energy and nitrogen in the rumen can lead to lower microbial growth and digestion. If

TDN:CP ratio exceeds 3:1 large losses of nitrogen occur, and the excess that is not utilized by ruminal microbes is excreted in the urine. For instance, Beever (1984) reported that some N in high-quality forages, such as tall fescue, is so rapidly degraded in the rumen that it is not incorporated into microbial protein and is lost as ammonia-N. Different types and timing of starch-based supplements produced variable results, most of the times impairing forage intake and digestibility. An alternative to high NSC-based supplements is the use of fibrous by-products, such as dried distillers grains (DDG), corn gluten feed (CGF) and soybean hulls (SH), which have less negative effect on ruminal pH (Klopfenstein and Owen, 1987) and depression on forage intake or digestibility (Bowman et al., 2004) compared to NSC-based supplements.

According to Bowman and Sanson (1996), supplementation of growing cattle with fibrous by-products, such as CGF and SH, at rates greater than 0.5% of BW daily (as-fed basis), lessened the negative impact on forage intake and digestibility compared to grain-based supplements. Gadberry et al. (2009, 2010) reported that fibrous by-products supplements, such as cottonseed cake or DDG, increased ADG of growing steers grazing bermuda grass by over 0.5 lb/d compared to non-supplemented steers. The TDN:CP ratio on this bermuda grass ranged between 4:1 and 5:1, indicating that forage energy content, not CP, is the limiting factor for growth (Moore et al., 1999; Beck et al., 2013, 2014). Therefore, the increased performance reported in this study can be related to the addition of energy in the diet. Performance of calves grazing tall fescue may be limited by unbalanced TDN:CP ratios of 2.5 to 5.2 during the growing season (Moore et al., 1999), which indicates a deficiency in ruminal energy availability for growth.

In summary, energy-based supplements containing adequate amounts of protein have been shown to improve cattle performance, principally when grazing low-quality forages (Kunkle et al., 2000; Bodine and Purvis, 2003). Energy supplements sources containing high NCS should not be provided in high amounts or less frequent than daily, due to the negative associative effects and lower ruminal pH, which decreases growth performance of cattle. Supplementation of low NSC-based fibrous by-products is an alternative to maximize growth performance of forage-fed beef cattle.

Frequency of supplementation

Up to 63% of annual production costs in beef cow/calf operations are associated with cattle feeding, including forage production and feed purchase (Miller et al., 2001). Expenses indirectly associated with feeding management, such as equipment, fuel and labor can also increase costs. Decreasing the frequency of supplementation from daily to 3 times weekly, for example, can help to reduce these costs by more than half. When supplementation frequency is reduced, the amount of supplement fed within each wk remains the same, but the amount of concentrate fed during each supplementation event is increased compared to daily supplementation (Drewnoski et al., 2014). Several studies evaluated the effects on growth performance of beef cattle fed forage-based diets and provided protein- and energy-based supplements less frequently, however results have been variable.

Frequency of protein supplementation. It has been shown that protein-based supplementation of beef cattle as infrequently as once weekly did not affect growth performance, forage intake and digestibility compared to daily protein supplementation

(Wettemann and Lusby, 1994; Huston et al., 1999; Kunkle et al., 2000, Farmer et al., 2001; Bohnert et al., 2002b; Schauer et al., 2005). This similar performance between daily or less frequent protein supplementation can be explained by the recycling of urea in the rumen, which provides N for ruminal microbes on days when protein is not supplemented and dietary protein concentration is low (Krehbiel et al., 1998; Bohnert et al., 2002a; Archibeque et al., 2007). In conclusion, decreasing the frequency of protein supplementation is a management alternative that can lower labor, equipment and fuel costs associated with supplementation, and can be implemented to beef cattle with no negative effects on growth performance.

Frequency of energy supplementation. Contrary to frequency of protein supplementation, decreasing the frequency of energy supplementation can be detrimental to beef cattle performance depending on forage quality and type of energy supplement provided. For cattle consuming low-quality forages, decreasing the frequency of energy supplementation (low- or high-NSC supplements) had negative effects on forage intake and growth performance (Kunkle et al., 2000; Cooke et al., 2007a, 2008). As mentioned previously, this negative impact on forage intake is associated with a reduction in ruminal pH (Hoover, 1986) and activity of cellulotic enzymes (Martin et. al., 2001), decreased bacterial attachment to fibrous material (Hiltner and Dehority, 1983) and an increase in lag time for digestion (Mertens and Loften, 1980).

Kartchner and Adams (1982) provided cracked corn supplementation at 0.3% of BW daily (1.5 kg of corn/d; as-fed basis) or 0.6% of BW on alternate days (3 kg of corn/d; as-fed

basis) to pregnant cows grazing on winter range pastures. Cows supplemented daily had a 2-fold greater BW gain than cows supplemented every other day (64 and 31 kg of BW gain, respectively) during a 70 d winter feeding period. Ruminal pH of fistulated steers that grazed with the cows was lower for alternate days supplementation compared to daily supplementation. The authors suggested that improved forage digestion may have been responsible for the greater performance in cows supplemented daily.

Chase and Hibberd (1989) studied the effects of level of corn and frequency of supplementation on the digestion and intake of low-quality hay (5.0% CP, 68.5% NDF and 46.2% ADF; DM basis) of beef cows. The corn-based supplements were offered daily or every other day, at weekly rates of 9.8 or 14 kg, respectively (as-fed basis). Frequency of supplementation did not affect daily hay OM intake (8.55 and 8.48 kg/d, respectively), but numerically reduced hay OM digestibility (48.1 and 46.6%, respectively) and total OM digestibility (54.6 and 53.2%, respectively) for daily or alternate days supplementation. The authors concluded that cattle supplemented daily utilized their diet more efficiently than cattle supplemented every other day.

Beaty et al. (1994) provided concentrate supplementation daily or 3 times weekly at the same weekly amount (concentrate DMI = 0.4%BW/d; 14.0 kg/wk) to fistulated beef steers (initial BW = 456 kg) consuming wheat straw. Concentrate consisted in four different supplements with varied CP concentrations (10, 20, 30 and 40% CP; DM basis) and ratio of soybean meal and sorghum grain in supplements. For each treatment (daily or 3 times weekly) steers were subdivided in groups to receive one of the four different CP level

supplements. Concentrate supplementation 3 times weekly reduced straw intake compared to daily concentrate supplementation (1.18 and 1.42% of BW, respectively), but improved total DM (54.2 and 49.6%, respectively) and NDF (54.4 and 51.1%, respectively) digestibility. This increase in total DM and NDF digestibility was, at least partially, due to the supplement being a higher proportion of the diet for 3 times weekly on the days both treatments were supplemented. Also, the lower ratio of straw:concentrate intake by animals supplemented 3 times weekly may have increased their total DM and NDF digestibility compared to animals supplemented concentrate daily. Increasing CP concentrations in the supplement increased straw DMI (1.20, 1.30, 1.38 and 1.32%), total DMI (1.63, 1.73, 1.82 and 1.76%), total DM digestibility (48.7, 50.3, 54.1 and 54.5%) and NDF digestibility (50.7, 52.9, 54.0 and 53.4%) for 10, 20, 30 and 40% of CP, respectively.

In a second study, Beaty et al. (1994) provided similar supplementation program as described above to beef cows (initial BW = 475 kg and BCS = 5.2) grazing dormant tallgrass prairie for a 105 d period. Cows supplemented daily instead of 3 times weekly lost less BCS (1.02 and 1.17, respectively) and BW (75.3 and 87.6 kg, respectively) during calving period. No differences on pregnancy rates (95.2% for both treatments) or carryover effect on calf ADG (0.93kg for both treatments) were observed among supplementation frequencies.

In a third experiment, Beaty et al. (1994) provided a grain based supplement for 111 d to beef cows (initial BW = 504 kg) on native range. The supplement was formulated in an as-fed basis (21% CP) containing 74% grain (either corn or sorghum), 23% soybean meal and 3% molasses at 0.48% of BW/d (14.8 kg/wk; DM basis) and supplemented daily or 3 times

weekly. Cows supplemented daily lost less BW during calving period compared to cows supplemented 3 times weekly (79.2 vs. 88.7 kg, respectively), in agreement with the experiment aforementioned.

La Manna (2002) provided supplementation of cracked corn to steers consuming chopped alfalfa hay (21% CP, 36% ADF of DM) daily (0.5% BW; as-fed basis), on alternate days (1% BW; as-fed basis) or every 3 d (1.5% BW; as-fed basis). Rumen pH was reduced as frequency of supplementation decreased (6.2, 6.0 and 5.7, respectively). Decreasing the frequency of supplementation reduced hay intake (11.5, 10.1, and 9.5 kg/d, respectively), but increased ADF and NDF digestibility of the total diet. It can be associated to decreased passage rate or corn making up a higher percentage of the diet. Average daily gain was lower for steers supplemented every 3 d (0.62 kg/d), but did not differ between the daily and alternate day supplementation (0.77 and 0.75 kg/d, respectively).

Loy et al. (2007) provided, to heifers fed forage-based diets (chopped grass hay; 8.2% CP; *ad libtum*), DDG-based supplements daily or every other day, at a weekly amount of 10.5 kg of DM (approximately 0.4% of BW daily or 0.8% of BW on alternate days). A pH collection was made from d9 to 14 of the study to represent supplementation (d 9, 11, 13) and non-supplementation (d 10, 12, 14) days for heifers in alternate-day treatments. No difference was observed on average ruminal pH (6.12 vs. 6.17, respectively), forage intake (1.69 vs. 1.66% of BW, respectively), and *in situ* rate of NDF disappearance (4.09 vs. 4.01% per h, respectively) between daily or alternate days supplementation.

Cooke et al. (2007b) studied the effect of decreasing supplementation frequency from daily (7x) or 3 times weekly (3x) providing a supplement (1% BW daily; DM basis) based on 75% citrus pulp and 25% cottonseed meal (87% DM; 19% CP and 70% TDN of DM) to growing beef steers being fed low quality warm season grass hay (9.1% CP and 54% TDN of DM). Steers supplemented daily tended to have greater BW gain than steers supplemented 3 times weekly (0.30 vs 0.18 kg/d, respectively).

In a second study, Cooke et al. (2008) utilized the same supplementation frequency treatments (daily or 3 times weekly) using wheat middling-based supplements in addition to SH, molasses and cottonseed meal (66.7% WM, 26.9% SH, 3.8% molasses and 2.7% cottonseed meal; DM basis) to growing heifers grazing warm season (bahiagrass, *Paspalum notatum*) pastures. Average daily gain decreased by less frequent supplementation (0.41 vs 0.33 kg for daily and 3 times weekly, respectively). Effect of frequency of supplementation on forage intake was not measured. Attainment of puberty and pregnancy were hastened for daily supplemented heifers. Approximately 48% of the heifers supplemented 3 times weekly were not pregnant at the end of the breeding season compared to only 36% of non-pregnant heifers for daily supplementation. The author stated that reproductive development and performance of replacement beef heifers are enhanced when low-starch energy supplements are offered daily instead of 3 times weekly.

Moriel et al. (2012) evaluated the effects of decreasing frequency of energy supplementation from daily (S7) to three times a week (S3) on performance and reproductive aspects of beef heifers fed low or medium quality hay (Stargrass: 8.3% CP; 50.5% TDN; and

Bermudagrass: 12.7% CP; 52% TDN; *ad-libtum;* respectively). Supplements consisted of 49.0% SH, 30.3% wheat middlings, 12.2% DDG, 4.50% molasses, 0.800% calcium carbonate and 3.20% canola pellets (DM basis) and was offered at weekly rates of 15.8kg of DM/heifer. Average daily gain did not differ between S7 and S3 heifers (0.27 and 0.25 kg, respectively), whereas overall hay DMI was decreased with less frequent supplementation (3.37 and 2.85 kg/d for S7 and S3 heifers, respectively), but did not differ between treatments on days that only S7 heifers received supplementation (S7 only; Tuesdays, Thurdays, Saturdays and Sundays; 3.38 and 3.15 kg for S7 and S3 heifers, respectively). Hay DMI was lower for S3 heifers when both treatments were supplemented (SUPALL; Mondays, Wednesdays and Fridays; 3.36 and 2.55 kg/d for S7 and S3 heifers, respectively). Attainment of puberty and pregnancy were delayed by decreasing the frequency of energy supplementation, agreeing with Cooke et al. (2008) results. At the end of the breeding season, approximately 38% of S7 heifers were pubertal, whereas only 17% of S3 heifers were pubertal. Final pregnancy rates, based on transrectal ultrasonography, did not differ between treatments (approximately 16.6% of pregnant heifers for both treatments); however, S7 heifers became pregnant earlier in the breeding season, whereas S3 heifers became pregnant later in the breeding season. Similar results were observed between hay quality (low vs. medium), indicating that daily supplementation of low-starch supplements also prevented daily oscillations in total DMI in heifers receiving low- or medium-quality hay.

Drewnoski et al. (2011) examined the effect of frequency of supplementation of a SH and CGF blend on hay intake and performance of growing steers (approximately 260 kg of BW) for 4 yr. Supplements consisted of 47% CGF, 47% SH, 4% molasses and 2% limestone

(as-fed basis). For the first 2 yr, treatments consisted of steers consuming *ad libitum* medium quality fescue hay (7-10% CP, 68% NDF and 40% ADF of DM) that was either not supplemented (HAY), supplemented daily (7X; 1% of BW; as-fed basis), or supplemented on Monday, Wednesday and Friday (3X; 2% of BW; as-fed basis). For yr 3 and 4, an additional treatment (2X) was added in which steers were supplemented twice a wk (on Mondays and Thursdays; 3% of BW; as-fed basis). Supplemented steers (7X and 3X) had greater overall (yr 1 - 4) ADG than non-supplemented steers (HAY) due to supplementation effect but no differences on ADG were observed within different supplementation frequencies (0.79, 0.76 and 0.24 kg/d for 7X, 3X and HAY, respectively). For yr 3 and 4, 7X and 3X steers had greater ADG than 2X and HAY steers (0.89, 0.87, 0.24 and 0.24 kg/hd daily, respectively). Overall hay DMI was reduced by supplementation, and further reduced by less frequent supplementation (5.4, 4.6 and 6.0 kg/hd daily for 7X, 3X and HAY steers, respectively). For yr 3 and 4, HAY and 2X steers had the greater hay DMI, followed by 7X steers; 3X steers had the least hay DMI (5.0, 4.4, 5.8 and 5.8 kg/hd daily for 7X, 3X, 2X and HAY steers, respectively). Overall feed efficiency was increased by supplementation, and was further increased by less frequent supplementation (0.098, 0.106 and 0.040 G:F for 7X, 3X and HAY steers, respectively). For yr 3 and 4, 3X steers tended to have greater feed efficiency than 7X steers; 2X and HAY steers had the least feed efficiency (0.115, 0.123, 0.054 and 0.054 for 7X, 3X, 2X and HAY steers, respectively). The authors reported that, when providing medium quality hay and supplementation of SH and CGF blend, steers can be supplemented as little as twice a wk without reducing performance.

Drewnoski and Poore (2012) evaluated the effects on ruminal fermentation and digestion of ruminally cannulated beef steers (approximately 362 kg of BW) fed medium quality fescue hay (9% CP, 67.1% NDF and 34.8% ADF) and receiving no supplementation (NS), or being supplemented with CGF and SH blend (90% DM, 14.6% CP) daily (SD; 1% BW; as-fed basis) or every other day (SA; 2% BW; as-fed basis). Hay DMI was reduced for SD and further reduced for SA compared to NS steers (4.52, 3.88 and 5.92 kg/hd daily, respectively). On supplementation day, mean ruminal pH for SA steers (6.14) was lower than those for both SD (6.30) and NS (6.54) steers, due to greater amount of supplement received on that day. On the day that only SD received supplement, ruminal pH of SD steers (6.30) was lower than SA (6.54) or NS (6.52) steers. Diet DM digestibility was increased by supplementation, however no differences were observed between supplementation frequencies (64.1, 64.6 and 57.9% for SD, SA and NS steers, respectively). Retention of N was also increased with supplementation, but not affected by frequency of supplementation (5.6, 5.5 and 3.3 g/d for SD, SA and NS steers, respectively). The authors stated that decreasing frequency of supplementation of a mix of CGF and SH did not impaired DM digestibility or N retention of growing beef steers.

In a third experiment of the same research group (Drewnoski et al. 2014), beef steers (approximately 287kg of BW) fed *ad libtum* medium quality hay (10% CP, 57% TDN, 66% NDF and 36% ADF) and receiving no supplementation (NS) or supplemented with a mix of CGF and SH (89.6% DM, 17.2% CP and 70% TDN of DM) daily (SD; 1% of BW; as-fed basis) or on alternate days (SA; 2% of BW; as-fed basis). Mean hay DMI of NS was greater than SD and SA (6.1, 4.5 and 4.3 kg/hd daily, respectively), but did not differ between

supplementation frequencies. Mean hay DMI of SA (approximately 1.2% of BW) on the day both groups were supplemented was lower than SD (approximately 1.5 % of BW), and NS mean hay DMI (approximately 2.1% of BW) was greater than both supplemented treatments. Hay DMI of SA (approximately 1.8% of BW) on non-supplemented days was greater than SD (approximately 1.5% of BW) and both were lower than NS (approximately 2.2% of BW). Average daily gain (0.90, 0.87 and 0.45 kg/hd daily for SD, SA and NS, respectively) and feed efficiency (0.123, 0.122 and 0.070 G:F for SD, SA and NS, respectively) was increased by supplementation but did not differ between supplementation frequencies.

Physiological effects of reduced supplementation frequency. Glucose is essential for ruminants and non-ruminants energy metabolism and consists in a small (6 carbons), polar, water-soluble monosaccharide. Glucose metabolic fates include generation of ATP via glycolysis and TCA cycle, and generation of NADPH through the hexose monophosphate shunt (Huntington, 1997). Blood glucose concentrations in beef cattle are positively related with feed intake and BW gain (Vizcarra et al., 1998; Hersom et al., 2004).

Insulin is a small peptide hormone synthetized by secretory granules in pancreatic beta-cells that primarily regulates glucose uptake by cells. In bovine insulin contains 51 amino acid residues and a molecular weight of 5.7 kDa (Smith, 1966). Several stimuli induce the conversion of proinsulin into active insulin, consequently releasing insulin into the bloodstream (Nelson and Cox, 2005). In addition to glucose uptake by cells, insulin also has an important effect on lipogenesis and cellular uptake of amino acids and some electrolytes (Austgen et al., 2003). Insulin is transported into the cell by GLUT4 transporter, inducing

glucose uptake by cells; after entering the cells, glucose enters glycolysis and the respiratory cycle. Insulin main effects are increasing production of ATP by cells, synthesis of glycogen in liver and muscle and lipogenesis in adipose, increasing anabolism and decreasing catabolism in the body. Even though the rate of insulin secretion in the pancreas is considered low, the amount secreted into the blood is extremely dependent on blood glucose concentrations, and as it increases blood insulin increases and vice-versa. Hence, insulin maintains body homeostasis due to the maintenance of blood glucose under constant concentrations. In addition, insulin secretion is also stimulated by gastrointestinal hormones and neural/paracrine mechanisms associated with feed intake (Nussey and Whitehead, 2001).

Insulin growth factor 1is a single chain polypeptide hormone molecule containing 70 amino acid residues with a molecular weight of approximately 7.6 kDa (McGuire et al., 1992; Etherton, 2004) and is primarily synthesized in the liver in response to growth hormone (GH; McGuire et al., 1992), resembling insulin on its structure (Gluckman et al., 1987) being this tissue responsible for the main source of circulating IGF-1 in the body (D'Ercole et al., 1984), however it can be synthesized by most, if not all, body tissues (Le Roith et al., 2001). Insulin growth factor 1 is essential for carbohydrates and protein metabolism and has an important role in cell growth and division due to its anabolic effects (Quin, 1992; Jones and Clemmons, 1995; Le Roith et al., 2001) and its secretion into the circulation occurs in a constant pattern (Thissen et al., 1994). Like insulin, IGF-1 has been shown to increase protein synthesis in skeletal muscle and reduce the rate of protein degradation (Florini et al., 1996). Six different types of insulin-like growth factor binding proteins (IGFBPs) that carries IGF-1 can be found in the body, being IGFBP-3 considered

the main one, since it is responsible for more than 90% of the IGF-I transported (Martin and Baxter, 1992). These IGFBPs are much larger in size than IGF-1 and besides transporting, can also retard IGF-I degradation and modulate the actions of IGF-I in target cells by increasing or inhibiting IGF-I activity (LeRoith et al., 2001). In addition to GH, IGF-I concentrations in blood were positively correlated to feed intake and BW (Bossis et al., 1999, 2000; Armstrong et al., 2001; Rausch et al., 2002), thyroid hormones (Burnstein et al., 1979) and plasma insulin concentration (Keisler and Lucy, 1996; Webb et al., 2004; Cooke et al., 2007a).

Cooke et al. (2007b) observed that mean plasma glucose and insulin concentrations were enhanced for steers fed low quality warm season grass hay and provided citrus pulp-based supplement 3 times weekly compared to steers supplemented every day (76.2 vs. 66.0 mg/dL of glucose and 0.60 vs. 0.46 ng/mL of insulin, respectively). However, no differences among supplementation frequency were found in mean plasma IGF-1 concentration (102.7 vs. 96.5 ng/mL for steers supplemented 3 times weekly or daily, respectively).

Moriel et al. (2012) provided low-starch energy supplements (49.0% SH, 30.3% wheat middlings, 12.2% DDG, 4.50% molasses, 0.800% calcium carbonate and 3.20% canola pellets) every day or 3 times weekly for heifers consuming low- or medium-quality forage-based diets. Heifers supplemented every day had less daily variation in hay DMI and subsequent plasma concentrations of glucose and IGF-I than heifers supplemented 3 times weekly. This lower variation in glucose and IGF-1 concentrations for heifers supplemented

daily also collaborated for the improved puberty achievement compared to heifers supplemented 3 times weekly.

Drewnoski et al. (2014) provided medium-quality hay and no supplementation (NS) or supplementation of CGF and SH blend daily (SD; 1% of BW; as-fed basis) or on alternate days (SA; 2% of BW; as-fed basis) to beef steers. Glucose and insulin data was reported as area under the concentration-time curves. It was reported that plasma insulin concentrations of beef steers were increased by less supplementation frequency on the days all received supplement (406 vs. 525 μIU/mL for SD and SA steers, respectively), but decreased on the days that only daily supplemented received supplementation (393 vs. 304 μIU/mL for SD and SA steers, respectively). However, no differences were found in plasma insulin concentrations of the two-day feeding cycle (800 vs. 829 μIU/mL for SD and SA steers, respectively). Plasma IGF-1 concentration was increased by less supplementation frequency (approximately 340 vs. 430 ng/mL for SD and SA steers, respectively). No differences by frequency of supplementation were found in plasma glucose concentrations for the two-day feeding cycle (4052 vs. 4127 mg/dL for SD and SA steers, respectively), concluding that performance of steers supplemented every other day with a SH and CGF blend did not differ from those supplemented daily.

In summary, decreasing the frequency of energy supplementation was detrimental to puberty achievement of beef heifers, but its effects on animal growth performance response have been varied and may depend on many factors, including forage quality and supplement

type. However, other studies investigating the impact of decreased energy supplementation frequency on immunity of beef cattle are unknown.

Immune System

The principal function of the immune system is to protect the organism from infection in order to maintain it in homeostasis. The immune system has evolved to protect the organism from pathogens and generates a variety of cells and molecules capable of specifically recognizing and eliminating foreign invaders and cancerous cells, all of which act together in a dynamic network (Kindt, 2007). In general, the immune system can be separated into three broad components: natural immunity, innate immunity, and adaptive or acquired immunity, all of which must be developed fully and functioning properly to provide adequate immunologic protection (Carrol and Forsberg, 2007).

Innate immune system. The innate immune system is considered the first line of defense against any pathogen in the body (Lippolis, 2008), independently of the class (bacteria, protozoa, virus, or fungi) and is less specific than the adaptive. The innate immunity includes physical (skin, tears and mucosal secretion), chemical (antimicrobial peptides, superoxide anion and nitric oxide), the complement system (Carroll and Forsberg, 2007) and cellular barriers. The innate immune system provides enough time for the adaptive system to develop a strong and effective response against a specific pathogen. The key cells of the innate immune system include phagocytic cells, such as neutrophils, monocytes, macrophages, and dendritic cells; include natural killer (NK) cells and cells that release inflammatory mediators, such as mast cells, basophils, and eosinophils (Kindt, 2007; Carroll

and Forsberg, 2007). Different than adaptive immunity, the innate immunity does not recognize every possible antigen infecting the organism and does not generate a long-term protective immunological memory (Murphy et al., 2008). Instead, the innate immune system recognizes highly conserved structures present in many different components of bacterial cell walls, such as lipopeptides and lipopolysaccharides (LPS; Parker et al., 2007; Lippolis, 2008), known as pathogen-associated molecular patterns (PAMPs) through the action of the toll-like receptors (TLR), which interacts with these PAMPs. The TLR are a family of cell receptors, located inside or on the surface of phagocytic cells, which bind to various molecules specific to pathogens, and act as some of the earliest surveillance mechanisms against infections. The TLR acts, upon stimulation, to encourage the cell to respond to infection and facilitates cellular responses via signaling pathways. Regulatory proteins called cytokines mediates the interactions among these cells.

Cytokines have multiple sources, targets and functions and have been found in several animal species including mammals, birds, fish, reptiles and starfish (Petersen et al., 2004). Cytokine response starts in neutrophils and macrophages, by activation of the transcription factor nuclear factor kappa beta (NFκB). The activation of the NFκB induces the gene expression of several important mediators of innate immune system, such as cytokines, chemokines, and co-stimulatory molecules (Murphy et al., 2008). Cytokines are chemical messengers that can regulate and modulate a variety of cell functions and physiological processes, including the inflammatory response (Kindt, 2007; Parker et al., 2007; Carroll and Forsberg, 2007; Lippolis, 2008). Cytokines that are released by phagocytic cells during an immune response are the major mediators of intermediary metabolism in

immunologically challenged animals (Klasing, 1988). The primary cytokines secreted by macrophages are: interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which are referred to as pro-inflammatory cytokines, due to their stimulation of an inflammatory response in the organism (Johnson, 1997). Carroll et al. (2009) observed enhanced circulating concentrations of these cytokines (IL-1, IL-6 and TNF-α) in beef calves after an acute bacterial LPS challenge. Similar results were reported after a neuroendocrine stress with infusion of corticotropin-releasing hormone (CRH; Cooke and Bohnert, 2011). An inflammation results in the activation of the acute-phase response, for the purpose of restore the homeostasis status in the damaged tissues within the infected organism.

Acute phase response. The acute-phase response (APR) is a complex series of reactions and physiological changes in an organism in response to infection, disease, stress or trauma. The APR is initiated to destroy the infective organism, to activate processes of reparation and to prevent further damages, in order to restore the infected body homeostasis, making it an important component of the innate immune system. The APR effects in the body includes febrile response, alterations in liver metabolism and gene regulation, in plasma mineral concentrations (such as Cu, Zn and Fe), increased circulating white blood cells, changes in behavior, such as lethargy, anorexia, hyperalgesia (Johnson, 1997), decreased social, sexual and aggressive behavior (Carrol et al., 2009, Cooke et al., 2009) and reduced ADG and feed efficiency (Arthington et al., 2013). The primary cell types associated with the beginning of the APR are tissue macrophages and blood monocytes (Gauldie, 1991), which releases pro-inflammatory cytokines, at the site of infection or inflammation. There is no specific cell type that is the exclusive target of most cytokines (Johnson, 1997; Kindt, 2007;

Parker et al., 2007; Lippolis, 2008). After stimulation of tissue macrophages and blood monocytes, IL-1, TNF-α, and subsequently IL-6 are released, stimulating APR.

Under normal circumstances, hepatocytes in the liver synthesize a range of acute-phase proteins (APP) at a relatively steady state, and they have different biological functions, such as enzymatic, proteinase inhibitors, coagulation, metal binding and transport proteins (Murata et al., 2004; Petersen et al., 2004; Carroll and Forsberg, 2007; Carroll, 2008). However, during an inflammatory response, the pro-inflammatory cytokines induces the production and release of APP, increasing its concentration. At this moment, most of liver function is dedicated to APP production (Carroll and Forsberg, 2007). Higuchi et al. (1994) demonstrated that hepatic synthesis of APP was stimulated by in vitro addition of glucocorticoids to cultures of bovine liver slices. Plasma concentrations increases following infection, and further pro-inflammatory cytokine stimulation of liver hepatocytes are referred to as positive APP, such as ceruloplasmin (Cp) and haptoglobin (Hp), whereas those for which plasma concentrations decline after an infection are referred to as negative APP. In addition some proteins that function as an APP in one species may not be an APP in another species (Carroll, 2008). The most studied APP in cattle are Cp, Hp, serum amyloid-A, and fibrinogen (Godson et al., 1995). For the purpose of the present studies, the focus of this review will be on Cp and Hp.

Ceruloplasmin is a copper-containing ferroxidase that oxidizes highly reactive iron (Fe^{+2}) to its non-toxic form (Fe^{+3}; Patel et al., 2002). The functions of Cp are to transport copper in the body (Eckersall and Conner, 1988) and to allow iron binding transferrin.

Ceruloplasmin integration in these metabolic pathways is essential to maintain iron and copper homeostasis (Mzhel'skaya, 2000). Ceruloplasmin is induced primarily by IL-6 and reaches its peak in serum concentrations 72 to 168 hr after the initiation of the APR and can remain enhanced as long as two weeks (Arthington et al., 2003; Eckersall and Bell, 2010). In situations of Cu deficiency, Cp levels are decreased (Mulhern and Koller, 1988) and it has been correlated with impaired immune system in cattle (Arthington et al., 1996). In this study, Arthington et al. (1996) observed that heifers in Cu-deficient diets had low Cu status at the end of the study and consequently lower plasma Cp levels than heifers in non-Cu-deficient diets, demonstrating that Cu status is highly correlated with Cp plasma concentrations.

Haptoglobin is an α2-globulin with a molecular weight of approximately 125 kDa (Petersen et al., 2004). Bovine Hp was found to consist of monomers of 16 to 23 kDa (α-chains) and 35 to 40 kDa (β-chains; Eckersall and Corner, 1990; Morimatsu et al., 1991) and to exist as a polymer in association with albumin with a molecular weight above 1000 kDa in cattle serum (Eckersall and Corner, 1990). Among the several functions that Hp has, the major one is to bind free hemoglobin released from damaged erythrocytes. It is known that Fe is essential to bacterial growth and development and this binding of Hp to hemoglobin restricts the availability of free iron to bacteria, decreasing further bacterial development due to lack of free Fe. As Cp, Hp is induced primarily by IL-6. Hp reaches its peak 24 to 48 after the initiation of the APR and may remain elevated as long as two weeks, also (Arthington et al., 2003; Eckersall and Bell, 2010).

Increased plasma APP concentrations, principally Cp and Hp, were found in cattle studies after weaning and transportation (Arthington et al., 2003, 2005 2008; Qiu et al., 2007; Araujo et al., 2010; Cooke et al., 2011, 2013; Guarnieri Filho et al., 2014), feed and water restriction (Cappellozza et al., 2012, 2014; Marques et al., 2012) induced inflammation challenges (Carroll et al., 2009b, 2011; Cooke and Bohnert, 2011; Cooke et al., 2012) and vaccination (Stokka et al., 1994; Arthington et al., 2013; Moriel and Arthington, 2013; Moriel et al., 2015; Artioli et al., 2015), leading to detrimental effects in feed intake and subsequently cattle performance; APP are also positive correlated with morbidity (Carter et al., 2002).

Adaptive immune system. Adaptive or acquired immunity, the second arm of the immune system, is responsible for adapting and building an immune response for each antigen found in the body (Carroll and Forsberg, 2007), and characterized by antibodies production and immunological memory creation. Adaptive immunity is recognized by its high specificity and capacity of recognizing and eliminating specific antigens (Kindt, 2007) with a dynamic antigen pathogen recognition system (Lippolis, 2008). The use of vaccines to protect animals from various pathogens is an example of adaptive immunity (Carroll and Forsberg, 2007). The adaptive immune system can be further subdivided into and cell-mediated and humoral. The T-lymphocytes (T-cells) provide protection against intracellular pathogens and tumor cells in cell-mediated immunity (Galyean et al., 1999). The B-lymphocytes (B-cells) mediate humoral immunity, which produce antibodies specific to antigens and become memory cells. For the purpose of the recent studies, this review will focus on humoral immune response.

Humoral immune response. The B-cells mature in the bone marrow and are the source of circulating antibodies and are activated when detect their specific foreign antigen. Activated B-cells proliferate and differentiate into antibody-secreting plasma cells and long-lived memory cells (Murphy et al., 2008). Following recognition and antigen attachment, the B-cell ingests the antigen and processes it for presentation of T-cells (Carrol and Forsberg, 2007). The B-cells have cell-surface immunoglobulin (Ig) molecules that, when activated, are secreted as soluble antibody against extracellular pathogens and act as receptors for antigens. The antibodies generated by B-cells recognize whole antigens, whereas the T-cell receptors recognize fragments of antigens presented by specialized molecules. The mature B- and T-cells that have not yet encountered their specific antigens are known as naïve T-cells (Murphy et al., 2008).

Dendritic cells are specialized antigen-presenting cells that are critical to the activation and maturation of naıve T-cells. The subtype (myeloid or plasmacytoid) of dendritic cells that responds to an infection can significantly affect the type of adaptive immune response. These dendritic cells subtypes not only are responsible for the activation of T-cells, but also for determining the type of T-cell response elicited (Lippolis, 2008). Activated T-cells proliferate and differentiate into effector T-cells (cytotoxic cells and helper cells) or memory T-cells (Murphy et al., 2008). The T-helper cells produce cytokines to help the other T- and B-cells to grow and divide themselves to produce more cells to fight future infections (Carrol and Forsberg, 2007). The T-helper cells are divided into T-regulatory cells, T-helper 1 (TH1), T-helper 2 (TH2) and T helper-17 (TH17). T-helper 1, TH2 and TH17 cells plays a role on the CD4+ co-receptor expression and stimulate inflammatory (TH1),

humoral or allergic (TH2), and acute (TH17) responses to pathogens. This initial adaptive immune response (TH1 or TH2) is controlled by the cytokine profile associated with the innate immune system (Carrol and Forsberg, 2007). Thus, the signal provided by dendritic cells is crucial to CD4+T-cells to initiate differentiation. The CD4+ T cells differentiate into TH1, when dendritic cells produce the pro-inflammatory cytokines interferon gamma (IFN-γ) and interleukin-1 (IL-12). However, it differentiates into TH2 when the anti-inflammatory cytokine interleukin-4 (IL-4) is produced. The TH1 cells further produce a pro-inflammatory cytokine profile, which supports cellular mediated immunity (Constant and Bottomly, 1997). In the other hand, the TH2 cells are the host immunity effectors against extracellular pathogens and promote humoral immunity and further antibody production by plasma cells derived from B-lymphocytes.

Antibodies take on various forms and are referred to as Ig, a type of protein that binds to antigens, which can be subdivided in 5 isotopes: IgG, IgM, IgA, IgD, and IgE. The most common immunoglobulin isotopes include IgM and IgG. The IgM isotopes are the first antibodies produced by the immune system in response to an infection. Even though they are the first antibodies to act on the scene following an infection, their affinity against antigen is relatively low (Carrol and Forsberg, 2007). The IgG are the most common Ig isotopes and have the greatest concentration among all Ig in cattle serum. In contrast to IgM, IgG are a highly specific and require additional time for their development.

Even though innate and adaptive immune systems are classified as distinct, do not operate independently, but rather in conjunction with one another and with help of molecules

and nonimmune cells in a complex network at the initiation of the inflammatory response (Lippolis, 2008).

Summary

In summary, the adoption of preconditioning has been shown to increase profitability of cow-calf farms by increasing calf reputation and sale price. Labor associated with feeding calves is one of the main reasons for producers not adopting preconditioning. However, decreasing the frequency of concentrate supplementation could be a strategy to overcome this obstacle. Infrequent energy supplementation of fibrous by-products, such as corn gluten feed and soybean hulls, is an alternative to decrease frequency of energy supplementation with less or no impacts on cattle performance. However, we are unaware of studies evaluating the effects of reduced supplementation frequency on the animal's immune system. Therefore, the focus of the studies described herein was to identify supplementation alternatives that would enable producers to decrease feeding labor and costs without compromising growth and health of calves. Hence, we evaluated the effects of decreasing only supplementation frequency (Chapter 2) or supplementation frequency and rate simultaneously (Chapter 3) on growth performance, physiological stress, and measurements of innate and humoral immune response of beef calves during a 42-d preconditioning period following vaccination.

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CHAPTER 2

Decreasing the frequency of energy supplementation from daily to three times weekly impairs growth and humoral immune response of preconditioning beef steers

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Introduction

Decreasing the frequency of energy supplementation from daily to three times weekly or alternate days decreases costs associated with labor and feeding, but it has either decreased (Cooke et al., 2007, 2008) or not affected growth performance of beef cattle (Moriel et al., 2012; Drewnoski et al., 2014). Discrepancies among those studies were attributed to differences in breed, supplement type and forage quality, but one similarity among them is the use of beef cattle around 10-12 months of age. Limited studies investigated the effects of decreasing the frequency of energy supplementation on growth of beef calves at different production stages, such as immediately after weaning and preconditioning (Drewnoski et al., 2011).

Preconditioning calves typically experience multiple processes, including weaning, vaccination and feedlot entry, that elicit an acute phase protein response (APR) and impair immunity and growth performance (Arthington et al., 2008, 2013). Fluctuations in nutrient intake due to decreased frequency of energy supplementation impacted metabolic parameters and decreased age at puberty of beef heifers (Cooke et al., 2008; Moriel et al., 2012), however, it is not known if fluctuations in nutrient intake caused by decreased frequency of concentrate supplementation impair the immunity of stressed calves during preconditioning. Our hypothesis was that decreasing the frequency of energy supplementation would further enhance the physiological stress and APR following vaccination leading to detrimental impacts on growth performance and immunity of preconditioning beef calves. Thus, this study evaluated the effects of decreasing the frequency of energy supplementation from daily to three times weekly, during a 42-d preconditioning period, on growth performance and

measurements of innate and humoral immune response following vaccination of beef calves.

Materials and Methods

The Institutional Animal Care and Use Committee of NC State University (14-054-A) approved all procedures for the experiment conducted at the Mountain Research Station (Waynesville, NC; 35.48° N, 82.99° W; elevation = 659 m) from October to November 2014. ***Animals, diets and sample collection.*** Angus steers (n = 24; 221 ± 6.3 kg; 177 ± 4 d of age) were weaned on d -7 and immediately transferred to a single drylot pen with daily free-choice access to long-stem tall fescue hay (*Lolium arundinaceum*; 17% CP and 58% TDN; DM basis) and concentrate DMI at 0.5% of BW from d -7 to 0 (50% soyhulls pellets:50% corn gluten pellets; DM basis). On d 0, steers were stratified by BW and age, and randomly allocated into 1 of 8 concrete floor pens (3 steers/pen; 18 x 4 m; 24 m^2/steer) in a half-covered feedlot facility. Treatments were randomly assigned to pens (4 pens/treatment) and consisted of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered either daily (**S7**) or three times weekly (**S3**; Monday, Wednesday and Friday) during a 42-d preconditioning period (d 0 to 42). Hence, days when all S7 and S3 steers received concentrate supplementation were defined as SUPPALL days (Monday, Wednesday and Fridays), whereas days that only S7 steers received concentrate supplementation were defined as S7ONLY days (Tuesday, Thursday, Saturday and Sunday). Concentrate and hay were offered separately in the same feed bunk at 0800 h. Nutritional composition of hay and concentrate used from d -7 to 42 are shown in Table 1. Concentrate offered was completely consumed within 1 h by S7 steers and within 6 h by S3 steers. Individual BW was measured before feeding on d 0 and 42, following 12 h of feed and water

withdrawal. Weekly concentrate DM offered (concentrate DMI = 1% of BW multiplied by 7 d) was estimated based on average shrunk BW of each pen on d 0, and readjusted on d 21 using average full BW of each pen obtained before feeding. Shrunk BW was not obtained on d 21 to not disturb feeding behavior and avoid an unnecessary physiological stress response due to shrink, which could affect plasma measurements and vaccine response (Marques et al., 2012). All steers were provided daily free-choice access to a complete mineral mix (RU-MIN 1600, Southern States, Richmond, VA; DM basis: 18.2% Ca, 0.72% K, 0.88% Mg, 0.76% S, 7.0% Na, 10.8% Cl, 2.9 % P, 29 mg/kg Co, 1,220 mg/kg Cu, 2,130 mg/kg Mn, 29 mg/kg Se and 2,530 mg/kg Zn) and water from d 0 to 42.

Hay DM offered and refused were obtained daily for each pen by drying samples of hay offered and refused in a forced-air oven at 56°C for 48 h. Daily DMI was determined by subtracting the daily hay DM refused from the daily hay DM offered. Samples of hay, concentrate and mineral mix offered were collected weekly and sent in duplicate to a commercial laboratory (Dairy One Laboratory, Ithaca, NY) for wet chemistry analysis of all nutrients (Table 1). Samples were analyzed for concentrations of CP (method 984.13; AOAC, 2006), ADF (method 973.18 modified for use in an Ankom 200 fiber analyzer; Ankom Technology Corp., Fairport, NY; AOAC, 2006) and NDF (Van Soest et al., 1991; modified for use in an Ankom 200 fiber analyzer; Ankom Technology Corp.). Concentrations of TDN were calculated as proposed by Weiss et al. (1992), whereas NEm and NEg were calculated using equations from NRC (2000).

On d 7, all steers were treated with doramectin for internal and external parasites (5 mL subcutaneous; Dectomax injectable, Zoetis Inc., Kalamazoo, MI), and vaccinated against

infectious bovine rhinotracheitis, bovine viral diarrhea Types 1 and 2 viruses, *Mannheimia haemolytica* (2 mL subcutaneous; Bovi Shield Gold One Shot; Zoetis Inc., New York, NY) and *clostridium* (2 mL subcutaneous; Ultrabac 7, Zoetis Inc., New York, NY). On d 21, steers received 2-ml subcutaneous boosters of Bovi Shield Gold 5 (Zoetis Inc., New York, NY) and Ultrabac 7. The vaccination and parasite control protocol described above was chosen in order to replicate the protocol utilized by the local preconditioning alliance (Mountain Cattle Alliance, Canton, NC; Moriel et al., 2015). The vaccination protocol was initiated 14 d after weaning and 7 d after feedlot entry to avoid the weaning- and feedlot entry-induced inflammatory response that could interfere with vaccine response.

Blood samples (10 mL) were collected via jugular venipuncture into sodium-heparin (158 USP) containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for plasma harvest 4 h after concentrate supplementation for 4 consecutive days in 2 wk (d 7, 8, 9 and 10 in wk 2, and d 21, 22, 23 and 24 in wk 4 of the study). The approach of collecting blood samples 4 h after feeding was utilized previously to correspond to the peak of ruminal fermentation and end products release after concentrate consumption (Cooke et al., 2008; Moriel et al., 2012). Days for blood collection were chosen to evaluate the post-vaccination energy and protein metabolism of S7 and S3 steers on days that all S7 and S3 steers received concentrate supplementation (SUPPALL; d 7, 9, 21 and 23) and days that only S7 steers received concentrate supplementation (S7ONLY; d 8, 10, 22 and 24). Additional blood samples (10 mL) were collected via jugular venipuncture into sodium-heparin (158 USP) containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for plasma harvest immediately after weaning (d -7), and 4 h after concentrate supplementation on d 0, 14, 28

and 35 to complement the blood collection schedule described previously and to characterize the APR of S7 and S3 steers. Blood samples (10 mL) from the jugular vein were collected into a tube containing no additives (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for serum harvest on d -7, 21 and 42 to evaluate serum antibody titers against BVDV-1b. Blood samples were immediately placed on ice following collection, and then centrifuged at 1,200 × g for 25 min at 4°C. Plasma and serum samples were stored frozen at -20°C until later laboratory analysis.

On d 0, steers were randomly selected from each pen for liver biopsy (2 steers/pen) on d 10 and 24, which corresponds to the vaccination-induced peak of inflammatory response based on plasma concentrations of haptoglobin (Moriel and Arthington, 2013; Arthington et al., 2013; Moriel et al. 2015). Liver samples were collected via needle biopsy, following the procedure described by Arthington and Corah (1995). Immediately following collection, 100 mg of wet tissue was stored in 1.5 mL of RNA stabilization solution (RNAlater, Ambion Inc., Austin, TX), kept on ice for 8 h, and then stored at -80°C, until analysis.

Laboratory analyses. Plasma concentrations of haptoglobin were determined in duplicate samples using a biochemical assay measuring haptoglobin-hemoglobin complex by the estimation of differences in peroxidase activity (Cooke and Arthington, 2013). Plasma ceruloplasmin oxidase activity was measured in duplicate samples by using a colorimetric procedure (Demetriou et al., 1974). Commercial quantitative colorimetric kits were used to determine the plasma concentrations of PUN (B7551; Pointe Scientific, Inc. Canton, MI) and glucose (G7521; Pointe Scientific, Inc., Canton, MI). Inter- and intra-assay CV for assays of

haptoglobin, ceruloplasmin, PUN and glucose were 3.4 and 9.7, 7.1 and 4.3, 3.1 and 6.9, 2.3 and 3.2%, respectively.

Plasma concentrations of cortisol and insulin were determined using a single chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). Plasma IGF-1 concentrations were determined using commercial enzyme-linked immunosorbent assay kits (SG100; R&D Systems, Inc., Minneapolis, MN) that were previously validated for bovine samples (Moriel et al., 2012). Intra-assay CV for assays of cortisol, insulin, glucose and IGF-1 were 3.3, 2.2, 3.2 and 4.5%, respectively. Inter-assay for IGF-1 assay was 4.3%.

Serum antibody titers against bovine viral diarrhea virus 1b (BVDV-1b) were determined by the Oklahoma Animal Disease and Diagnostic Laboratory using a virus neutralization test (Rosenbaum et al., 1970). Serum titers against BVDV-1b were reported as the log base 2 of the greatest dilution of serum that provided complete protection of the cells (lowest and greatest tested dilution = 1:4 and 1:256, respectively). For the seroconversion analysis, samples with serum neutralization value of < 4 were considered negative and assigned a value of 0, whereas samples with serum neutralization value ≥ 4 were considered positive and assigned a value of 1. Then the assigned values (0 or 1) were used to calculate the positive seroconversion (% of steers with positive serum neutralization) to BVDV-1b (Richeson et al., 2008; Moriel et al., 2015)

Detailed description of procedures for mRNA isolation and tissue gene expression are described in Cappellozza et al. (2014). Briefly, total RNA was extracted from liver tissue samples using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). Extracted

RNA was quantified via UV absorbance (UV Mini 1240; Shimadzu Scientific Instruments, Inc., Columbia, MD) at 260 nm, incubated (2.5 μg) at 37°C for 30 min in the presence of RNase-free (DNase; New England Biolabs Inc., Ipswich, MA), and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random hexamers (Applied Biosystems, Foster City, CA). Real-time PCR was completed using the FAST SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers (20 pM each; Table 2) with the StepOne Real-time PCR system (Applied Biosystems). At the end of each RT-PCR, amplified products were subjected to a dissociation gradient (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) to verify the amplification of a single product by denaturation at the anticipated temperature. A portion of the amplified products were purified with the QIAquick PCR purification kit (Qiagen Inc.; Valencia, CA) and sequenced at the Oregon State University - Center for Genome Research and Biocomputing to verify the specificity of amplification. All amplified products represented only the genes of interest. Responses were quantified based on the threshold cycle (CT) and normalized to cyclophilin CT examined in the same sample and assessed at the same time as the targets. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$), as described by Ocón-Grove et al. (2008).

Statistical analyses. Except for seroconversion, all data were analyzed as a completely randomized design using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 9.3) with Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. Pen was the experimental unit, and steer(pen) and pen(treatment) were included as random effects in all analysis, except for analyses of DM and nutrient intake that included only pen(treatment) as random effect. All measurements

(growth performance, intake, plasma and serum measurement) obtained on d 0 were included as covariate only when $P \leq 0.05$, and will be described below. Feed efficiency, ADG and mean total intake of DM, mean daily intake of CP and NEg were tested for fixed effects of treatment. Within each wk of the study, daily DMI data was pooled by SUPPALL and S7ONLY days to simplify data analyses, interpretation and report. Daily intake of DM (concentrate, hay and total), CP and NEg data were analyzed as repeated measures and tested for fixed effects of treatment, day (SUPPALL and S7ONLY), wk of the study and resulting interactions, using pen(treatment) as the subject. Body weight, plasma measurements, serum BVDV-1b titers and tissue mRNA expression were analyzed as a repeated measures and tested for fixed effects of treatment, day, wk of the study, and resulting interactions. The covariance structures were chosen using the lowest Akaike information criterion. The unstructured covariance structure was used for all analysis, except for liver mRNA expression of haptoglobin (Toeplitz structure), BW from d 0 to 42, plasma IGF-1 concentrations (Compound symmetry structure), and plasma concentrations of cortisol, haptoglobin and ceruloplasmin (Autoregressive structure). Positive seroconversion to BVDV-1b were analyzed as repeated measures using the GLIMMIX procedure of SAS with pen(treatment) and steer(pen) as random effects. All results are reported as least-squares means. Data were separated using LSD if a significant preliminary F-test was detected. Significance was set at $P \leq 0.05$, and tendencies if $P > 0.05$ and ≤ 0.10.

Results

Body weight at weaning (d -7) did not differ between treatments ($P = 0.75$), but was included as a covariate ($P < 0.0001$) for the statistical analysis of BW. Treatment x day effect

was detected for BW from d 0 to 42 ($P \leq 0.01$; Table 3), as S7 steers had greater BW than S3 steers on d 42 ($P = 0.001$). Overall ADG and total DMI were greater for S7 vs. S3 steers ($P \leq 0.02$), whereas overall G:F did not differ between treatments ($P = 0.14$; Table 3). A treatment x day x wk was detected for hay DMI ($P < 0.0001$; Figure 1). On SUPPALL days, hay DMI was greater for S7 vs. S3 steers from wk 1 to 6 ($P < 0.0001$). On S7ONLY days, hay DMI was greater for S3 vs. S7 steers from wk 1 to 6 ($P \leq 0.05$), except for wk 2 in which hay DMI did not differ between treatments ($P = 0.68$; Figure 1). Hay DMI of S3 steers was less on SUPPALL vs. S7ONLY days ($P < 0.0001$), whereas hay DMI of S7 steers did not differ on SUPPALL vs. S7ONLY days ($P \geq 0.13$), except for wk 2 in which hay DMI of S7 steers was less on SUPPALL vs. S7ONLY days ($P = 0.004$; Figure 1). Total hay DMI (d 0 to 42) was less for S3 vs. S7 steers ($P = 0.002$; Table 4).

Effects of treatment x day ($P < 0.0001$), but not treatment x wk x day and treatment x wk ($P \geq 0.30$), were detected for daily intake of concentrate DM, total DM, CP and NEg (Table 4). Steers offered concentrate 3 times weekly had greater daily total DMI on SUPPALL days ($P = 0.05$), but less on S7ONLY days ($P = 0.002$) compared to S7 steers. On SUPPALL days, daily CP intake tended to be greater ($P = 0.07$), whereas daily NEg intake was greater ($P = 0.001$) for S3 vs. S7 heifers. On S7ONLY days, daily CP and NEg intake was greater for S7 vs. S3 steers ($P \leq 0.002$). Hence, mean daily CP and NEg intake were greater ($P \leq 0.03$) for S7 vs. S3 steers.

Plasma concentrations of cortisol, haptoglobin and ceruloplasmin on d 0 did not differ between treatments ($P \geq 0.23$), but were included as covariates ($P \leq 0.03$) for the statistical analyses of plasma concentrations of cortisol, haptoglobin and ceruloplasmin, respectively. A

tendency for treatment x day effect was detected (P = 0.07) for plasma concentrations of cortisol, which did not differ between treatments from d 9 to 23 and 28 to 35 ($P \geq 0.22$), but were greater for S3 vs. S7 steers on d 7, 8 and 28 ($P \leq 0.03$; Figure 2a). Effects of time ($P <$ 0.0001), but not treatment x day of study ($P \geq 0.17$), were detected for plasma concentrations of ceruloplasmin and haptoglobin (Figure 2b). However, treatment effects were detected for mean plasma concentrations of haptoglobin (P = 0.004), but not for mean plasma concentrations of ceruloplasmin (P = 0.48). Mean plasma concentration of haptoglobin was greater for S3 vs. S7 steers (0.91 vs. 0.69 ± 0.048 mg/mL, respectively).

Effects of treatment x day x wk and treatment x wk were not detected for plasma concentrations of glucose, IGF-1, PUN and insulin ($P \geq 0.19$). However, effects of treatment x day were detected for plasma concentrations of glucose, IGF-1 and PUN ($P \leq 0.01$), but not for insulin (P = 0.12). Therefore, data for plasma concentrations of glucose, IGF-1 and PUN were presented as plasma concentrations of each measurement immediately before (d 0) and 1, 2 and 3 d after vaccination, which represent the average plasma results of d 7 and 21 (first and second rounds of vaccination, respectively), 8 and 22, 9 and 23, and 10 and 24 of the study, respectively. Regardless of treatment, plasma concentrations of glucose decreased on d 2 and 3 vs. 0 and 1 ($P \leq 0.006$), relative to vaccination, whereas plasma concentrations of IGF-1 and PUN decreased ($P < 0.0001$) on d 1, 2 and 3 vs. 0, relative to vaccination. Steers provided concentrate supplementation daily tended (P = 0.07) to have greater plasma concentrations of glucose on d 2, and had greater ($P \leq 0.002$) plasma concentrations of IGF-1 on d 0 and plasma concentrations of PUN on d 1 and 3, relative to vaccination, compared to S3 steers (Table 5). Effect of time ($P < 0.0001$), but not treatment (P = 0.71), was detected

for plasma concentrations of insulin, which did not differ on d 1 and 3 ($P = 0.14$), greatest on d 2 and least on d 4 ($P \leq 0.05$), relative to vaccination (Table 5).

Effects of treatment x day and day were not detected ($P \geq 0.12$) for mRNA expression of haptoglobin, IGF-1 and pyruvate carboxylase. Treatment effect was detected for mRNA expression of pyruvate carboxylase ($P = 0.03$), but not for haptoglobin and IGF-1 ($P \geq 0.42$). Mean hepatic mRNA expression of pyruvate carboxylase was greater for S7 vs. S3 steers (Table 6). Positive seroconversion and serum BVDV-1b titers on d -7 did not differ between treatments ($P \geq 0.23$), but were included as covariates ($P \leq 0.003$) for the seroconversion and serum BVDV-1 titers statistical analyses, respectively. A tendency for treatment x time effect was detected ($P = 0.06$) for positive seroconversion to BVDV-1b, which did not differ between treatments on d 28 ($P = 0.55$), but was greater for S7 vs. S3 steers on d 42 ($P = 0.05$; Table 7). Effect of treatment ($P = 0.03$), but not treatment x time ($P = 0.14$), was detected for serum BVDV-1b titers, which was greater for S7 compared to S3 steers (Table 7).

Discussion

Published data on frequency of energy supplementation observed variable responses in ADG of beef steers and heifers. In the current study, decreasing the frequency of energy supplementation from 3 to 7 times weekly decreased overall ADG of beef steers by 21%, which is in agreement with others (Cooke et al., 2008; Loy et al., 2008). Decreasing the frequency of energy supplementation from daily to 3 times weekly decreased ADG by 18% of beef heifers fed low-quality warm-season forages and low-starch, wheat middling-based energy supplements (Cooke et al., 2008) and decreased ADG by 10% of beef steers fed low-quality native grasses and dry rolled corn- or dry distillers grains plus soluble-based

supplements (Loy et al., 2008). In contrast, decreasing the frequency of low-starch concentrate supplementation from daily to 3 times weekly (Moriel et al., 2012; Drewnoski et al., 2011) or alternate days (Drewnoski et al., 2014) did not affect ADG of beef heifers and steers fed medium-quality forages. Discrepancies among results are likely due to differences in supplement composition, breed, gender, location, forage species and quality, and potentially resulting interactions among these factors. Discussing each of those factors is beyond the scope of this manuscript, and hence our discussion will focus on comparing the differences between our results and those from Drewnoski et al. (2011), who used similar supplement composition (corn gluten feed and soyhulls mix), supplementation frequency (7 or 3 times weekly), production stage (immediately after weaning) and beef steers of same breed as in our study.

The observed difference on daily hay intake between S7 and S3 steers was the primary factor impacting differences in growth performance. Daily hay DMI of S3 steers decreased by 53% on SUPPALL days and increased by 10% on S7ONLY days compared to S7 steers, leading to an overall reduction on intake of total hay DM, CP and NEg. Drewnoski et al. (2011) reported that hay DMI was decreased by approximately 32% when all steers received concentrate supplementation, but hay DMI was similar on days that only S7 steers received concentrate supplementation. Despite the low-starch supplements used in this study, this response on daily hay DMI was expected because supplements often decrease forage DMI when TDN:CP ratio is less than 7 and supplemental TDN is greater than 0.7% of BW (Moore et al., 1999). However, concentrate supplementation-induced depression in forage intake is greater as forage quality increases (Horn and McCollum, 1987). Hence, differences

observed in the magnitude of hay DMI reduction due to concentrate supplementation between our study and Drewnoski et al. (2011) may be attributed to differences on average forage quality (17 vs. 8.4% CP of DM, respectively). In addition, as part of our experimental design, all S7 and S3 steers received 2 rounds of vaccinations against pathogens associated with bovine respiratory disease that have been shown to elicit APR and decrease growth performance (Arthington et al., 2013; Moriel and Arthington, 2013; Moriel et al., 2015). Steers utilized by Drewnoski et al. (2011) were vaccinated at a fairly long time before the study began. However, they were implanted with 36 mg of Zeranol and treated for internal and external parasites before starting the study. Hence, our results are in agreement with our hypothesis that growth performance of beef calves is impaired by decreasing the frequency of energy supplementation from daily to 3 times weekly during a preconditioning period including rounds of vaccination.

Weaning, feedlot entry and vaccination elicit an APR leading to increased hepatic synthesis of acute-phase proteins (i.e. haptoglobin and ceruloplasmin), depressed feed intake, enhanced muscle and fat mobilization (Johnson, 1997), and decreased feed efficiency of beef cattle (Arthington et al., 2013). Also, an increase in plasma concentrations of cortisol reduced DMI (Allen et al., 2009), induced an APR and increased plasma haptoglobin concentrations (Cooke and Bohnert, 2011), whereas plasma concentrations of haptoglobin increased following vaccination of beef steers against *M. haemolytica* (Arthington et al., 2013; Moriel et al., 2015) and may be used as an indicator of inflammation when plasma concentrations are $\geq$ 0.11 mg/mL (Tourlomoussis et al., 2004). In the present study, S3 steers had greater plasma concentrations of cortisol on d 7, 8 and 24 of the study, and had mean plasma

concentrations of haptoglobin 31.8% greater than S7 steers. These responses agree with our hypothesis and indicate that decreasing the frequency of energy supplementation exacerbated the physiological stress (as indicated by greater plasma concentrations of cortisol), leading to a heightened vaccine-induced APR (as indicated by the greater plasma concentrations of haptoglobin) and less growth performance compared to daily energy supplementation of preconditioning beef calves. In addition, the fact that only treatment effects, and not treatment x time effect, was detected for plasma concentrations of haptoglobin indicates that S3 steers had greater plasma concentrations of haptoglobin even before vaccine administration. An increasing number of studies indicated that feeding beef cattle high grain-based diets led to an accumulation of microbial endotoxins in the ruminal fluid that induced a general nonspecific inflammatory response (Berry et al., 2004; Jafari et al., 2006). For instance, plasma haptoglobin concentrations of beef steers peaked after 3 to 9 wk of feeding backgrouding and finishing starch-based diets containing (DM basis) 76% wheat (Gohzo et al., 2006) and 45 or 95% barley grain (Ametaj et al., 2009). Although the concentrate utilized in the present study had low-starch concentrations, it is possible that the greater concentrate consumption of S3 vs. S7 steers on SUPPALL days resulted in the accumulation of endotoxins in the ruminal fluid and increased plasma concentrations of haptoglobin. Further studies need to be conducted to support this rationale.

To further explore the effects of frequency of energy supplementation on APR, liver biopsies were collected to evaluate the hepatic mRNA expression of haptoglobin. We are unaware of published studies describing the hepatic mRNA expression of haptoglobin following vaccination of beef cattle. Hence, we decided to collect liver samples 3 d after

vaccination, which corresponds to the post-vaccination peak of plasma haptoglobin concentrations as indicated previously (Arthington et al., 2013; Moriel and Arthington, 2013; Moriel et al., 2015). Hepatic mRNA expression of haptoglobin did not differ in our study, suggesting that decreasing the frequency of energy supplementation does not affect hepatic mRNA expression of haptoglobin 3 d after vaccination. However, the greater mean plasma haptoglobin concentrations of S3 vs. S7 steers indicates that the hepatic synthesis of haptoglobin was affected and that the increase on mRNA synthesis of haptoglobin occurred within 3 d of vaccination. Further studies are warranted to characterize and identify the peak of hepatic haptoglobin mRNA expression of beef cattle following an inflammatory challenge.

Although APR is essential for early defense mechanism in response to cellular injury (Eckersall and Conner, 1988), nutrient demand is increased to accommodate the synthesis of acute-phase proteins, immune cells and gluconeogenic precursors (Reeds and Jahoor, 2001). In order to support an immunological response, muscle protein and fat reserves are mobilized (Jahoor et al., 1999) and absorbed AA are shifted from growth towards hepatic uptake (Reeds et al., 1994) leading to a negative correlation with growth performance (Qiu et al., 2007). Indeed, circulating cortisol stimulates degradation of hepatic, adipose and muscle cells (Nelson and Cox, 2005). Thus, the greater plasma concentrations of haptoglobin and cortisol of S3 vs. S7 steers may indicate that nutrient mobilization from body energy and protein reserves to support the immune system were increased by decreasing the frequency of energy supplementation and might further explain the greater growth performance of S7 vs. S3 steers.

Insulin-like growth factor 1 is an essential constituent of multiple systems controlling growth (Le Roith et al., 2001), with liver as the primary source of circulating IGF-1 (Yakar et al., 1999). Circulating concentrations of IGF-1 increases with increasing nutrient intake and growth rate (Elsasser et al., 1989; Moriel et al., 2012, 2015). Also, PUN concentration is an indicator of protein intake in forage-fed cattle (Hammond, 1997), with optimal levels for growing cattle varying between 11 to 15 mg/dL (Byers and Moxon, 1980). In the current study, plasma concentration of IGF-1 was greater for S7 vs. S3 steers immediately before vaccination, whereas PUN concentrations were greater for S7 vs. S3 steers on d 1 and 3, relative to vaccination, which likely reflect the differences on nutrient (CP and NEg) intake and further explain the greater growth performance of S7 vs. S3 steers. However, plasma concentrations of IGF-1 and PUN decreased for 3 d following vaccination, regardless of treatment, as previously reported by others (Moriel and Arthington, 2013; Moriel et al., 2015). Also, hepatic mRNA expression of IGF-1 did not differ between treatments on d 3, relative to vaccination, which explains the similar plasma concentrations of IGF-1 that day. Proinflammatory cytokines released during APR induce a state of IGF-1 resistance, which inhibits the anabolic effects of IGF-1 and facilitates energy and protein mobilization from body stores (O'Connor et al., 2008). Hence, this decrease in plasma concentrations of IGF-1 and PUN following vaccination further supports that nutrients were being partitioned to support the immune system rather than growth, and that S7 steers likely had less mobilization of body energy and protein reserves than S3 steers.

Drewnoski et al. (2014) investigated the variation on the area under the curve (AUC) of plasma concentrations of insulin, glucose and PUN for 48 h after steers were offered low-

starch concentrate supplementation daily or on alternate days. Only plasma insulin AUC was affected and steers offered daily supplementation had decreased plasma insulin AUC on days that both treatments received supplementation, but increased plasma insulin AUC when only those steers received supplementation. In contrast, Cooke et al. (2008) and Moriel et al. (2012) reported that plasma concentrations of glucose and insulin were greater for steers receiving concentrate supplementation 3 times weekly (S3) vs. daily supplementation (S7) on days that only S7 received supplementation, but similar plasma concentrations of glucose and insulin on days that both received concentrate supplementation. Those authors attributed the differences on plasma concentrations of glucose and insulin to the pattern of nutrient intake of each treatment, as both insulin and glucose are influenced positively by rate of nutrient intake (Vizcarra et al., 1998), and to the time required for synthesis and activation of gluconeogenic enzymes to substantially increase the magnitude of hepatic synthesis and release of glucose. In support of this rationale, hepatic mRNA expression of pyruvate carboxylase (one of the key glugoneogenic enzymes; Jitrapakdee et al., 2008) in the current study was greater for S7 vs. S3 steers on d 10 and 24 of the study, which corresponds to days that only S7 received concentrate supplementation. However, plasma concentrations of glucose differed only on previous days (d 9 and 23 of the study), which correspond to days when both treatments received supplementation.

Reasons for not detecting differences in plasma insulin concentrations and additional differences on plasma glucose concentrations may be attributed to the inflammatory state and APR induced by vaccination. Acute phase response alters carbohydrate metabolism by inhibiting de novo synthesis of glucose (Gifford et al., 2012) and increasing insulin synthesis

(Anderson et al., 2001) likely to decrease hepatic utilization of carbohydrates to meet the demand of peripheral tissues. For instance, hyper metabolic states that occur during the acute phase of illness impact carbohydrate and lipid metabolism (Chioléro et al., 1997), whereas LPS infusions depleted carbohydrate stores, caused hypoglycemic states for 10 h in cattle (Kushibiki et al., 2000) and inhibited in vitro hepatic expression of gluconeogenic enzymes (Jones and Titheradge, 1993). In the current study, plasma concentrations of insulin increased on d 1 and gradually decreased below baseline levels on d 2 and 3, relative to vaccination, whereas plasma concentrations of glucose decreased on d 2 and 3, relative to vaccination, regardless of treatment assignment. These effects in plasma concentrations of glucose and insulin, in combination with the decrease in plasma concentrations of IGF-1 and PUN after vaccination, support the rationale that nutrients were partitioned to support immunity rather than growth. In addition, the release of glucocorticoids stimulates gluconeogenesis (Carroll and Fosberg 2007), and hence, S3 steers may have increased glucose synthesis due to greater plasma cortisol concentrations, which may have prevented the detection of differences on plasma concentrations of glucose and insulin compared to S7 steers.

Neutralizing serum antibody titers may be used as an indicator of immune protection, disease prevention and vaccine efficacy in calves (Howard et al., 1989; Bolin and Ridpath, 1995; Richeson et al., 2008). The ability of an animal to response to vaccination varies from animal to animal, and depends on environmental and genetic factors, maternal antibody concentrations (Downey et al., 2013), timing of vaccination after feedlot entry (Richeson et al., 2008) and MP supply (Moriel et al., 2015). Duff and Galyean (2007) highlighted that few studies focused on the interaction between vaccination and nutrition. Thus, we explored the

potential effects of decreasing the frequency of energy supplementation on post-vaccination antibody production of preconditioning steers. In the present study, positive seroconversion and serum titers against BVDV-1b were less for S3 vs. S7 steers indicating that decreasing the frequency of energy supplementation lessened the vaccine response, which might lead to less immune protection against BVDV-1b and greater chances of developing bovine respiratory diseases complex (BRDC). For instance, the majority of BRDC cases occur within 30 d post-weaning or 14 d relative to feedlot entry (Kirkpatrick et al., 2008), whereas calves with serum BVDV-890 neutralizing titers > 4 (log base 2 scale) did not develop severe clinical signs of fever, leukopenia and diarrhea (Bolin and Ridpath, 1995). Downey et al. (2013) reported that BVDV antibody titers increased by 0.068 titer units (log base 2) for every 1 kg increase on ADG during the first 21 d after vaccination. Therefore, the greater mean daily CP intake (Moriel et al., 2015), and consequently overall ADG (Downey et al., 2013), of S7 vs. S3 steers likely contributed with the differences observed on serum BVDV-1b titers. In addition, cortisol may induce immune suppression effects (Salak-Johnson and McGlone, 2007), weaken the innate immune response (Dai and McMurray, 1998) and block the cytokine secretion by $CD4^{+}$T helper 1 and 2 that are involved on antibody production (Salak-Johnson and McGlone, 2007). Hence, the exacerbated physiological stress experienced by S3 steers led to greater plasma concentrations of cortisol than S7 steers and may have decreased the communication between innate and humoral immune response causing a decreased antibody production against BVDV-1b.

Summary

In summary, decreasing the frequency of energy-based, low-starch concentrate

supplementation from daily to three times weekly decreased growth performance, increased plasma concentrations of haptoglobin and cortisol, and decreased vaccine-induced antibody production against BVDV-1b of recently weaned beef steers during a 42-d preconditioning period. Taken together, our results suggest that decreasing the frequency of energy supplementation during preconditioning and vaccination are not recommended because it might decrease growth performance, vaccine response and potentially immune protection against pathogens associated with bovine respiratory disease.

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Table 1.1: Average chemical composition of ground tall fescue hay and concentrate[1].

Item	Tall fescue hay	Concentrate[2]
DM, %	91.2	89.6
	---------- DM basis ----------	
CP, %	17.4	17.0
ADF, %	34.4	26.5
NDF, %	57.7	47.4
TDN[3], %	58.0	72.0
NE_m[4], Mcal/kg	1.14	1.67
NE_g[4], Mcal/kg	0.60	1.10
Ca, %	0.51	0.41
K, %	2.53	1.48
Mg, %	0.32	0.32
Na, %	0.05	0.20
P, %	0.38	0.58
Cu, mg/kg	8	6
Fe, mg/kg	358	252
Mn, mg/kg	68	24
Mo, mg/kg	0.40	1.4
Zn, mg/kg	31	58

[1]Hay and concentrate samples were collected weekly and sent in duplicate to a commercial laboratory for wet chemistry analysis (Dairy One Laboratory, Ithaca, NY).

[2]Same concentrate was used from weaning to end of study (d -7 to 42), and consisted of 50% soybean hull pellets and 50% corn gluten feed pellets (DM basis).

[3]Calculated as described by Weiss et al. (1992).

[4]Calculated using the equations proposed by the NRC (2000).

Table 2.1: Nucleotide sequence of bovine-specific primers used in the quantitative real-time reverse transcription PCR to determine the hepatic mRNA expression of haptoglobin, IGF-1, pyruvate carboxylase and cyclophilin.

Target gene	Primer sequence[1]	Accession number
Haptoglobin		
Forward	GTC TCC CAG CAT AAC CTC ATC TC	AJ_271156
Reverse	AAC CAC CTT CTC CAC CTC TAC AA	
IGF-1		
Forward	CTC CTC GCA TCT CTT CTA TCT	NM_001077828
Reverse	ACT CAT CCA CGA TTC CTG TCT	
Pyruvate carboxylase		
Forward	CCA ACG GGT TTC AGA GAC AT	NM_177946.3
Reverse	TGA AGC TGT GGG CAA CAT AG	
Cyclophilin		
Forward	GGT ACT GGT GGC AAG TCC AT	NM_178320.2
Reverse	GCC ATC CAA CCA CTC AGT CT	

[1]Primer sequences obtained for haptoglobin (Hiss et al., 2004), IGF-1 and pyruvate carboxylase (Cooke et al., 2008), and cyclophilin (Cappellozza et al. 2014).

Table 3.1: Growth performance of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered daily (S7) or three times weekly (S3) during a 42-d preconditioning period (n = 4 pens/treatment; 3 steers/pen).

	Treatments[1]			
Item	**S7**	**S3**	**SEM**	***P*-value**
Body weight[2], kg				Treatment x day
d 0	218[a]	218[a]	2.31	0.01
d 42	273[b]	262[a]		
				Treatment
ADG (d 0 to 42), kg/d	1.30	1.03	0.040	0.02
Total DMI (d 0 to 42), kg	252	222	3.9	0.01
G:F[3] (d 0 to 42)	0.22	0.20	0.010	0.14

[a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

[1]From d 0 to 42, steers were provided a similar weekly concentrate amount (DMI = 1% of BW multiplied by 7 d) offered either daily (S7) or three times weekly (S3; Mondays, Wednesdays and Fridays) at 0800 h.

[2]Body weights obtained after 12 h of feed and water withdrawal. Body weight at weaning (d -7) did not differ between treatments ($P = 0.75$), but was included as a covariate ($P < 0.0001$) for BW statistical analysis.

[3]Estimated by dividing total BW gain by total DMI from d 0 to 42.

Table 4.1: Ingredient and nutrient intake of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered either daily (S7) or three times weekly (S3) during a 42-d preconditioning period (n = 4 pens/treatment; 3 steers/pen).

	Treatments[1]			
Item[2]	**S7**	**S3**	**SEM**	***P*-value[3]**
Concentrate DMI, kg/d				
SUPPALL	2.31	5.18	0.184	<0.0001
S7ONLY	2.31	0	0.184	<0.0001
P-value[4]	1.00	<0.0001		
Total DMI, kg/d				
SUPPALL	6.03	6.93	0.270	0.05
S7ONLY	6.12	4.22	0.270	0.002
P-value[4]	0.33	<0.0001		
CP intake, kg/d				
SUPPALL	1.04	1.18	0.046	0.07
S7ONLY	1.05	0.69	0.046	0.002
P-value[4]	0.88	<0.0001		
NEg intake, Mcal/d				
SUPPALL	4.71	6.63	0.247	0.001
S7ONLY	4.76	2.51	0.247	0.0005
P-value[4]	0.51	<0.0001		
				Treatment
Total hay DMI, kg	156	129	2.0	0.002
Mean CP intake[5], kg/d	1.02	0.90	0.016	0.01
Mean NEg intake[5], Mcal/d	4.69	4.22	0.085	0.03

[1]From d 0 to 42, steers were provided daily free-choice access to ground tall fescue hay and a similar weekly concentrate amount (DMI = 1% of BW multiplied by 7 d) that was offered either daily (S7) or three times weekly (S3; Monday, Wednesday and Friday) at 0800 h.

[2]SUPPALL = days when all S3 and S7 steers were offered concentrate; S7ONLY = days that only S7 steers received concentrate.

[3]Comparison of treatments within each day.

[4]Comparison of day within each treatment.

[5]Determined by multiplying the total DMI of hay and concentrate by their respective concentration of CP or NEg, and then divided by 42 d.

Table 5.1: Plasma concentrations of glucose, insulin and IGF-1 of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered daily (S7) or three times weekly (S3) during a 42-d preconditioning period[1] (n = 4 pens/treatment; 3 steers/pen).

	Day relative to vaccination[2]					
Item	**0**	**1**	**2**	**3**	**SEM**	***P*-value**
Glucose, mg/dL						Treatment x day
S7	77.7[a]	80.3[a]	75.1[b]	73.8[b]	2.89	0.01
S3	81.1[a]	81.8[a]	70.4[b]	73.7[b]	2.89	
P-value[3]	0.41	0.63	0.07	0.94		
IGF-1, ng/mL						
S7	106.5[a]	75.8[b]	65.5[c]	63.1[c]	4.76	0.007
S3	87.8[a]	68.7[b]	55.7[c]	63.9[b]	4.76	
P-value[3]	0.008	0.30	0.16	0.91		
PUN, mg/dL						
S7	12.1[a]	8.9[b]	9.2[b]	9.3[b]	0.54	<0.0001
S3	13.1[a]	6.1[c]	9.4[b]	5.4[c]	0.54	
P-value[3]	0.23	0.002	0.78	<0.0001		
						Day
Insulin, pmol/L	13.9[a]	20.3[b]	12.2[a]	10.3[c]	1.98	<0.0001

[a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

[1]Steers were vaccinated with Bovi Shield Gold One Shot and Ultrabac 7 (Zoetis Inc., New York, NY) on d 7, and Bovi Shield Gold 5 (Zoetis Inc., New York, NY) and Ultrabac 7 on d 21.

[2]Data presented as plasma concentrations of each measurement immediately before (d 0) and 1, 2 and 3 d after vaccination, which corresponds to the mean plasma results of d 7 and 21 (first and second rounds of vaccination, respectively), 8 and 22, 9 and 23, and 10 and 24 of the study, respectively.

[3]Comparison of treatments within each day (S7 vs. S3 steers).

Table 6.1: Mean hepatic mRNA expression of haptoglobin, IGF-1 and pyruvate carboxylase of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered daily (S7) or three times weekly (S3) during a 42-d preconditioning period[1].

	Treatments			
Item	S7	S3	SEM	*P*-value
Hepatic genes	---- *Relative fold change*[2] ----			
Haptoglobin	3.12	3.38	0.552	0.75
IGF-1	1.86	2.06	0.172	0.42
Pyruvate carboxylase	2.05	1.53	0.128	0.03

[1]Steers were randomly selected within each pen (2 steers/pen) for a liver biopsy on d 10 and 24, which corresponds the vaccination-induced peak of inflammatory response base on plasma haptoglobin concentrations.

[2]Responses were quantified based on the threshold cycle (CT) and normalized to cyclophilin CT examined in the same sample and assessed at the same time as the targets. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$), as described by Ocón-Grove et al. (2008).

Table 7.1: Positive seroconversion and serum antibody titers against bovine viral diarrhea viral type 1b of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered daily (S7) or three times weekly (S3) during a 42-d preconditioning period (n = 4 pens/treatment; 3 steers/pen).

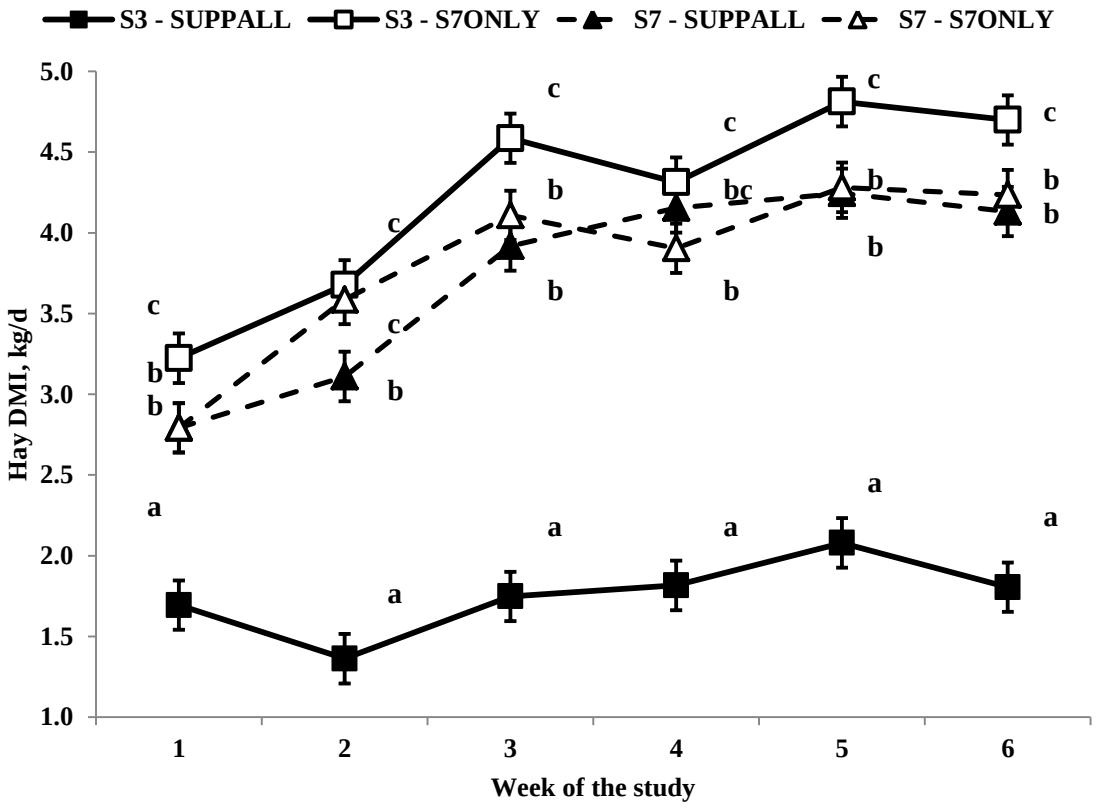
	Treatments			
Item	**S7**	**S3**	**SEM**	***P*-value**
Bovine viral diarrhea virus type 1b				
Seroconversion[2], %				Treatment x day
d 21	12.8[a]	20.5[a]	8.81	0.06
d 42	100.0[b]	78.8[a]		
				Treatment
Mean titers[2], log base 2	2.51	1.46	0.306	0.03

[a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

[1]Steers were vaccinated with Bovi Shield Gold One Shot and Ultrabac 7 (Zoetis Inc., New York, NY) on d 7, and Bovi Shield Gold 5 (Zoetis Inc., New York, NY) and Ultrabac 7 on d 21.

[2]Positive seroconversion and serum BVDV-1b titers on d -7 did not differ between treatments ($P \geq 0.23$), but were included as a covariate ($P \leq 0.003$) for the seroconversion and serum BVDV-1 titers analyses, respectively.

Figure 1.1: Daily hay DMI (kg/d) of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered daily (S7) or three times weekly (S3) during a 42-d preconditioning period.



A treatment x day x wk was detected for hay DMI ($P < 0.0001$). When all S7 and S3 steers received concentrate supplementation (SUPPALL), hay DMI was greater for S7 vs. S3 steers from wk 1 to 6 ($P < 0.0001$). When only S7 steers received supplementation (S7ONLY), hay DMI was greater for S3 vs. S7 steers from wk 1 to 6 ($P \leq 0.05$), except for wk 2 in which hay DMI did not differ ($P = 0.68$) between treatments. Hay DMI of S3 steers was less on SUPPALL vs. S7ONLY days, whereas hay DMI of S7 steers was similar between SUPPALL

vs. S7ONLY days, except for wk 2 in which hay DMI was less on SUPALL vs. S7ONLY days (Figure 1). [a-c]Within wk, means without a common superscript differ ($P \leq 0.05$).

Figure 2.1.: Plasma concentrations of cortisol (a), haptoglobin and ceruloplasmin (b) of steers provided daily free-choice access to ground tall fescue hay and similar weekly concentrate amount offered daily (S7) or three times weekly (S3) during a 42-d preconditioning period.

Figure 2.1.a:

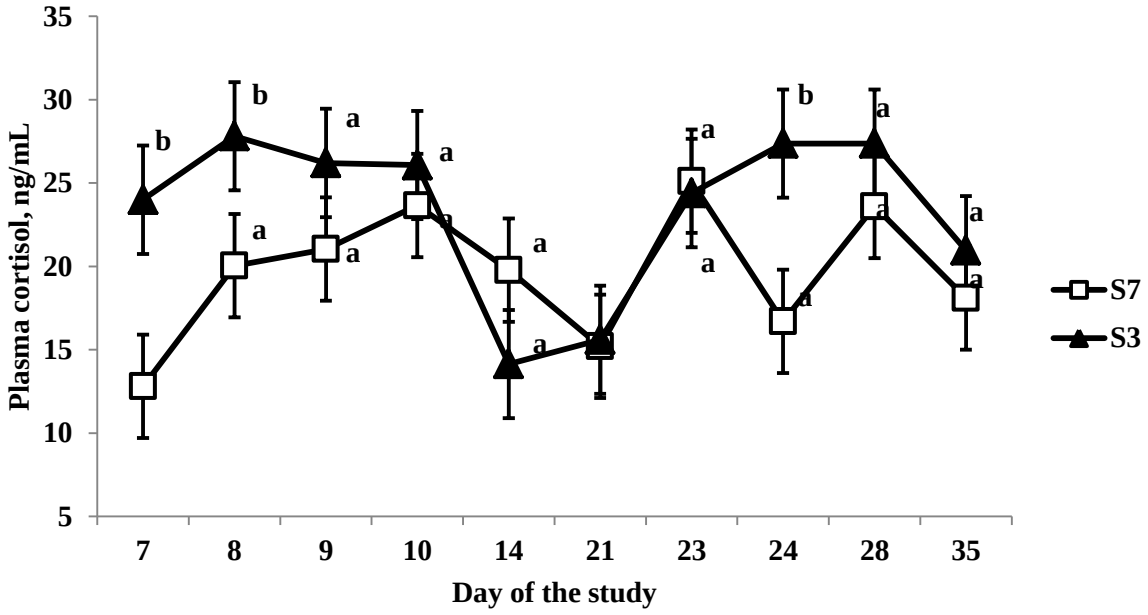
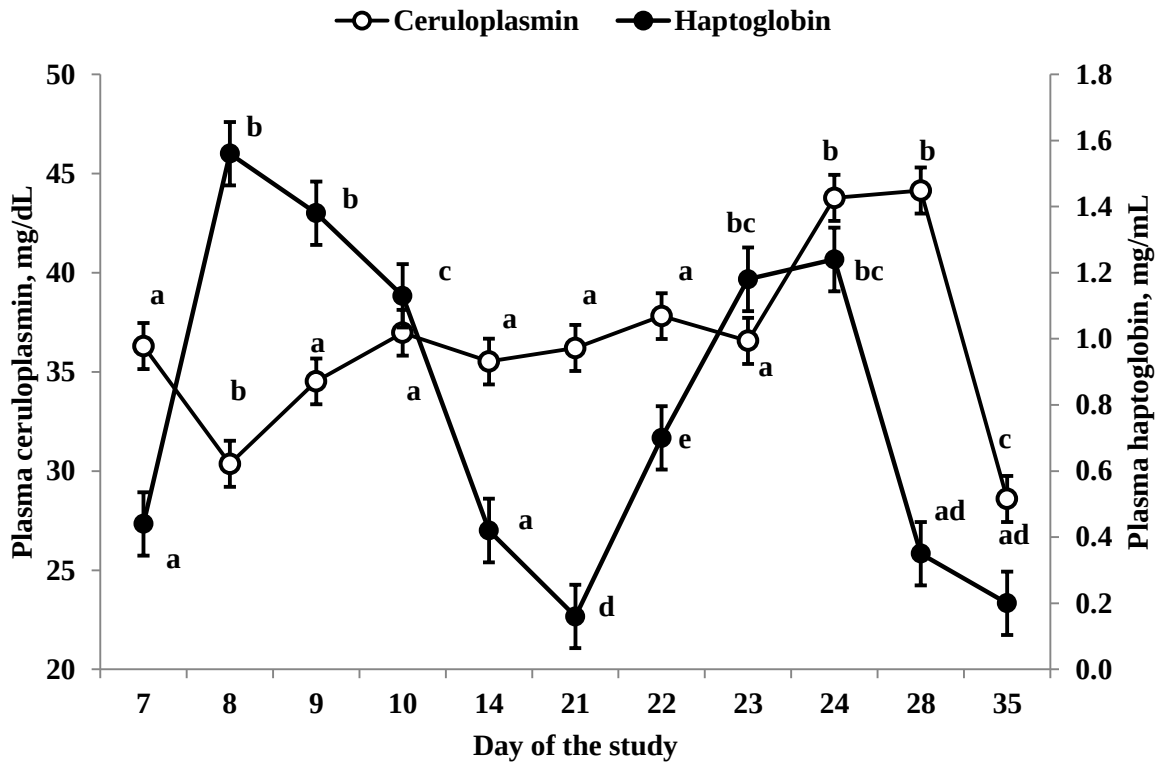


Figure 2.1.b:



A tendency for treatment x day effect was detected (P = 0.07) for plasma concentrations of cortisol, which were similar between treatments from d 9 to 23 and 28 to 35 ($P \geq 0.22$), but were greater on d 7, 8 and 28 ($P \leq 0.03$) for S3 vs. S7 steers. Effects of time ($P < 0.0001$), but not treatment x day ($P \geq 0.17$), were detected for plasma concentrations of ceruloplasmin and haptoglobin. [a-d]Within day (Figure 2a) or across days (Figure 2b), means without a common superscript differ ($P \leq 0.05$).

CHAPTER 3

Decreasing the frequency and rate of wet brewers grains supplementation did not impact growth but reduced humoral immune response of preconditioning beef heifers.

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Introduction

Wet brewers grains (WBG) are byproducts of barley brewing. Due to the removal of sugar and starch after malting and mashing processes, WBG contains greater concentrations of trace minerals than the foundation grains (Westendorf and Wohtl, 2002; Homm et al., 2008), and was an effective substitute for ground corn in supplements for preconditioning beef calves (Moriel et al., 2015a). Preconditioning beef calves typically experience processes, such as weaning, vaccination and feedlot entry that induce an acute phase protein response (APR) and impair immunity and growth (Arthington et al., 2013). Recently, we demonstrated that decreasing the frequency of concentrate supplementation from daily to 3 times weekly reduced feeding costs, but impaired growth and humoral immunity of preconditioning beef steers (Artioli et al., 2015). Hence, it is crucial to identify nutritional strategies that would enable the reduction on frequency of supplementation to decrease feed costs, without impairing growth and immunity of calves.

Growth and immunity of calves described above were likely impaired due to a greater reduction on hay DMI and fluctuations in nutrient intake of steers offered concentrate 3 vs. 7 times weekly (Artioli et al., 2015), which was expected because supplements often decrease forage DMI when supplemental TDN intake is greater than 0.7% of BW (Moore et al., 1999). We hypothesized that reducing the frequency of WBG supplementation would not impact growth and immunity of beef heifers if supplementation rate was also decreased, leading to less reduction on hay DMI and variation in nutrient consumption. Hence, our objectives were to evaluate the growth, trace mineral status and vaccine-induced innate and humoral

immunity of preconditioning beef heifers supplemented WBG at two supplementation rates (0.5 and 1.0% of BW) and frequencies (3 vs. 7 times weekly).

Materials and Methods

The Institutional Animal Care and Use Committee of NC State University (protocol #15-090-A) approved all procedures for the experiment conducted at the Mountain Research Station (Waynesville, NC; 35.48° N, 82.99° W; elevation = 659 m) from July to August 2015.

Animals, diets and sample collection. Angus heifers (n = 36; 213 ± 2 kg of BW; 254 ± 7 d of age) were weaned on d -14, immediately allocated into a single 22-ha tall fescue pasture (Lolium arundinaceum; 16% CP and 59% TDN; DM basis), provided concentrate DM at 0.5% of BW (50:50 soy hulls and corn gluten pellets; 17% CP and 72% TDN; DM basis) and free-choice access to white salt without trace mineral fortification for 14 d. On d 0, heifers were stratified by BW and age, and randomly assigned to 1 of 12 concrete floor pens (3 heifers/pen; 18 x 4 m; 24 m2/heifer) in a half-covered drylot feeding facility. Treatments were randomly assigned to pens (3 pens/treatment), in a 2 × 2 factorial design, and consisted of heifers provided daily free-choice access to ground tall fescue hay and supplemented with WBG at 0.5 or 1.0% of BW (DM basis). Within each supplementation rate, heifers were then assigned to receive similar weekly concentrate amount (weekly WBG DMI = 0.5 or 1% of BW multiplied by 7 d) that was offered either daily (7X) or 3 times weekly (3X; Monday, Wednesday and Friday) from d 0 to 42.

Hay and WBG were offered separately in the same feed bunk at 0800 h. Daily WBG offered was adjusted daily to account for alterations on DM concentration, whereas weekly

WBG offered was estimated based on average shrunk BW of each pen on d 0, and readjusted on d 21 using average full BW of each pen obtained before feeding. Individual BW was measured before feeding on d 0 and 42, following 12 h of feed and water withdrawal. Shrunk BW was not obtained on d 21 to not disturb feeding behavior and avoid an unnecessary physiological stress response due to shrink that could interfere with plasma measurements and vaccine response (Marques et al., 2012). A complete mineral mix (RU-MIN 1600, Southern States, Richmond, VA; Average composition, DM basis: 18.2% Ca, 0.72% K, 0.88% Mg, 0.76% S, 7.0% Na, 10.8% Cl, 2.9 % P, 29 mg/kg Co, 1,220 mg/kg Cu, 2,130 mg/kg Mn, 29 mg/kg Se and 2,530 mg/kg Zn) was top-dressed daily over the supplement at a rate of 0.114 g/heifer from d 0 to 42.

Hay and WBG DM offered and refused were obtained daily for each pen by drying samples of hay and WBG offer and refusal in a forced-air oven at 56°C for 48 (hay) or 72 h (WBG). Daily DMI was determined by subtracting the daily hay and WBG DM refused from the daily hay and WBG DM offered. Samples of hay, WBG and mineral mix offered were collected daily and pooled within each wk (1 to 6), and then sent in duplicate to a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY) for wet chemistry analysis of all nutrients (Table 1). Samples were analyzed for concentrations of CP (method 984.13; AOAC, 2006), ADF (method 973.18 modified for use in an Ankom 200 fiber analyzer; Ankom Technology Corp., Fairport, NY; AOAC, 2006) and NDF (Van Soest et al., 1991; modified for use in an Ankom 200 fiber analyzer; Ankom Technology Corp.). Concentrations of TDN were calculated as proposed by Weiss et al. (1992), whereas NEm and NEg were calculated using equations from NRC (2000).

On d 0, all heifers were treated with doramectin for internal and external parasites (5 mL subcutaneous; Dectomax injectable, Zoetis Inc., Kalamazoo, MI). On d 14, heifers were vaccinated against infectious bovine rhinotracheitis (IBR), bovine viral diarrhea virus (BVDV) types 1a and 2, Mannheimia haemolytica (2 mL subcutaneous; Bovi Shield Gold One Shot; Zoetis Inc., New York, NY) and clostridium (2 mL subcutaneous; Ultrabac 7, Zoetis Inc., New York, NY). On d 28, heifers received 2-ml subcutaneous boosters of Bovi Shield Gold 5 (Zoetis Inc., New York, NY) and Ultrabac 7. The vaccination protocol described above was chosen to replicate the protocol utilized by the local preconditioning alliance (Mountain Cattle Alliance, Canton, NC; Moriel et al., 2015c; Artioli et al., 2015). The vaccination protocol was initiated 14 d after feedlot entry to avoid the feedlot entry-induced inflammatory response that could interfere with vaccine response (Richeson et al., 2008).

Blood samples (10 mL) were collected via jugular venipuncture into sodium-heparin (158 USP) containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for plasma harvest 4 h after WBG supplementation on d 14, 15, 16, 17, 21, 28, 29, 30, 31, 35 and 42. The approach of collecting blood samples 4 h after feeding was utilized previously to correspond to the peak of ruminal fermentation and end products release after concentrate consumption (Moriel et al., 2012, 2015c; Artioli et al., 2015), and to correspond with days that all heifers received WBG supplementation (d 14, 16, 28 and 30) and days that only 7X heifers received WBG supplementation (d 15, 17, 29 and 31). Additional blood samples (10 mL) from jugular vein were collected into tube containing no additives (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for serum harvest on d 0 and 42 to evaluate serum antibody

titers against IBR, BVDV-1a and 2. Blood samples were immediately placed on ice following collection, and then centrifuged at 1,200 □ g for 25 min at 4oC. Plasma and serum samples were stored frozen at -20oC until later laboratory analysis.

Liver samples (100 mg of tissue wet weight) were collected via needle biopsy from all heifers on d 0 and 42, following the procedure described by Arthington and Corah (1995), and then stored at -80°C until later laboratory analyses. Samples were then assessed for trace mineral concentrations at Michigan State University Diagnostic Center for Population & Animal Health (Braselton et al., 1997). Liver trace mineral concentrations on d 0 were included to covariately-adjust liver trace mineral concentrations on d 42. Liver samples were collected only on d 0 and 42 because: (1) our goal was to evaluate the final liver trace mineral concentrations of heifers after receiving WBG supplementation for 42 d at different rates and frequencies, and (2) to avoid a surgery-induced inflammatory response in the middle term of the study that could interfere with vaccine response. Hence, the analyses of trace mineral consumption were calculated by multiplying the mean weekly DMI of hay, WBG and mineral mix by the respective weekly mean concentration of each trace mineral present in hay, WBG and mineral mix.

Laboratory analyses. Plasma concentrations of haptoglobin were determined in duplicate samples using a biochemical assay assessing haptoglobin-hemoglobin complex by the estimation of differences in peroxidase activity (Cooke and Arthington, 2013). Plasma concentrations of cortisol were determined using a single chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA).

Intra- and inter-assay CV for assays of haptoglobin and cortisol were 2.0 and 8.0, and 2.9 and 2.7%, respectively.

Serum antibody titers against IBR, BVDV-1a and -2 were determined by the Oklahoma Animal Disease and Diagnostic Laboratory using a virus neutralization test (Rosenbaum et al., 1970). Serum titers were reported as the log base 2 of the greatest dilution of serum that provided complete protection of the cells (lowest and greatest tested dilution = 1:4 and 1:256, respectively). For the seroconversion analyses, samples with serum neutralization value of < 4 were considered negative and assigned a value of 0, whereas samples with serum neutralization value ≥ 4 were considered positive and assigned a value of 1. Then the assigned values (0 or 1) were used to calculate the positive seroconversion (% of steers with positive serum neutralization; Richeson et al., 2008; Moriel et al., 2015c; Artioli et al., 2015).

Statistical analyses. Except for seroconversion, all data were analyzed as a completely randomized design using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 9.3) with Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. Pen was the experimental unit, and heifer(pen) and pen(treatment) were included as random effects in all analyses, except for analyses of hay, WBG, total and trace mineral consumption that included only pen(treatment) as random effect. Feed efficiency, ADG and mean total DMI were tested for fixed effects of supplementation frequency, rate and frequency × rate. Within each week of the study, daily DMI data was pooled by days that all 3X and 7X heifers were fed WBG (Mon, Wed and Fri) and days that only 7X heifers were fed WBG (Tue, Thu, Sat and Sun) to simplify data

analyses, interpretation and report. Daily intake of DM (WBG, hay and total), CP and NEg data were analyzed as repeated measures and tested for fixed effects of supplementation frequency, rate, week of the study, day of supplementation and all resulting interactions, using pen(treatment) as the subject. Body weight, plasma and serum measurements were analyzed as repeated measures and tested for fixed effects of frequency, rate, day of supplementation, week of the study (except for BW analyses), and resulting interactions. Compound symmetry covariance structure was used for the analyses of BW and serum titers whereas autoregressive 1 was used for the analyses of plasma concentrations of cortisol and haptoglobin as these covariance structures generated the lowest Akaike information criterion. Positive seroconversion to IBR, BVDV-1a and -2 were analyzed as repeated measures using the GLIMMIX procedure of SAS with pen(treatment) and heifer(pen) as random effects. All results are reported as least-squares means. Data were separated using PDIFF if a significant preliminary F-test was detected. Significance was set at $P \leq 0.05$, and tendencies if $P > 0.05$ and ≤ 0.10.

Results

Effects of frequency × rate × day of study, frequency x rate and frequency were not detected ($P \geq 0.21$) for BW, ADG, total DMI and G:F from d 0 to 42 (Table 2). A tendency for rate of supplementation effect was detected ($P = 0.07$) for mean BW, which was greater for heifers supplemented with WBG at 1.0 vs. 0.5% of BW (237 vs. 233 ± 1.3 kg, respectively).

Effect of frequency × rate × day of supplementation was detected ($P < 0.0001$) for WBG DMI (% relative to initial offer; Table 3). Heifers supplemented with WBG at 0.5% of

BW daily consumed 100% of the initial WBG DM offered, whereas 7X1.0 heifers consumed in average 99.6% of daily WBG DM offered, resulting in a loss of 0.4% of WBG DM offered (Table 3). On days of supplementation (Mon, Wed and Fri), 3X0.5 heifers consumed 99.8% of initial WBG DM offered, and did not consume the remaining 0.2% of WBG DM on the next day, and thus, WBG refused had to be discarded before the next WBG supplementation event. On days of supplementation (Mon, Wed and Fri), 3X1.0 heifers consumed 73.1% of initial WBG DM offered. On the following days (Tue, Thu, Sat and Sun), 3X1.0 heifers consumed only 19.5 of the 26.9% of WBG DM remaining from the previous day, resulting on a loss of 7.4% of WBG DM offered that was discarded (Table 3).

Effects of frequency × day of supplementation (Table 4) and rate of supplementation, but not frequency × rate × day of supplementation and rate × day of supplementation ($P \geq 0.42$), were detected for hay DMI ($P \leq 0.02$). Regardless of supplementation rate, 7X heifers had similar daily hay DMI throughout the week, whereas 3X heifers had greater hay DMI on days that they did not receive WBG supplementation compared to days that WBG was provided ($P < 00001$). However, hay DMI of 7X and 3X heifers did not differ regardless of the day of the week ($P \geq 0.18$; Table 4). Mean hay DMI was greater for heifers supplemented with WBG at 0.5 vs. 1.0% (3.21 vs. 2.58 ± 0.156 kg/d, respectively).

Effects of frequency × day of supplementation, but not frequency × rate × day of supplementation, rate × day of supplementation, frequency × rate, frequency and rate of supplementation ($P \geq 0.16$), were detected for intake of total DM, CP and NEg ($P < 0.0001$; Table 4). Regardless of supplementation rate, 7X heifers had similar daily intake of total DM, CP and NEg throughout the week ($P \geq 0.12$), whereas 3X heifers had greater intake of

total DM, CP and NEg on days that they received WBG supplementation compared to days that WBG was not provided ($P < 0.0001$). On days of supplementation, however, intake of total DM, CP and NEg were greater for 3X vs. 7X heifers ($P \leq 0.001$), whereas intake of total DM, CP and NEg were less for 3X vs. 7X heifers on days that only 7X heifers were fed WBG ($P \leq 0.01$; Table 4). Effects of supplementation rate were detected ($P \leq 0.02$) for intake of total CP and NEg, which were less for heifers supplemented with WBG at 0.5 vs. 1.0% of BW (0.77 vs. 1.02 ± 0.030 kg/d of CP, and 2.97 vs. 3.74 ± 0.116 Mcal/d of NEg, respectively).

Liver trace mineral concentrations on d 0 were included to covariately-adjust liver trace mineral concentrations on d 42. Effects of frequency × rate and frequency were not detected ($P \geq 0.18$) for daily intake and liver concentrations of trace minerals. Effects of rate of supplementation were detected for daily intake of Cu, Mo, Se and Zn ($P < 0.0001$), and liver concentrations of Co and Se ($P \leq 0.02$), but not for the remaining trace minerals ($P \geq 0.31$; Table 5). Heifers supplemented with WBG at 1.0% of BW had greater intake of Cu, Mo, Se and Zn, less liver Co concentrations, but greater liver Se concentrations than heifers fed WBG at 0.5% of BW. Effects of supplementation rate, but not frequency and frequency × rate ($P \geq 0.59$), were detected for mean daily S intake, which was greater ($P = 0.001$) for heifers fed WBG at 1.0 vs. 0.5% of BW (13.7 vs. 11.1 ± 0.33 g/d, respectively).

Plasma concentrations of haptoglobin and cortisol on d 0 did not differ among treatments ($P \leq 0.29$), but were included as covariates ($P \leq 0.04$). Effects of frequency of supplementation × day of the study ($P = 0.05$; Figure 1), but not frequency × rate × day of the study and rate of supplementation × day of the study ($P \geq 0.61$), were detected for plasma

haptoglobin concentrations. Plasma haptoglobin concentrations were greater for 3X vs. 7X heifers on d 14 (P = 0.006), but greater for 7X vs. 3X heifers on d 16 (P = 0.03; Figure 1). Effects of frequency of supplementation × day of the study (P = 0.05; Figure 2a) and rate of supplementation × day of the study (P = 0.05; Figure 2b), but not frequency x rate x day of the study and frequency x rate of supplementation (P ≥ 0.25), were detected for plasma concentrations of cortisol. Heifers fed WBG 3 times weekly had greater (P = 0.04) plasma cortisol concentrations on d 15 compared to 7X heifers. Heifers fed WBG at 1.0% of BW (DM basis) had greater plasma concentrations of cortisol on 17 (P = 0.05) and tended to have greater plasma cortisol concentrations on d 15 (P = 0.06) and 35 (P = 0.09) compared to heifers fed WBG at 0.5% of BW (DM basis).

Effects of frequency × rate × day of supplementation, frequency × day of supplementation, rate × day of supplementation, frequency × rate, rate and frequency were not detected (P ≥ 0.30) for positive seroconversion for IBR (44.4, 44.4, 44.4 and 50.0 ± 4.81% for 3X0.5, 7X0.50, 3X1.0 and 7X1.0 heifers, respectively), BVDV-1a (44.4, 50.0, 38.9 and 44.4 ± 5.30% for 3X0.5, 7X0.50, 3X1.0 and 7X1.0 heifers, respectively) and BVDV-2 titers (50.0, 55.6, 50.0 and 50.0 ± 2.78% for 3X0.5, 7X0.50, 3X1.0 and 7X1.0 heifers, respectively). Effects of frequency × rate × day of supplementation, frequency × rate, rate and frequency were not detected (P ≥ 0.15) for serum titers against IBR, BVDV-1a and -2. However, a tendency for effects of frequency × day of study was detected (P = 0.06) for serum BVDV-1a titers (Table 6), which was greater for 7X vs. 3X heifers on d 42 (P = 0.04). Effects of rate × day of study were detected for serum titers against BVDV-2 and IBR (P ≤

0.05), which both were greater ($P \leq 0.04$) for heifers supplemented with WBG at 1.0 vs. 0.5% on d 42 (Table 6).

Discussion

Previously, decreasing the frequency of energy supplementation from daily to 3 times weekly reduced ADG of beef steers and heifers by 10 to 21% (Cooke et al., 2008; Loy et al., 2008; Artioli et al., 2015). In the current study, decreasing the frequency of WBG supplementation from daily to 3 times weekly did not decrease ADG of heifers (regardless of supplementation rate), which is partially in agreement with our hypothesis and in accordance with others (Moriel et al., 2012; Drewnoski et al., 2011, 2014a), but it is in contrast with Artioli et al. (2015), even though these authors utilized the same preconditioning and vaccination protocols used in the current study. Discrepancies among our results and those from Artioli et al. (2015) are probably related to differences on supplement composition (high-moisture WBG vs. grain pellet-based concentrate), although breed, gender, location, forage species and quality, and potential resulting interactions among these factors may have also played a role.

High moisture supplements cause different feeding behavior compared to high-DM, grain-based supplements leading to different outcomes on forage intake (Arthington et al., 2004; Cooke et al., 2007). For instance, Arthington et al. (2004) reported that beef heifers had complete consumption of dry range cube-based supplement within 1 h after feeding, whereas heifers fed isocaloric, isonitrogenous, high-moisture molasses-based supplement had longer period for total supplement consumption (approximately 48 h). In agreement, Artioli et al. (2015) reported that steers supplemented 3 times weekly with grain-based concentrate

(50:50 soy hulls corn and gluten feed pellets) at 1% of BW consumed 100% of the supplement offered within 6 h after supplementation, whereas in the current study, 3X1.0 heifers consumed 73.1% of supplement DM on days of supplementation and only 19.5% on the next day. Consequently, hay DMI of 3X1.0 heifers was 20.5% less on days of WBG supplementation, whereas in the study of Artioli et al. (2015), hay DMI of steers fed concentrate at 1% of BW 3 times weekly was 57.1% less on days of WBG supplementation vs. days that WBG was not provided. Despite the low-starch concentration of WBG, this reduction on daily hay DMI was expected because supplements often decrease forage DMI when TDN:CP ratio is less than 7 and supplemental TDN is greater than 0.7% of BW (Moore et al., 1999). Thus, the high-moisture characteristics of WBG likely caused rumen fill effects and limited supplemental DM intake of 3X heifers on days of supplementation, whereas the relatively high spoilage rate of WBG (Moriel et al., 2015b) resulted in an offensive odor (and likely taste) that reduced intake of WBG left from previous day. This combination of high moisture and high spoilage rate of WBG caused smaller daily variation on daily supplemental DMI throughout the week and decreased the magnitude of supplement-induced reduction on hay DMI, leading to similar total DMI and ADG compared with 7X heifers. It is also important to highlight that due to the slower consumption and high spoilage rate of WBG, 0.2 and 7.4% of initial WBG DM offered were not consumed by 3X0.5 and 3X1.0 heifers, respectively, and had to be discarded. This supplement wastage needs to be accounted for when evaluating the economic feasibility of using WBG supplementation.

Due to the removal of sugar and starch after malting and mashing processes, WBG contains greater concentrations of minerals than the unprocessed grains (Westendorf and

Wohtl, 2002). Dietary concentrations of Cu, Fe, Mn, Mo and Zn increased linearly as WBG was gradually added from 0 to 45% of diet DM provided to feedlot beef heifers (Homm et al., 2008), whereas serum Se concentration was greater for feedlot beef heifers fed WBG at 34 vs. 0% of diet DM (Crickenberger and Johnson, 1982). In the current study, the concentrations of Mo, Cu, Se and Zn were greater, whereas Co concentrations were less for WBG vs. tall fescue hay (Table 1), which explains the observed differences on intake of these minerals and on liver concentrations of Co and Se as WBG supplementation rate increased (Table 6). Liver concentrations of Co was less for heifers supplemented with WBG at 1.0 vs. 0.5% of BW, which reflects the numerically less intake of Co caused by the reduction on hay DMI as WBG supplementation rate increased. Liver Se concentrations were greater for heifers supplemented with 1.0 vs. 0.5% of BW, which is in agreement Crickenberger and Johnson (1982) who observed that adding WBG (34 or 62% of diet DM) to a corn-silage based diet increased serum Se concentrations of growing beef heifers. However, liver concentrations of the remaining trace minerals were not affected by WBG supplementation rate. Wet brewers grains contain greater S concentrations than corn and fescue hay (Moriel et al., 2015a). Dietary S concentrations above 0.30% of DM may reduce Cu and Se bioavailability by associating with Mo in the rumen (Suttle, 1974; Mason, 1990; NRC, 2005). Consequently, the impact of WBG supplementation rate on dietary S-induced absorption of Cu and Se needs to be addressed. In the current study, estimated dietary S concentrations (including contributions from mineral mix) were 0.25 and 0.29% of diet DM for heifers supplemented with WBG at 0.5 and 1.0% of BW, respectively, which is close to the low end range of maximum tolerable limit for S in beef cattle diets (NRC, 2005;

Drewnoski et al., 2014b). Therefore, dietary S intake might have impacted Cu and Se as consistently reported in the literature, but it was not sufficient to prevent an increase on liver Se concentrations and decrease liver Cu concentrations of heifers supplemented with WBG at 0.5 or 1.0% of BW. Further studies evaluating the impact of greater WBG supplementation rates on growth and trace mineral status are warranted, but based on optimal dietary S concentrations to avoid reduction of intestinal Cu and Se absorption, WBG supplementation rate above 1.0% might not be recommended.

In addition, frequency of WBG supplementation used in the current study did not affect liver concentrations of all trace minerals. Hence, the liver capacity to store trace minerals was not impacted by providing WBG supplementation 3 vs. 7 times weekly. Plausible explanations for the lack of differences on liver concentrations of most trace minerals, due to WBG supplementation rate and frequency, may be an adequate initial liver concentration of trace minerals and metabolic homeostasis (Miller, 1975). All heifers were nursing their dams and had free-choice access to a commercial trace mineral-fortified salt-based supplement from birth to weaning. Liver trace mineral concentrations on d 0 imply that heifers were in adequate trace mineral status at the start of the study. For instance, liver Mo concentrations of all heifers were slightly above the normal range of 2 to 3 mg of Mo/kg of tissue DM (Anke et al., 1985). Also, animals metabolically compensate for mineral imbalances to maintain cellular concentrations of trace minerals within narrow limits (McDowell, 1989, 1992; Underwood and Suttle, 1999). Therefore, the adequate liver trace minerals status and homeostatic control mechanisms of mineral absorption likely prevented further absorption and storage of trace minerals of heifers fed WBG at different

supplementation rates (0.5 or 1.0% of BW) and frequencies (3 vs. 7 times weekly). Further studies evaluating the impact of decreasing the frequency of WBG supplementation on liver capacity to store trace minerals, in heifers with trace mineral deficiency, are warranted.

Weaning, feedlot entry and vaccination stimulate an APR leading to increased hepatic synthesis of acute-phase proteins (i.e. haptoglobin; Moriel et al., 2015c). Although cortisol can directly reduce synthesis of pro-inflammatory cytokines by leucocytes (Kelley, 1988), acute increases in circulating cortisol, such as during a stress or CRH challenge, can indirectly stimulate an inflammatory response (Higuchi et al., 1994; Cooke and Bohnert, 2011) and increase plasma haptoglobin concentrations (Cooke and Bohnert, 2011), which may be used as an indicator of inflammation when plasma concentrations are ≥ 0.11 mg/mL (Tourlomoussis et al., 2004). Therefore, the greater plasma haptoglobin concentrations of 3X vs. 7X heifers on d 15 may be partially associated with the acute rise on pre-vaccination plasma cortisol concentrations. This response was previously reported by our research group (Artioli et al., 2015) and occurred regardless of supplementation rate (0.5 or 1.0% of BW on a DM basis), which partially agrees with our hypothesis and confirms that decreasing the frequency of supplementation exacerbates the physiological stress (as indicated by greater plasma concentrations of cortisol and haptoglobin before vaccination). Feeding beef cattle high grain-based diets led to an accumulation of microbial endotoxins in the ruminal fluid that induced a general nonspecific inflammatory response and increased synthesis of acute-phase proteins (Zebeli et al., 2010). For instance, plasma haptoglobin concentrations of beef steers peaked after 3 to 9 wk of feeding starch-based diets containing (DM basis) 45 or 95% barley grain (Ametaj et al., 2009). Although WBG has relatively low-starch concentrations,

the greater supplement DMI of 3X vs. 7X heifers on days that WBG was provided to all heifers likely induced an accumulation of endotoxins in the ruminal fluid and increased hepatic synthesis of haptoglobin. This rationale might also explain the greater plasma cortisol concentrations of heifers fed WBG at 1.0 vs. 0.5% of BW (DM basis), although plasma concentrations of haptoglobin were not impacted by WBG supplementation rate. Further studies need to be conducted to evaluate the rationale of reduced frequency of supplementation causing accumulation of ruminal endotoxin.

It is interesting to notice that although 3X heifers had greater plasma haptoglobin concentrations before vaccination, the post-vaccination plasma haptoglobin concentrations were greater for 7X vs. 3X heifers on d 16 and did not differ between these treatments for the remainder of the study. Previously, our laboratory showed that reducing the frequency of energy supplementation (7 vs. 3 times weekly) increased pre- and post-vaccination plasma concentrations of haptoglobin (Artioli et al., 2015). Reasons for the discrepancy on vaccination-induced plasma concentrations of haptoglobin between the current and previous studies (Artioli et al., 2015) might be associated with the differences on supplement DM concentration (21.2 vs. 90% of DM, respectively) and time to consume the entire supplemental DM offered. Steers in the study of Artioli et al. (2015) consumed the grain pellet-based supplement within 6 h of feeding, whereas in the current study, heifers consumed the WBG offered within 24 to 48 h of feeding, which may have slowed the ruminal fermentation process. Hence, it is possible that the accumulation of endotoxins in the ruminal fluid, and consequently the APR-induced synthesis of acute phase proteins, was lessened in the current study. In support of this rationale, mean plasma concentrations of

haptoglobin reported by Artioli et al. (2015) and the current study were 0.80 vs. 0.44 mg/mL, respectively, even though the vaccination and preconditioning protocols were similar.

Neutralizing serum antibody titers may be used as an indicator of immune protection, disease prevention and vaccine efficacy in calves (Howard et al., 1989; Bolin and Ridpath, 1995; Richeson et al., 2008). Vaccination response differs from animal to animal, and depends on environmental and genetic factors, maternal antibody concentrations (Downey et al., 2013), timing of vaccination after feedlot entry (Richeson et al., 2008), metabolizable protein supply (Moriel et al., 2015c), energy concentration of maternal diet offered during late-gestation (Moriel et al., 2016) and frequency of supplementation (Artioli et al., 2015). In the present study, serum titers against BVDV-1a, but not BVDV-2 and IBR, were less for heifers fed WBG 3 vs. 7 times weekly, which is in agreement with our previous study (Artioli et al., 2015) and confirms that decreasing the supplementation frequency lessened the vaccine response. More importantly, the current study also demonstrated that decreasing the frequency of WBG supplementation reduced vaccine-induced BVDV-1a titers regardless of supplementation rate (0.5 or 1.0% of BW; DM basis). The lessened vaccine response of heifers fed 3 vs. 7 times weekly may have resulted in less immune protection against BVDV-1a and greater chances of developing bovine respiratory diseases. For instance, the majority of bovine respiratory disease cases occur within 30 d post-weaning or 14 d relative to feedlot entry (Kirkpatrick et al., 2008), whereas calves with serum BVDV-890 neutralizing titers > 4 (log2 scale) did not develop severe clinical signs of fever, leukopenia and diarrhea (Bolin and Ridpath, 1995). Similar to our previous study (Artioli et al., 2015), the lessened vaccine response may be associated with the greater pre-vaccination plasma cortisol concentrations

of heifers fed WBG 3 vs. 7 times weekly. Cortisol may induce immune suppression effects (Salak-Johnson and McGlone, 2007), weaken the innate immune response (Dai and McMurray, 1998) and block the cytokine secretion involved on antibody production (Salak-Johnson and McGlone, 2007). Hence, the exacerbated physiological stress experienced by reducing the frequency of WBG supplementation may have decreased the communication between innate and humoral immune response causing a decreased antibody production against serum BVDV-1a. The exact reasons for the lack of treatment effects on serum BVDV-2 and IBR titers are not known, and further research is needed to elucidate this lack of response.

Although heifers fed WBG at 1.0% of BW (DM basis) had greater plasma cortisol concentrations (consequently, greater likelihood for causing immune suppression effects), serum titers against BVDV-2 and IBR were greater for heifers fed WBG at 1.0 vs. 0.5% of BW (DM basis). Previously, we demonstrated that serum titers against BVDV-1a were boosted by increasing dietary concentrations of metabolizable protein offered to beef steers (115 vs. 85% of daily requirements; Moriel et al., 2015c). Hence, the greater total CP intake of heifers fed WBG at 1.0 vs. 0.5% of BW (1.02 vs. 0.77 kg of CP/d, respectively) might have contributed to the greater post-vaccination serum BVDV-2 and IBR titers. In addition, adequate dietary Se concentration is vital for immune function (Arthur et al., 2003) and might affect the resistance to infection in ruminants (Suttle and Jone, 1989). Although the contribution of other trace minerals cannot be disregarded, the greater dietary intake and liver concentrations of Se of heifers supplemented with WBG at 1.0% of BW may further explain the greater serum titers against BVDV-2 and IBR compared to heifers supplemented with

WBG at 0.5% of BW. In agreement, beef heifers administered a single injection of trace minerals (60, 10, and 15 mg/mL of Zn, Mn, and Cu, as disodium EDTA chelates, and 5 mg/mL of Se, as sodium selenite) had greater liver Se concentrations and serum titers against porcine red blood cells compared to heifers injected with 0.9% saline solution (Arthington et al., 2014). Also, serum titers against M. haemolytica and Escherichia coli were increased by i.m injections of Se in beef calves (0, 25 and 50 mg of Se; Droke and Loerch, 1989) and dairy cows, respectively (0 or 0.1 mg of Se/kg of BW; Panousis et al., 2001).

Summary

In summary, this experiment demonstrated that regardless of supplementation rate, decreasing the frequency of WBG supplementation from daily to three times weekly did not affect growth, but increased pre-vaccination plasma cortisol concentrations, post-vaccination plasma haptoglobin concentrations, and decreased vaccine-induced antibody production against BVDV-1a of beef heifers during a 42-d preconditioning period. Collectively, our results suggest that decreasing the frequency of energy supplementation during preconditioning and vaccination are not recommended as it might lead to less immune protection against pathogens associated with bovine respiratory disease. Furthermore, these results indicate that increasing the WBG supplementation rate from 0.5 to 1.0% of BW increased pre- and post-vaccination plasma concentrations of cortisol, but did not affect growth or contribute to immune suppression.

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Table 1.2: Average weekly chemical composition of ground tall fescue hay and wet brewers grains (WBG) provided to heifers from d 0 to 42[1].

Item	Tall fescue hay	WBG
DM, %	92.6	21.2
	---------- DM basis ----------	
CP, %	12.0	32.4
ADF, %	41.1	25.0
NDF, %	64.1	50.6
TDN[2], %	56.0	72.5
NE_m[3], Mcal/kg	1.07	1.71
NE_g[3], Mcal/kg	0.52	1.09
Ca, %	0.40	0.24
K, %	1.98	0.08
Mg, %	0.23	0.18
Na, %	0.01	0.003
P, %	0.27	0.62
Co, mg/kg	0.37	0.17
Cu, mg/kg	6.0	21.0
Fe, mg/kg	426	165
Mn, mg/kg	100	47
Mo, mg/kg	0.40	2.65
S, %	0.19	0.39
Se, mg/kg	0.03	0.65
Zn, mg/kg	26	86

[1]Hay and WBG samples were collected daily, pooled within each wk, and sent in duplicate to a commercial laboratory for wet chemistry analysis (Dairy One Forage Laboratory, Ithaca, NY).

[2]Calculated as described by Weiss et al. (1992).

[3]Calculated using the equations proposed by the NRC (2000).

Table 2.2: Growth performance of heifers provided, in a 2 × 2 factorial design, wet brewers grains (WBG) supplementation rate at **0.5** or **1.0%** of BW (DM basis) that was offered either daily (**7X**) or 3 times weekly (**3X**; Monday, Wednesday and Friday) from d 0 to 42 (n = 3 pens/treatment; 3 heifers/pen)[1].

	Treatment					
Item	**3X0.5**	**7X0.5**	**3X1.0**	**7X1.0**	**SEM**	***P*-value**
BW[2], kg						Freq. × rate × day
d 0	212	213	214	213	2.0	0.43
d 42	255	253	258	262	3.4	
d 0 to 42						Freq. × rate
ADG, kg/d	1.01	0.96	1.04	1.15	0.104	0.43
Total DMI, kg	185	174	192	196	10.2	0.48
G:F[3]	0.23	0.23	0.23	0.25	0.011	0.58

[a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

[1]From d 0 to 42, heifers were provided daily free-choice access to ground tall fescue hay.

[2]Body weight obtained after 12 h of feed and water withdrawal.

[3]Estimated by dividing total BW gain by total DMI from d 0 to 42.

Table 3.2: Supplemental wet brewers grains (WBG) DMI of heifers provided, in a 2 × 2 factorial design, WBG supplementation rate at **0.5** or **1.0%** of BW (DM basis) that was offered either daily (**7X**) or 3 times weekly (**3X**) from d 0 to 42 (n = 3 pens/treatment; 3 heifers/pen).

	Treatment					***P*-value[2]**
Item[1]	**3X0.5**	**7X0.5**	**3X1.0**	**7X1.0**	**SEM**	**Freq. × rate × day**
WBG DMI[3], % of initial DM offered						
Mon, Wed, Fri	99.8[b]	100.0[b]	73.1[a]	99.9[b]	0.90	<0.0001
Tue, Thu, Sat, Sun	0.0[a]	100.0[c]	19.5[b]	99.4[c]	0.90	
P-value[4]	<0.0001	1.00	<0.0001	0.68		

[a-c]Within supplementation day, means without a common superscript differ ($P \leq 0.05$).

[1]Monday, Wednesday and Friday = days when all 3X and 7X heifers received WBG supplementation; Tuesday, Thursday, Saturday and Sunday = days that only 7X heifers received WBG supplementation.

[2]*P*-value for effect of frequency × rate × day of supplementation.

[3]Dry matter intake of WBG relative to initial WBG DM offered.

[4]Treatment comparison within each day.

Table 4.2: Ingredient and nutrient intake of heifers provided, in a 2 × 2 factorial design, wet brewers grains (WBG) supplementation rate at **0.5** or **1.0%** of BW (DM basis) that was offered either daily (**7X**) or 3 times weekly (**3X**) from d 0 to 42 (n = 3 pens/treatment; 3 heifers/pen).

	Supplementation frequency				***P*-value**
Item[1]	**3X**	**7X**	***P*-value**[2]	**SEM**	**Freq. × day**[3]
Hay DMI, kg/d					
Mon, Wed, Fri	2.56	2.89	0.18	0.160	<0.0001
Tue, Thu, Sat, Sun	3.22	2.91	0.20	0.160	
P-value[4]	<0.0001	0.74			
Total DMI, kg/d					
Mon, Wed, Fri	5.58	4.40	0.001	0.166	<0.0001
Tue, Thu, Sat, Sun	3.81	4.59	0.01	0.166	
P-value[4]	<0.0001	0.55			
CP intake[5]*, kg/d*					
Mon, Wed, Fri	1.30	0.85	<0.0001	0.031	<0.0001
Tue, Thu, Sat, Sun	0.55	0.88	<0.0001	0.031	
P-value[4]	<0.0001	0.21			
NEg intake[5]*, Mcal/d*					
Mon, Wed, Fri	4.66	3.20	<0.0001	0.120	<0.0001
Tue, Thu, Sat, Sun	2.25	3.30	0.0001	0.120	
P-value[4]	<0.0001	0.12			

[1] Monday, Wednesday and Friday = days when all 3X and 7X heifers received WBG supplementation; Tuesday, Thursday, Saturday and Sunday = days that only 7X heifers received WBG supplementation.

[2]Comparison of frequency of WBG supplementation within each day.

[3]*P*-value for effects of supplementation frequency × day of supplementation.

[4]Comparison of day within each WBG supplementation frequency.

[5]Calculated as daily DMI of hay and WBG multiplied by respective CP and NEg concentrations.

Table 5.2: Intake and liver concentrations of trace minerals of heifers provided wet brewers grains (WBG) supplementation rate at **0.5** or **1.0%** of BW (DM basis) from d 0 to 42.

	Supplementation rate			
Item	**0.5%**	**1.0%**	**SEM**	***P*-value**
Intake[1]	*------ mg/d (DM basis) ------*			
Co	4.68	4.61	0.063	0.45
Cu	180	197	1.12	<0.0001
Fe	1549	1436	72.4	0.31
Mn	615	597	17.0	0.48
Mo	4.04	6.40	0.089	<0.0001
Se	4.08	4.7	0.014	<0.0001
Zn	462	530	4.8	<0.0001
Liver Concentration[2]	*------ mg/kg (DM basis) ------*			
Co	0.29	0.24	0.012	0.01
Cu	321	356	24	0.31
Fe	517	543	138	0.87
Mn	13.3	15.5	2.08	0.44
Mo	3.73	3.87	0.126	0.39
Se	2.2	2.54	0.098	0.02
Zn	195	181	10.4	0.34

[1]Trace mineral consumption was calculated by multiplying the mean weekly DMI of hay, WBG and mineral mix by the respective weekly mean concentration of each trace mineral present in hay, WBG and mineral mix.

[2]Covariate-adjusted for liver concentration of the respective trace mineral on d 0 ($P \leq 0.05$), except for Fe and Mn ($P \geq 0.27$).

Table 6.2: Serum antibody titers against infectious bovine rhinotracheitis, bovine viral diarrhea viral types 1a and 2 of heifers provided, in a 2 × 2 factorial design, wet brewers grains (WBG) supplementation rate at **0.5** or **1.0%** of BW (DM basis) that was offered either daily (**7X**) or 3 times weekly (**3X**) from d 0 to 42 (n = 3 pens/treatment; 3 heifers/pen).

	Supplementation frequency			***P*-value**	**Supplementation rate**			***P*-value**
Item	**3X**	**7X**	**SEM**	**Freq. × day[2]**	**0.5%**	**1.0%**	**SEM**	**Rate × day[3]**
Bovine viral diarrhea virus type-1a titers, log_2								
d 0	0.00[a]	0.00[a]	0.270	0.06	0.00	0.00	0.270	0.82
d 42	3.26[a]	4.17[b]	0.280		3.78	3.66	0.280	
Bovine viral diarrhea virus type-2 titers, log_2								
d 0	0.00	0.08	0.224	0.93	0.08[a]	0.00[a]	0.224	0.04
d 42	7.13	7.18	0.224		6.74[a]	7.58[b]	0.224	
Infectious bovine rhinotracheitis titers, log_2								
d 0	0.00	0.00	0.186	0.38	0.00[a]	0.00[a]	0.186	0.05
d 42	3.20	2.87	0.186		2.76[a]	3.31a	0.186	

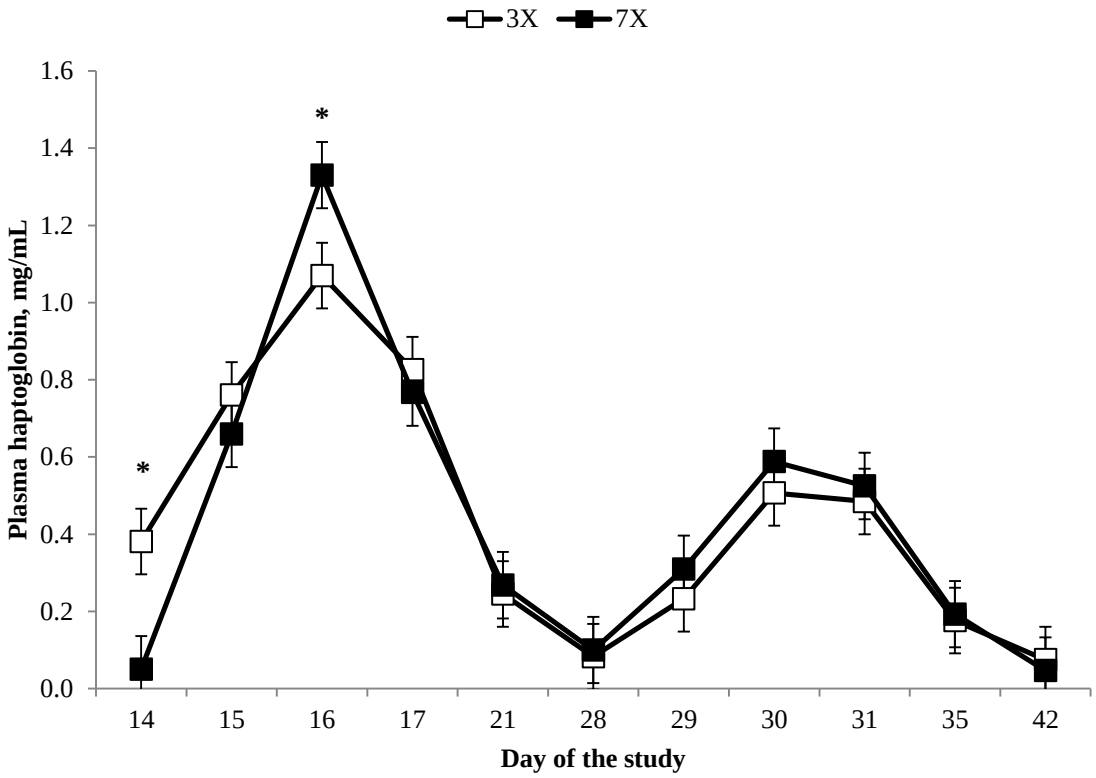
[a-b]Within a row, means without a common superscript differ ($P \leq 0.05$).

[1]Heifers were vaccinated with Bovi Shield Gold One Shot and Ultrabac 7 (Zoetis Inc., New York, NY) on d 14, and Bovi Shield Gold 5 (Zoetis Inc., New York, NY) and Ultrabac 7 on d 28.

[2]*P*-value for effects of supplementation frequency × day of the study.

[3]*P*-value for effects of supplementation rate × day of the study.

Figure 1.2: Plasma haptoglobin concentrations of heifers provided, in a 2 × 2 factorial design, wet brewers grains (WBG) supplementation rate at 0.5 or 1.0% of BW (DM basis) that was offered either daily (7X) or 3 times weekly (3X) from d 0 to 42 (n = 3 pens/treatment; 3 heifers/pen).



Means were covariate adjusted to plasma concentrations of haptoglobin on d 0 (P = 0.04). Effects of frequency of supplementation × day (P = 0.05), but not rate of supplementation × day (P = 0.61), were detected for plasma haptoglobin concentrations. Plasma haptoglobin concentrations were greater for 3X vs. 7X heifers on d 14 (P = 0.006), but greater for 7X vs. 3X heifers on d 16 (P = 0.03). *Within day, means without a common superscript differ ($P \leq 0.05$).

Figure 2.2: Plasma cortisol concentrations of heifers provided, in a 2 × 2 factorial design, wet brewers grains (WBG) supplementation rate at 0.5 or 1.0% of BW (DM basis) that was offered either daily (7X) or 3 times weekly (3X) from d 0 to 42 (n = 3 pens/treatment; 3 heifers/pen).

Figure 2.2.a:

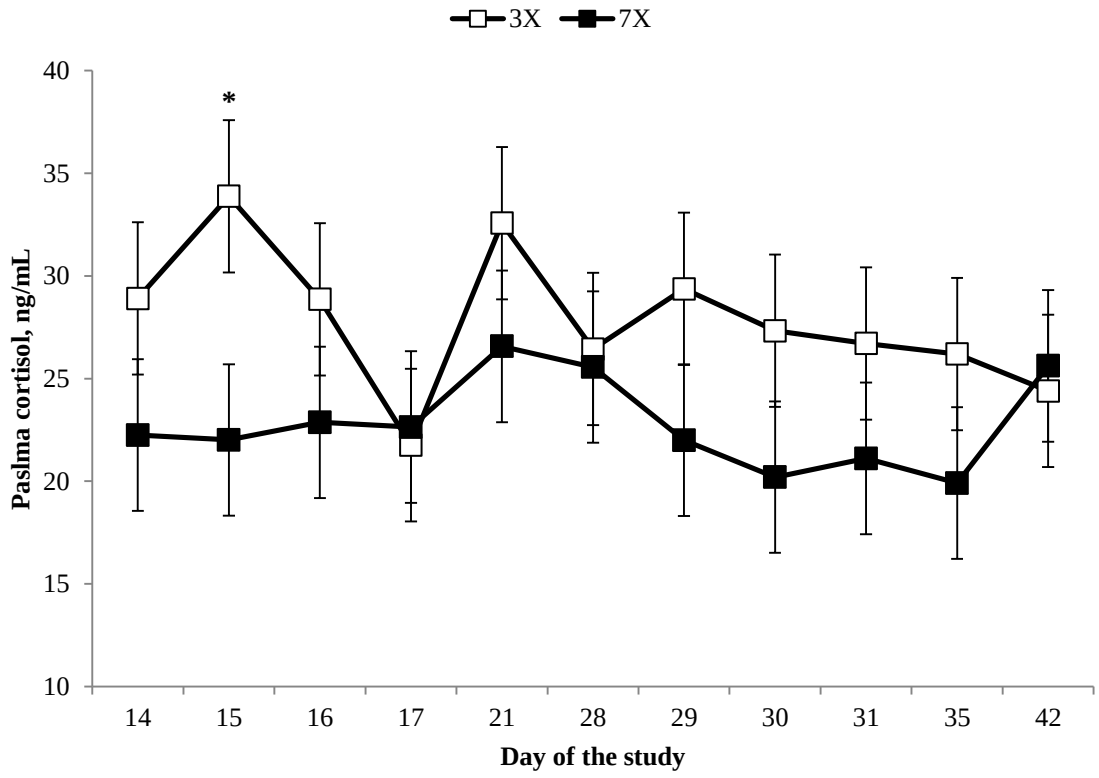
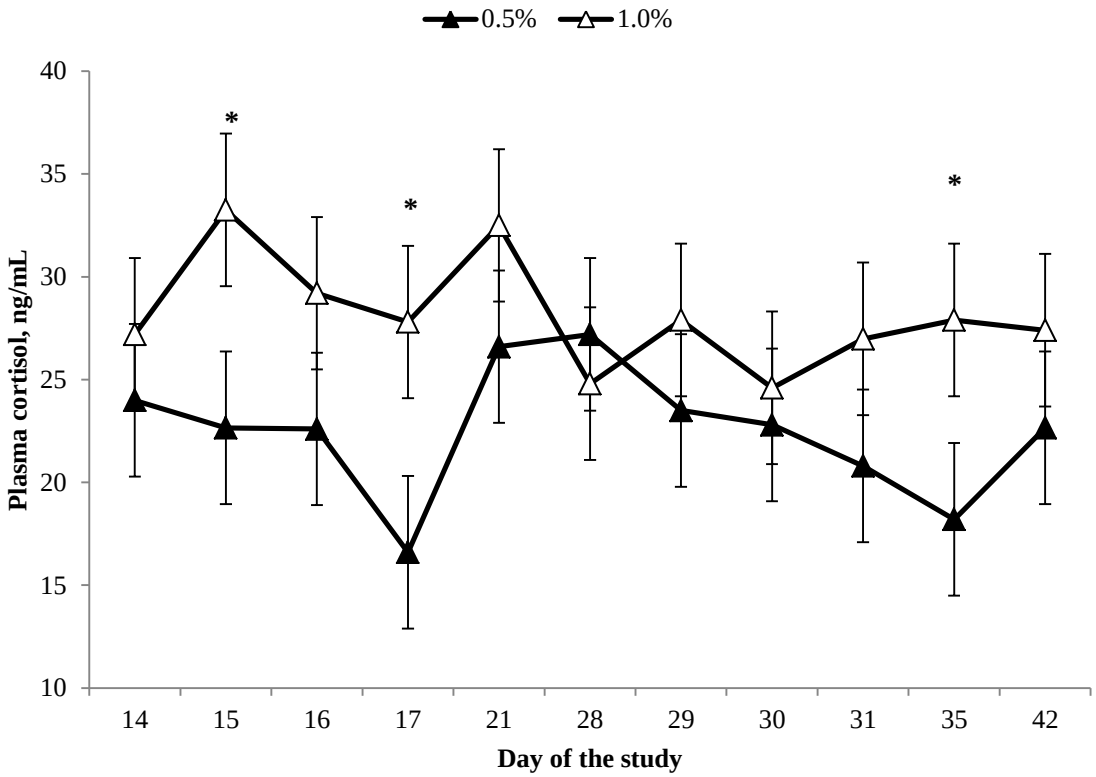


Figure 2.2.b:



Means were covariate adjusted to plasma concentrations of cortisol on d 0 ($P = 0.04$). Effects of frequency of supplementation × day ($P = 0.05$; Figure 2a) and rate of supplementation × day ($P = 0.05$; Figure 2b) were detected for plasma concentrations of cortisol. Heifers fed WBG 3 times weekly had greater ($P = 0.04$) plasma cortisol concentrations on d 15 than heifers supplemented daily. Heifers fed WBG at 0.5% of BW (DM basis) had greater plasma concentrations of cortisol on d 17 ($P = 0.05$) and tended to have greater plasma cortisol concentrations on d 15 ($P = 0.06$) and 19 ($P = 0.09$) compared to heifers fed WBG at 1.0% of BW (DM basis). *Within day, means without a common superscript differ ($P \leq 0.05$).