# EFFECTS OF FORAGE QUALITY VARIATION ON LACTATING DAIRY COWS

**THESIS**

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By

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**Abstract**

Day to day variation in forage quality, e.g. neutral detergent fiber (NDF) concentration, is substantial on dairy farms, potentially costly, and presents challenges for sampling the forage accurately. We hypothesized that variation in forage NDF concentration (FNDF) and diet variation caused by sampling error will result in decreased milk production over time, affect partitioning of nutrients, and decrease feed efficiency. Twenty-four Holstein cows averaging 73 days in milk were used in 8 concurrent replicated 3x3 Latin squares with 21 d periods. The treatments were 1) Control, 2) Variable, and 3) Overreacting. All 3 treatments were fed similar diets on average, being 24.8% FNDF, 48.2% total forage, and the forage containing 67% alfalfa silage (45% NDF) and 33% grass silage (64% NDF). Control was consistent in total forage, FNDF, and proportion of alfalfa and grass silages fed from day to day. Variable changed daily (random pattern) in proportion of alfalfa and grass silages fed which resulted in a FNDF standard deviation of 2.4 and range of 6.4 over the 21 d period. Overreacting varied in a 5 day cyclic pattern in total forage concentration resulting in a FNDF standard deviation of 2.6 and range of 7.1 over the 21 d period. Overreacting (25.1 kg/d) had higher dry matter intakes (DMI) compared to the Control (24.5 kg/d) and Variable (24.3 kg/d). Milk production (42.8 kg/d) and

gross feed efficiency were not affected by treatment. Milk production, diet sorting, DMI, milk urea nitrogen, and milk fatty acid concentrations were affected by treatment by day interactions. Milk fat (3.49%) and protein (2.80%) concentrations were not affected by treatment or treatment by day interactions. Lipolytic and lipogenic enzymes mRNA abundance in subcutaneous adipose tissue were unaffected by treatment, but were generally higher for Variable and Overreacting. Milk fatty acid markers for cellulolytic bacteria (*iso*-14:0, *iso*-15:0, *iso*-16:0), ruminal propionate (15:0) and lipolysis (18:0, long chain fatty acids) were affected by treatment by day interactions and generally followed the expected response to individual day rations and/or DMI changes. Variable had lower DMI and milk production than Control on 4 and 1 d during the 21 d period. In contrast, Variable had higher daily DMI on 1 d and milk production on 3 d compared to Control. Daily DMI for Overreacting was never lower than Control but milk yield was lower on 1 d of the 21 d period. Daily DMI and milk yields were higher on 5 d and 6 d for Overreacting compared to Control. Daily decreases for milk yields by Variable and Overreacting followed sustained 4 and 5 d of feeding a higher FNDF diet compared to Control. In contrast, increased daily milk yields versus Control for Variable and Overreacting were more frequent (3 and 6 d) and responsive to sustained diet changes (3 and 2 d). Cows in peak lactation showed no cumulative negative effects of variable FNDF for DMI, milk production, and gross feed efficiency. Changes of lipolysis indicators suggested the ability was present to buffer short term diet aberrations and/or changing DMI.

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## Chapter 1: Literature Review

## Introduction

One kg of milk (3.5% fat and 3.0% protein) contains 0.68 Mcal of net energy for lactation (**NEL**; NRC, 2001). One Mcal of NEL costs $0.19 in today's (Sept 2012) Ohio dairy feed market versus $0.08 in Sept 2003 (St-Pierre, 2012; St-Pierre, 2003). A modern dairy cow (BW ~ 625 kg) producing 45 kg of milk requires 41 Mcal of NEL per day which costs $7.79 using Sept 2012 NEL price versus $3.28 in Sept 2003. Feed costs now account for 82% of operating costs in dairy production (USDA-ERS, 2012). Improving the efficiency of energy utilization has been a concern for centuries (Reynolds et al., 2011) and improvements have obvious economic and environmental impacts. Efficiency can be defined using various methods;

*Gross Feed Efficiency (GFE):* Milk yield divided by DMI.

*Net Energy Efficiency (NEE)*: Efficiency of converting metabolizable energy (**ME**) into net energy (**NE**).

*Residual Feed Intake (RFI):* Observed energy intake minus the predicted energy intake required for the observed performance, e.g. growth, milk yields (Koch et al., 1963).

Increased DMI and milk production has greatly increased GFE as a lesser percent of feed energy intake is needed for maintenance energy; this is referred to as "dilution of maintenance" (Bauman et al., 1985; VandeHaar and St-Pierre, 2006). However, short-term GFE estimates can be biased, as failing to account for changes in body stores leads to selection of cows that have the ability to mobilize significant amounts of tissue depending on their stage in lactation (Vallimont et al., 2011). NEE accounts for the ability to convert ME energy into NEL and dairy cows are approximately 0.6 efficient (Moe et al., 1971). The difference in intake of NEL above or below what is expected (predicted) based on recovered energy change (milk production, growth) is referred to as RFI (Koch et al., 1963).

High DMI can decrease the efficiency of conversion of gross energy (**GE**) to digestible energy (**DE**) through diet digestibility depression and follows the law of diminishing returns when increasing intake of nutrients (Brody, 1945; Tyrell and Moe, 1975; NRC, 2001). The diminishing returns of increasing DMI makes continued improvement in GFE, challenging, considering that a cow needs to consume 7X maintenance to produce 90 kg/d of milk (3.5% fat) (VandeHaar and St-Pierre, 2006).

## Background

Energy losses happen during nutrient digestion, absorption, storage, mobilization, the synthesis of ATP, and the use of ATP for work functions, e.g. milk energy (Baldwin, 1968). A substantial portion of GE is undigested and lost as fecal energy (**FE**) and is usually the largest and most variable energy loss (Weiss, 2007). Losses of urine and methane energy occur during the metabolism of DE and are accounted for with

metabolizable energy (**ME**) which reflects the energy available for metabolism by the animal (Armsby, 1917). Net energy (**NE**) equals ME minus the energy loss by transforming energy into NE (entropy, thermogenesis). The energy loss during transformation of energy to NE or maintenance needs is heat increment (or diet induced thermogenesis) (Weiss, 2007). Heat increment and maintenance energy are the total heat production of an animal and can include physical activity. Table 1 illustrates the energy losses of lactating cows in one representative study (Tine et al., 2001).

Heat production and FE represent the greatest loss of energy (**Table 1**) and this has normally been observed in energy balance experiments of lactating dairy cows (Yan et al., 1997; Kirkland and Gordon, 1999). A 5% reduction in heat production would result in approximately 1.7 more Mcal available for milk energy, or $0.32, respectively, using the 2012 NEL price (St-Pierre, 2012). A cow that has reduced heat production of ~ 5% may save $117 per year. A 29% reduction in methane energy is required to achieve a similar gain which is a large change that may affect other efficiency related variables, e.g. level of milk production. Heat producing processes, e.g. tissue metabolism, stress, and protein turnover, have been identified as being different in energy losses from animal to animal and groups of animals and are estimated to account for 37% of feed efficiency variation in the beef population (Herd et al., 2004).

## US Energetic System (NRC, 2001)

The NRC (2001) estimates the diet DE by estimated diet digestibility, nutrient composition of feeds, and DMI. The level of intake and diet digestibility determines the digestibility depression estimate. The calculated DE of a diet is always equal to or less

than the weighted average DE of the ingredients (NRC, 2001). Positive associative effects may skew estimates of DE resulting in greater diet DE than the weighted average DE of the ingredients. The ME of a diet is calculated based on the diet DE concentration and diet ether extract concentration. High fiber and high protein diets will have lower efficiency for DE to ME because higher methane and urinary losses are to be expected; however, protein concentrations are not considered in DE to ME efficiency by the NRC (2001). Diet NEL is calculated based on the diet ME concentration and ether extract concentration of the diet. Long chain fatty acids (**LCFA**) are considered to be converted from ME to NEL with 77% efficiency (Andrew et al., 1991) and the NRC (2001) assumes 80% efficiency. Van Es (1975) showed that for every unit decrease in diet ME concentration (Mcal/kg), a corresponding 0.4 percentage unit decrease in efficiency of ME to NE milk energy occurs. Diets with lower ME concentrations are assumed to have higher heat loss, e.g. fermentation, increased mastication, increased gastrointestinal tract (**GIT**) size, and therefore reduced efficiency for ME to NEL (Reynolds et al., 1991; NRC, 2001).

The ME utilization efficiencies for maintenance and milk energy are considered the same (i.e., 0.64) and efficiency for replenishment of body tissue energy is 0.75 efficient (Moe, 1981; NRC, 2001). Body tissue energy utilization for milk production is assumed to be 0.82 efficient (Moe, 1981). Overall use of body tissue energy to support milk production is 0.615 efficient (0.75 body tissue replenishment * 0.82 mobilization) and accounting for the cost of maintaining the additional body tissue stores prior to mobilization should be considered (Moe, 1981).

## Energetics

Numerous attempts have been made to prove that the efficiency of ME to NE has changed or should be calculated differently with simpler (Tolkamp and Kyriazakis, 2009) or more complex models (Kebreab et al., 2003; Strathe et al., 2011). There is considerably less diet variation in the conversions of DE to ME or ME to NE than during the conversion of GE to DE (Bauman et al., 1985; Reynolds, et al., 2011) and most models empirically estimate these conversions (DE to ME, ME to NE). Energetic systems determine requirements for NE based on estimates of efficiency for ME utilization (Tolkamp and Kryrizakis, 2009).

$NE_{function} = ME * k_{function}$

Where k = efficiency, $k_l$ = milk production, $k_m$ = maintenance, $k_g$ = body tissue gain, $k_t$ = milk production energy derived from body tissue

The NRC (2001) considers NE to be used with the same efficiency for maintenance and milk production; however, diet ME and fat concentration are considered in the efficiency calculation. Kebreab et al. (2003) used a nonlinear Mitscherlich function to estimate $k_l$, $k_g$, and $k_t$ efficiencies. The $k_l$ and $k_t$ efficiencies were much lower than the NRC (2001) and the $k_g$ efficiency much higher (**Table 2**). The lower efficiencies were supported using a more complex model (Strathe et al., 2011). Tolkamp and Kryziaakis (2009) proposed simply using the same efficiency ($k_l$, $k_g$, $k_m$) except for milk energy obtained from tissue. At ad libitum intakes, ME efficiency is considered independent of feed quality by some (Tolkamp and Kryziakis, 2009) which is different than the NRC (2001). Strathe et al. (2011) found a smaller effect than expected but still a

significant effect of diet metabolizability on $k_l$. As can be observed (Table 2), differences in efficiency estimates exist, especially for deriving estimates of $k_t$ and $k_g$.

Determination of ME to NE efficiency requires calculation of ME used for maintenance, ME lost as heat increment, and recovered energy (Agnew and Yan, 2005). Differences in measurement or calculation of the amount of energy used for maintenance will greatly affect estimates of NE efficiencies (Moe and Tyrell, 1975). Two methods have been used to estimate maintenance; measured fasting heat production (**FHP**) and regressing recovered energy on ME intake and considering the intercept to be maintenance. Regressing recovered energy on ME intake is problematic because it requires estimates of $k_l$, $k_m$, and possibly $k_g$ and $k_t$ if the animal changes BW. Multi-collinearity becomes a problem when estimating multiple independent variables that are related, e.g. $k_l$, $k_m$, $k_g$, when using multiple regression analysis (Strathe et al., 2011).

The NRC (2001) estimates NEL maintenance requirements to be 0.08 NE Mcal/kg $BW^{0.75}$ which includes a 10 percent activity allowance. The NRC (2001) estimate is the same as was observed in non-lactating, non-pregnant, mature cows that averaged 0.073 NE Mcal/kg $BW^{0.75}$ nearly 50 ys ago (Flatt et al., 1965). Some suggest there is considerable evidence that the modern dairy cow's maintenance energy requirements are greater (Agnew and Yan, 2005).

Yan et al.,(1997) summarized 221 measured energy balances of Holstein/Friesian lactating dairy cows conducted between 1992 and 1995 and using multiple linear regression estimated a maintenance requirement of 0.102 NE Mcal/kg $BW^{0.75}$ (Assumed $k_m = 0.64$). Increasing dietary grass silage increased maintenance estimates which were

0.076, 0.104, and 0.113 NE Mcal/kg $BW^{0.75}$ for diets containing <50%, 51 to 99%, and 100% silage GE as a percent of diet GE (Yan et al., 1997). However, the change in NE maintenance was calculated using a constant $k_m$. This could also suggest that the $k_m$ is dependent on diet as the NRC (2001) assumes. Kirkland and Gordon (1999) fed cows a diet containing 18% straw and 82% concentrate and measured heat production using indirect calorimetry (total of 36 energy balance trials); the average maintenance was 0.0933 NE Mcal/kg $BW^{0.75}$. Notably, the diet was 21.3% CP, which is abnormally high and should decrease ME to NE efficiency (Oldham, 1984).

Multiple linear regression or other techniques put forth by Agnew and Yan (2000), Yan et al. (1997), and Kebreab et al. (2003) have resulted in higher estimates of maintenance; 0.095, 0.102, and 0.090 versus estimates of 0.073, 0.078, and 0.075 NE Mcal/kg $BW^{0.75}$ by earlier researchers (Flatt et al., 1965; Moe et al., 1970; Van Es et al., 1975), respectively. Measured FHP of non-lactating, non-pregnant cows (intakes of 9.95 and 10.55 NE Mcal/d) resulted in estimates of 0.0913 and 0.0975 NE Mcal/kg $BW^{0.75}$ (Birnie, 1999). Dry cows had an energy balance of 0.1 Mcal/d and measured heat production of 0.138 ME Mcal/kg $BW^{0.75}$ (Tine et al., 2001), and if a $k_m$ of 0.64 is assumed, a 0.0886 NE Mcal/kg $BW^{0.75}$ maintenance requirement. Recent measured FHP studies suggest the NRC (2001), using the 0.08 NE Mcal estimate, may underestimate maintenance by a maximum of 0.0175 NE Mcal per metabolic body weight (**MBW**) unit, a 22% increase. Multiple linear regression techniques on recovered energy suggest that the maximum difference is 0.022 NE Mcal per MBW unit compared to the NRC (2001), a 28% increase.

Reasons for suggesting that maintenance energy has changed include genetics, proportion of live weight as protein mass has increased, and proportion of visceral organs of live weight has increased (Johnson et al., 1990; Agnew and Yan, 2005). Estimates of NE for maintenance may have changed as well, suggesting improved modeling is needed (e.g. discounted DE is too high). Strathe et al. (2011) used year as a covariate to model genetic progress in changes in maintenance; genetic progress was not significant ($P$=0.45). Recent reviews on feed efficiency (RFI) have suggested that ruminants with improved feed efficiency do not have substantial differences in body composition (more protein mass) but that most of the variation exists in basic metabolism processes (Herd and Arthur, 2009). Agreement in predictions of NE efficiency from ME and how to estimate multiple efficiencies of NE (body tissue, milk, and maintenance) is lacking and more research might be needed (Table 2).

## Heat Increment

Heat increment represents losses of energy during nutrient absorption, transportation, and metabolism within an animal (Baldwin, 1968). Protein synthesis and turnover, $Na^+$-$K^+$-ATPase pump, substrate cycles, RNA turnover, and urea synthesis are some major mammalian energy losses (Rolfe and Brown, 1997). Some have suggested that these basal metabolic processes represent large opportunities for improving RFI; Herd and Arthur (2009) observed that protein turnover, tissue metabolism, and stress accounting for 37% of the observed variation of RFI in beef cattle. Ferrell (1986) suggested that variation of the visceral tissue and energy expenditures by visceral tissues represent opportunity for improvement. Indeed, variation in ME required for

maintenance exists between cows, with Van Es (1972) estimating a CV of 5 to 10%, and Hotovy et al. (1991) showing a range of 0.10 to 0.115 ME Mcal/kg $^{BW0.75}$ in beef animals.

## Protein Synthesis and Turnover

The cost of protein synthesis per peptide bond formation in vitro is estimated to be 5 ATP equivalents; 2 ATP for AA activation, 1 ATP for bond formation, 1 ATP for translocation, and 1 ATP for transportation, RNA synthesis, and other errors (Wright et al., 2005). Protein degradation is also costly and costs at least one ATP equivalent per peptide bond (Milligan and McBride, 1985). Regarding maintenance energetic processes, protein associated costs are thought to be the greatest requirement for ATP (Rolfe and Brown, 1997). Protein turnover in the gastrointestinal tract (GIT) is high, estimated to be 500 g/d in a 200 kg steer at a daily cost of 450 kcal (Baldwin, 1968), and is likely substantially greater in high producing dairy cows. For 1.2 kg/d synthesis of milk protein, the mammary gland must synthesize approximately 3.93 kg/d of protein to account for protein turnover in the mammary gland (Hanigan et al., 2009). Turnover of nucleic acids, e.g. RNA, in the mammary gland was shown to occur every 11 d (cows producing 12 kg/d of milk) and that enzymes vary widely in turnover with half-lives between 1 d to 24 d (Palmquist et al., 1971). More recent work has placed the cost of maintaining milk protein synthesis at 46% of the ATP required for overall mammary maintenance metabolism (Hanigan et al., 2009).

The cellular maintenance cost of whole body protein turnover as a percentage of maintenance energy expenditure in cows is estimated to be 9 to 12 % compared to 2 to 4% for lipid synthesis and turnover (Baldwin, 1980). Every 18 d, the lactating cow

synthesizes the equivalent of the protein mass of the entire body (Lobley, 2003), and the amount of synthesized protein that is retained or secreted in milk is small, approximately 33% (Lapierre et al., 2002), given the high turnover. Increased protein mass of an animal would increase energetic cost more than adipose mass due to the high cost of protein turnover. Non-lactating, non-pregnant cows that were fatter versus leaner in body condition had a lower maintenance requirement per kg of MBW than the leaner cows (Birnie et al., 2000) which provides evidence that cows with increased proportion of tissue as adipose versus protein (muscle) have reduced maintenance requirements.

Protein metabolism in hind limbs of yearling steers divergently selected for high or low growth rates are different (Oddy et al., 1998). Oxygen uptake was 36% greater for the low growth steers in the hind limb but there was no difference in protein gain (Oddy et al., 1998). High growth steers had lower protein synthesis and degradation rates than the low growth steers, suggesting a higher protein turnover by the low growth steers. Also, when intake was increased from maintenance to 1.6X maintenance, an increase in oxygen uptake by the hind limb muscle was observed only in the low growth steers. If these savings are extrapolated for the entire body muscle, approximately 70% of the differences in feed efficiency between the high and low growth steer lines were accounted for by the increased oxygen uptake related to the differences in protein metabolism (Oddy et al., 1998). Identification of why these large differences in protein turnover are occurring, e.g. genetics, may greatly reduce the energetic cost of protein turnover.

Energy plays an important role in mediating protein turnover and diet formulation can minimize use of AA for energy and decrease protein turnover. Cows fed a high energy, low protein diet, produced 24 g/d more milk protein N versus cows fed a low energy, low protein diet (Rius et al., 2010). Numerical increases in plasma alanine (12 g/d) and glutamine (5 g/d) were observed in the cows fed high energy and may indicate sparing for energy use (Rius et al., 2010). Alanine and glutamine are direct links between energy and protein metabolism (Wright et al., 2005), due to the importance of transfer of nitrogen and carbon (TCA cycle), supply of energy, and nucleic acid synthesis. For cows that consumed equal amounts of nitrogen, post-ruminally infusion with starch resulted in an increase (5 to 16%) in tissue retention of nitrogen and a reduction of heat production (2 percentage units) as a percent of consumed ME (Reynolds et al., 2001).

Other factors affecting turnover of protein are physiological status, microRNAs, and possibly some amino acids. Protein turnover as a percent of total protein in the liver of a lamb was 8.4% and 5.3% at 10 and 100 d of age (37% change), respectively (Baldwin, 1980). MicroRNAs are small noncoding RNAs (21 to 25 bp in length) that have broad regulatory functions in animals (Lau et al., 2001). Recently, micoRNAs have been identified as potent regulators of post transcriptional gene expression (Ambros, 2004), and microRNAs can affect protein synthesis and turnover of thousands of genes (Selbach, 2008). Branched chain amino acids, particularly leucine, have shown the ability to exert control on translational activity, impact the mTOR signaling pathway, and interact with energy, e.g. via insulin, when regulating protein synthesis in non-ruminants (Millward, 2012).

## Visceral Tissue

The portal drained viscera (**PDV**) and liver account for approximately 40 to 55% of body oxygen usage in lactating dairy cows and roughly 10 and 3% of body mass, respectively (Drackley et al., 2006). The gastrointestinal tract (**GIT**) tissue mass markedly increases during early lactation (Baldwin et al., 2004; Reynolds et al., 2004). Digestion, absorption, and synthesis of proteins, glucose, and urea occur at high rates in the PDV and liver; blood flow through the liver can be up to 3,000 L/h in early lactation cows (Drackley et al., 2006). In humans, reductions in the size of the GIT, e.g. liver, spleen, and kidney, are associated with a reduced metabolic heat production during aging (20 to 80 years) (Manini, 2010).

Dietary nutrients can have an effect on heat increment of the GIT. Growing beef heifers fed two different diets, high forage or high concentrate, consumed approximately equal amounts of ME (limit fed) and differed in heat production and recovered energy (NE) (Reynolds et al., 1991). The growing beef heifers were also fed differing levels of DM, low (~4 kg/d) and high (~7 kg/d) (Reynolds et al., 1991) Approximately 80 and 61% of the differences in energy for NE was accounted for by the changes in PDV oxygen use between the high forage and high concentrate diets at high and low intakes, respectively. Differences in diet NE energy concentrations were likely due to differing level of DMI and the cost of digesting and metabolizing these differences in DMI (Reynolds et al, 1991).

Some studies have not found differences in ME efficiency in response to diet ME concentrations. Cows that were fed a 70% or a 30% concentrate diet ad libitum had

similar measured heat production (percent of ME intake) (Xue et al., 2011). However, the DE to ME efficiencies were improved with the high concentrate diet (Xue et al., 2011), indicating reductions in methane and urinary energy losses. Some suggest that metabolizability does not have an effect on ME to NE efficiency when animals have ad libitum access to feed (Tolkamp and Kyriazakis, 2009; Xue et al., 2011); the Reynolds et al. (1991) study did not feed for ad libitum intakes.

The GIT changes in size and quantity of total heat produced in response to the functional workload, e.g. DMI or ME intake (Johnson et al., 1990) and lactation (Baldwin et al., 2004). However, use of energy, e.g. oxygen usage per gram of tissue, may not be related to ME intake, but rather tissue size as the tissue size does respond to ME intake (Johnson et al., 1990). The GIT mucosa of lactating (32 kg/d) and dry cows appear to use the same amount of oxygen per gram of tissue *in vitro* (McBride and Milligan, 1984; Johnson et al., 1990). The lack of a substantial change in oxygen uptake per gram of tissue (liver and GIT mucosa) was also observed in sheep (McBride and Milligan, 1985) and rats (Burin et al., 1988) at differing intakes. The energetic cost of the gut appears to be highly related to DMI, but the gut may not use more energy per unit of GIT tissue during times of differing intakes which may provide partial support that high producing cows do not become less efficient, e.g. more oxygen usage per gram of tissue.

Assuming GIT energy needs are directly related to DMI and size of tissue, increased milk production should not change the ME to NE efficiency. Flatt (1966) predicted based on the few measurements with cows consuming energy above 4X maintenance that no depression in ME to NE efficiency occurred. Holstein cows were

fed a high concentrate diet with intakes of 46.4 NE Mcal/d versus cows fed a low concentrate diet with intakes of 34.6 NE Mcal/d, and the efficiency of ME use for milk production was equivalent, 0.65 for both treatments (Yan et al., 2006). Regression of the relationship between ME intake and milk energy output with nonlinear functions does not perform well or are substantially better than using a linear function (Kebreab et al., 2003). Regression of 701 cow energy balance observations showed that with an intake range of 0.24 NE Mcal/kg $BW^{0.75}$ to 0.60 NE Mcal/kg $BW^{0.75}$, milk energy output was approximately linear in response to intake and that metabolic efficiency likely is unaffected by increased ME intake (Kebreab et al., 2003; Strathe et al., 2011).

## $Na^+$-$K^+$-ATPase Pump

A major energetic cost of cells are sodium potassium transport pumps with estimated costs being 19 to 28% of ATP usage and might be the highest contribution to cellular maintenance energy requirements (Rolfe and Brown, 1997; Milligan and McBride, 1985). Three sodium ions are pumped out of the cell and simultaneously two potassium ions enter the cell at the cost of 1 ATP to create a sodium gradient that allows nutrients to be imported into the cell. During lactation, $Na^+$-$K^+$-ATPase pump respiration rate of the intestinal epithelium was 3.6 ul $O_2$/mg of dry weight per hour versus 2.5 ul $O_2$/mg per hour of dry cows (McBride and Milligan, 1984). Sheep that were starved, fed at maintenance, or above maintenance had $Na^+$-$K^+$-ATPase transport pump respiration of 2.47, 4.48, and 6.15 ul O2/mg per hour, respectively (McBride and Milligan, 1985). McBride and Milligan (1985) estimated that in major energy expending tissues, e.g. liver and intestinal epithelium, the $Na^+$-$K^+$-ATPase transport pump will expend over one-third

of total cellular in vitro respiration and respiration energetic costs. The energetic cost varies with physiological stage and DMI, suggesting maintenance may vary as well.

Some examples of changing the energetic costs of the $Na^+$-$K^+$-ATPase pump include thermal temperature and dietary Na concentration. Gregg and Milligan (1982) showed that skeletal muscle $Na^+$-$K^+$-ATPase activity is related to thermal environment as would be expected and total oxygen respiration increased by 50% when the thermal temperature was 1° C versus 25° C and the $Na^+$-$K^+$-ATPase transport pump accounted for 79% of this increased respiration. In chickens fed high Na diets (2X requirements), feed efficiency decreased as well as the affinity of the $Na^+$-$K^+$-ATPase pump compared to chickens fed required dietary Na concentrations (Gal-Garber et al., 2003) which indicated an energetic cost of kidney excretion of Na and/or decreased nutrient uptake efficiency by the $Na^+$-$K^+$-ATPase pump. In chickens that were fed low Na diets (36% of requirements), $Na^+$-$K^+$-ATPase pump activity, feed efficiency, and Na affinity decreased (Gal-Garber et al., 2003), indicating Na was needed for absorption of nutrients. Obesity in humans and mice has been related to reductions in $Na^+$-$K^+$-ATPase pump activity (Rolfe and Brown, 1997) which would reduce heat production energy losses, though the relationship has not been fully explored.

## Substrate Cycles

Providing the optimal diet and end products of digestion (e.g. propionate, acetate) to maximize efficiency of energy transformation through substrate cycles and between body stores and milk energy is important (Moe, 1981; Baldwin et al., 1980; Bauman et al., 1985). Theoretical efficiency of ME use for milk energy is 0.76 and is different for

the synthesis of fat, protein, and lactose (0.84, 0.72, and 0.78, respectively; Baldwin, 1968). Absorbed VFA (acetate, butyrate, and propionate) contribute up to 70% of ME supply in ruminants and VFA such as propionate versus acetate can have specific effects on metabolic pathway efficiency and flux (e.g. gluconeogenesis). The theoretical conversion efficiencies for carbohydrate to body fat, lipid to body fat, protein to body fat, and protein to body protein are different: 0.80, 0.96, 0.66, and 0.86, respectively (Blaxter, 1989). Application of a mechanistic model (Baldwin, 1980) by simulating varying dietary acetate, propionate, lipid, and protein inputs yields different efficiencies for milk production or growth, suggesting room for improvement (Johnson et al., 2003). Depending on the nutrients supplied, ME requirements for maintenance can be up to 20% different in required ME due to differences in substrate cycling and efficiency of use (Baldwin, 1980).

Modeling these theoretical relationships of varying substrates and product yields and efficiency is difficult, e.g. nutrient driven interactions causing shifts in substrate cycling (Mills et al., 2001). Individual VFA have substantially different energetic efficiencies for fattening sheep (Blaxter, 1989), however, animals are usually fed mixed diets. Predicting the response of animals (milk production and composition) when extreme diet changes in composition or supply occur (short term feed restriction) can be substantially wrong by some models (Kebreab et al., 2009). Recent work has provided information on what occurs regarding nutrient regulation of biochemical pathways. Feeding steers increasing amounts of dietary propionate (pelleted sodium propionate) altered lipid metabolism related gene networks and increased N retention, suggesting that

a particular nutrient can play an influential role in modifying adipose tissue metabolism, gene transcription, and possibly changing efficiency through enhanced cell formation and function (Baldwin et al., 2012).

Conclusive evidence suggesting a relationship between diet and energetic efficiencies, ME to NE, has not been found when modeling large datasets (Agnew and Yan, 2000). A range of diet forage concentration of over 50 percentage units did not affect ME to milk NE estimates (Yan et al., 1997). As mentioned earlier, over 70% of ME is obtained from three VFA (propionate, acetate, and butyrate), and variation in end products of digestion of mixed diets might be less than expected in typical diets. When different diets are fed, differences in VFA production are usually observed. Feeding a 100% hay versus 90% concentrate diet to dairy cows changed the acetate to propionate (**A:P**) ratio from 4.1 to 2.2 (Russell, 1998) and feeding 72% versus 52% concentrate diet in another study changed the A:P ratio from 3.2 to 1.8 (Agle et al., 2010). Increase or decrease in NE intake can occur when the A:P ratio is reduced. Feeding 60% versus 91% diet concentrate to lactating dairy cows resulted in VFA production of 21.4 and 26.1 Mcal/d, respectively (Sutton et al., 2003). These diet changes are atypical as most dairy diets are between 40 to 60% concentrate (Mertens, 2009). While there is evidence that the A:P ratio can be substantially change which would possibly affect substrate cycling and energetic efficiency, implementing these extreme dietary changes are usually not practical (negative associative effects). Instead, dairy diets may have less than expected variation of produced VFA composition in typical diets which would reduce the need for mechanistically modeling VFA efficiencies for end products, e.g. milk production. Also,

if variation of A:P ratio exists, greater propionate absorption has been shown to lead to increased oxidation in the liver of dairy cows (Allen, 2000) which would lessened the expected benefit from more propionate and improved substrate cycling efficiency.

Substantial evidence exists for considering and modeling the effects of changing diet CP concentrations on energy efficiency. Microbes degrade protein in the rumen which produces ammonia. Some of the ammonia is incorporated into microbial protein and some is absorbed by the portal vein and/or absorbed through rumen epithelium and then subsequently absorbed by the liver (Lewis et al., 1957; Reynolds, 2007). The liver converts most of the absorbed ammonia (or excess rumen undegradable protein (**RUP**)) to urea at a net cost of 1 ATP per ammonia ion to safely eliminate the ammonia and to maintain normal blood pH (Lobley et al., 1995). Decreased energy efficiency was observed in high CP diets fed in excess of requirements (Moe et al., 1972). One kg of extra diet CP is estimated to cost the equivalent of 0.72 Mcal of NEL (Oldham, 1984). Some have suggested that the cost of converting ammonia to urea is not the reason for the decreased efficiency, but that the cost of maintaining the infrastructure and catabolism activities to handle excess amino acid supply reduces energy efficiency (Reynolds et al., 2011). Indeed, the synthesis of urea requires a second N atom through an amino acid, usually aspartate, which may require deamination and reduces the amino acids available for protein synthesis in the liver (Wright et al., 2005). However, providing adequate CP is important for maintaining DMI, digestible DM, efficient metabolizable protein use, and microbial protein yield, which are all factors that also impact energy efficiency.

In summary, substrates and substrate cycles differ widely in theoretical efficiencies and may influence gene transcription networks. Besides dietary fat, accounting for these theoretical differences and effects on efficiencies has not proved beneficial and is difficult. The lack of benefit from a mechanistic model might be because typical mixed diets and the products of their digestion do not vary substantially in profile but only in supply (intake) and as mentioned earlier, feeding 70% or 30% concentrate diet had no effect on ME to NE efficiency (Xue et al., 2011).

## Mitochondria

Mitochondria are subcellular organelles of eukaryotes that generate the bulk of ATP for cellular metabolism and are integral for not only energetics but amino acid and lipid metabolism, cell signaling, and apoptosis (Meisinger et al., 2008). Mitochondria are responsible for the majority of cellular oxygen consumption, estimated to be 90% (Rolfe and Brown, 1997). The mitochondrial electron transport chain is made up of five cytochrome complexes that facilitate pumping protons ($H^+$ ions) for creating a gradient that leads to ATP production. Uncoupling proteins (**UCP**) act against the proton gradient and allow protons to be leaked out of the inner membrane prior to being coupled to ATP synthesis (Rousset et al., 2004). A relationship between one or more of these UCP and improved feed efficiency has been shown in sheep, mice, chickens, and beef cows (Ojano-Dirain et al., 2007; Kelly et al., 2011; Murphy, 2012; Sharifabadi et al., 2012). In mammals, 20% of the oxygen used for ATP synthesis is thought to be respired by a leaky membrane leading to wasted heat production, estimated to be 80% of the heat generated by the cell on average (Rolfe and Brown, 1997). However, this "wasted respiring" due to

leaking protons is important for thermogenesis (maintaining body temperature depending on environmental temperatures) and preventing massive increases of ATP production that would have major downstream effects due to the rising cellular ATP concentrations (Rousset et al., 2004).

Twenty-five generations of selection for high and low heat production loss were conducted on mice and heat production differed by 56 % after selection (Murphy, 2012). The low heat production mice had improved mitochondrial efficiency by improved respiratory control and reduced uncoupling (leaking) of hydrogen ions (Murphy, 2012). In sheep, greater activity of the five respiratory cytochrome complexes were observed in the more feed efficient sheep (Sharifabadi et al., 2012), indicating less leaking of protons. During a compensatory growth period in steers, microarrays revealed up-regulation of genes encoding mitochondrial complex proteins, suggesting that improved mitochondrial ATP synthesis efficiency was part of the reason for the improved feed efficiency (Connor et al., 2010). Uncoupling protein 3 mRNA abundance (**UCP3**) tended to be increased in low feed efficient versus high feed efficient beef heifers that had similar muscle composition (Kelly et al., 2011). Low feed efficient broilers had lower respiration chain coupling activity, higher $H_2O_2$ production, and higher protein oxidation when compared to the more feed efficient broilers (Ojano-Dirain et al., 2007), which somewhat disagrees with the previous study where UCP3 (related to reduced reactive oxygen species production (**ROS**) was increased in low feed efficient animals.

During oxidative phosphorylation, ATP is synthesized by being coupled to a proton gradient which is imposed by the flow of electrons and the electrons are

eventually accepted by oxygen which then sometimes forms $O_2^-$ anions (ROS), 1 to 2% of consumed oxygen on average (Andreyev et al., 2005). Up to 9 opportunities/sites for production of ROS may exist during oxidative phosphorylation (Andreyev et al., 2005). The ROS are then converted to $H_2O_2$ by zinc-manganese superoxide dismutase in the mitochondria (Andreyev et al., 2005). The $H_2O_2$ can be a marker of ROS production and also plays important roles in signal transduction (Andreyev et al., 2005; Murphy, 2009). However, ROS contribute to oxidative stress, apoptosis, aging, and damage to various compounds, e.g. protein, DNA, etc (Murphy, 2009).

UCP2 and UCP3 appear to be less related to adaptive thermogenesis and more to ROS production and basal metabolism versus UCP1 (Rousset et al., 2004). Mild uncoupling by UCP2 and UCP3 may decrease ROS production by reducing the membrane potential and allowing more protons to flow across the inner membrane (Rousset et al., 2004). The reduced form of glutathione has been shown to regulate UCP2 and UCP3 and when bound to UCP2 and UCP3, decreases uncoupling activity (Mailloux et al., 2011) which increases ROS production. Feed efficiency might be related to decreased UCP2 and UCP3 activity but this may lead to increased oxidative stress by increased ROS production. A recent microarray experiment found 21 genes were differentially expressed (greatest difference) between high and low feed efficient bulls (Chen et al., 2011) and the 21 genes then underwent quantitative PCR. Using principal component analysis, the more efficient bulls had greater expression of xenobiotic pathway related genes; this pathway protects the liver against oxidative stress (Chen et al., 2012). From the several recent studies, we can possibly conclude that

reduced uncoupling of the mitochondrial membrane does occur in high feed efficient animals which may lead to increased ROS production, and subsequently results in increased expression of members of the xenobiotic pathway.

## Nucleotide Turnover

Protein synthesis is energetically costly as discussed earlier and the cost of synthesizing and turnover of DNA and RNA cannot be ignored in mammalian cells. The average human liver cell has roughly 13 million ribosomes compared to only 13,000 ribosomes in an average bacterial cell and requires roughly 200,000 times more energy to maintain this machinery for protein synthesis (Lane and Martin, 2010). In rat liver cells, 10% of the liver's ATP consumption was attributed to RNA synthesis and turnover (Smith et al., 2000). In mammalian cells, mRNA median half-lives are estimated to be 9 h compared to protein median half-lives of 46 h (Schwanhausser et al., 2011), and mRNA turnover is higher than tRNA and rRNA, which are all higher than DNA (Rolfe and Brown, 1997). RNA turnover has been estimated at 9.0 and 4.8% per d in the mammary glands of rats and cows (Palmquist et al., 1971). Recent research with goat mammary glands have shown a high energetic cost of total protein turnover which includes RNA turnover associated costs (Hanigan et al., 2009).

Turnover of mRNA is important for regulation of gene expression for basal and regulatory event responses and for maintaining quality mRNA (Maquat and Carmichael, 2001). Low mRNA turnover is associated with increased aging and metabolic problems in humans. Some environmental stressors such as caloric restriction enhances micro RNAs activity and turnover of mRNA which enables cells to better handle stress related

problems (Mori et al., 2012). Micro RNAs can repress protein synthesis and decrease mRNA concentrations in the cell through degradation (Guo et al., 2010). In growing beef cattle, feeding increasing amounts of propionate increased expression not only of lipogenic pathway related enzymes but also lipolytic pathway related enzymes in the adipose tissue. The changes in both pathways may indicate enhanced mRNA expression and turnover and improved cell formation and function (Baldwin et al., 2012). Dairy cows 30 d prepartum to 49 d postpartum were overfed or underfed energy prepartum compared to a control diet and showed much greater expression of the ribosome pathway at multiple time points in both the overfed and underfed cows compared to the control cows (normal energy intakes; Bionaz and Loor, 2012). The increased ribosomal pathway expression likely indicated greater enzyme synthesis and possibly turnover, which the authors speculated to result in greater energetic costs (Bionaz and Loor, 2012). Increased turnover of nucleotides (adipose tissue as an example) is energetically costly and reducing turnover of adipose tissue may reduce whole body energy maintenance expenditures by 3 to 5% in dairy cows (Baldwin et al., 1980).

## Other Heat Increment Costs

Diet induced thermogenesis and physical activity are also major energetic costs that are variable and can reduce energy efficiency (Herd et al., 2004). Diet induced thermogenesis is the increase in metabolic heat production in response to increases in absorption and metabolism of nutrients. Increased synthesis of storage compounds, e.g. glycogen, increased generation of ATP and respiration, fermentation, swallowing and rumination of food, and increased urea synthesis all increase heat production when food

is absorbed, compared to fasting when heat production is much lower (Rolfe and Brown, 1997). Dietary changes can affect diet induced thermogenesis, especially if unbalanced in non-ruminants (high diet protein concentrations); however, thermogenesis has an evolutionary adaptive mechanism of maintaining body temperature. For example, diet induced thermogenesis is linked to the regulation of UCP in mitochondria (Chang-rong et al., 2008) and the extent of proton leaking caused by diet induced thermogenesis may be related to maintaining body temperature.

Mating selection for reduced heat production in mice resulted in the mice being two times less active and the reduced activity explained 35% of the differences in heat production between the mice lines (Mousel et al., 2001). Bulls that were selected for feed efficiency were shown to walk 6% less and spent less time eating and ruminating, however, these differences only accounted for 5% of the increased feed efficiency (Richardson et al., 2000). Grazing dairy cows that produced similar daily milk yields (43 kg/d) as confinement cows, had a 17% greater daily NE cost (1.6 Mcal/d for 625 kg BW cow) because the grazing cows lied down less, walked more, and spent more time eating (Kaufmann et al., 2011). Animal to animal variation and management likely represent opportunities for changing the heat production associated with physical activity.

## Genetic Variation: Partitioning of Nutrients

Theoretical efficiencies for substrate cycling differ widely for pathways (Baldwin et al., 1980) and partitioning of nutrients, e.g. body fat mobilization, may be different from cow to cow and possibly affected by diet (Clark and Davis, 1983). Improvements in efficiency have been made through use of exogenous bovine growth hormone, which

alters partitioning and metabolism of nutrients (Bauman et al., 1985). Friggens et al. (2007) calculated energy balance on 322 cows and 637 lactation records and found variation in patterns of body energy change during lactation and that the variation is partially genetically driven within breed. Large differences in mobilization and deposition of body energy across breeds (Danish Holstein, Danish Red, and Jersey breeds) and parity also occurred (Friggens et al., 2007). Genetic merit has been shown to be related to adipose regulation and more specifically hormone sensitive lipase activity (Smith and McNamara, 1990) and genetics may affect dairy cows that differ in their partitioning of nutrients (McNamara, 2012). McNamara (2012) evaluated 126 cows fed the same diet in early lactation (0-120 DIM) using the Molly model (Baldwin, 1995; McNamara and Baldwin, 2000) and found differences in prediction of body fat accretion, body protein accretion, and visceral protein accretion among animals and that the use of energy for specific substrate cycles can vary up to 100% from cow to cow. Changes in body protein accretion, e.g. visceral protein, would be energetically costly given the low efficiency of synthesis and the high cost of maintenance of protein tissue.

## Summary

The cost of providing feed energy for milk energy production has increased dramatically in recent years and heat producing processes (minus maintenance) may account for roughly 24.3% of consumed GE (versus only 5.7% as methane) (Table 1). Energetic costs of maintenance in the modern cow may have increased, however, estimates vary widely. Heat increment, the cost of transforming ME into NE or maintenance, likely has not changed much over the past 100 y (Johnson et al., 2003),

despite increases in milk yields. Theoretical opportunities for manipulating heat increment cost have been proposed, but modeling and identification of practical strategies to reduce heat production have been somewhat elusive.

Most mechanistic nutrition models are unable to practically model these heat producing processes and predict responses to nutrients; e.g. products of nutrient digestion, responses to deliberate diet changes (feed restriction), and shifts in nutrient partitioning (lipolysis). For example, increasing small intestine absorption of *trans*-10, *cis* 12 CLA changed nutrient metabolism by decreasing de novo milk fat synthesis and increased adipose tissue accretion (Harvatine et al., 2009). Imposing a dietary deficiency of one amino acid, histidine, increased mammary blood flow by 33% and histidine transport activity 43 fold in lactating goats (Bequette et al., 2000). Modeling the cow's response to nutrients is difficult and requires further research.

Future research is needed to incorporate what we know about these heat producing processes and their response to nutrients (feed variation), genetics (ability to mobilize body fat), physiological changes (early pregnancy, lactation), and regulation (gene expression,) into a system biology modeling approach. Once we are able to practically model the system biology of a cow and predict responses, identification of strategies for optimizing the transformation ME energy into milk energy might be possible.

Short term diet variation is expected to reduce milk production and GFE. Alterations in diet that are abrupt are expected to adversely affect rumen microorganisms, digestion, and absorption of nutrients. These consequences should result in a decreased

conversion of diet GE into DE. Decreased digestion and other unknown effects may lead to decreased DMI over time. Changes in substrate cycling, partitioning of nutrients, protein synthesis and turnover, and nucleotide turnover represent heat producing reactions that possibly altered and possibly negatively affected by feed variation. Over time, continued variation of dietary nutrients should decrease consumption of diet energy and efficiency of energy conversion into milk energy by lactating dairy cows.

Table 1. Energy losses and the effects of changing the efficiency of energy use in lactating dairy cows (adapted from Tine et al., 2001)

| Item[1,2,3] | Mcal/d | % of GE Intake | 5% Improved | 25 % Improved |
|---|---|---|---|---|
| Gross Energy Intake | 101.6 | | | |
| Fecal Energy | 31.1 | 31% | $0.30 | $1.48 |
| Urine Energy | 4.0 | 4% | $0.04 | $0.19 |
| Methane Energy | 5.8 | 6% | $0.06 | $0.28 |
| Heat Production | 34.6 | 34% | $0.33 | $1.64 |
| Milk Energy | 22.7 | 22% | | |
| Tissue Energy | 3.3 | 3% | $0.03 | $0.16 |

[1]Adapted from Tine et al., 2001**,** BW~600 kg, Milk Production ~ 32 kg/d.
[2]Total NEL intake was 35.9 Mcal/d, which costs $6.82 per day when using Sept. 2012 NEL prices (St-Pierre, 2012).
[3]Savings resulting from a 5 or 25% reduction in the corresponding energy expenditure, $0.19 per NEL Mcal (Sept. 2012, St-Pierre, 2012).

Table 2. Proposed ME energetic efficiencies from recent reviews

| | NRC(2001) | Kebreab et al., 2003 | Tolkamp and Kyriazakis, 2009 | Strathe et al., 2011 |
|---|---|---|---|---|
| Efficiency[1] | | | | |
| $k_l$[2] | 0.64 | 0.55 | 0.60 | 0.58 |
| $k_t$[3] | 0.82 | 0.66 | 0.84 | 0.69 |
| $k_g$[4] | 0.75 | 0.83 | 0.60 | 0.89 |

[1]Efficiency = Proportion of ME converted to NE function
[2]$k_l$ = milk energy derived from diet ME
[3]$k_t$ = milk energy derived from body tissue ME
[4]$k_g$ = body tissue energy gain derived from diet ME

# Chapter 2: Introduction

Forages usually have the highest inclusion rates of feedstuffs in diets for lactating cows and are often the most economical source of nutrients (corn silage) for milk production (Mertens, 2006; Weiss, 2010). However, variation in nutrient composition of forages is considerable. As an example, haycrop silage NDF concentration had an average SD of 2.4 and a range of 8.5 units over 14 d periods on 8 dairy farms (Weiss et al., 2012a).

Forage NDF is a good predictor of intake and milk production of lactating dairy cows (Waldo, 1986; Allen, 2000). In high producing dairy cows limited by physical fill, excess FNDF concentrations decreases DMI, energy intake, and milk production over time. Decreasing diet FNDF concentration results in the opposite response, increased DMI and milk production if effective fiber is adequate and starch concentrations are not excessive (Allen, 2000). Low concentrations of forage NDF usually are associated with reduced effective fiber and increased fermentable carbohydrates which can increase the risk of reduced ruminal pH, altered ruminal biohydrogenation, and reduced milk fat concentrations (Zebeli et al., 2008).

Short term undersupply of a particular nutrient is theorized to result in a reduction of milk production similar to model predictions (Fadel et al., 2006). Safety factors are commonly practiced on farms and are strongly encouraged as safety factors increase the likelihood of maximum milk production (St-Pierre and Thraen, 1999). For example, when formulating a diet for FNDF concentrations, low (less than 19%) and high concentrations (greater than 25%) should be avoided to decrease the risk of acidosis and decreased DMI and milk production, respectively (Allen, 2000; Kohn, 2006; NRC, 2001).

Another diet formulation issue that is related to feed variation is sampling. Obtaining a representative sample and determining if the nutrient composition has indeed changed in a timely manner is difficult when the feed is highly variable (Weiss et al., 2012a). The most common method is sampling once a month and believing the single sample results with limited consideration for the previous samples, distribution of the nutrient values, and laboratory errors (St-Pierre and Cobanov, 2007). This can result in "overreacting", and using sample results that are not representative of the feed population within a particular storage structure over time.

Oscillating diet LCFA concentrations resulted in reduced DMI and milk production over time compared to consistent diet LCFA concentrations (Weiss et al., 2012b). The LCFA variation study suggests nutrient variation may have negative cumulative effects as some have hypothesized (Weiss and St-Pierre, 2009). In contrast, nutrient oscillation of diet CP has not resulted in cumulative negative effects on nutrient utilization and CP oscillation may actually improve nutrient utilization (Cole et al., 2003;

Archibeque et al., 2007). Also, abrupt changes in diet DM percent and diet forage concentrations did not result in cumulative negative effects on milk production in mid-lactation dairy cows (McBeth, 2012).

We hypothesize that day to day variation in diet FNDF will decrease DMI, milk production, feed efficiency, and affect partitioning of nutrients in high producing dairy cows. We also hypothesize that imposed diet formulation changes in diet FNDF that mimic poor sampling would have similar effects.

# Chapter 3: Materials and Methods

## Cows and Diets

All procedures involving animals were approved by The Ohio State University Institutional Animal Care and Use Committee. Twenty-four Holstein cows were blocked by parity (2 squares of primiparous and 6 squares of multiparous) and milk production and randomly assigned to one of 3 treatment sequences in 8 orthogonally replicated 3x3 Latin squares. Four squares started the experiment together (group 1) and a second group of four squares started the experiment 3 weeks later (group 2). Period length was 21 d and the entire experiment was completed in 84 d. At the beginning of the experiment, BW and DIM averaged 645 kg (+/- 64kg) and 73 d (+/-11d). Cows were housed in individual tie stalls, fed once daily, and milked twice daily. Diets were fed as a TMR at approximately 0300 hr for a target of about 5% refusal. Actual refusal averaged 4.1% across all treatments and cows with an overall standard deviation of 4.2% (Appendix: **Figure 36**).

The treatments were: control (**CON**), variable (**VAR**), and over-reacting (**ORR**). On average, all treatment diets were similar to CON. The CON contained two different alfalfa silages, a grass silage, and a corn grain-based concentrate mix (**Table 3**). The

grass silage was a mix of mature cool season grasses. Two independent alfalfa silages (stored in separate Harvestore silos (Engineered Storage Products Company, DeKalb, IL)) were used to reduce the variation of alfalfa silage composition. Grass silage (stored in a bottom unloading concrete silo) was used to cause changes in forage quality. Alfalfa silage B was fed until day 10 of period 3 (group 1) when the silo became empty. The CON diet was formulated to have a constant 24.8% FNDF day to day with a constant forage mixture and forage to concentrate ratio (**Table 4, Figures 1-4**).

The VAR diet was designed to be variable in FNDF concentration from day to day but to average 24.8% FNDF (Table 4, Figures 1-4) over the 21 d period. The FNDF concentration was varied by changing the proportion of alfalfa and grass silage, but the forage to concentrate ratio was held constant (Table 4, Figures 1-4). Alfalfa and grass silages daily inclusion rates were determined randomly via Monte Carlo uniform distribution simulation prior to the experiment (Figures 1-2). The same pattern was used for all periods (Appendix: **Figures 29-31**).

The ORR diet was designed to have four changes in forage to concentrate ratio and to average 24.8% FNDF concentration over the period and have a constant forage mixture (Table 4, Figures 1-4). The ORR diet mimicked using erroneous sample results to formulate a diet to maintain a target FNDF concentration when the NDF concentration of the silage really did not change. Using the hypothetically erroneous silage NDF samples resulted in four changes in forage as percent of the diet and diet FNDF concentrations (**Figure 5**). On day 1 to day 5, the forage percent of the diet was higher than the CON diet and represents formulating a diet with a lower than actual NDF silage

sample result (Figure 5). On day 6 to day 10, the forage percent of the diet was lower than the CON diet and represents formulating with a higher than actual NDF silage sample (Figure 5). On day 11 to day 15 and day 16 to day 20, a lower than actual and a higher than actual NDF sample result, respectively, were used resulting in a higher and lower forage percent of the formulated diet compared to the CON diet **(**Figure 5).

## Production Trial Sampling and Analysis

Silages were sampled daily and assayed for DM by drying for approximately 24 h at 100° C to adjust the diet for changes in silage DM. Single d samples for period 1 (group 1) and an average of 2 d samples (taken on consecutive days) for period 2 (group 1) and continuing for the entire experiment (group 1 and group 2) were used for adjusting TMR for changes in silage DM concentrations. Adjustments of the as-fed diet inclusion rates of the silages only occurred if the calculated FNDF, % of diet DM, changed by 0.25 units or more because of changes in silage DM.

Day to day changes in silage NDF concentrations were expected and would affect imposed dietary treatments. Therefore, NDF concentration of the silage was monitored during the experiment and adjustments in diet formulation were done when necessary. Silages were sampled twice weekly on consecutive days for period 1, period 2, and until day 10 of period 3 for group 1. Starting on day 11 of period 3 and continuing until group 2 finished the experiment, silages were sampled 3 d/wk (Monday, Wednesday, and Friday). Silages were dried at 100° C for 24h and then ground through a 1mm screen (Wiley Mill, Arthur A. Thomas, Philadelphia, PA) and analyzed in duplicate for NDF (Ankom200 Fiber Analyzer, ANKOM Technology, Fairport, NY) with sodium sulfite

and amylase (Sigma A3306, Sigma Diagnostics, St. Louis, MO). Adjustments of the forage inclusion rates were made if the 2 d average NDF analyses resulted in change of FNDF of 0.30 units or more. If diet grass silage NDF as a proportion of total silage NDF changed by more than 0.4 percentage units, adjustments in inclusion rates of alfalfa and grass silage were made. Starting on d 11 of period 3 (group 1) and continuing for the remainder of the experiment, adjustments of the diet were based on a 3 d weighted average NDF concentrations; 50% weight on the most recent sample and 25% weight on each of the previous two samples.

Variation in diet composition occurs primarily through diet inclusion and nutrient composition, and we monitored ingredient inclusion daily (entire experiment) and silage nutrient composition for 80 d of the 84 d experiment by compositing the 80 d samples into 16 samples that were assayed for DM, CP, NDF, ash, and lignin. Silage samples were taken daily and composited for 5 d sub periods (day 1-5, day 6-10, day 11-15, and day 16-20) of each experimental period. Silage composite samples were dried at 55° C for 48h and then ground through a 1mm screen (Wiley Mill, Arthur A. Thomas, Philadelphia, PA). Samples were analyzed for DM (100° C oven for 48h), in duplicate for NDF (Ankom200 Fiber Analyzer, ANKOM Technology, Fairport, NY) with sodium sulfite and amylase (Sigma A3306 Diagnostics, St. Louis, MO), ash (600° C overnight), and particle size (as-fed basis) using the Penn State Particle Separator (**PSPS**) (Lammers et al., 1996). Silage composite samples were further analyzed in duplicate for CP and lignin using wet chemistry procedures by Cumberland Valley Analytical Services (Hagerstown, MD). Silage composite samples (5 d sub-periods) were composited into

period samples (n=3) for group 1, periods 1-3, and analyzed by wet chemistry for acid-detergent insoluble CP, 30-h *in vitro*- NDF digestibility (Goering and Van Soest, 1970), Ca, P, Mg, K, Na, Fe, Mn, Zn, and Cu by Cumberland Valley Analytical Services (Hagerstown, MD). Silage composite samples (5 d sub-period composites) were composited into 4 samples by period (group 1's period 1 to 3, and group 2's period 3) and analyzed for long chain fatty acids (**LCFA**) (Weiss and Wyatt, 2003).

Concentrate samples were taken weekly and composited by period. Concentrate samples were analyzed for DM (100° C oven for 48h), in duplicate for NDF (Ankom200 Fiber Analyzer, ANKOM Technology, Fairport, NY) with sodium sulfite and amylase (Sigma A3306 Diagnostics, St. Louis, MO), ash (600° C overnight), and starch (Weiss and Wyatt, 2000). Crude protein, Ca, P, Mg, K, Na, Fe, Mn, Zn, and Cu were analyzed using wet chemistry procedures by Cumberland Valley Analytical Services (Hagerstown, MD).

## Feed Refusals

Refusals were sampled on day 3, 6, 9, 12, 15, 18, and 21 of each period and assayed for DM (100° C for 48h). Refusal DM content was not measured daily, but we hypothesized that the VAR and ORR diets resulted in daily changes in refusal DM content because of changes in ingredient inclusion rates (e.g., silage or concentrate) that was related to diet DM concentration and as-fed refused amount. Diet DM concentration and refusal as-fed amounts were used as independent variables in multiple or simple linear regression to estimate refusal DM concentration by day for each cow. If the independent variables (diet DM content or refusal as-fed amount) were significant ($P <$

0.05), the equation using diet DM and/or refusal as-fed amount as independent variables was used to estimate daily refusal DM concentration. If the model was not significant, the average of the 7 samples (within cow) collected during the period were used as the refusal DM concentration for each day of the respective period. Refusal daily DM concentrations were estimated for 26 cow periods using linear regression and 46 cow periods had constant refusal daily DM concentration based on the average of seven refusal DM samples.

## Sorting Behavior

Feed was mixed using a fork in the feed bunk 4 h after feeding, sampled, and analyzed in duplicate using the Penn State Particle Separator (**PSPS**) (Lammers et al., 1996) on day 5, 6, 10, 11, and 15 for group 1 multiparous cows (n=9) during all 3 periods. Particle sizes of duplicate TMRs for each treatment were also assayed on those days. The proportion of as-fed mass on each screen (top screen, middle screen, or pan) of the TMR was divided by the proportion of respective screen of the samples collected 4 h after feeding to evaluate sorting (Leonardi and Armentano, 2003). In addition, the TMR was constructed and particle size analyzed on day 18 for group 1, periods 1-3, and day 5, 10, 15, and 18 for group 2, period 3, to show the assayed TMR particle size over the entire experiment (Appendix: **Figure 33**).

## Milk Production

Cows were milked twice daily (0200 and 1300 hr) and weights were recorded. Milk was sampled (a.m. and p.m) on day 2, 5, 6, 7, 10, 12, 13, 15, 17, and 20 of each

period and assayed for milk fat, protein, lactose (B2000 Infared Analyzer, Bentley Instruments, Chaska, MN), and milk urea nitrogen (**MUN**) (Skalar SAN Plus segmented flow analyzer, Skalar Inc., Norcross, GA) by DHI Cooperative Inc. (Columbus, OH). A subsample of milk (p.m.) from group 1 and group 2 multiparous cows (n=18) was taken on day 2, 5, 7, 10, 12, 15, 17, and 20 of each period and assayed for fatty acids using a 2-step procedure for methylation (Jenkins, 2000) with separation by gas-liquid chromatography using a CP-SIL88 capillary column (100 m x 0.25 mm x 0.2-µm film thickness; Varian Inc., Palo Alto, CA). The selection of sampling days for milk composition measurements was based on the ORR 5 day changes in forage concentration of the diet (Figure 5). Day 2, 7, 12, and 17 represents the second d after an abrupt change in forage concentration of the diet and day 5, 10, 15, and 20 is the last day of a five day cycle of higher or lower diet forage concentrations. Cows were weighed on 2 consecutive days prior to the experiment and on the last 2 days of each period. Cows were body condition scored by three independent people (averaged) at the beginning of the experiment and on day 21 of each period.

## Adipose Biopsies

Subcutaneous adipose tissue biopsies from the tail-head region were obtained from 8 multiparous cows on day 20 of each period approximately 5 h after feeding (0800 hr) using a procedure similar to Fincham et al. (2009). The biopsy site alternated sides between experiment periods. Before biopsy, the cow was placed in a chute equipped with a head restraint and the tail-head region was clipped and cleaned with a surgical scrub. Lidocaine (2%; 10 mL) was used for local anesthesia and a 3 to 4 cm sagittal

incision was made in the skin near the gluteal area immediately ventral to the tail head with a #10 scalpel blade. Subcutaneous adipose tissue (approximately 500 mg) was excised aseptically using a combination of blunt and sharp dissection with metzenbaum scissors. Excised samples were immediately evaluated visually and tissue that did not appear to be adipose (fascia) was removed with a scalpel. Remaining adipose tissue was snap frozen in liquid nitrogen and stored at -80° C. The incision was then closed with surgical staples and a topical antibiotic ointment was applied. Incision sites were monitored daily for infection (none observed) and skin staples were removed 10 d later.

Subcutaneous adipose tissue could not be obtained from one cow for all three sampling periods and data from a different cow were removed for period 3 due to mastitis. This resulted in only 8 cows for periods 1 and 2, and 7 cows for period 3, respectively.

Total RNA was extracted from snap-frozen adipose samples using a method recently described by Swank (2012). Briefly, samples (mean= 90 mg; SD = 8.5) were lysed using a TissueLyser LT (Qiagen; Valencia, CA). Each sample was placed in a 2 mL microcentrifuge tube containing a 5 mm stainless steel bead and 1000 μL of buffer RLT (Qiagen; Valencia, CA); mechanical and chemical lysis were achieved by operating the TissueLyser LT for 5 min at 50 Hz. Tissue lysates were then processed using RNeasy mini kits (Qiagen; Valencia, CA) to extract total RNA from biopsy samples using the procedures recently described by Rinaldi et al. (2010). RNA purity and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies;

Rockland, DE). The 260/280 ratio averaged 1.97 (SD=0.09), the 260/230 ratio averaged 1.27 (SD=0.72), and yield averaged 0.03 µg total RNA/mg tissue (SD=0.017).

Single-stranded cDNA synthesis was done according to the method of Swank (2012). For each sample, 0.5 µg of RNA (contained in 11.3 µL) were first denatured in DNase/RNase free water for 12 min, at 70°C; samples were then placed on ice for 3 min. Reverse transcription followed immediately. Reverse transcription master mix (8.7 µL) contained: 4 µL of M-MLV RT 5x buffer (Promega; Madison, WI), 2 µL of 0.1 *M* dithiothreitol, 1 µL 10 m*M* dNTP mix (Promega; Madison, WI), 1 µL of oglio(dT)15 primer, 0.5 µL M-MLV reverse transcriptase (Promega; Madison, WI), and 0.2 µL of RNase inhibitor (Promega; Madison, WI). The entire volume of master mix was added to each well of denatured RNA (11.3 µL) in a 96 well PCR plate for a final reaction volume of 20 µL (Bio-Rad; Herculese, CA). Reverse transcription was carried out in an iQ5 Multicolor reverse transcription PCR Detection System (Bio-Rad; Hercules, CA) with cycle conditions at 40°C for 1 h, followed by 95°C for 10 min. Resultant cDNA was then diluted 1:1 with addition of 20 µL of DNase/RNase free water. The cDNA was vortexed, aliquoted (5 µl each) into tubes, and stored at -20°C until further use.

For later tests of primer efficiency and reference gene stability, two samples of 1:1 cDNA stock (9 µl each) were combined for each treatment (CON, VAR, ORR). These 3 pooled stock aliquots were each further diluted to 1:10, 1:100, and 1:1000 using DNase/RNase free water and stored at -20°C until further use.

Due to limited adipose cDNA availability from animals in this experiment, previously collected and stored cDNA (extracted and processed as described above) from bovine mammary fat pad (Essselburn, 2012) was used in initial testing of gene primers.

Genes evaluated included; abhydrolase domain containing 5 (ABDH5), acetyl-coenzyme A carboxylase alpha (ACACA), adipose triglyceride lipase (ATGL), fatty acid synthase (FASN), fasting-induced adipose factor (FIAF), hormone-sensitive lipase (HSL), leptin (LEP), lipoprotein lipase (LPL), perilipin (PLIN), stearoyl-CoA desaturase (SCD), and thyroid hormone responsive spot 14 (S14) (**Table 5**). Housekeeping genes evaluated were adenylosucciante lyase (ADSL), glyceraldehyde 3-phosphate dehyrodgenase (GAPDH), kelch-like ECH-associated protein 1 (KEAP1), ribosomal protein S9 (RPS9), and tripartite motif containing 41 (TRIM41) (Table 5).

Primer sequences for HSL (Sumner and McNamara, 2007), FIAF (Mamedova et al., 2010), ABHD5, FASN, S14, LPL, LEP, ACACA, SCD (Peng, 2011), RPS9 and GAPDH (Swank, 2012), were obtained from previous studies; sequences are presented in (Table 3**).** Primer sequences for ATGL, PLIN, KEAP1, TRIM41, and ADSL (**Table 6**) were designed using Primer 3 software (v. 0.4.0). Newly designed primer sequences were queried in the *bos taurus* genome database, which is maintained online by the National Center for Biotechnology Information (NCBI) via use of the BLAST tool. This confirmed specificity of designed primers for genes of interest.

Primer efficiencies were determined for each primer pair by the use of serial dilutions of cDNA (1:1, 1:10, and 1:100), using the following equation: percent

efficiency = $(10^{(-1/slope)} - 1) * 100$. Primer efficiency across all genes averaged 91% (SD = 8.6%, Minimum = 76%) (**Table 7**).

Quantitative reverse transcription PCR (qPCR) was performed on all samples in duplicate using a master mix of 0.25 ul for each forward and reverse primer, respectively, 10 ul of iQ SYBR Green Supermix (Bio-Rad; Hercules, CA), and 8.5 ul of RNase/DNase free water. The resulting master mix was added to 1 ul of cDNA (1:1 stock) from each respective sample for a total reaction volume of 20 ul. Assays were performed using an iQ5 Mulicolor Real Time PCR Detection System (Bio-Rad; Hercules, CA) with the following conditions; 1 cycle at 95°C for 5 min, 40 repeating cycles of 95°C for 10 s (denature) and then either 55, 57.5, or 60°C for 45s (anneal and extend; Table 7). In addition to cDNA samples from experimental cows, each assay included controls for each gene. These were no template controls and no reverse transcriptase controls; when used, 1 ul of the respective control was added to the master mix instead of cDNA. The no template control was RNase/DNase free water; the no reverse transcriptase control was sample that underwent the reverse transcription protocol without the enzyme; in essence, no cDNA. Samples were repeated (qPCR) if the resulting CV was greater than 2.8%. Average duplicate sample SD and CV were 0.19 and 0.66% Ct values. If duplication of samples did not occur within the same amplification run (e.g. 2 Ct values), the sample result Ct was not used for data analysis.

Melting curve analyses were performed after each qPCR assay to determine whether primer dimers or genomic DNA contamination were present during the assay. Melting curves for PLIN indicated that primer dimers occurred. Several attempts were

made to optimize assay conditions for this gene, to no avail. Therefore, PLIN was excluded from the study.

In addition to melting curve analyses, two other quality control checks were included in qPCR experiments - product size verification and product sequence determination. For product size verification, representative samples of qPCR products for each primer pair were electrophoresed on a 2% agarose gel to confirm purity and size of each amplicon. All qPCR products were visualized as single bands that corresponded with predicted product size (Appendix: **Figures 38-40**). Sequencing of qPCR products took place at the Molecular and Cellular Imaging Center (MCIC; OARDC, Wooster, OH). For this, qPCR products for each gene (n=1) were purified with a MinElute PCR Purification Kit (Cat. #28004, Qiagen; Valencia, CA). DNA concentrations of eluents were then measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Rockland, DE); each sample was diluted to 0.6 ng/ul per 100 bp. Per MCIC instructions, forward primers for each gene (from unused primer stock tubes) were diluted to 5ng/ul. Diluted DNA eluents (6 ul) and the appropriate forward primer (3 ul) were combined. This process was repeated for all samples to be sequenced; samples were then delivered to MCIC for sequencing. Returned sequence data were then compared to the predicted *bos taurus* sequence for each gene by use of the online BLASTN tool from NCBI and homology algorithm (Altschul, 1997); sequence homology averaged 98% (Minimum = 94%) (Appendix: **Table 26**).

Target genes of interest were normalized to the geometric mean of ADSL and KEAP1 (target gene Ct – reference genes CT = $\Delta$ Ct) (Vandesompele et al., 2002). The

Ct values for ADSL, KEAP1, Trim41, RPS9, and GAPDH were evaluated using GeNorm software (Vandesompele et al., 2002). The M stability values were 0.476 (ADSL), 0.476 (KEAP1), 0.814 (Trim41), 0.895 (RPS9), and 1.399 (GAPDH). Based on M stability values, the decision was made to use ADSL and KEAP1 as reference genes. Lower M values indicate higher stability and Peng (2011) found the M value to be approximately 0.26 and 0.22 for ADSL and KEAP1 in non-lactating, non-pregnant dairy cows, which is more stable that what we observed. ADSL and KEAP1 were determined to be unaffected by treatment and period using a mixed model and have been shown to be stable reference genes in other studies (Peng, 2011; Ji et al., 2012). Delta Ct data were transformed to $2^{(-\Delta Ct)}$ to represent fold differences relative to the reference genes (Velayudhan et al., 2008). Data was not normally distributed (Shapiro Wilk's Test), so all genes were then log(e) transformed after multiplying by 100 prior to statistical analysis.

A different statistical analysis of gene expression data was also performed using the geometric mean of the reference genes (ADSL and KEAP1) as a covariate (Martinez et al., 2004). Normalizing data with a regression based approach allows for a non-zero intercept which adapts to changes in the relationship between the target genes and geometric mean of the reference genes (Poehlman, 2002). Target gene Ct value LSM were estimated using a mixed model by using the reference genes as a covariate. Results will not be discussed, but are presented in the appendix (Appendix: **Tables 24 and 25**).

## Statistical Analysis

One cow on the ORR treatment was diagnosed with clinical mastitis and was removed on day 16 of period 3. Data from period 3 for this cow were discarded except

for sorting data. Based on data analysis, there was evidence that one cow was eating from the feed bunk of another cow that was on a different experiment during period 2, day 17-21, and for the entire duration of period 3. DMI was estimated for the experimental cow for day 17-21 of period 2. The difference between the day 1 to day 16 of period 2 average DMI and the observed day 17-21 for the 2 cows was calculated. The differences then were averaged across the 2 cows, and then added to the observed DMI for day 17 to 21 of period 2 for each cow. Period 3 DMI was discarded for this cow. A second cow also appeared to be eating from the feed bunk of another cow on this experiment. For the cow that was probably eating feed from the other cow, the period 3 milk production and components and DMI data were removed as the cow was consuming another treatment diet than what was assigned. For the cow that was having its food eaten by another cow, the period 3 DMI was removed but not period 3 milk production as the cow was consuming the assigned treatment diet. For statistical analysis, 70 cow-periods were used for milk production and components (CON=24, VAR=23, ORR=23), and 68 cow-periods were used for intake related measurements (CON=22, VAR=23, ORR=23).

Diet nutrient composition was determined using daily feed data, daily DM samples, 5 d composites for silages, and period composites for concentrate; this resulted in 462, 483, and 483 rations for the CON, VAR, and ORR, respectively. Daily $NE_L$ intake was calculated using the NRC (2001). Period average BW by cow, diet composition for the experimental day, and daily DMI (for calculating the discount factor) were used. Milk energy concentration was determined based on the average DHI

analyses of the 4 sub-periods, day 1-5, day 6-10, day 11-15, day 16-21. Energy balance was calculated by subtracting daily milk energy yield and maintenance requirements (period average BW) from daily $NE_L$ intake.

The experimental design was a replicated 3 x 3 Latin Square with two groups of replicated squares, four squares within group, three periods, and three treatments. Six statistical models were used to analyze the data using the MIXED procedure of SAS (v9.3; Cary, NC). The average CV (within cow-period) for milk production, milk composition, sorting behavior, and DMI were calculated. The degrees of freedom listed are not adjusted for missing observations (these were considered in the actual analysis) and prior to the Kenward-Rodgers adjustment.

Period average CVs were analyzed with the following model and Fisher's protected LSD ($P<0.10$) with Kenward-Rodgers adjustment were used to compare treatment period CVs.

$$Y_{ijklm} = \mu + T_i + L_j + TL_{ij} + g_k + p_l + c_{m(k)} + e_{ijklm}$$

Where: $Y_{ijklm}$ = dependent variable

$\mu$ = population mean

$T_i$ = fixed effect of treatment (2 df)

$L_j$ = fixed effect of parity (1 df)

$TL_{ij}$ = fixed effect of treatment and parity interaction (2 df)

$g_k$ = random effect of group (1 df)

$p_l$ = random effect of period (2 df)

$c_{m(k)}$ = random effect of cow (group) (22 df)

$e_{ijklm}$ = residual error (42 df)

Treatment means and treatment by day means were determined for milk production, milk composition, DMI, and diet composition. The chosen covariance structure of the residual errors was compound symmetry which was based on the Bayesian Information Criterion. Treatment by day means were compared using Fisher's Protected LSD only if treatment by day interaction was significant ($P$<0.10) using the SLICE option.

$$Y_{ijklmn} = u + T_i + D_j + L_k + TD_{ij} + TL_{ik} + TLD_{ijk} + g_l + p_m + c_{n(l)} + cp_{nm} + e_{ijklmn}$$

Where: $Y_{ijklmn}$ = dependent variable

$T_i$ = fixed effect of treatment (2 df)

$D_j$ = fixed effect of day, repeated measure (20 df)

$L_k$ = fixed effect of parity (1 df)

$TD_{ij}$ = fixed effect of treatment by day interaction (40 df)

$TL_{ik}$ = fixed effect of treatment by parity interaction (2 df)

$TLD_{ijk}$ = fixed effect of treatment by parity by day interaction (40 df)

$g_l$ = random effect of group (1 df)

$p_m$ = random effect of period (2 df)

$c_{n(l)}$ = random effect of cow (group) (22 df)

$cp_{nm}$ = random effect of cow by period interaction (46 df)

$e_{ijklmn}$ = residual error (1336 df)

Treatment means and treatment by day means for milk fatty acids were determined using the following model (only multiparous cows were sampled for milk

fatty acids). The covariance structure of the residual error was compound symmetry. Treatment by day means were compared using Fisher's Protected LSD only if treatment by day interaction was determined to be significant ($P$<0.10) using the SLICE option.

$$Y_{ijklmn} = \mu + T_i + D_j + TD_{ij} + g_k + p_l + b_{m(g)} + c_{n(m)} + cp_{nl} + e_{ijklmn}$$

Where: $Y_{ijklmn}$ = dependent variable

$\mu$ = population mean

$T_i$ = fixed effect of treatment (2 df)

$D_j$ = fixed effect of day (7 df)

$TD_{ij}$ = fixed effect of day by treatment interaction (14 df)

$g_k$ = random effect of group (1 df)

$p_l$ = random effect of period (2 df)

$b_{m(g)}$ = random effect of block (group) (4 df)

$c_{n(m)}$ = random effect of cow (block) (12 df)

$cp_{nl}$ = random effect of cow by period interaction (36 df)

$e_{ijklmn}$ = residual error (354 df)

Gene expression data representing fold change were log transformed and analyzed using the following model (only 8 multiparous cows in group 1 were sampled). Cow and period were considered fixed effects because the number of observations was too small to estimate random effects, predicting gene expression in other cows was not a goal, and inference was only for detecting whether differences occurred in these cows and the periods of this experiment. Fishers Protected LSD was only used to compare treatment and period means if treatment and period were significant ($P$<0.10).

$Y_{ijk} = u + T_i + P_j + C_k + PT_{ij} + e_{ijk}$

Where $Y_{ijk}$ = dependent variable

$\mu$ = population mean

$T_i$ = fixed effect of treatment (2 df)

$P_j$ = fixed effect of period (2 df)

$C_k$ = fixed effect of cow (7 df)

$PT_{ij}$ = fixed effect of period x treatment (4 df)

$e_{ijk}$ = 9 df

Feed sorting data were averaged across period within cow and treatment and the period CVs were analyzed using the following model (only 9 multiparous cows in group 1 were sampled).

$Y_{ijkl} = \mu + T_i + p_j + b_k + c_{l(k)} + e_{ijkl}$

Where: $Y_{ijkl}$ = dependent variable

$\mu$ = population mean

$T_i$ = fixed effect of treatment (2 df)

$p_j$ = random effect of period (2 df)

$b_k$ = random effect of block (2 df)

$c_{l(k)}$ = random effect of cow (block) (6 df)

$e_{ijkl}$ = residual error (15 df)

Treatment means and treatment by day means were estimated on feed sorting using the following model. Treatment by day means were compared using Fisher's

Protected LSD only if treatment by day interaction was determined to be significant ($P < 0.10$) using the SLICE option.

$$Y_{ijklm} = \mu + T_i + D_j + TD_{ij} + p_k + b_l + c_{m(l)} + cp_{mk} + e_{ijklm}$$

Where: $Y_{ijklm}$ = dependent variable

$\mu$ = population mean

$T_i$ = fixed effect of treatment (2 df)

$D_j$ = fixed effect of day (4 df)

$TD_{ij}$ = fixed effect of treatment by day interaction (8 df)

$p_k$ = random effect of period (2 df)

$b_l$ = random effect of block (2 df)

$c_{m(l)}$ = random effect of cow (block) (6 df)

$cp_{mk}$ = random effect of cow by period interaction (16 df)

$e_{ijklm}$ = residual error (95 df)

# Chapter 4: Results and Discussion

No parity by treatment interactions were found and parity effects were as expected (e.g., milk yields were less for primiparous compared with multiparous) and will not be discussed.

## Forage Composition

Differences in silage composition were substantial and important for implementing the VAR treatment (Table 3). The alfalfa silages, A and B, were much lower in NDF concentrations and higher in $NE_L$ 3X than the grass silage **(**Table 3). The assayed ADICP percent of DM of the alfalfa silages A and B were greater than tabular values (NRC, 2001) and resulted in less than expected differences in $NE_L$ concentrations compared to the grass silage (Table 3). The particle size distribution varied between the silages with the alfalfa silages having less top screen particles than the grass silage (Table 3).

## Forage Composition Variation

The CV for silage single day DM samples were similar to slightly less than the average CV that was observed in 8 farms over 14 d periods for haycrop silage but much

less than the CV of 14.7% observed in farms over a 12 month period (Weiss et al., 2012a) (**Table 8**, **Figures 8-10**). If a 7 d simple moving average is applied to the data for alfalfa silage A and B, and grass silage the range is reduced by 10, 5, and 10 units respectively, a reduction of 30, 34, and 50% (Figures 8-10). The largest 1 day change was 14.2 units by alfalfa silage B (Figure 8).

Single day sample NDF concentration variation was similar for alfalfa silage A and grass silage but much higher for alfalfa silage B (Table 8, **Figures 11-13**). The silo for alfalfa silage B was nearly empty and the feeding rate per day was higher than the other silage feeding rates which may have increased the NDF concentration variation. The NDF CV for alfalfa silage A and grass silage are lower than what was observed over 14 d periods in haycrop silage and 67% and 64% less than the CV over a 12 month period in alfalfa silage (Weiss et al., 2012a). The largest change between consecutive samples (5 days apart) was 11.0 units by alfalfa silage B (Figure 12). The largest change on consecutive day samples was 7.8 NDF units by grass silage. During a 7 d feeding period and 3 samples per silage, all silages decreased in NDF concentration by an average of 8.5 units (Figures 11-13).

Daily silage samples were composited into 5 d samples and analyzed for DM and NDF. Five day composites reduced the CV by 12 to 37% and range by 28 to 58% for DM and NDF versus single day samples. The reduction in variation is likely from sampling error and/or the effect of averaging day samples. Independent daily duplicate samples of haycrop silage reduced variation by 13 to 25% in a previous study (Weiss et

al., 2012). The five day composites reduced the variation in NDF concentrations, but trends are evident from the data which indicate control limit changes should be detected.

From Feb 5 to Feb 26, the 5 day composites increased in NDF concentration for 4 consecutive samples (Figure 13), a 6.9 unit change. In comparison, from Feb 7 to Feb 29, the single day samples increased in NDF concentration for 5 of the single day samples and decreased for 2 of the single day samples for a cumulative change of 9.8 units. In large herds, the optimal estimated control limit change is a 1.2 SD change (average of 2 samples) (St-Pierre and Cobanov, 2007). Using SD estimates from the 5 d samples and single day samples of NDF concentration over the entire experiment, we evaluated when a "control limit change" of 1.2 SD (average of 2 samples) from Feb 5 to Feb 29 for the grass silage would occur. Of the four 5 d composite samples, 3 samples would be considered false alarms (Feb 10, 16, and 26) and 1 sample (Feb 21) would have indicated a population change in NDF concentration. Of the seven 1 day samples, 5 samples would be considered false alarms (Feb 8, 14, 21, 28, and 29) and 2 samples (Feb 15 and 22) would have indicated a population change in NDF concentration. The single day samples resulted in more control limit changes and the change was quicker (Feb 15 and 22) versus the 5 day samples (Feb 21). Five day composite samples may be useful for reducing sampling error but detecting "control limit changes" might be delayed which is also potentially costly.

In summary, the data showed that sampling of silage on a given day for DM or NDF could result in a wide range of possible values with a range of 20.1 and 11.9 units for DM and NDF over the 3 month period. Generally, variation was similar or less than

what was observed over 14 d period on dairy farms but much less than a 12 month period. The experimental silages represented 1 and/or 2 cuttings versus a 12 month period likely representing different years and/or multiple cuttings of haycrop silage.

## Diet Composition

The objective of the VAR was to evaluate forage quality variation and this was achieved by varying concentrations of alfalfa and grass silage of the diet. The average NDF and CP concentrations of the forage fed were nearly equal for the CON and VAR treatments for the 84 d experiment (**Table 9**). The VAR forage SD for NDF and CP were higher than the most variable commercial farm in a previous study (Weiss et al., 2012a). The VAR forage $NE_L$ 3X concentration SD was slightly higher than the CON forage, suggesting the NRC(2001) predicted similar $NE_L$ variation regardless of the grass or alfalfa silage percent of the forage (Table 9).

The objective of the ORR was to evaluate changes in forage to concentrate ratio caused by formulation errors that resulted from errant samples (Figure 5). The composition and variation of the forage of the ORR was nearly equal to the CON treatment (Table 9). The diet forage concentration SD of the ORR was increased by 129% and the range by 125% versus the CON forage concentrations (Table 4). Changes in forage concentrations can result in adverse changes in physical effective fiber and nonstructural carbohydrate intake (Stone, 2004).

Diet FNDF concentration SD and range were increased by 85 and 49% in the VAR and 100 and 85% in the ORR versus the CON (**Table 12**). The SD and range for

diet CP were increased by 163% and 138% in the VAR versus the CON but the SD and range for the ORR diet was similar to the CON diet (Table 12).

The diet $NE_L$-3X or $NE_L$-adjusted for intake were similar in variation regardless of treatments (Table 12). Ash and lignin contain no energy and the diet concentrations of these were negatively correlated to diet FNDF concentrations in the VAR diet (Appendix: **Table 28**) which will reduce NEL changes from changing diet FNDF concentrations. Also, diet NEL adjusted for intake had a weaker relationship with diet FNDF than diet NEL 3X which shows that changing DMI also reduced the diet NEL variation (Appendix: Table 28). ORR diet NEL variation was slightly greater than the CON and VAR diets as major nutrients, FNDF, ash, lignin, were positively related and reflect changing dietary forage concentrations in the diet (Appendix: **Table 29**). Variation in major nutrients, e.g. NDF, lignin, and ash change estimates of NEL (Weiss, 1997), but major nutrients of the experimental diets were not highly correlated, which greatly reduced the diet NEL variation. Changing DMI likely reduced the variation even more by reducing the calculated digestibility depression when DMI decreased while increasing the digestibility depression when DMI increased which altogether has opposing effects when NEL-3X changes in response to more or less diet fiber concentrations (Appendix: **Tables 27-29**).

## Diet Particle Size Composition

The average distribution of as-fed particles was nearly equal for the three treatment diets (**Table 13**). Compared to previous haycrop silage based diets, the as-fed particles on the top screen is lower than some studies (Calberry et al., 2003; Bhandari et

al., 2007; Bhandari et al., 2008) but is within the recommended range of 2 to 8% for the top screen (Heinrichs and Kononoff, 2002). The CV of the top screen was increased greatly for the VAR and ORR compared to the CON.

## Diet Sorting

**Table 14** presents the period particle sorting results at 4 h after feeding. This time after feeding was chosen because it is the time when a diet is expected to show the greatest extent of sorting (Maulfair et al., 2010). Data represent only 5 d of the 21 d period, and the VAR had 3 days of lower grass diet concentrations, 1 day of similar grass diet concentrations, and only 1 day of high grass concentrations compared to the CON (Figure 3). Based on the top screen selection, excessive sorting occurred 4 h after feeding for all treatments and the sorting was higher than what has been observed in some other studies (Bhandari et al., 2008). However, sorting of diets is reduced the longer feed is left in the bunk and by 24 h, might be similar regardless of the original diet particle sizes (Maulfair et al., 2010). The VAR selected more for the top screen particles ($P<0.01$) than the CON (Table 14). Reducing the particle size of the top screen by reducing the forage of the diet may increase the selection of the top screen particles by making the concentrate more accessible, e.g. less long particles (De Vries, 2007). Also, when 10% of the fed diet is on the top screen versus 4% of a different diet (e.g. less grass silage), a similar 2 percentage unit change from sorting (decrease) will result in vastly different selection estimates, 80% versus 50 % sorting selection. Reducing the forage of diet, e.g. less long particles, (De Vries, 2007) and/or reducing fed diet top screen

particles, e.g. less grass silage, affects sorting selection, and in our experiment, changing grass silage likely had similar effects.

The CV of middle screen selection was higher for the VAR versus CON ($P$=0.03), indicating treatment increased variation in selection of middle screen particles, but treatment did not affect the CV of the other sorting indices (Table 14). Middle screen selection likely was more variable due to the changing particle size of VAR diet (Table 13) as the cows changed their sorting behavior in response to the daily diet. Sorting behavior can respond immediately to a diet change in forage to concentrate ratio (within 1 day) (De Vries, 2007). The lack of increased sorting CVs for the top screen by the VAR might be a result of the high CV and range for the CON diet or because only a small percentage of the diet mass was present on the top screen (~8%) (Table 13).

Treatment effects on top and middle screen sorting ($P$<0.05, $P$<0.05) were observed on given days of the experimental period (**Figures 14-16**). The VAR sorted the diet more on days grass silage was lower in concentration than the CON (day 5, 6, and 15; $P$<0.05). The ORR also tended to follow the same pattern, more sorting when diet forage was reduced (day 10, $P$=0.04). Decreasing the particle size of the top screen by decreasing forage content of the diet increased sorting selection of the top screen (De Vries, 2007; McBeth, 2012) and changing the particle size of the diet by changing grass concentration and/or reducing the fed diet mass on the top screen may have the same effect. Middle screen sorting was decreased when grass silage and forage concentration increased on day 11 for the VAR and ORR compared to the CON ($P$=0.02, 0.05) and increasing forage decreases middle screen sorting (De Vries, 2007). In summary, the

imposed daily treatment diets resulted in sorting activity that in most cases agreed with other studies and indicated the cows quickly changed their sorting behavior when differing daily dietary treatments were imposed. Diet particles retained on the top screen are positively related to chewing time, mean ruminal pH, and milk fat percent in most situations (Yansarie et al., 2004; Yang and Beauchemin, 2006; Yang and Beauchemin, 2009).

## Intake

Period average DMI was not different between the CON and VAR ($P$=0.65) but the ORR consumed more DMI than the CON and VAR ($P$=0.06, $P$=0.02; **Table 15**). Variation of daily DMI within cow (CV) was not affected by treatment over 21 d ($P$=0.17). However, the imposed daily treatments of changing grass or forage concentration resulted in expected treatment by day effects for DMI and NEL intake ($P$<0.01, $P$<0.01). In high producing cows, physical fill usually limits DMI, and increasing diet FNDF (forage or grass) should lower DMI (Allen, 2000). When decreasing diet FNDF (forage or grass), DMI increases in most situations for high producing cows as well (Allen, 2000).

The VAR diet NEL concentration was significantly higher on day 14, 15, and 21 ($P$<0.05) compared to the CON (**Figure 18**) when the grass silage percent of the forage was 0%, 4%, and 2%, respectively (Figure 2). The highest concentration of grass silage for the VAR treatment were fed on day 1 and 19, and calculated diet NEL concentration did not differ. Lower DMI reduces the digestibility discount (NRC, 2001) and high concentrations of grass silage reduced DMI for several days. The ORR diet NEL

concentration was less for day 2-5 ($P$<0.03) and day 11-15 ($P$<0.01) and greater for day 17-20 ($P$<0.05) than the CON diet NEL. The changing ORR diet NEL generally followed the changing dietary forage pattern, except for day 6 to 10 when the increased DMI resulted in no gain in calculated diet NEL concentration versus the CON diet NEL.

DMI was lower for the VAR during 4 days of the 21 d experiment, day 10, 13, 19, and 20 ($P$<0.06; **Figure 17**) and the VAR grass silage percent of the diet was higher by 20 units or more compared to the CON diet on these days. Increasing the ratio of grass silage to red clover silage decreases intake linearly (Moorby et al., 2009). The highest FNDF concentration of VAR diet occurred on day 10, and only 2 consecutive days of the 21 d period was the FNDF greater than 26% (Figure 4). The largest 1 d intake decrease in DMI by the VAR cows was 2.6 kg of DM on day 13, resulting in a decrease of 3.6 NE Mcal (Figure 18). The intake of NEL by the VAR cows was lower on the same days that DMI was lower, day 10, 13, 19, and 20 ($P$<0.02), and was a function of reduced DMI as the calculated energy concentration of the diet did not differ on these days.

The DMI of the VAR was only higher on day 6 ($P$=0.04; Figure 17) but the VAR diet grass concentration was 20 units lower during 4 d compared to the CON cows and diet. Two consecutive days of 0 and 4% of the forage as grass silage (day 14 and 15) did not elicit an increase in DMI by the VAR cows compared to the CON cows ($P$=0.74 and 0.14), respectively. The largest 1 d increase in DMI by the VAR cows was 2 kg on day 14 compared to day 13, and was a change of 3.1 Mcal (Figure 18).

Grass silage is strongly related to physical fill as it is less fragile and more buoyant in the rumen than legume silage. These factors should increase retention time

and decrease particle size reduction in the rumen with grass particles compared to legume silage (Allen, 2000; Kammes and Allen, 2012c). The grass silage of this experiment was longer in particle length than the alfalfa silages. The 30-h IVNDF of the grass silage was low (41 %) as is expected with mature grass silage. The rumen turnover time of indigestible NDF is high for grass silage versus alfalfa silage (Kammes and Allen, 2012a) and longer particles of grass silage increases the turnover time more and decreases passage to the duodenum (Kammes and Allen, 2012b). The slower turnover, digestion, and rumen removal of grass silage, especially indigestible NDF, likely resulted in the high grass silage diets taking several days for the physical filling constraint to be reduced, which is apparent given the no change in DMI following 2 d of reduced grass silage on day 14 and 15. The VAR cows daily DMI responded to increased grass silage more than increased alfalfa silage (4 d vs. 1 d) as the negative filling effect of grass silage likely is not confined to 1 day.

On average, DMI by cows on the ORR was greater than the other treatments ($P$=0.06; Table 15). The DMI was not different from the CON for day 1-5 or day 11-15 indicating that the physical fill of the increased forage was not limiting DMI even at higher diet FNDF concentrations (Figure 17). However, the cows responded to higher concentrate and reduced forage on day 6- 10 and day 16-20 with greater DMI being observed on day 6, 7, and 8 ($P$<0.03) and on day 19 and 20 ($P$<0.06) compared to the CON (Figure 17). ORR intake of NEL was reduced during the one higher forage diet cycle (day 11-15) for day 12-15 ($P$<0.02) compared to the CON and reflects the lower diet NEL concentration and numerical lower intakes by the ORR. Intake of NEL was

increased every day of the decreased forage cycles ($P< 0.04$) and was a function of increased DMI for day 6-10 and increased DMI and diet NEL concentrations for day 16-20.

Diet forage concentrations changes affect physical fill and satiety mechanisms through changes in gut fill, altered VFA production, changing ruminal pH, and liver metabolism of end products such as propionate (Hoover, 1986; Allen, 2000). Increasing the forage to concentrate ratio by 23 units decreased intake 1.7 kg/d (Voelker et al., 2003) and we observed an average numerical decrease of 0.6 kg/d (day 11 to d 15), suggesting the response was not large enough to be significant. The ORR consumed 1.3 kg/d more than the CON when forage was reduced (day 16 to 20). In high producing cows, time spent ruminating and reducing particle size of NDF may be the limiting DMI factor versus physical fill and removal of indigestible NDF in moderate producing cows (Voelker et al., 2003). The top screen as-fed particle increased 20% during the high forage diet cycle (day 11-15) but decreased 32% during the lower forage cycle (day 16-20) compared to the CON. The DMI increase when more concentrate was fed indicates the cows were likely restricted by physical fill, possibly particle size reduction, and removal of indigestible NDF versus the fermentation and absorption of acids (Allen, 2000).

## Milk Production

Period average milk production and the daily variation within cow (CV) was not affected by treatment ($P$=0.34 and $P$=0.24), respectively (Table 15). Energy-corrected milk (**ECM**) and efficiency per unit of DMI was similar across treatments ($P$=0.21 and

*P*=0.38), respectively. The period average milk, fat, protein, and lactose, percentages and yields, and milk urea nitrogen (**MUN**) were not affected by treatment (Table 14). Variation in milk component percentages and MUN variation within cow (CV) were not affected by treatment.

The imposed daily treatments of changing grass or forage concentration did result in treatment day interactions for MUN and yields of milk, ECM, and milk components as would be expected. However, no treatment day interaction was observed for milk component percentages.

The VAR had only 1 d of lower milk production, day 21 (*P*=0.06; **Figure 19**), and this followed 4 consecutive days when the FNDF concentration was 1.5 units or greater than the CON. Grass silage as percent of the diet forage was 66% on day 1, 59% on day 10, and 56% on day 13 versus the CON diet and failed to elicit a change in milk yield compared to the CON. Substituting grass silage for alfalfa silage and /or increasing the diet FNDF should decrease DMI and milk production (Allen, 2000; Dewhurst et al., 2003) but this occurred only following 4 days of higher grass silage and FNDF concentrations than the CON diet.

The VAR cows increased milk production compared to the CON on day 8, 9, and 17 (*P*<0.02; Figure 19). Day 8 and 9 were preceded by 3 and 4 days in which the VAR grass silage diet concentration was reduced compared to the CON. Grass concentrations of the VAR were reduced 3 days preceding the increase in milk yield on day 17 compared to the CON (*P*=0.02). One day decrease to 23% FNDF for the VAR on day 12 failed to elicit a milk production increase on day 13 or 14. The VAR appeared to

increase milk production compared to the CON as would be expected when fed lower diet concentrations of grass silage and FNDF (Allen, 2000), but the increase only occurred if the diet change was 3 days or more in length.

Milk production for the ORR decreased on day 16 ($P$=0.04) and this followed 5 days of a higher forage diet (day 11 to 15) compared to the CON. The ORR milk production was not affected by the higher forage diet (day 1 to 5; day 11 to 15) versus the CON (Figure 19).

Milk production by the ORR was greater than the CON on days 8, 9, and 18-21 ($P$<0.05; Figure 19). Increased milk production occurred on days when the diet forage was reduced (day 6 to 10; day 16- 20) compared to the CON. However, increased milk production did not occur until the 2$^{nd}$ and 3$^{rd}$ day of reduced dietary forage. The ORR had reduced milk production on day 16 (1.9 kg, $P$ = 0.04) compared to CON but responded to 2 d of reduced forage with 2.6 kg more milk compared to the CON on day 18. The change in milk production from day 16 to day 18 by the ORR cows was 4.6 kg/d. Milk production is highly responsive to changing diet forage concentrations in high producing cows (Voelker et al., 2003).

MUN concentration of the milk was affected by the VAR treatment on day 7, being higher in concentration ($P$=0.009), but no other days were found to be significant (**Figure 21**). On day 6, the VAR cows were fed a diet containing 98% of the forage as alfalfa silage and the diet CP concentration was 19.1%, the highest day of the period, compared to the CON, and was also preceded by 2 d of higher VAR diet CP. The MUN concentration of the VAR cows did not decrease when the diet was 16.0% CP on day 19,

but was numerically lower on day 20. Increasing diet CP in legume silage based diets from 15.1 to 18.4% results in large increases in MUN (Broderick, 2003). These MUN changes were not observed from our short term (1 to 2 d) fluctuations in diet CP. MUN is a good predictor of urinary N excretion and is highly related to dietary CP concentrations (Kauffman and St-Pierre, 2000; Nousiainen et al., 2004). On average, the treatment diets had the same CP concentrations, except variation was increased in the VAR. Improvements in the retention and utilization of dietary CP during oscillation of dietary crude protein (2 d cycles) have been shown in growing sheep and beef cattle (Cole et al., 2003; Archibeque et al., 2007). Lower period average MUN for VAR was not observed (lower urinary N excretion), however, the diet CP changes were more random rather than in an oscillating pattern.

The ORR cows MUN concentration was affected by treatment on day 7, 12, and 15 compared to the CON cows ($P<0.09$; Figure 21), but the diet CP concentration of the ORR was similar to CON for all 21 d (Figure 5). Increasing dietary energy without changing diet CP concentrations reduces MUN in cows fed haycrop silage based diets (Broderick, 2003). The ORR was lower in forage concentration (day 6 to 10), NEL intake was increased (day 6 to 10; $P<0.03$), and MUN was lower on day 7 ($P=0.09$) compared to the CON. When ORR diet forage concentration increased (day 11 to 15), MUN was higher on day 12 ($P= 0.05$) compared to the CON, however, this did not occur on day 15 as the MUN was lower than the CON ($P=0.04$). In CON, MUN increased on d 15 compared to the CON period average (12.0 vs. 10.9), making the comparison with ORR questionable. The ORR had decreased forage concentrations (day 16- 20), increased

NEL concentrations (day 16 to 20), and increased NEL intake (day 16 to 20; $P<0.02$), however, no changes in ORR MUN versus CON were observed ($P>0.19$) (Figures 3, 18, 22). The lack of a decreased MUN (day 16 to 20) is unknown, though the corn-based concentrate contained higher RUP concentrations versus the forage, and use of fermentable carbohydrates for microbial protein may have been maximized.

Treatment day interactions were not present for milk fat, milk protein, and lactose percent and milk energy concentration (**Figure 20**). The increased dietary starch (~30%) and decreased forage of the ORR (day 16 to 20) had no effect on milk fat percent compared to the CON. Decreasing diet forage NDF is expected to decrease effective fiber intake and alter biohydrogenation which decreases milk fat percent (Armentano and Pereira, 1997). Changes in the particle size of the VAR by reducing the grass concentration of the diet led to more sorting selection of the top pan on day 5 and 6 ($P<0.03$) but no change was observed in VAR milk fat percent for day 5, 6, or 7 compared to the CON as would be expected when the level of dietary effective fiber changes (Yang and Beauchemin, 2006).

Previous studies evaluating short term variation in diet forage composition and quantity fed were not found in the literature. For both treatments, milk production was reduced only 1 day versus 3 and 6 days of milk production increases for the VAR and ORR compared to the CON. When diet variation resulted in a higher FNDF ration, cows responded less than expected suggesting cows are resilient to short term changes perhaps via mobilization of body fat. Previously, a 51% restriction in DMI for 3 weeks only decreased milk production 3 kg/d which resulted in a 15 Mcal/d average NEL deficit and

a 56 kg BW loss (over 3 wks) which shows an extreme ability to mobilize body fat (Gross et al., 2011). In another experiment, cows producing 39 kg/d of milk were restricted in DMI by 48% which decreased milk yield by 10 kg/d and caused a NEL deficit of 8.2 Mcal/d on d 4 following feed restriction (Koltes and Spurlock, 2012). In our study, the effects of diet variation on milk production were only observed following 4 and 5 d of less digestible diets being fed which suggests we should evaluate the effects of negative dietary FNDF changes not in the construct of a 24 h day but over a much longer period, perhaps 96 h or more. When diet digestibility presumably increased (lower FNDF), milk production increased more closely to what was expected from the imposed dietary changes.

Short term changes in diet FNDF composition were expected to have cumulative negative effects resulting in decreasing milk yields over time. Deliberate restriction of NEL intake has not decreased milk yield as much as expected because cows mobilize body lipid stores (Velez and Donkin, 2005; Carlson et al., 2006; Moyes et al., 2009; Gross et al., 2011; Koltes and Spurlock, 2012). Furthermore, realimentation occurs rapidly, suggesting no negative carryover effect of the imposed NEL intake restriction (Gross et al., 2011). Within 1 wk following energy restriction (51% decreased DMI), cows produced similar milk yields as the control cows (no energy restriction) (Gross et al., 2011). Within 3 and 4 d, cows that were restricted by 50% of DMI returned to similar energy balance and milk production, respectively, as the control cows (no energy restriction) (Velez and Donkin, 2005). In our experiment, changing forage type of FNDF concentrations also did not have a cumulative negative effect or carryover effect. The

VAR and ORR produced the most milk on day 17 and day 20 of the 21 d period, respectively, suggesting repeated dietary assaults (FNDF changes) did not result in negative carryover effects.

## Energy Expenditures

No treatment differences were found in overall body weight change (**BWC**) or BCS change ($P$=0.26, $P$=0.65; **Table 17**). The cows gained weight in all treatments ($P$<0.06), indicating that they were in positive energy balance. When accounting for the period BWC, calculated NEL balance was negative, suggesting errors were present in the estimate as NEL balance cannot be negative when the cows are gaining BW. Accurately measuring BWC is difficult, as gut fill in dairy cows is estimated to be 15% of BW (NRC, 2001) and DMI and diet composition were much more variable for the VAR and ORR cows. A 1 kg change in DMI increased BW by an estimated 4 kg (Chilliard et al., 1991) and a change in intake caused by feed restriction dramatically reduces BW, 67 kg in 2 d (Argenas et al., 2003). Cows consuming grass silage versus alfalfa silage have higher rumen contents weights, 18 kg in one study (Dewhurst et al., 2003), and 10 kg in a study where intake of NDF was similar (Kammes and Allen, 2012c) compared to alfalfa silage. For these reasons, only maintenance and milk were used for calculating NEL use.

Average NEL use/kg DMI did not differ across treatments ($P$=0.30), suggesting no negative effects on conversion of DM to NE, e.g. digestion (Table 17). Average NEL balance was not affected by treatment ($P$=0.60) but the imposed daily treatments did result in treatment day interactions as would be expected considering the DMI and milk production changes. Changes in calculated $NE_L$ balances are supposed to indicate

mobilizing or replenishment of body tissue stores, however, unknown changes in diet composition, digestion and absorption of nutrients would cause erroneous calculated NEL balances.

The VAR had lower energy balance compared to the CON (average of 2.4 Mcal/d) on day 10, 13, 17, 19, and 20 ($P<0.10$; **Figure 22**). The ORR had lower energy balance compared to the CON on day 11, 12, 13, and 15 ($P \leq 0.10$) (average of 1.9 Mcal/d). The VAR and ORR calculated daily NEL deficits suggest the ability to produce more milk than expected, suggesting mobilization of body tissue NEL occurred or an inaccurate NEL balance calculation. The ORR showed the ability to produce similar milk yields as the CON (day 11 to 15) while having an increased NEL deficit compared to the CON for the 5 d period (day 11 to 15). Other studies have shown that cows have the ability to mobilize tissue NEL and have severe energy deficits following 3 wk, 5 d, 7 d, or 7 d of feed restriction (Carlson et al., 2006; Moyes et al., 2009; Gross et al., 2011; Koltes and Spurlock, 2012).

Greater NEL balance by the VAR versus the CON was not observed while the VAR did consume more NEL on day 6, 12, and 15 ($P<0.09$; Figure 22). The ORR had 3 days of greater NEL balance versus the CON cows (day 6, 16, and 17; $P<0.001$) and occurred on the first day and second day of lower forage compared to the CON, suggesting a time lag response in milk NEL from increased NEL intake. Improved NEL intake by the VAR and ORR resulted in more milk NEL on 3 days (day 7 to 8, day 17; $P<0.04$) and 8 days (day 7 to 10, day 18 to 21; $P<0.08$) compared to the CON, respectively. The increased NEL milk suggest replenishment of body tissue stores was

not occurring even though body tissue NEL was apparently mobilized on some days. Increased NEL intake by high producing cows was partitioned entirely to milk but much less for moderate producing cows as some NEL went towards body tissue replenishment (Voelker et al., 2003). The partitioning of NEL intake and body tissue NEL to milk NEL while not partitioning NEL intake to replenish body tissue NEL may result in the VAR and ORR losing body weight and/or BCS if the treatments lasted for a longer time period.

In summary, the VAR and ORR each had 4 days of reduced NEL intake and 5 and 4 days of reduced NEL balance compared to the CON. In comparison, the VAR and ORR had 3 and 10 days of increased NEL intake but only 0 and 3 days of increased energy balance versus the CON, respectively. Day to day treatment responses (more days lower in calculated NEL balance) suggests that even at high levels of milk production (43 kg/d) and low BCS (2.8), the transient ability to mobilize body tissue stores is present.

## Milk Fatty Acids

Generally, average milk fatty acid concentrations were not affected by treatment ($P > 0.13$; **Table 18**) with only 2 fatty acids approaching significance, *iso*-16:0 ($P=0.09$) and *cis*-9, *trans*-11 CLA ($P=0.07$). The daily or 5 day imposed treatment diet changes resulted in 22 milk fatty acid treatment by day interactions as would be expected ($P<0.10$).

C18:1, *trans*-10 concentration was affected on 1 day of the experiment, being greater in concentration for the ORR on day 20 compared to CON ($P=0.04$; **Figure 23**). C18:1, *trans*-10 is a biohydrogenate intermediate of an altered pathway and is positively

associated with SARA, increased *trans*-10, *cis* 12 CLA, and milk fat depression, respectively (Kalscheur et al., 1997; Peterson et al., 2003; Colman et al., 2010). *Iso* C16 concentration decreased on d 20 and is negatively related to *trans*-10, C18:1 (Colman et al., 2010). The ORR diet forage was reduced and starch was increased from day 16 to 20 compared to the CON. *Trans*-10, C18:1 has been shown to increase more following 7 days of feeding an acidosis type diet versus 2 days of feeding, which would agree with our response not being observed until day 5 of reduced dietary forage (Colman et al., 2010). Variation in grass silage in the VAR affected diet particle size, sorting, and DMI, but we observed no relationship with changes in VAR cows' *trans*-10, C18:1 milk fat concentration which suggests no alteration of rumen biohydrogenation and/or rumen pH changes.

Odd branch chain fatty acids (OBCFA) have been shown to be markers of rumen function and microbial synthesis and are synthesized by rumen bacteria (Wu and Palmquist, 1991; Vlaeminck et al., 2006; Fievez et al., 2012). We observed treatment by day interactions for *iso* C14:0, *iso* C15:0, and *iso* C16:0 which are primarily synthesized by rumen cellulytic bacteria and can predict acetate production and are negatively related to subclinical acidosis (SARA) (Vlaeminck et al., 2006; Coleman et al., 2010). Alterations in synthesis of OBCFA by rumen bacteria are not related to dietary supply of nutrients but are reflective of the abundance of specific bacterial populations (French et al., 2012; Fievez et al., 2012). The VAR had increased *iso*-C14:0 and *iso* C16:0 on day 12 and 20 ($P<0.09$) and *iso* C15:0 on day 20 ($P=0.02$) (**Figure 24**). These increases in VAR OBCFA were preceded by 2 and 3 d of higher diet grass silage and FNDF

concentrations compared to the CON. The ORR also had increased *iso* C14:0 during every sampling day when diet forage was increased compared to the CON (day 2, 5, 12, and 15; P<0.01). The ORR milk *iso* C15:0 and *iso* C16:0 followed a similar trend, though not as pronounced compared to the CON. These OBCFA fatty acid isomers appeared to be responsive to increased dietary fiber, suggesting the ability for the cellulytic bacteria to transiently increase in population numbers. Variation in grass silage and diet forage (except day 7 and 20) did not appear to harm the cellulytic population as minimal decreases in the OBCFA markers were observed when compared to the CON and/or when diet FNDF decreased.

The VAR and ORR had increased concentrations of C15:0 on 1 day each (day 7 and 20; $P<0.03$) following 3 days and 5 days of a lower fiber diet, respectively, compared to the CON (**Figure 25**). The ORR had increased C17:0 concentration on day 15 ($P=0.04$) during 5 d of a higher forage diet compared to the CON. Linear odd chain fatty acids, C15:0 and C17:0, are formed through elongation of propionate or valerate and de novo synthesis can occur by rumen bacteria, the mammary gland, and other tissues (Vlaeminck et al., 2006; Dewhurst et al., 2007; French et al., 2012). Milk fat C15:0 concentrations are positively correlated to plasma propionate changes (Massart-Leen et al., 1983; French et al., 2012) and are a predictor of rumen propionate production (Bhagwat et al., 2012). Milk fat C17:0 concentrations are positively related to plasma propionate as well, however, a positive relationship with adipose mobilization has also been shown (Cranix et al., 2008; French et al., 2012). Our results indicate during times of prolonged decreases in diet grass silage or diet forage, NEL intake increased and

C15:0 increased. During prolonged increases in diet forage or grass silage compared to the CON, we observed increased C17:0 indicating adipose mobilization (numerically for the VAR).

Total LCFA (C18 and greater) concentration was lower in the VAR when a lower fiber diet was fed for the preceding 3 days (day 7; $P<0.02$) and higher when a greater FNDF diet was fed for the preceding 3 days (day 20; $P<0.10$) compared to the CON (**Figure 26**). Milk fat C18:0 concentrations were increased in the VAR (day 2, 12, and 20; $P<0.04$) and ORR (d 12 and 15; $P<0.05$) when a greater FNDF diet was fed compared to the CON. Cows mobilizing body tissue NEL have increased LCFA and C18:0 in early lactation (Stoop et al., 2009) so our results suggest that mobilization of body tissue NEL was occurring when higher FNDF diets and NEL intake was reduced.

Changes in daily milk fatty acid concentration suggest that short term changes in diet fiber concentration illicit changes in rumen cellulytic bacteria but do not have immediate effects on altered rumen biohydrogenation. Changes in C17:0, C18:0, and LCFA concentrations suggest that mobilization of body tissue NEL was occurring and agrees with the 5 and 4 days of reduced NEL balance for the VAR and ORR compared to the CON.

## Gene Expression

Adipose tissue genes related to lipolysis and lipogenesis were measured via mRNA abundance using QT-PCR. No treatment or period treatment interactions were found to be significant (**Table 20**). Gene mRNA abundance was affected by the fixed effect of cow for S14 and ATGL ($P<0.10$) and also period for LPL, FASN, SCD, S14,

and ABHD5 ($P$<0.04). Gene mRNA abundance changes were analyzed using the geometric mean of 2 reference genes, ADSL and KEAP1. ACACA expression was determined on only 16 samples due to lack of repeatability and data were not analyzed statistically (no degrees of freedom).

Sampling of adipose tissue occurred on day 20, and calculated NEL balance on day 19 was 2.6 Mcal less for the VAR cows ($P$<0.01) and 1.1 Mcal more for the ORR ($P$=0.25) compared to the CON. On the sampling day (day 20), NEL balance was 1.6 Mcal less for the VAR ($P$=0.10) and 0.3 Mcal more for the ORR ($P$=0.73). The largest numerical log fold treatment difference was LEP for both the VAR and ORR compared to the CON ($P$=0.20). Numerical log fold increases greater than 0.75 were observed for LPL and HSL in the VAR, and SCD and S14 in the ORR compared to the CON. The CON were numerically lower in all genes compared to the VAR and ORR, except for S14 in the VAR and FIAF in the ORR. Numerical changes in gene expression (>0.75 log fold) compared to the CON will be discussed.

Cows that experience negative NEL balance, e.g. feed restriction, in mid lactation have rapid body weight loss, increased plasma NEFA, and increased transcriptional expression and phosphorylation of genes related to lipolysis (Valez and Donkin, 2005; Carlson et al., 2006; Elkins and Spurlock, 2009; Gross et al., 2011; Koltes and Spurlock, 2012). LEP gene expression is related to NEL balance (Block et al., 2001) and regulation of appetite (Ingvartsen and Boisclair, 2001) and is decreased during short term feed restriction (Block et al., 2003; Koltes and Spurlock, 2012). Early lactation cows exhibited large reductions in plasma LEP during negative NEL balance, and positive

energy balance increases plasma LEP (Block et al., 2001; Harvatine et al., 2009; Gross et al., 2011). However, we observed numerically higher increases in the VAR cows, which were in transient negative NEL balance. Severe feed restriction did not decrease expression of adipose LEP following day 1 of feed restriction (Koltes and Spurlock, 2012), and administration of growth hormone increased expression of LEP mRNA (Houseknecht et al., 2000). Growth hormone secretion is related to negative NEL balance (Samuelsson et al., 1996; Bradford and Allen, 2008). While the numerical LEP treatment changes may or may not agree with previous findings, the increased expression may also be a response to the increased variation in NEL intake.

HSL is a rate-limiting lipolytic-related enzyme in beta adrenergic protein kinase A (**PKA**) stimulated lipolysis and is primarily regulated at the phosphorylation level (Koltes and Spurlock, 2011; McNamara, 2012). HSL cleaves fatty acids from the glycerol backbone of diglycerides and allows lipolysis to proceed to glycerol and fatty acids (Zechner et al., 2009). HSL mRNA abundance increases during peak milk production and is lower in late lactation (Sumner and McNamara, 2007).

ATGL (cofactor is ABHD5) is also a rate-limiting lipolytic enzyme that cleaves off the first fatty acid of triglycerides yielding diglycerides (Zechner et al., 2009). However, in early lactation and negative NEL balance states, cows have reduced protein abundance of ATGL and ABHD5 (Koltes and Spurlock, 2011). ATGL activity might be dependent on basal lipolysis needs and is likely more influential in late lactation cows (Koltes and Spurlock, 2011). We observed a numeric increase in HSL that was much larger than the change in ATGL and ABHD5 in the VAR cows and agrees with Koltes

and Spurlock (2011) that HSL is related to PKA stimulated lipolysis and ATGL to basal lipolysis. The VAR cows also had higher numerical HSL expression than the ORR cows, and the ORR cows were calculated to be in positive energy balance.

LPL is the rate-limiting step for uptake of fatty acids from circulating chylomicrons and very low density proteins (Weinstock et al., 1997). Fasting-induced adipose factor (**FIAF**), also known as angiopoietin-related protein (Kersten et al., 2000), is a potent signal to prevent fat storage and activate fat mobilization (Mandard et al., 2006). FIAF also inhibits LPL (Koltes and Spurlock, 2012). FIAF transcription expression was increased on day 1 and day 3 following feed restriction and administration of bovine growth hormone, respectively (Koltes and Spurlock, 2012). In the VAR cows, we observed a larger numeric change in LPL and not in FIAF compared to the CON. The relationship we observed (increased LPL, no change FIAF) suggests that the transient negative NEL balance (day 19 and 20) did not elicit FIAF expression as some have found in response to energy balance changes (Koltes and Spurlock, 2012). However, the increased LPL expression maybe a result of the daily changes in NEL balance, and the lack of change in FIAF resulted in no inhibitory effects on LPL expression.

Stearoyl-CoA desaturase (SCD) inserts the delta 9 double bond in saturated fats and allows for biosynthesis of unsaturated fatty acids in adipocytes and is important for storage of fat and cell membrane fluidity (Ntambi, 1995). Increased energy balance increases SCD transcription expression (Harvatine et al., 2009; Loor, 2010) and we observed increased numeric expression of SCD in the ORR cows (calculated transient

positive energy balance) versus the CON and VAR cows. Thyroid response hormone (S14) is highly responsive to energy balance changes and is an important regulator for transcription of enzymes related to lipogenesis (Cunningham et al., 1998). Increasing energy balance in cows increased S14 mRNA abundance (Harvatine et al., 2009; Loor, 2010). S14 and SCD both numerically increased in the ORR cows that were in transient increased energy balance, which is in agreement with the previous studies and suggest some retention of energy in adipose tissue was occurring.

While the experiment was not designed with gene expression change as the primary measurement and necessary power to detect change, we did observe numeric increases by several key enzymes in response to the diet variation and/or possibly short term energy balance changes. Environmental stressors (caloric restriction) in humans increase expression of miRNAs (dicer miRNAs) within days and are thought to increase turnover of mRNA (Mori et al., 2012). MiRNAs are small RNAs that are critical for gene regulation and binding of miRNAs to mRNA can repress protein synthesis and decrease mRNA concentrations in the cell through degradation (Guo et al., 2010). Cows fed restricted or an excess of energy exhibited large increases in the ribosomal activity pathway in the liver and adipose tissue compared to normal fed cows (Bionaz and Loor, 2012). The results may indicate the need for enhanced translation of genes in response to the differing levels of energy intake (Bionaz and Loor, 2012). Feeding more pelleted propionate to beef steers increased the expression of not only adipose lipogenic enzymes but also lipolytic enzymes and indicated possibly enhanced mRNA expression and/or turnover (Baldwin et al., 2012). The increased numeric transcription expression we

observed in nearly all the genes for the VAR and ORR compared to the CON might be the result of increased variation in energy balance requiring greater lipolytic and lipogenic enzymes transcription and/or turnover. However, further research is needed to confirm this hypothesis.

The experimental cows adipose tissue were sampled at 3 different stages in lactation (Latin square design); 99, 121, and 142 DIM (**Table 21**). As mentioned earlier, mRNA abundance of 5 genes were significantly affected by period but no treatment period interactions were present (Appendix: Table 22). The expression of 4 of the 5 genes (LPL, FASN, SCD, S14) increased from period 1 to period 3 ($P<0.02$) and these enzymes are related to lipogenesis and fat accretion. The other gene, ABHD5 expression increased from period 1 to 3 as well ($P=0.01$). ABHD5 is related to basal lipolysis and is thought to be more highly expressed in late lactation (Koltes and Spurlock, 2012). The other genes related to lipolysis (ATGL, HSL, FIAF) were unaffected by period. Related to changes in mobilization of tissue energy, milk LCFA concentrations, BCS changes, and BWC had significant period effects and followed the trend of the cows going into positive energy balance and gaining BW. Our results suggest that there were substantial changes in adipose gene expression between 99 and 142 DIM for these experimental cows.

## Conclusion

We observed substantial variation in single day samples of DM and NDF concentrations in haycrop silage and 5 day composites greatly reduced the variation. The VAR treatment successfully imposed daily variation of haycrop silage NDF

concentration that was more than what is observed on most dairy farms and the ORR treatment imposed 5 day changes in dietary forage that would result from errant NDF sample analyses. On average, DMI, milk production, milk fatty acid profiles, ECM/DMI, and gene expression were unaffected by treatment (except positive effect on DMI for ORR). No cumulative negative effects were documented from our imposed treatments of dietary FNDF and/or forage concentration variation. Milk production was reduced for only 1 day each for the VAR and ORR versus the CON and this occurred during sustained 4 and 5 days of feeding a higher fiber diet. The less than expected days of reduced milk production were likely mediated by changes in partitioning of nutrients and mobilization of body tissue energy. Changes in milk fatty acid concentrations and numerical changes of adipose gene expression indicated the transient ability to mitigate some of the NEL intake variation. The cows also had the ability to increase milk production when fed a lower fiber diet with the response being quicker and more frequent than the negative milk production responses. Altogether, daily variation or 5 d variation in forage NDF and/or forage to concentrate ratio of the experimental diets had no cumulative negative effects and high producing cows appear to have the transient ability to buffer repeated short term bouts (1 to 3 d) of higher fiber diets and/or reduced energy intake.

# Table

**Table 3**. Composition of silages (DM basis)

| | Alfalfa Silage A | | Alfalfa Silage B | | Grass Silage | |
|---|---|---|---|---|---|---|
| | **Mean** | **SD** | **Mean** | **SD** | **Mean** | **SD** |
| Major Nutrients[1] | n=16 | | n=10 | | n=16 | |
| DM, % | 44.1 | 2.98 | 50.7 | 3.28 | 43.4 | 2.87 |
| CP, % | 18.3 | 0.64 | 21.5 | 1.61 | 11.1 | 0.33 |
| NDF, % | 45.3 | 1.39 | 44.4 | 2.15 | 64.1 | 2.07 |
| Lignin, % | 8.62 | 0.55 | 8.56 | 1.12 | 7.11 | 0.69 |
| Ash, % | 9.15 | 0.23 | 12.14 | 0.43 | 8.14 | 0.54 |
| $NE_L$, 3X Mcal/kg[2] | 1.18 | | 1.10 | | 1.01 | |
| Other Nutrients[3] | n=3 | | n=3 | | n=3 | |
| LCFA, % | 2.16 | 0.08 | 1.98 | 0.25 | 1.69 | 0.11 |
| IVNDF, % of NDF[4] | 37.4 | 3.08 | 37.2 | 2.38 | 41.8 | 1.73 |
| ADICP, % | 2.11 | 0.21 | 2.90 | 0.36 | 1.81 | 0.12 |
| Ca, % | 1.08 | 0.02 | 1.11 | 0.06 | 0.56 | 0.03 |
| P, % | 0.33 | 0.03 | 0.40 | 0.03 | 0.31 | 0.02 |
| K, % | 2.85 | 0.11 | 3.58 | 0.33 | 2.55 | 0.26 |
| Mg, % | 0.25 | 0.01 | 0.31 | 0.01 | 0.21 | 0.01 |
| Particle Size[5] | n=15 | | n=9 | | n=15 | |
| Top Screen, % | 14.6 | 3.0 | 26.0 | 4.9 | 33.7 | 5.2 |
| Middle Screen, % | 54.2 | 1.6 | 40.4 | 3.9 | 45.9 | 4.2 |
| Pan, % | 31.2 | 2.1 | 33.7 | 2.5 | 20.4 | 1.9 |

[1]Determined from 5-d composite samples, % DM.
[2]NRC (2001).
[3]Determined from period composite samples.
[4]*In-vitro* NDF disappearance during a 30-h incubation (Goering and Van Soest, 1970).
[5]Determined from 5 d composite samples, % as-fed,using Penn State Particle Separator (Lammers et al., 1996).

**Table 4**. Major ingredient composition and variability of the diets

| | CON (n[1]=462) | | VAR (n=483) | | ORR (n=483) | |
|---|---|---|---|---|---|---|
| Ingredient | | | | | | |
| **% of Diet DM** | **Mean** | **CV, %** | **Mean** | **CV, %** | **Mean** | **CV, %** |
| Alfalfa Silage A | 21.2 | 39 | 21.6 | 54 | 21.4 | 39 |
| Alfalfa Silage B | 11.0 | 74 | 10.2 | 90 | 10.8 | 77 |
| Grass Silage | 16.1 | 8 | 16.1 | 70 | 16.2 | 13 |
| Concentrate | 51.8 | 4 | 52.0 | 4 | 51.6 | 9 |
| Total Forage | 48.2 | 4 | 48.0 | 5 | 48.4 | 10 |
| **% of Forage DM** | | | | | | |
| Alfalfa Silage %[2] | 66.7 | 4 | 66.4 | 35 | 66.6 | 4 |
| Grass Silage | 33.3 | 8 | 33.6 | 69 | 33.4 | 8 |

[1]Number of cow diets used to determine the mean and CV.
[2]Alfalfa silage is the sum of alfalfa silage A and B.

**Table 5**. Gene abbreviation and name

| Gene Abbreviation | Gene Name |
|---|---|
| ABHD5 | abhydrolase domain containing 5 |
| ACACA | acetyl-coenzyme a carboxylase alpha |
| ADSL | adenylosucciante lyase |
| ATGL | adipose triglyceride lipase |
| FASN | fatty acid synthase |
| FIAF[1] | fasting-induced adipose factor |
| HSL | hormone-sensitive lipase |
| KEAP1 | kelch-like ECH-associated protein 1 |
| LEP | leptin |
| LPL | lipoprotein lipase |
| PLIN | perilipin |
| RPS9 | ribosomal protein S9 |
| SCD | stearoyl-CoA desaturase |
| S14 | thyroid hormone responsive spot 14 |
| TRIM41 | tripartite motif containing 41 |

[1]FIAF is also known as angiopoiten-related protein (Kersten et al., 2000).

**Table 6**. Gene accession number, gene, primer direction, sequence, and amplicon size of primers

| Accession # | Gene | Primers[1] | Primers (5'-3') | bp[2] |
|---|---|---|---|---|
| NM_001076063.1 | ABHD5 | F. | ctgcagatgatgtgggaaagc | 100 |
| | | R. | gactgcctggttctcgtgtca | |
| AJ132890 | ACACA | F. | catcttgtccgaaacgtcgat | 101 |
| | | R. | cccttcgaacatacacctcca | |
| NM_001102377.1 | ADSL | F. | aacagcagatcggctcaagt | 142 |
| | | R. | agtgtgcgttcaaaccactg | |
| FJ897536.1 | ATGL | F. | agctcaagaacaccatcacg | 121 |
| | | R. | cgcaggttgaactggatg | |
| CR552737 | FASN | F. | acctcgtgaaggctgtgactca | 92 |
| | | R. | tgagtcgaggccaaggtctgaa | |
| NM_001046043.2 | FIAF | F. | gatggctccgtggactttaacc | 102 |
| | | R. | ggatgtgatgcaccttctccag | |
| NM_001080220.1 | HSL | F. | gagtttgagcggatcattca | 101 |
| | | R. | tgaggccatgtttgctagag | |
| NM_001101142.1 | KEAP1 | F. | tgatggtaacaccgactcca | 129 |
| | | R. | gcatagatatgcccgtcgat | |
| BT020625 | LEP | F. | ggctttggccctatctgtctta | 124 |
| | | R. | gagacggactgcgtgtgtga | |
| BC118091 | LPL | F. | acacagctgaggacacttgcc | 101 |
| | | R. | gccatggatcaccacaaagg | |
| NM_001083699.1 | PLIN | F. | agagttcctcctccctccag | 148 |
| | | R. | tgtggtctggaaggtgtgtc | |
| DT860044 | RPS9 | F. | cctcgaccaagagctgaag | 108 |
| | | R. | cctccagacctcacgtttgttc | |
| AY241933 | SCD | F. | tcctgttgttgtgcttcatcc | 101 |
| | | R. | ggcataacggaataaggtggc | |
| AY656814 | S14 | F. | ctaccttcctctgagcaccagttc | 151 |
| | | R. | acacactgaccaggtgacagaca | |
| NM_001206165.1 | TRIM41 | F. | gcgctatctgcctcgattac | 135 |
| | | R. | cctcttcctcccggtctaac | |

[1]Primer direction (F: forward, R: reverse).
[2]Amplicon size in base pairs (bp).

**Table 7**. Quantitative PCR performance of genes measured in adipose tissue and annealing temperature

| Gene | Median Ct[1] | Median ΔCT[2] | Slope[3] | Efficiency %[4] | Temp.[5] |
|---|---|---|---|---|---|
| ABHD5 | 28.26 | 1.08 | 3.51 | 92.8 | 57.5 |
| ACACA | 32.76 | 5.40 | 3.18 | 106.3 | 55.0 |
| ADSL | 26.81 | | 3.46 | 94.5 | 57.5 |
| ATGL | 28.13 | 0.63 | 3.57 | 90.5 | 57.5 |
| FASN | 29.48 | 1.93 | 3.19 | 105.9 | 60.0 |
| FIAF | 30.14 | 2.58 | 3.71 | 85.9 | 57.5 |
| HSL | 31.28 | 3.20 | 3.62 | 88.8 | 55.0 |
| KEAP1 | 28.65 | | 3.65 | 87.8 | 57.5 |
| LEP[6] | 34.52 | 5.27 | 3.81 | 83.0 | 57.5 |
| LPL | 27.89 | 0.51 | 3.43 | 95.5 | 57.5 |
| RPS9 | 22.30 | | 4.07 | 76.2 | 57.5 |
| SCD | 27.85 | 0.55 | 3.44 | 95.4 | 55.0 |
| S14 | 25.75 | -2.29 | 3.77 | 84.2 | 60.0 |

[1]The median Ct is calculated using all samples and all cows.
[2]The median ΔCt is calculated as [Ct gene – geometrical mean of ADSL and KEAP1] for all samples and all cows and M stability values were 0.48 each for ADSL and KEAP1 respectively (Vandesomple et al., 2002).
[3]Slope of the standard curve; dilutions were 1:1, 1:10, and 1:100.
[4]Efficiency is calculated as $[(10^{-1/\text{slope}} - 1) * 100]$.
[5]Temp = annealing temperature, °C.
[6]LEP standard curve was calculated from dilutions of 1:1 and 1:10.

**Table 8**. Silage DM and NDF for single day samples

| **DM, %** | Alfalfa Silage A n=91[1] | Alfalfa Silage B n=58 | Grass Silage n=91 |
|---|---|---|---|
| Mean | 45.1 | 51.6 | 44.0 |
| SD | 3.4 | 4.0 | 3.6 |
| CV | 7.6% | 7.8% | 8.1% |
| Minimum | 38.8 | 44.4 | 35.6 |
| Maximum | 53.1 | 60.6 | 55.7 |
| | | | |
| **NDF, % DM** | n=31 | n=18 | n=31 |
| Mean | 46.2 | 46.6 | 64.9 |
| SD | 1.7 | 3.5 | 2.5 |
| CV | 3.6% | 7.6% | 3.9% |
| Minimum | 42.1 | 42.2 | 59.4 |
| Maximum | 49.7 | 54.1 | 70.0 |

[1]Number of days sampled.

**Table 9**. Assayed nutrient composition and calculated variability of the total forage mixture fed

| **Nutrient Composition**[1] | **CON** | **VAR** | **ORR** |
|---|---|---|---|
| | n=84[2] | n=84 | n=84 |
| **NDF, % DM** | | | |
| Mean | 51.3 | 51.3 | 51.3 |
| SD | 1.35 | 4.42 | 1.36 |
| Minimum | 48.6 | 43.2 | 48.7 |
| Maximum | 53.5 | 59.4 | 53.5 |
| Range | 4.9 | 16.2 | 4.8 |
| **CP, % DM** | | | |
| Mean | 16.6 | 16.5 | 16.6 |
| SD | 0.64 | 2.05 | 0.65 |
| Minimum | 15.1 | 13.3 | 15.1 |
| Maximum | 18.7 | 21.7 | 18.7 |
| Range | 3.6 | 8.4 | 3.6 |
| **NEL, 3X, Mcal/kg**[3] | | | |
| Mean | 1.10 | 1.10 | 1.10 |
| SD | 0.02 | 0.04 | 0.02 |
| Minimum | 1.05 | 1.01 | 1.05 |
| Maximum | 1.17 | 1.20 | 1.17 |
| Range | 0.12 | 0.19 | 0.12 |

[1]The forages, alfalfa versus grass forage, were deliberately changed daily in the Variable treatment. The Control and Overreacting treatment forage mixtures were to be constant as possible in nutrient composition variation.
[2]Number of treatment days.
[3]NRC (2001).

**Table 10**. Average ingredient composition of diets, % DM

| Ingredient | CON | VAR | ORR |
|---|---|---|---|
| Alfalfa Silage A | 21.2 | 21.6 | 21.4 |
| Alfalfa Silage B | 11.0 | 10.2 | 10.8 |
| Grass Silage | 16.1 | 16.1 | 16.2 |
| Corn Grain, ground | 35.2 | 35.4 | 35.1 |
| Dried Distiller Grains | 4.10 | 4.12 | 4.09 |
| Corn Gluten Meal | 3.60 | 3.62 | 3.59 |
| Treated soybean meal[1] | 2.91 | 2.93 | 2.90 |
| Soybean Meal, 48% CP | 0.88 | 0.89 | 0.88 |
| Soybean Hulls | 1.73 | 1.73 | 1.72 |
| Fat, Animal-Vegetable Blend | 0.96 | 0.96 | 0.95 |
| Tallow | 0.48 | 0.48 | 0.48 |
| Limestone | 0.59 | 0.60 | 0.59 |
| Trace Mineral Salt | 0.50 | 0.50 | 0.50 |
| Dicalcium phosphate | 0.18 | 0.18 | 0.18 |
| Magnesium Oxide | 0.02 | 0.02 | 0.02 |
| Trace Mineral and Vitamin Premix[2] | 0.60 | 0.60 | 0.60 |

[1]Aminoplus®, Ag Processing, Inc., Omaha, NE.

[2]Contained 57.4% biotin (220mg/kg) premix (DSM Nutritional Products, Inc., Parsippany, NJ), 21.1% Sodium selenite premix (200mg/kg), 6.4% Zinc-methionine complex premix (Zinpro 100, Zinpro Corp., Eden Prairie, MN), 0.5% copper sulfate, 7.7% Vitamin E premix (44 IU/g), 5.1% Vitamin D premix (3,000 IU/g), and 1.9% Vitamin A premix (30,000 IU/g).

**Table 11**. Major nutrient concentrations and variability in diet

| Nutrient Composition, % DM | CON n=462[1] | | VAR n=483 | | ORR n= 483 | |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| n=84 samples[2,3] | | | | | | |
| DM, % | 60.7 | 1.7 | 60.7 | 2.4 | 60.7 | 2.4 |
| | | | | | | |
| n=16 composite samples[2,3] | | | | | | |
| NDF, % | 33.3 | 1.1 | 33.2 | 2.3 | 33.3 | 1.9 |
| Forage NDF, % | 24.8 | 1.3 | 24.6 | 2.4 | 24.9 | 2.6 |
| CP, % | 17.4 | 0.38 | 17.4 | 1.0 | 17.4 | 0.39 |
| Ash, % | 6.95 | 0.31 | 6.92 | 0.40 | 6.95 | 0.37 |
| Lignin, % | 4.55 | 0.30 | 4.55 | 0.39 | 4.57 | 0.43 |
| NEL, Mcal/kg[4] | 1.50 | 0.04 | 1.50 | 0.05 | 1.49 | 0.05 |
| NEL, 3X, Mcal/kg[5] | 1.57 | 0.02 | 1.57 | 0.03 | 1.57 | 0.05 |
| Starch, %[6] | 27.4 | 1.2 | 27.6 | 1.2 | 27.4 | 2.4 |

[1]Number of cow diets used to determine the average and SD nutrient composition.
[2]Daily samples of silages for DM and 5-d composite samples of other major nutrients.
[3]Concentrate DM and major nutrients determined from 4 composite samples.
[4]NRC(2001), using daily feed intake, feed composition, and discount factor and period average BW.
[5]NRC(2001), using daily diet data and a constant discount factor.
[6]NRC (2001) book values were used for the silages.

**Table 12**. Average concentrations of other dietary nutrients

| **Nutrient Composition, % DM** | **CON** | **VAR** | **ORR** |
|---|---|---|---|
| | Mean | Mean | Mean |
| MP allowable milk (kg/d)[1] | 47.8 | 47.5 | 49.1 |
| RDP[1] | 10.1 | 10.1 | 10.1 |
| RUP[1] | 7.2 | 7.2 | 7.3 |
| Acid detergent insoluble CP, % | 1.06 | 1.04 | 1.06 |
| Long-chain fatty acids (LCFA), % | 3.29 | 3.29 | 3.28 |
| Ca, % | 0.97 | 0.97 | 0.97 |
| P, % | 0.40 | 0.40 | 0.40 |
| K, % | 1.71 | 1.70 | 1.72 |
| Mg, % | 0.22 | 0.21 | 0.22 |

[1] MP allowable milk: RDP, and RUP calculated using NRC (2001), treatment average. DMI, assayed nutrients, and NRC (2001) values of protein fractions and digestion rates.

**Table 13**. Particle size of diets, as-fed basis

| | **CON (n=22)[1]** | | | **VAR (n=22)** | | | **ORR (n=22)** | | |
|---|---|---|---|---|---|---|---|---|---|
| PSPS, %[2] | Mean | CV | Range[4] | Mean | CV | Range | Mean | CV | Range |
| Top screen | 8.1 | 26.3 | 7.9 | 7.2 | 43.9 | 10.1 | 7.8 | 29.8 | 8.4 |
| Middle screen | 35.6 | 6.9 | 9.0 | 35.8 | 6.8 | 9.3 | 36.0 | 8.4 | 10.0 |
| Pan | 56.3 | 3.2 | 5.8 | 57.0 | 7.4 | 13.7 | 56.2 | 6.8 | 13.2 |

[1]Diets were analyzed on 22 separate days.
[2] Penn State Particle Separator (Lammers et al., 1996).
[3]Maximum – minimum observed values.

**Table 14**. Effects of treatment on particle selection of the diet 4 h after feeding

| | Treatment[1] | | | | *P*[2] | | |
|---|---|---|---|---|---|---|---|
| | CON | VAR | ORR | SEM | Trt. | D | D*Trt. |
| Particle Selection %[3] | (n=9) | (n=9) | (n=9) | | | | |
| Top Screen Selection | 64.3[a] | 53.2[b] | 60.5[a] | 4.61 | 0.003 | 0.24 | 0.05 |
| Top Screen Selection CV, %[4] | 28.0 | 29.4 | 25.2 | 4.91 | 0.65 | | |
| Middle Screen Selection | 92.9 | 94.2 | 95.9 | 2.81 | 0.15 | 0.03 | 0.05 |
| Middle Screen Selection CV, % | 5.0[a] | 8.3[b] | 6.3 | 2.30 | 0.10 | | |
| Pan Selection | 119.8 | 120.8 | 118.2 | 2.22 | 0.44 | 0.42 | 0.42 |
| Pan Selection, CV % | 6.6 | 6.4 | 5.6 | 0.64 | 0.36 | | |

[a-b]Means within a row with different superscripts differ (P <0.04).
[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2]Fixed effect of treatment (Trt), day (D), and D by Trt interaction.
[3]Selection % = 100* (x of TMR / x at 4 h after feeding), where x = % on top, middle, or pan (Leonardi and Armentano, 2003).
[4]CV= calculated from 5 samples within cow.

**Table 15**. Effects of treatments on intake and milk production

| | Treatment[1] | | | | *P*[2] | | |
|---|---|---|---|---|---|---|---|
| | CON | VAR | ORR | SEM | Trt | D | Trt*D |
| DMI, kg/d[3] | 24.5[a] | 24.3[a] | 25.1[b] | 0.54 | 0.05 | <0.01 | <0.01 |
| DMI CV %[4] | 6.1 | 7.4 | 6.7 | 0.60 | 0.17 | | |
| Milk kg/d[5] | 42.8 | 43.1 | 43.6 | 1.18 | 0.34 | <0.01 | <0.01 |
| Milk % CV | 5.1 | 5.9 | 5.2 | 0.46 | 0.24 | | |
| ECM kg/d[6] | 42.1 | 42.5 | 42.9 | 1.30 | 0.21 | <0.01 | <0.01 |
| ECM/DMI[3] | 1.72 | 1.76 | 1.72 | 0.04 | 0.38 | <0.01 | <0.01 |

[a-b]Means within a row with different superscripts differ (P <0.06).
[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2] Fixed effect of treatment (Trt), day (D), and trt by day interaction (Trt*D).
[3]22, 23, and 23 CON, VAR, and ORR cow periods, respectively.
[4]CV= calculated within cow.
[5]24, 23, and 23 CON, VAR, and ORR cow periods, respectively.
[6]ECM = 3.5% energy corrected milk = (12.82 x fat (kg)) + (7.13 x protein (kg)) + (0.323 x milk (kg).

**Table 16**. Effects of treatment on milk composition

| | Treatment[1] | | | | *P*[2] | | |
|---|---|---|---|---|---|---|---|
| | CON n=24 | VAR n=23 | ORR n=23 | SEM | Trt | D | Trt*D |
| Milk fat, % | 3.49 | 3.51 | 3.54 | 0.19 | 0.74 | <0.001 | 0.86 |
| Milk fat CV %[3] | 8.3 | 8.7 | 7.9 | 1.25 | 0.82 | | |
| Milk fat, kg/d | 1.50 | 1.52 | 1.54 | 0.09 | 0.46 | <0.001 | <0.001 |
| Milk protein, % | 2.80 | 2.80 | 2.78 | 0.05 | 0.65 | <0.001 | 0.18 |
| Milk protein CV % | 2.9 | 2.8 | 2.7 | 0.22 | 0.74 | | |
| Milk protein, kg/d | 1.20 | 1.21 | 1.21 | 0.04 | 0.64 | <0.001 | <0.001 |
| Milk lactose, % | 4.80 | 4.80 | 4.80 | 0.05 | 0.99 | <0.001 | 0.46 |
| Milk lactose CV % | 1.2 | 1.3 | 1.3 | 0.13 | 0.47 | | |
| Milk lactose, kg/d | 2.05 | 2.07 | 2.09 | 0.08 | 0.47 | <0.001 | <0.001 |
| MUN, mg/dL | 10.92 | 11.09 | 10.87 | 0.43 | 0.30 | 0.002 | <0.001 |
| MUN CV % | 13.4 | 14.4 | 14.0 | 3.33 | 0.87 | | |

[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2] Fixed effect of treatment (Trt), day (D), and trt by day interaction (Trt*D).
[3]CV= Calculated within cow.

**Table 17**. Effects of treatment on calculated NEL expenditures

| | Treatment[1] | | | | *P*[2] | | |
|---|---|---|---|---|---|---|---|
| | CON | VAR | ORR | SEM | Trt | D | Trt*D |
| NEL[3] | | | | | | | |
| Milk, Mcal/d[4] | 28.6 | 28.9 | 29.2 | 0.97 | 0.26 | <0.001 | <0.001 |
| Intake, Mcal/d[5] | 36.8 | 36.7 | 37.5 | 0.67 | 0.11 | <0.001 | <0.001 |
| Use, Mcal/d[6] | 38.7 | 39.0 | 39.3 | 0.93 | 0.30 | <0.001 | <0.001 |
| Balance, Mcal/d[7] | -1.9 | -2.3 | -1.8 | 0.85 | 0.60 | <0.001 | <0.001 |
| Use/ DMI, Mcal/kg | 1.59 | 1.62 | 1.58 | 0.03 | 0.28 | <0.001 | <0.001 |
| BW, kg | 634 | 635 | 632 | 11.7 | 0.20 | | |
| BWC, kg/d | 0.31 | 0.63 | 0.51 | 0.15 | 0.26 | | |
| BCS[8] | 2.8 | 2.8 | 2.8 | 0.07 | 0.92 | | |
| BCS, change/period | 0.01 | 0.05 | 0.07 | 0.06 | 0.65 | | |

[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2]Fixed effect of treatment (Trt), day (D), and treatment by day interaction (Trt*D).
[3]NRC (2001).
[4]24, 23, and 23 CON, VAR, and ORR cow periods, respectively.
[5]22, 23, and 23 CON, VAR, and ORR cow periods, respectively.
[6]NEL use = (0.08 * BW^0.75 (average BW per period)+ Milk $NE_L$).
[7]NEL balance = NEL intake – NEL use.
[8]Determined by three independent evaluators.

**Table 18**. Effects of treatment on milk fatty acid concentrations

| | Treatment[1] | | | | *P*[2] | | |
|---|---|---|---|---|---|---|---|
| **Fatty Acid**, g/100g fatty acids[3] | **CON** n=18 | **VAR** n=17 | **ORR** n=17 | **SEM** | **Trt** | **D** | **Trt*D** |
| 4:0 | 3.94 | 3.99 | 3.93 | 0.14 | 0.40 | <0.01 | 0.48 |
| 6:0 | 2.57 | 2.64 | 2.58 | 0.07 | 0.36 | <0.01 | 0.97 |
| 8:0 | 1.34 | 1.38 | 1.35 | 0.06 | 0.50 | <0.01 | 0.67 |
| 10:0 | 2.83 | 2.90 | 2.87 | 0.17 | 0.74 | <0.01 | 0.07 |
| 12:0 | 3.09 | 3.15 | 3.13 | 0.20 | 0.79 | <0.01 | 0.04 |
| *iso*-13:0 | 0.02 | 0.02 | 0.02 | 0.0008 | 0.69 | <0.01 | 0.06 |
| *anteiso*-13:0 | 0.08 | 0.08 | 0.08 | 0.0064 | 0.84 | <0.01 | 0.06 |
| 13:0 | 0.09 | 0.10 | 0.10 | 0.015 | 0.11 | <0.01 | 0.76 |
| *iso*-14:0 | 0.09 | 0.09 | 0.09 | 0.0053 | 0.12 | <0.01 | <0.01 |
| 14:0 | 10.18 | 10.24 | 10.22 | 0.41 | 0.96 | <0.01 | 0.05 |
| *iso*-15:0 | 0.18 | 0.18 | 0.18 | 0.0032 | 0.87 | <0.01 | <0.01 |
| *anteiso*-15:0 | 0.37 | 0.38 | 0.38 | 0.011 | 0.38 | <0.01 | 0.01 |
| 14:1 | 0.94 | 0.94 | 0.96 | 0.08 | 0.83 | <0.01 | <0.01 |
| 15:0 | 0.83 | 0.82 | 0.84 | 0.05 | 0.63 | <0.01 | <0.01 |
| *iso*-16:0 | 0.22 | 0.23 | 0.23 | 0.0092 | 0.09 | 0.02 | <0.01 |
| 16:0 | 25.48 | 25.49 | 25.45 | 0.42 | 0.99 | <0.01 | <0.01 |
| *iso*-17:0 | 0.36 | 0.35 | 0.35 | 0.13 | 0.25 | <0.01 | <0.01 |
| 16:1& *anteiso*-17:0 | 1.79 | 1.74 | 1.77 | 0.08 | 0.63 | 0.09 | 0.94 |
| 17:0 | 0.63 | 0.63 | 0.63 | 0.011 | 0.62 | <0.01 | <0.01 |
| 17:1 | 0.22 | 0.22 | 0.21 | 0.02 | 0.53 | <0.01 | 0.17 |
| 18:0 | 11.41 | 11.68 | 11.65 | 0.40 | 0.25 | <0.01 | <0.01 |
| *trans*-6 & 8, 18:1 | 0.61 | 0.59 | 0.61 | 0.03 | 0.59 | <0.01 | 0.18 |
| *trans*-9, 18:1 | 0.46 | 0.46 | 0.45 | 0.04 | 0.84 | <0.01 | 0.43 |
| *trans*-10, 18:1 | 1.38 | 1.24 | 1.33 | 0.20 | 0.59 | <0.01 | 0.01 |
| *trans*-11, 18:1 | 1.58 | 1.50 | 1.57 | 0.13 | 0.14 | <0.01 | 0.04 |
| *trans*-12, 18:1 | 0.63 | 0.63 | 0.65 | 0.03 | 0.44 | <0.01 | <0.01 |
| *cis*-9, 18:1 | 22.75 | 22.49 | 22.51 | 0.89 | 0.80 | <0.01 | <0.01 |
| *cis*-11, 18:1 | 0.62 | 0.59 | 0.60 | 0.04 | 0.33 | <0.01 | 0.98 |
| 18:2 | 3.59 | 3.57 | 3.52 | 0.09 | 0.47 | <0.01 | 0.20 |
| 20:0 | 0.19 | 0.19 | 0.19 | 0.004 | 0.29 | <0.01 | <0.01 |
| 20:1 | 0.13 | 0.13 | 0.13 | 0.005 | 0.65 | 0.16 | 0.01 |
| 18:3 | 0.61 | 0.60 | 0.61 | 0.01 | 0.67 | <0.01 | <0.01 |
| *cis*-9,*trans*-11 CLA[4] | 0.77 | 0.72 | 0.75 | 0.05 | 0.07 | <0.01 | 0.61 |
| CLA, other[4] | 0.023 | 0.023 | 0.024 | 0.003 | 0.94 | 0.01 | 0.84 |
| *trans*-10, *cis*-12 CLA[5] | n.d. | n.d. | n.d. | n.d. | n.d | n.d. | n.d |

[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2]Fixed effect of treatment (Trt), day (D), and Trt by day interactions (Trt*D).
[3]Number of carbons: number of double bonds.
[4]CLA = Conjugated Linoleic Acid.
[5] *trans*-10, *cis*-12 CLA concentration was not determined due to repeatability problems.

**Table 19**. Effects of treatment on milk fatty acid categories

| | **Treatment**[1] | | | | ***P***[2] | | |
|---|---|---|---|---|---|---|---|
| **Fatty Acid**, g/100g fatty acids | **CON** n=18 | **VAR** n=17 | **ORR** n=17 | **SEM** | **Trt** | **D** | **Trt*D** |
| SMFA[3] | 25.0 | 25.3 | 25.1 | 0.84 | 0.74 | <0.01 | 0.22 |
| C16[4] | 25.7 | 25.7 | 25.7 | 0.42 | 0.99 | <0.01 | <0.01 |
| LCFA[5] | 44.8 | 44.4 | 44.6 | 1.07 | 0.88 | <0.01 | <0.01 |

[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2]Fixed effect of treatment (Trt), day (D), and trt by day interactions (Trt*D).
[3] SMFA = short medium chain fatty acids, sum of even chain fatty acids < C16:0.
[4]C16:0 + *iso*-C16:0.
[5]LCFA = sum of C18 and greater.

**Table 20**. Effects of treatment on relative transcription mRNA log abundance ($2^{\wedge(-\Delta Ct)}$) compared to reference genes in adipose tissue

| | Treatment[1] | | | | *P*[2] | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene[3,4]** | **CON** | **VAR** | **ORR** | **SEM** | **Trt** | **Cow** | **Period** | **Period*Trt** |
| LPL | 4.06 | 4.84 | 4.61 | 0.63 | 0.61 | 0.15 | 0.03 | 0.53 |
| FASN | 3.39 | 3.54 | 3.90 | 0.69 | 0.86 | 0.11 | 0.02 | 0.24 |
| SCD | 4.49 | 5.02 | 5.70 | 0.62 | 0.62 | 0.26 | 0.03 | 0.48 |
| S14 | 6.52 | 6.39 | 7.40 | 0.74 | 0.58 | 0.09 | 0.01 | 0.20 |
| ATGL | 4.31 | 4.47 | 4.47 | 0.35 | 0.92 | 0.03 | 0.28 | 0.15 |
| ABHD5 | 3.75 | 4.17 | 4.05 | 0.23 | 0.44 | 0.25 | 0.02 | 0.32 |
| HSL | 2.00 | 3.10 | 2.66 | 0.77 | 0.18 | 0.38 | 0.18 | 0.37 |
| LEP | -2.08 | 0.26 | 1.79 | 0.60 | 0.20 | 0.32 | 0.19 | 0.30 |
| FIAF | 2.75 | 2.91 | 2.72 | 0.18 | 0.69 | 0.12 | 0.09 | 0.05 |

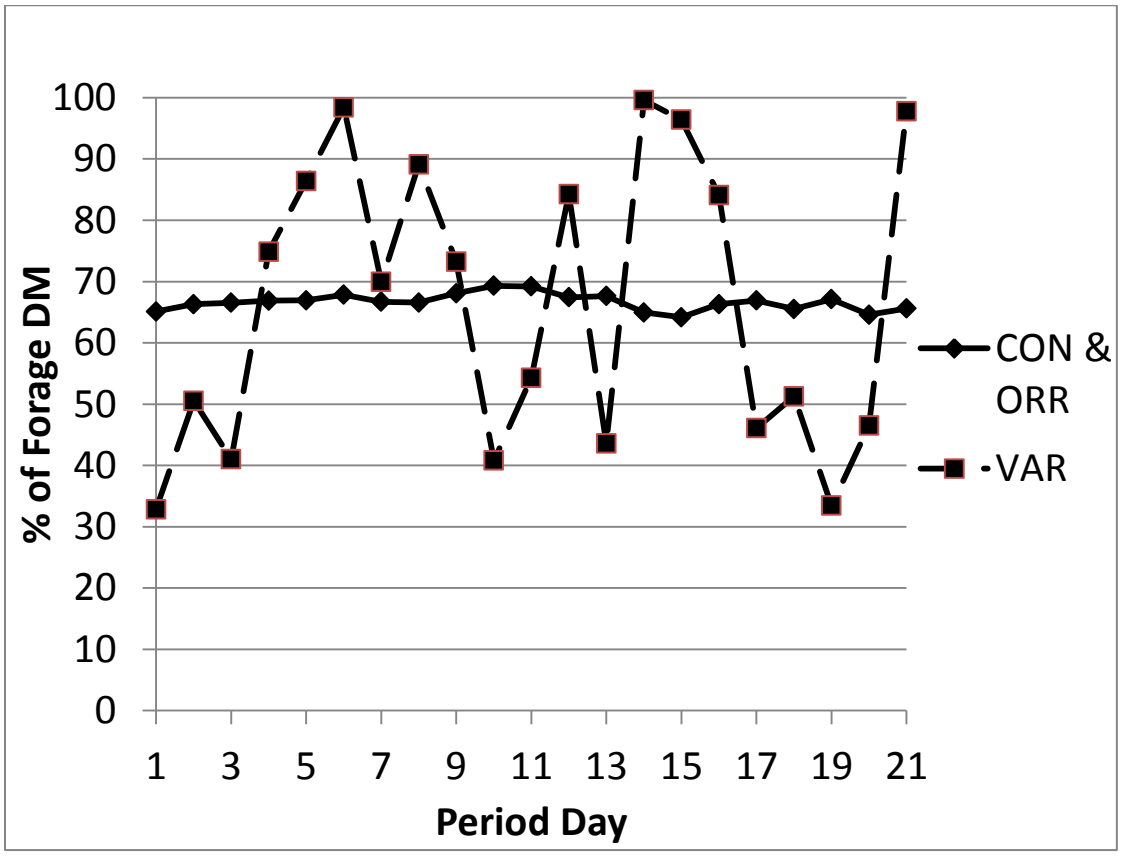
[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2]Fixed effect of treatment, cow, period, and period by treatment interaction.
[3] Number of cows per treatment; 8, 8, and 7 CON, VAR, and ORR cows for LPL, FASN, S14, ATGL, and FIAF; 7, 8, and 7 CON, VAR, and ORR cows for SCD; 7, 7, and 6 CON, VAR, and ORR cows for ABHD5 and HSL; 6, 7, and 6 CON, VAR, and ORR cows for LEP.
[4]Lipoprotein lipase (LPL), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), thyroid hormone responsive SPOT 14 (S14), adipose triglyceride lipase (ATGL), abhydrolase domain containing 5 (ABHD5), hormone-sensitive lipase (HSL), leptin (LEP), and fasting-induced adipose factor (FIAF).

**Table 21**. Effect of period on relative transcription mRNA log abundance ($2^{\wedge(-\Delta Ct)}$) compared to reference genes in adipose tissue

| | Period[1] | | | | *P*[2] | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene[3,4,5,6]** | **Period 1** | **Period 2** | **Period 3** | **SEM** | **Period** | **1*2** | **1*3** | **2*3** |
| LPL | 3.13 | 4.38 | 5.99 | 0.63 | 0.03 | 0.15 | 0.01 | 0.10 |
| FASN | 1.83 | 4.08 | 4.93 | 0.69 | 0.02 | 0.03 | 0.01 | 0.39 |
| SCD | 3.12 | 5.08 | 7.02 | 0.81 | 0.03 | 0.08 | 0.01 | 0.12 |
| S14 | 4.57 | 7.11 | 8.64 | 0.74 | 0.01 | 0.03 | 0.004 | 0.17 |
| ATGL | 4.08 | 4.82 | 4.35 | 0.35 | 0.28 | 0.13 | 0.58 | 0.35 |
| ABHD5 | 3.26 | 4.03 | 4.68 | 0.27 | 0.02 | 0.03 | 0.01 | 0.11 |
| HSL | 1.43 | 2.72 | 3.60 | 0.89 | 0.18 | 0.17 | 0.10 | 0.44 |
| LEP | -1.80 | -0.23 | 2.01 | 0.68 | 0.19 | 0.22 | 0.13 | 0.21 |
| FIAF | 2.46 | 2.86 | 3.06 | 0.18 | 0.09 | 0.11 | 0.04 | 0.45 |

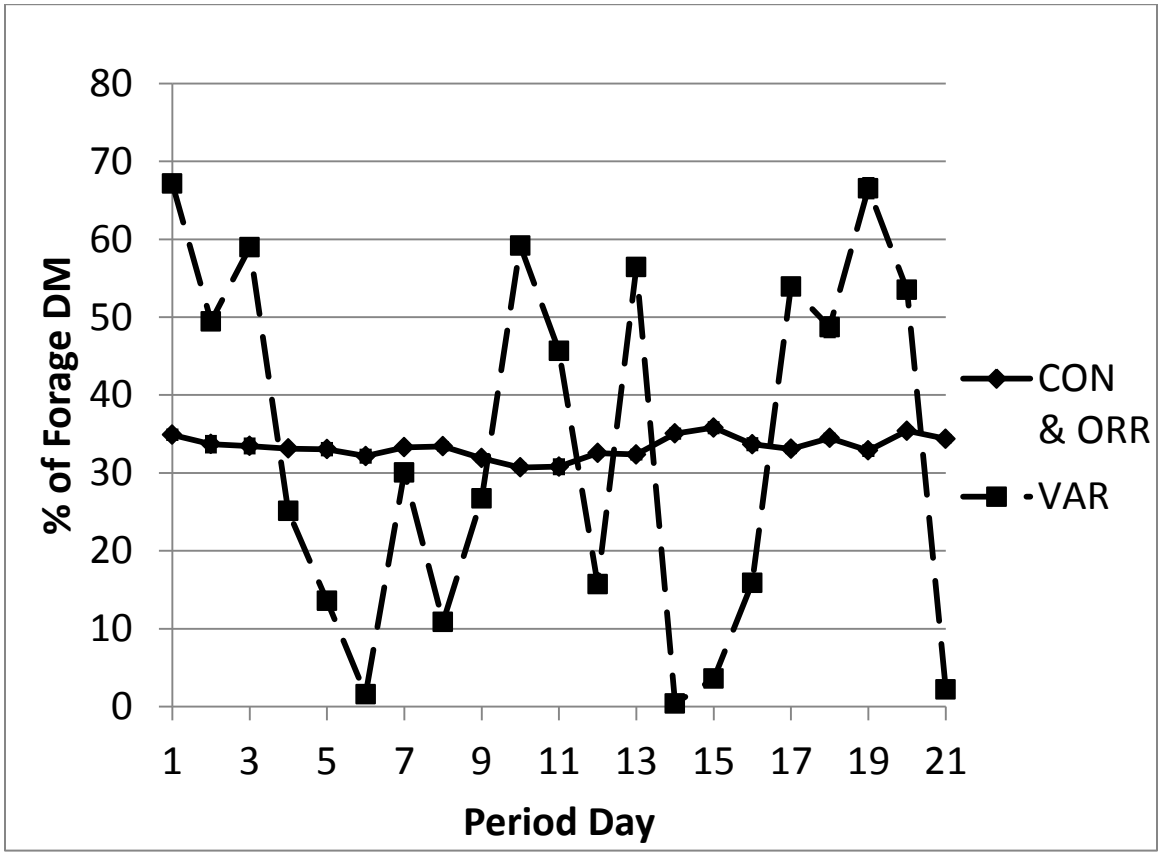
[1]Cows were sampled during 3 different stages in lactation; Period 1 = 99 DIM, Period 2 = 120 DIM, and Period 3 = 141 DIM.
[2]Treatment comparisons, Period 1 vs. Period 2 (1 vs. 2), Period 1 vs. Period 3 (1 vs. 3), and Period 2 vs. Period 3 (2 vs. 3) and the overall effect of period.
[3]Number of cows for Period 1 are 8 cows for LPL, FASN, SCD, S14, ATGL, ABHD5, HSL, and FIAF and 7 cows for LEP.
[4]Number of cows for Period 2 are 8 cows for all genes.
[5]Number of cows for Period 3 are 7 cows for LPL, FASN, S14, and FIAF; 6 cows for SCD; and 4 cows for ABHD5, HSL, and LEP.
[6]Lipoprotein lipase (LPL), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), thyroid hormone responsive SPOT 14 (S14), adipose triglyceride lipase (ATGL), abhydrolase domain containing 5 (ABHD5), hormone-sensitive lipase (HSL), leptin (LEP), and fasting-induced adipose factor (FIAF).

## Figures



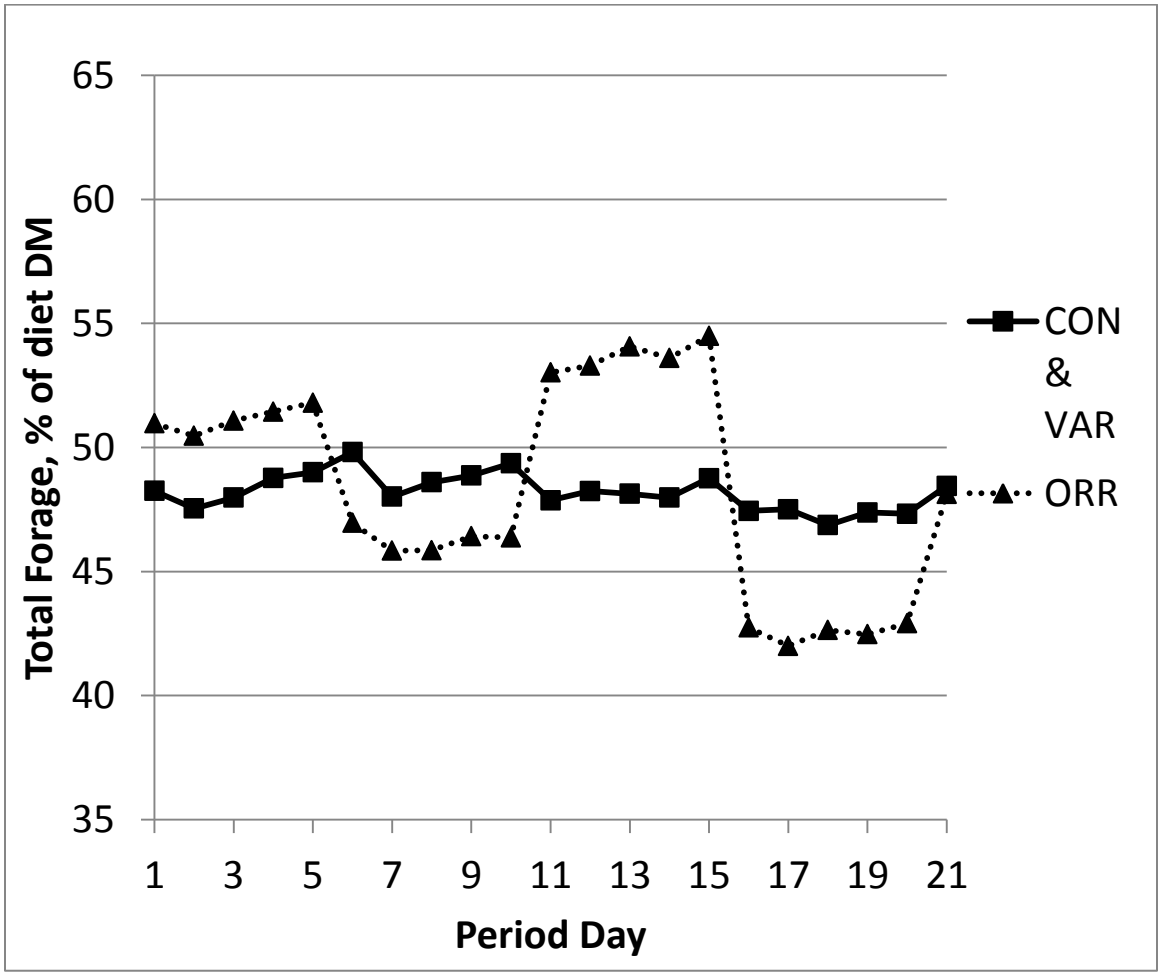

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.

**Figure 1**. Change in total alfalfa silage in treatment diets.



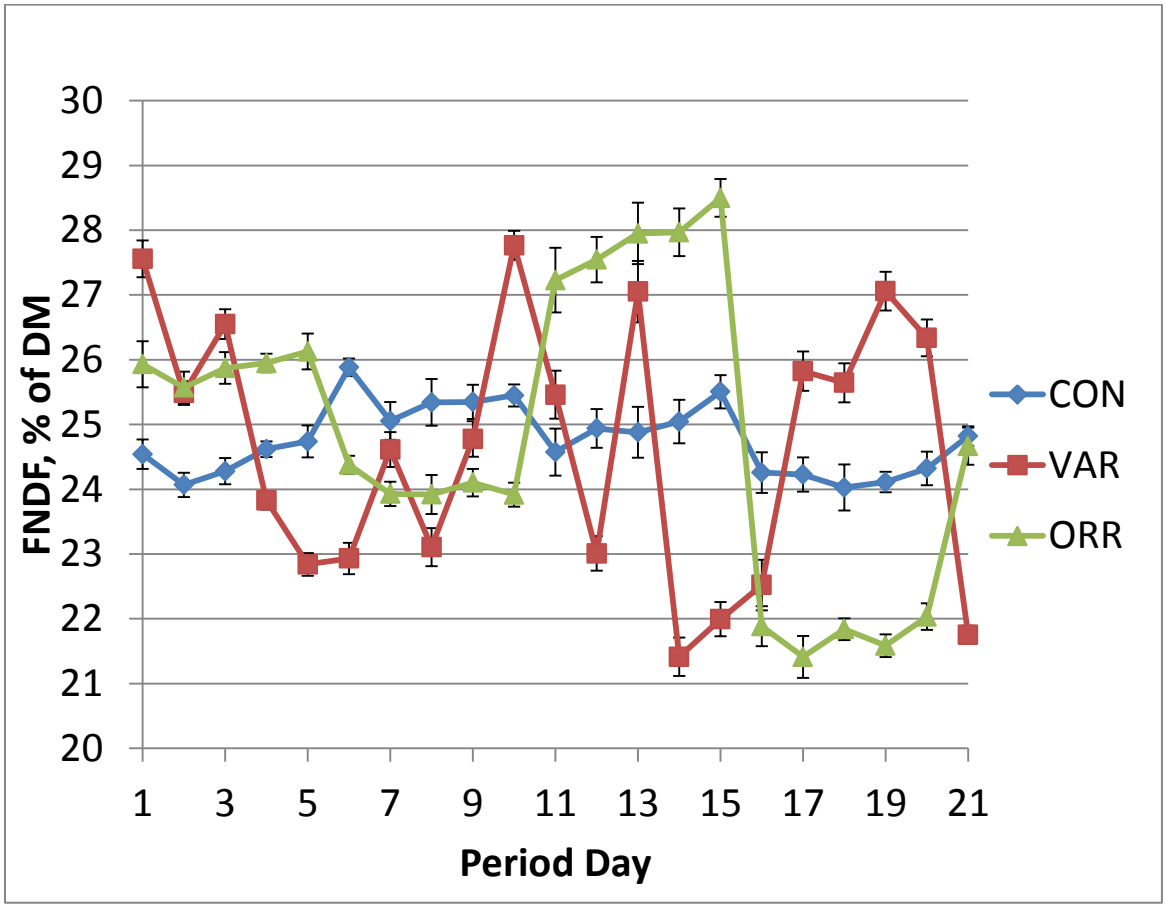

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.

**Figure 2**. Change in grass silage in treatment diets.




Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.

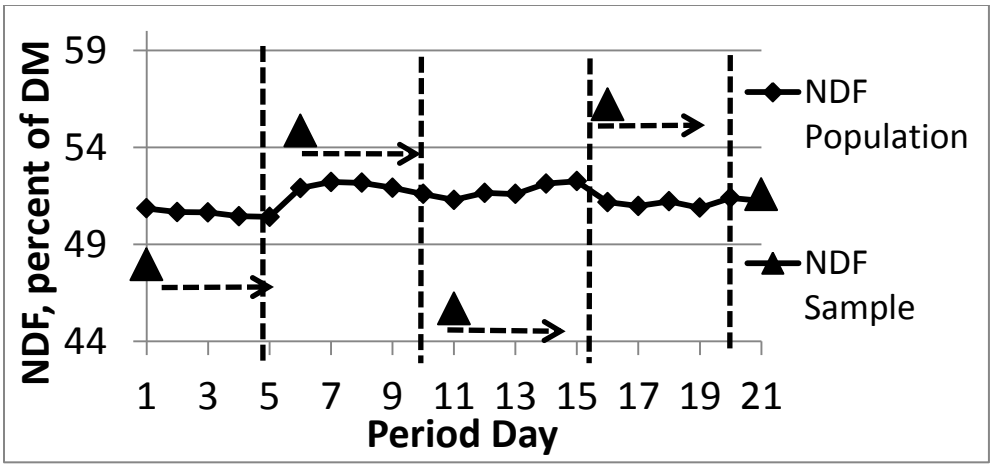
**Figure 3**. Change in total forage in treatment diets.



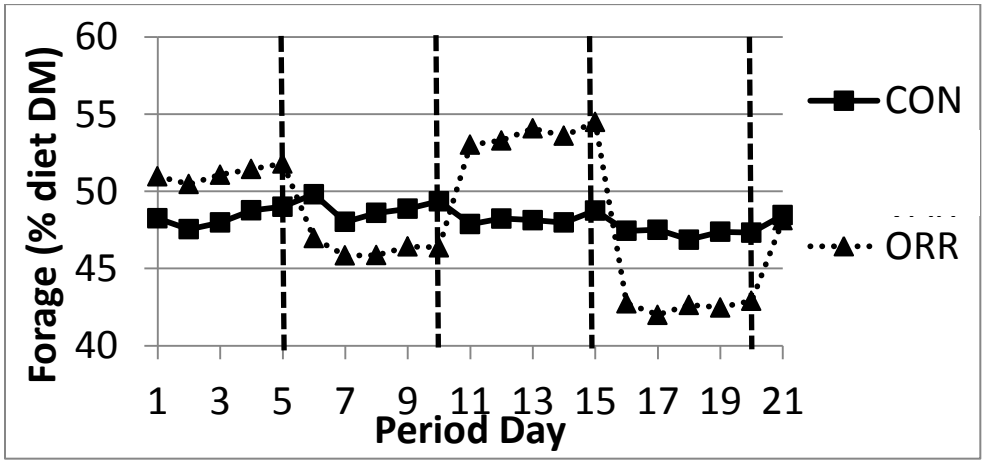

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.

**Figure 4**. Change in forage NDF in treatment diets.

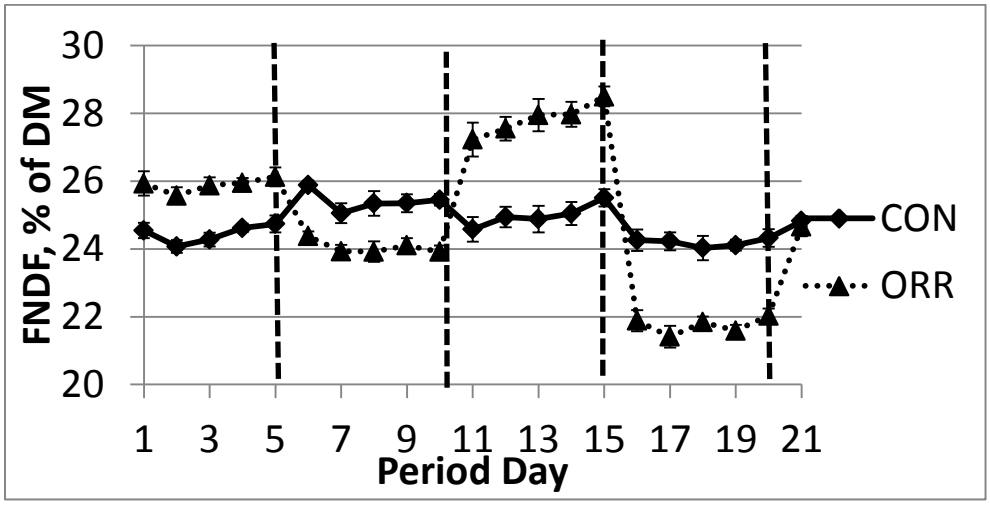
A.




B.



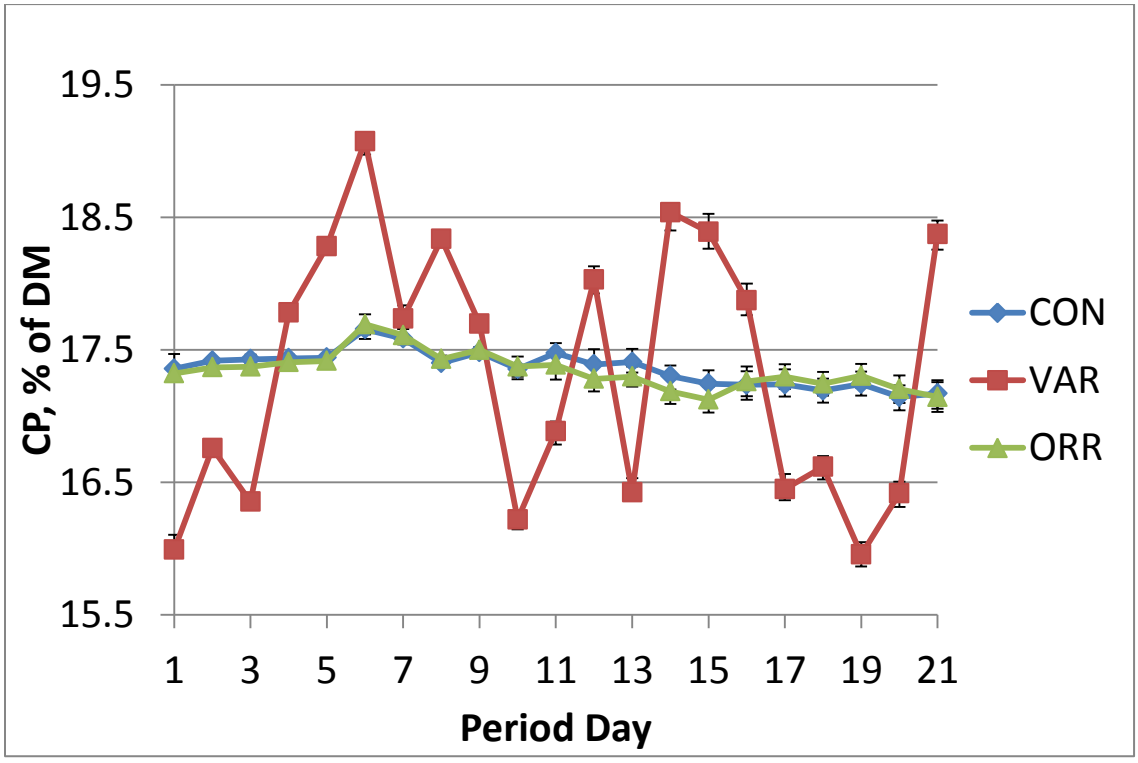

C.




The ORR treatment diet was designed to have 4 hypothetical NDF samples (A), day 1, 6, 11, and 16, that resulted in formulation of greater or less diet forage concentrations (B). The silage population NDF concentration was not representative the hypothetical samples

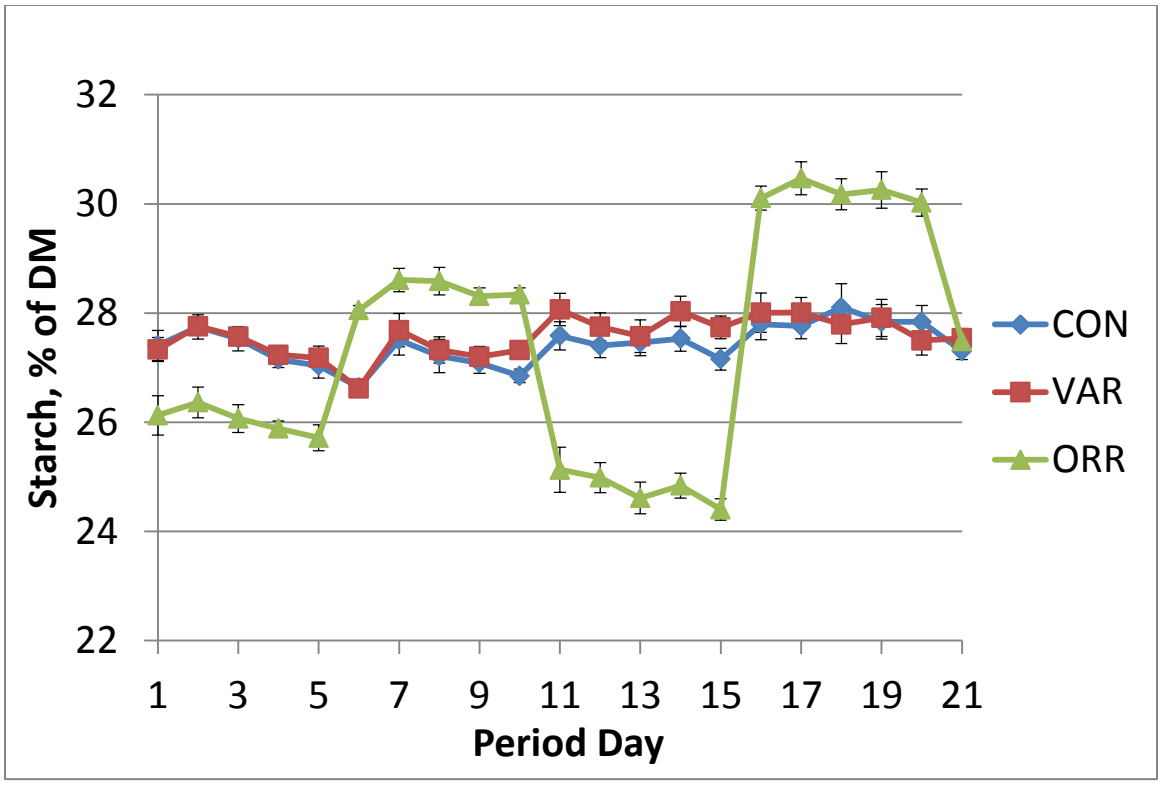
(A). Since the hypothetical samples were errant, the changes in diet forage concentrations resulted in 4 abrupt changes in diet FNDF (C) compared to the CON.

**Figure 5**. ORR treatment, hypothetical NDF samples (A), total forage (% of diet DM) (B), and forage NDF (% of diet DM) (C).




Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.

**Figure 6**. Change in CP of treatment diets.




Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.

**Figure 7**. Change in starch of treatment diets.

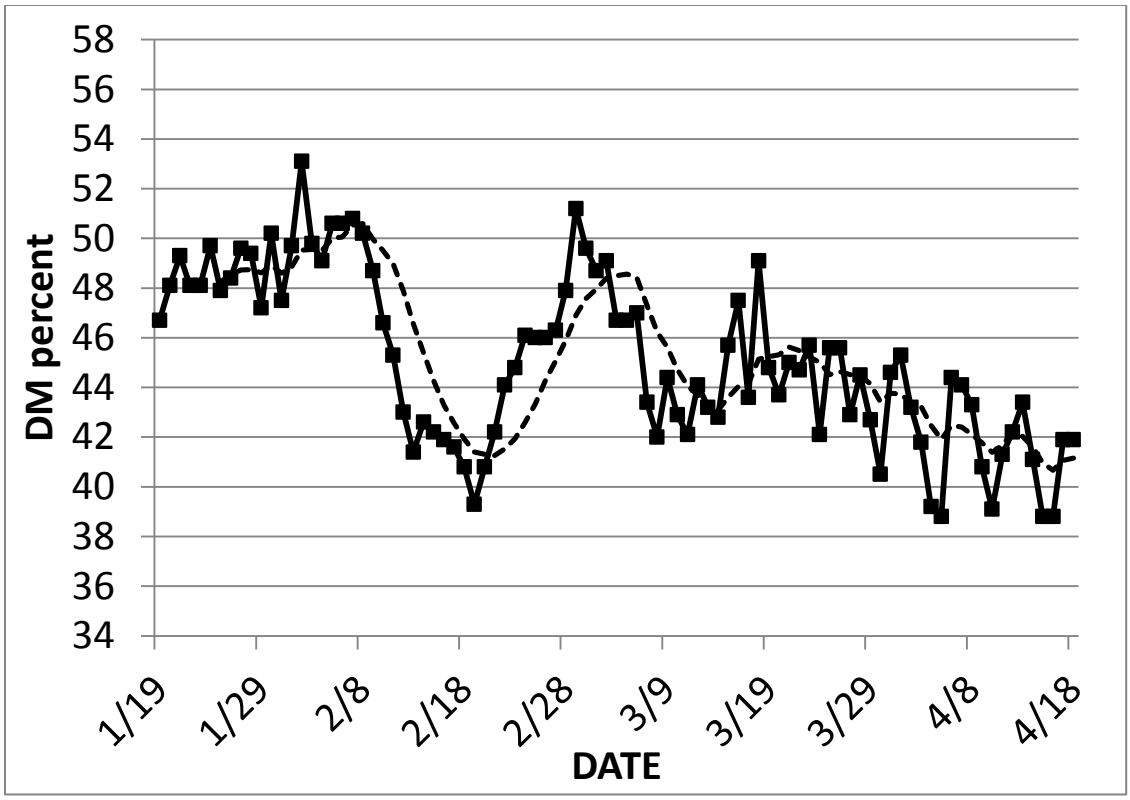



**Figure 8**. Changes in DM concentration in alfalfa silage A and a 7-d moving average (dashed line) of DM concentrations.

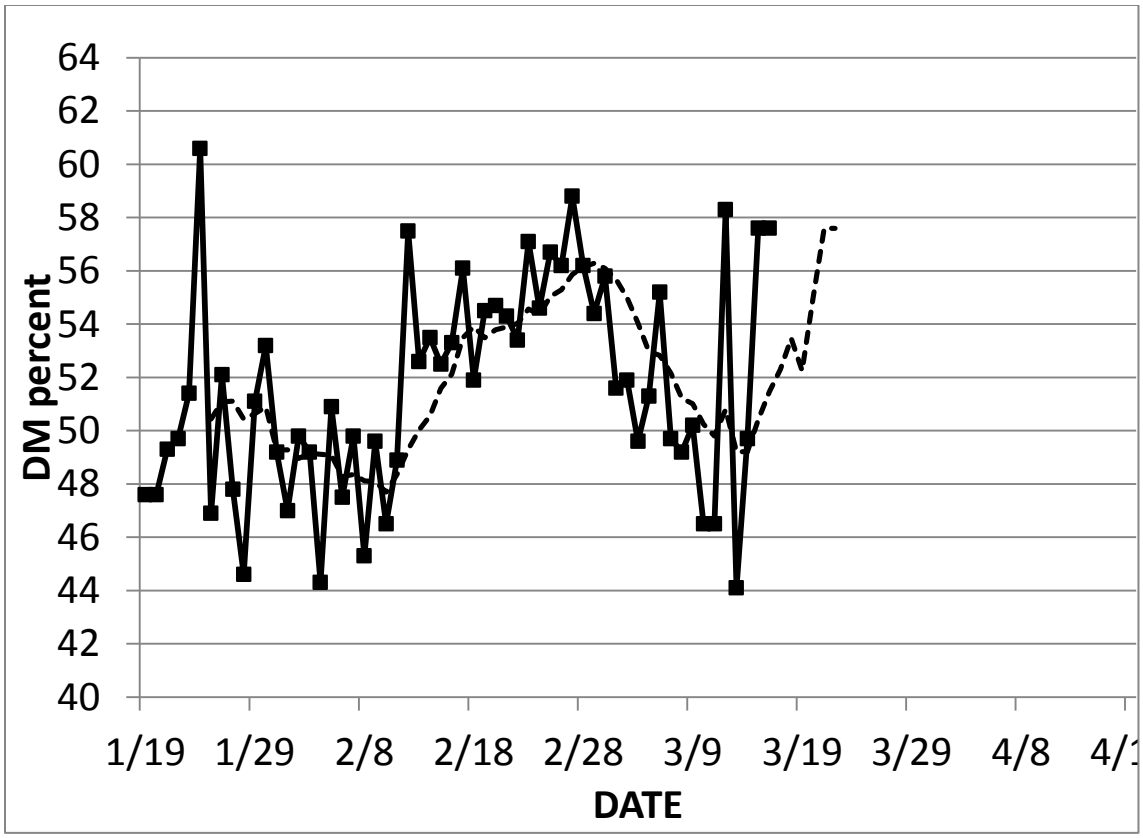



**Figure 9**. Changes in DM concentration in alfalfa silage B and a 7-d moving average (dashed line) of DM concentrations.

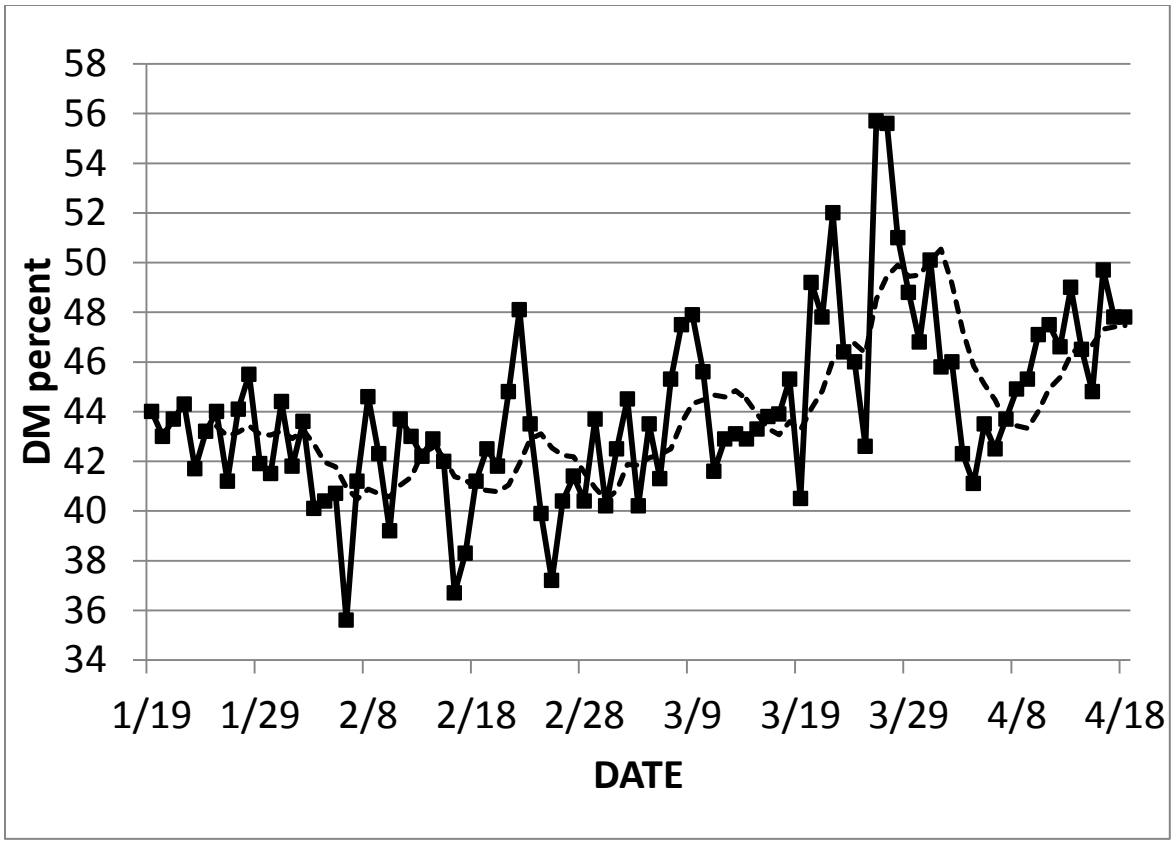
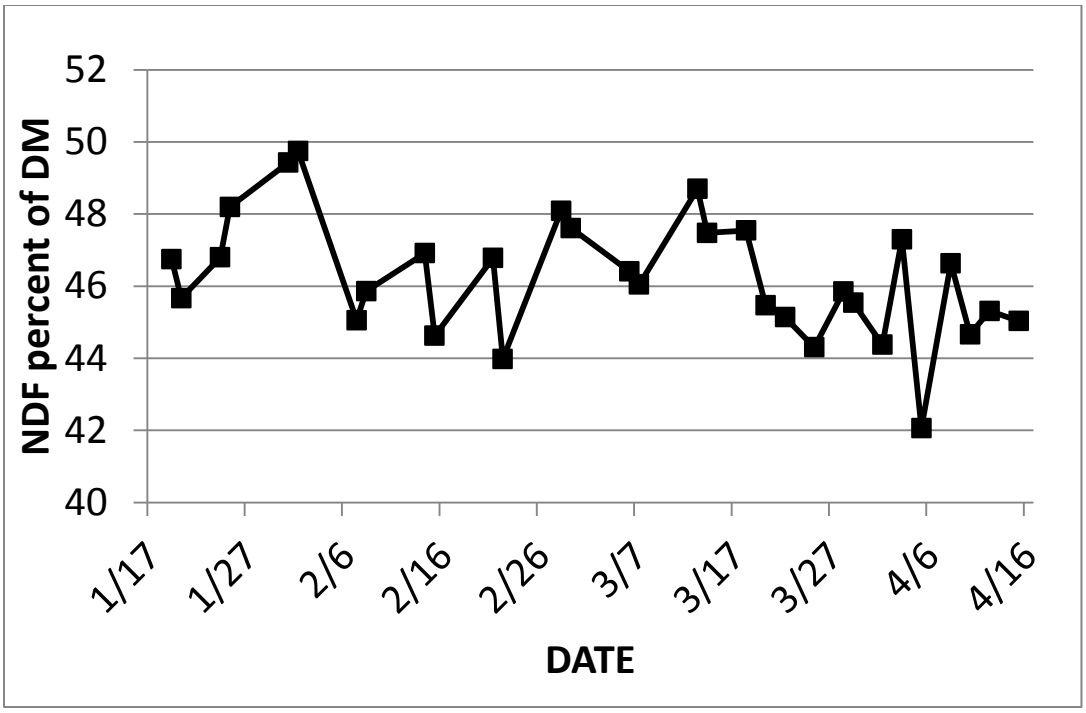



**Figure 10**. Changes in DM concentration in grass silage and a 7-d moving average (dashed line) of DM concentrations.

A.




B.

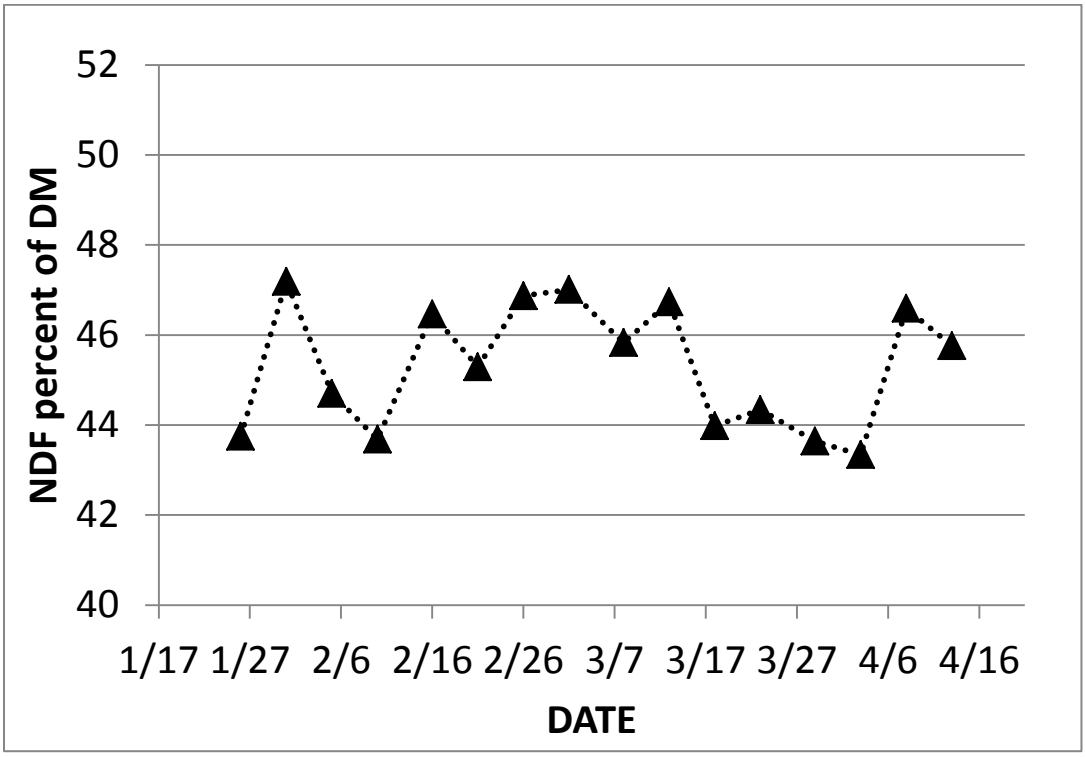
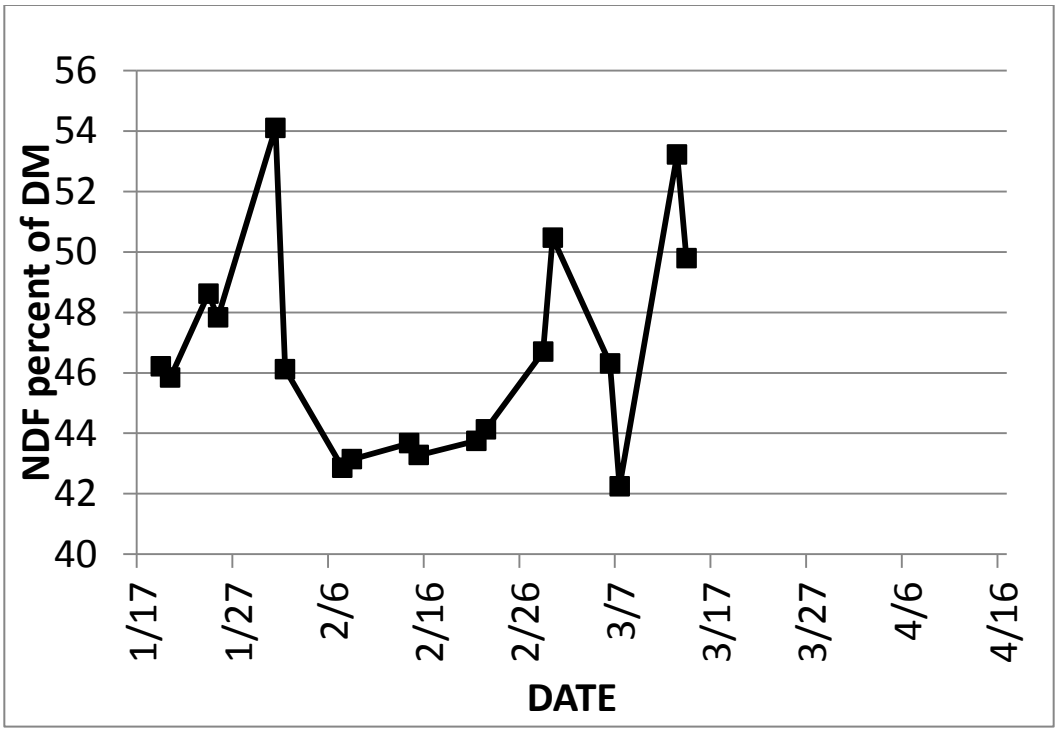



**Figure 11**. Concentrations of NDF in alfalfa silage A on individual days (A) or using 5 d composite samples (B).

A.




B.

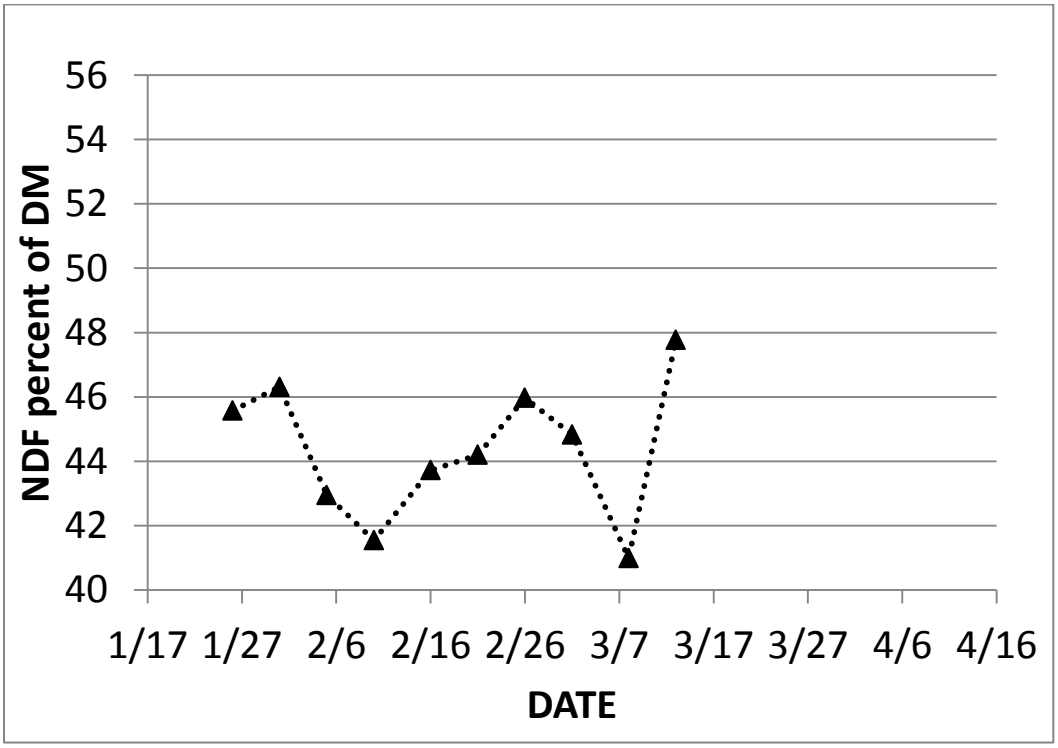
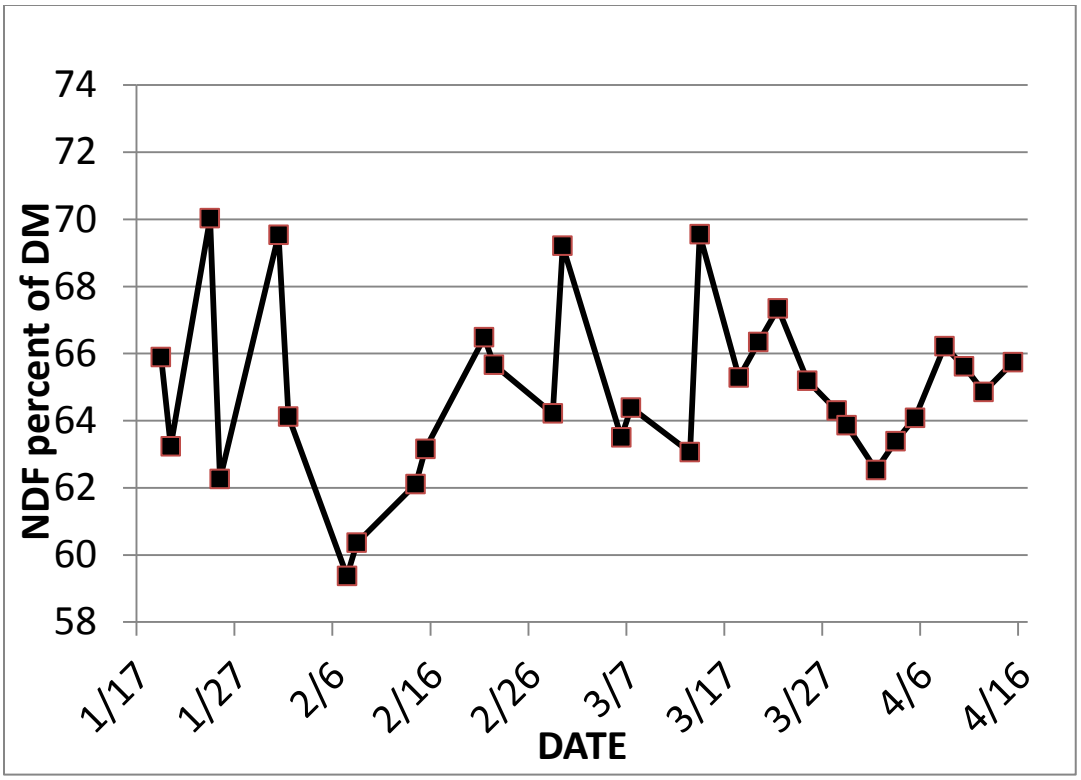



**Figure 12**. Concentrations of NDF in alfalfa silage B on individual days (A) or using 5 d composite samples (B).

A.




B.

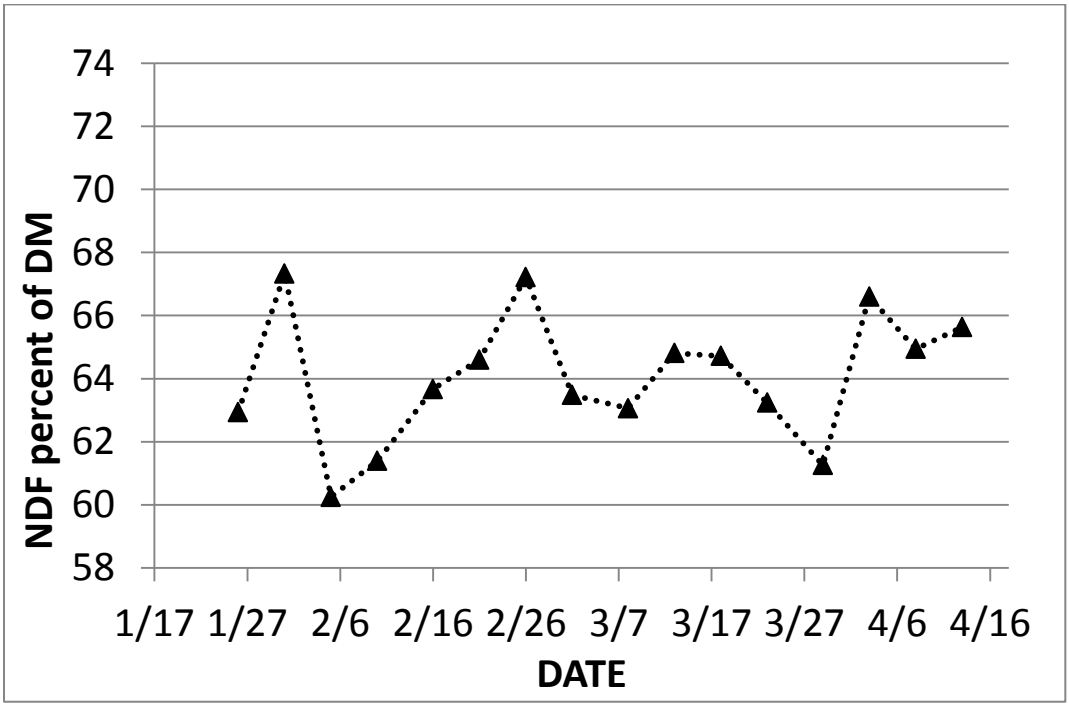
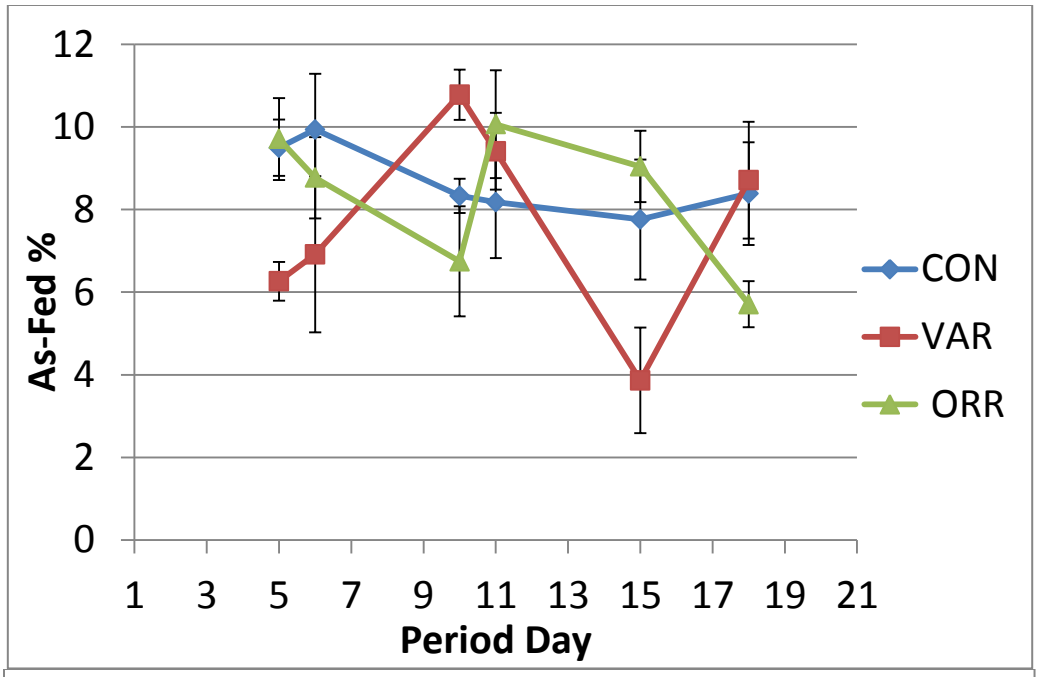
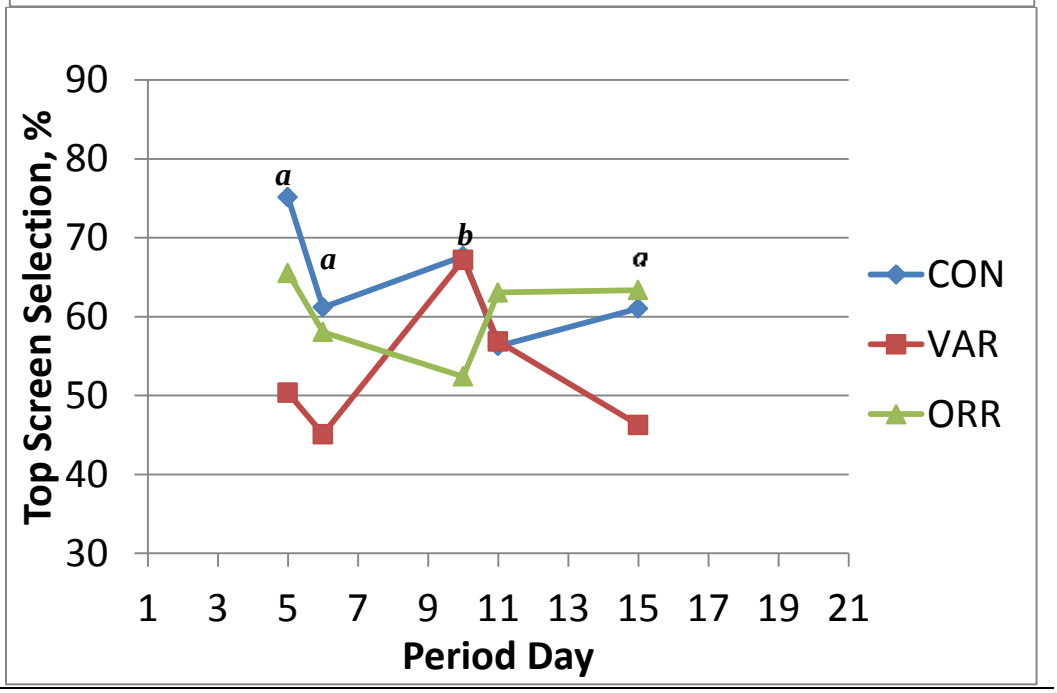



**Figure 13**. Concentrations of NDF in grass silage on individual days (A) or using 5 d composite samples (B).

A.



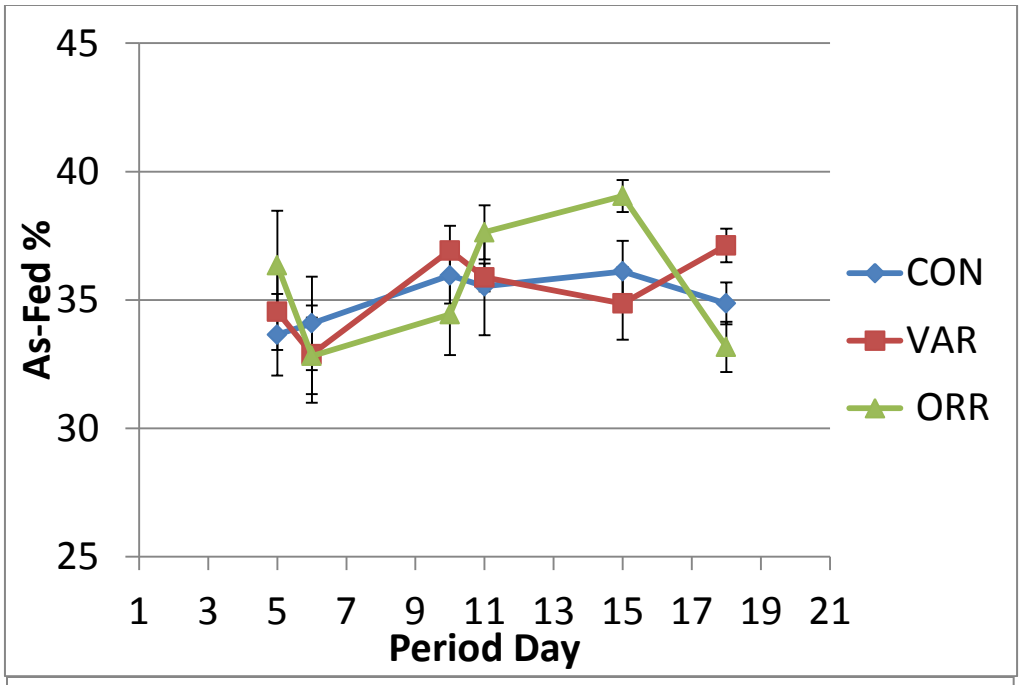

B.



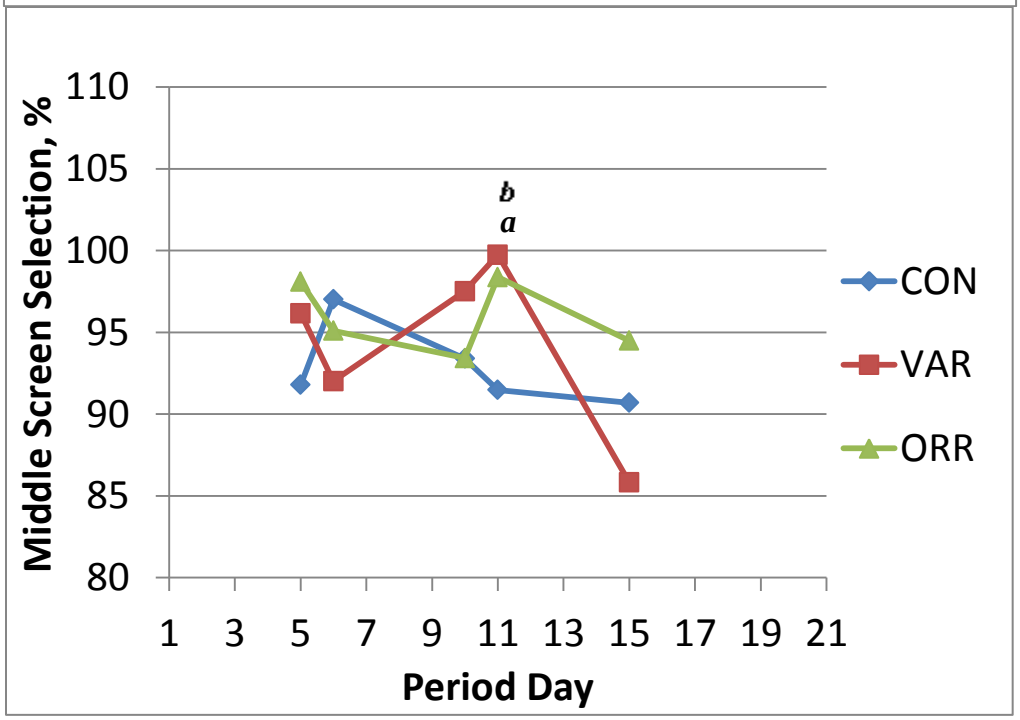

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR treatments $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 6.7 (B)**

**Figure 14.** Effects of treatment on diet top screen as-fed particles (A), and daily 4 h selection of top screen particles (B).

A.



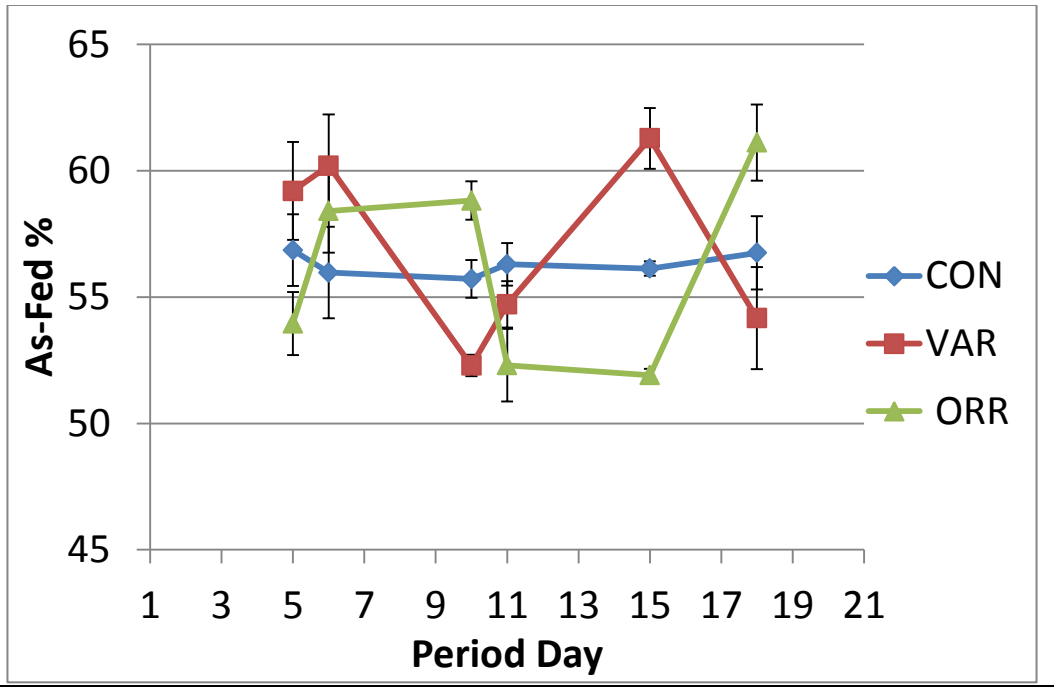

B.



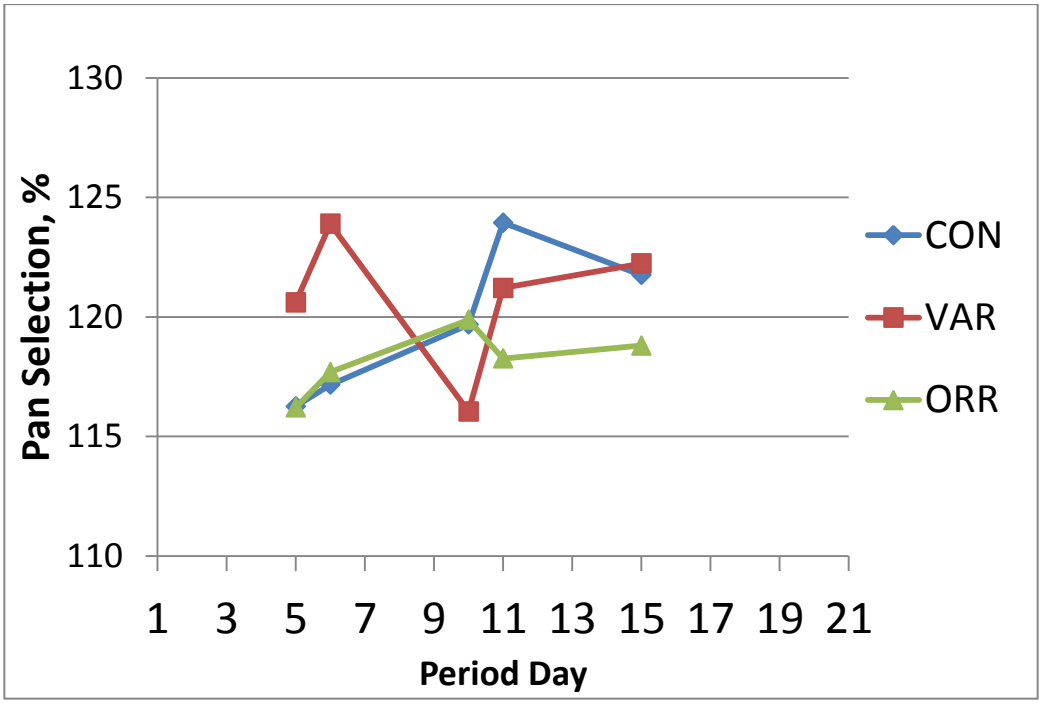

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR treatments $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 3.6 (B)**

**Figure 15**. Effects of treatment on diet middle screen as-fed particles (A), and daily 4 h selection of middle screen particles (B).

A.



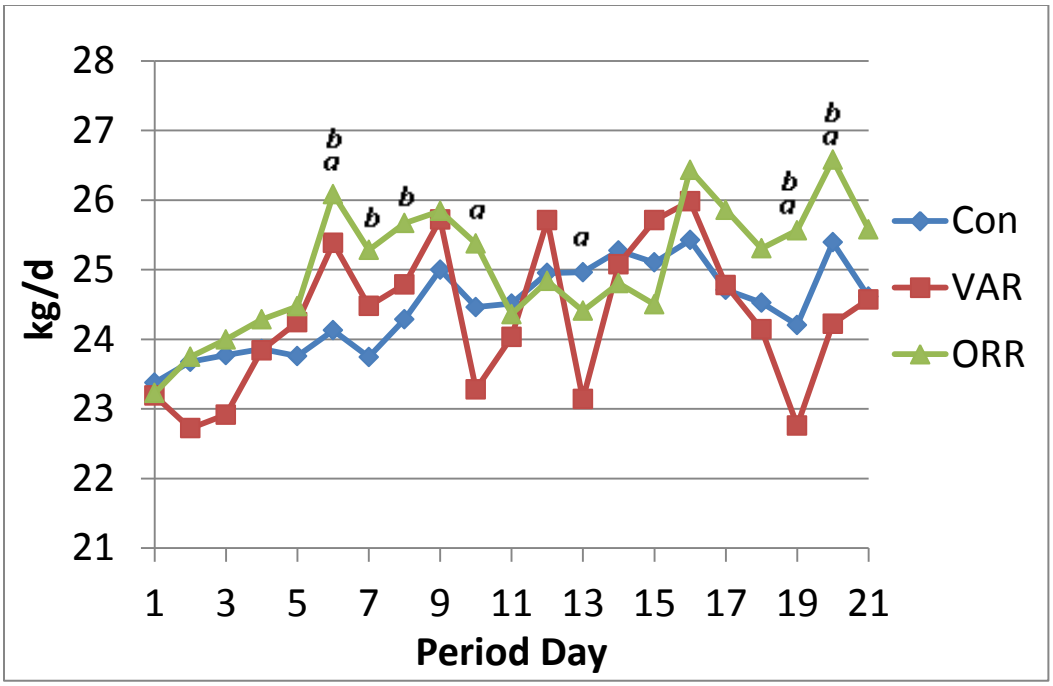

B.



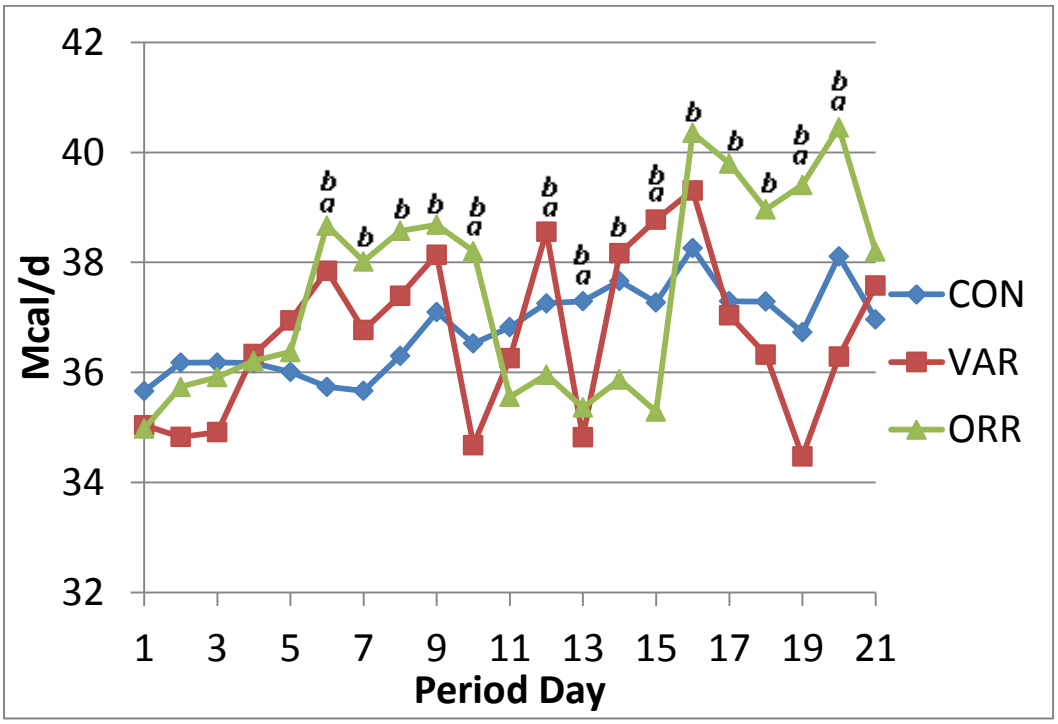

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR treatments $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 3.2 (B)**

**Figure 16**. Effects of treatment on diet pan as-fed particles (A), and daily 4 h selection of pan particles (B).

A.



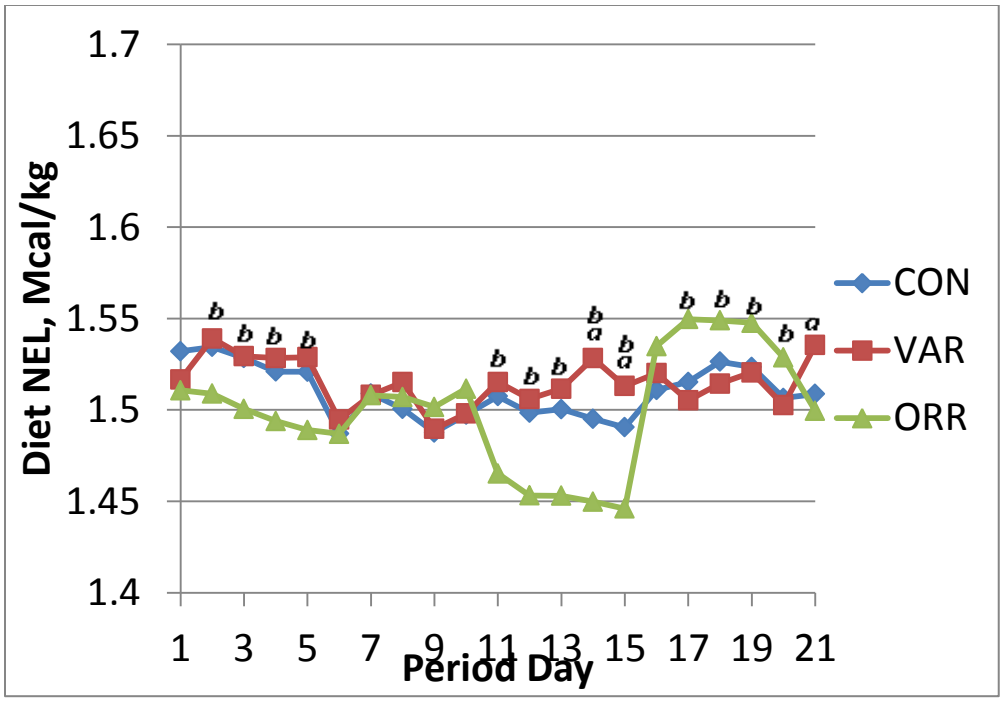

B.



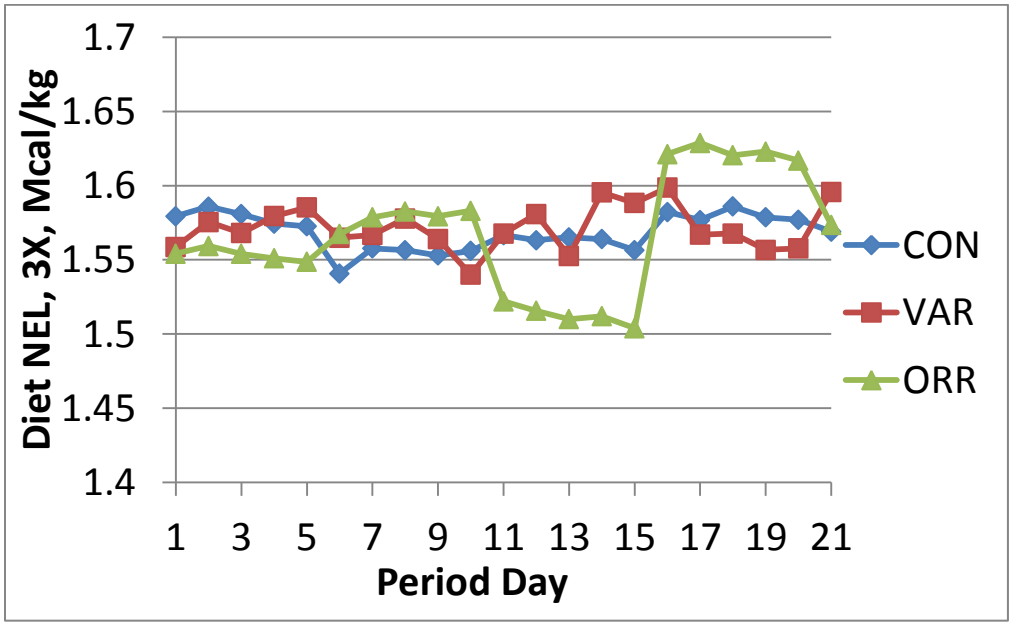

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 0.66 (A), 0.81 (B).**

**Figure 17**. Effects of treatment on daily DMI (A) and daily NEL intake (B).

A.



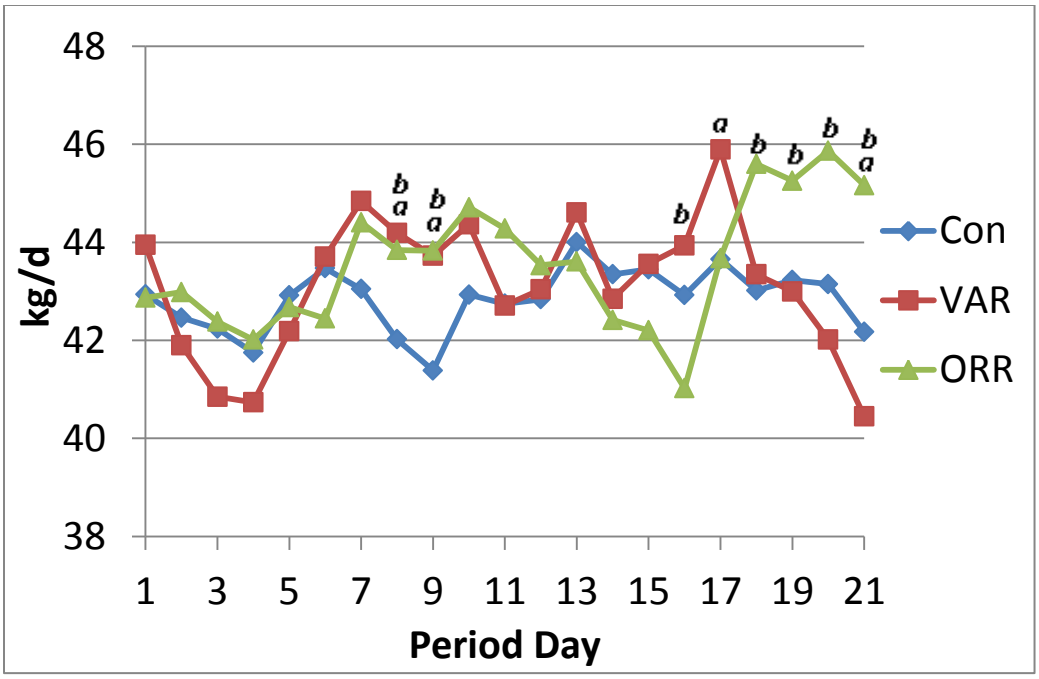

B.



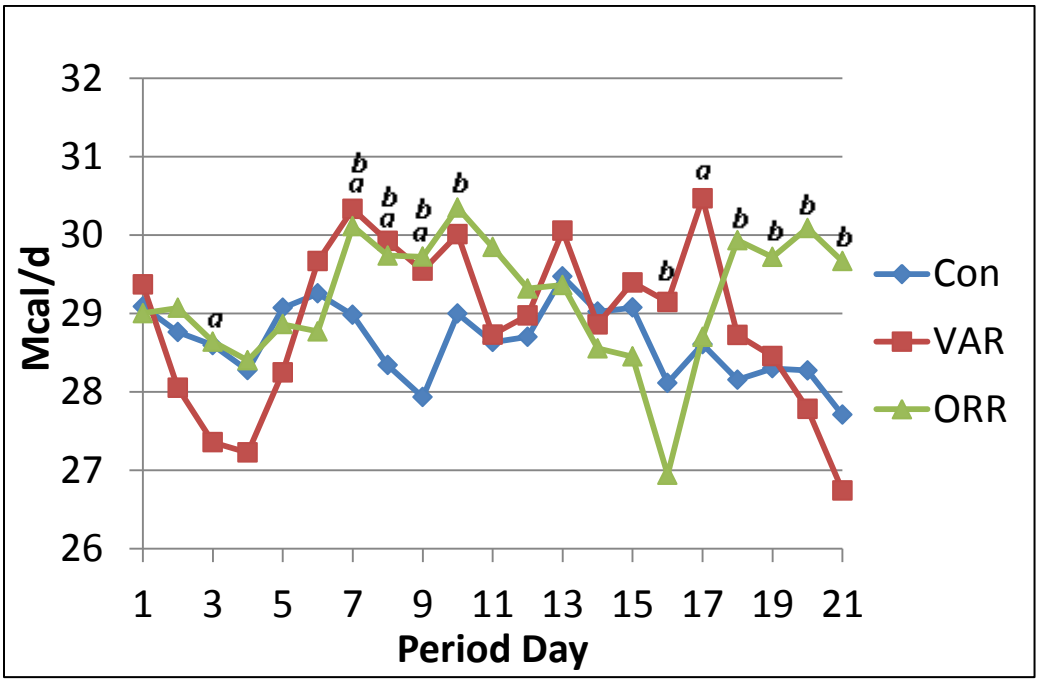

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Diet NEL is calculated using actual DMI and NEL 3X is calculated using a constant 3X level of intake. Treatment comparisons; *a*= CON vs. VAR *P*<0.1; *b*= CON vs ORR treatments *P*<0.1. **SEM = 0.01 (A), 0.01 (B).**

**Figure 18**. Effects of treatments on diet NEL concentration (discount adjusted for DMI) (A) and diet NEL 3X concentrations (3X discount) (B).

A.



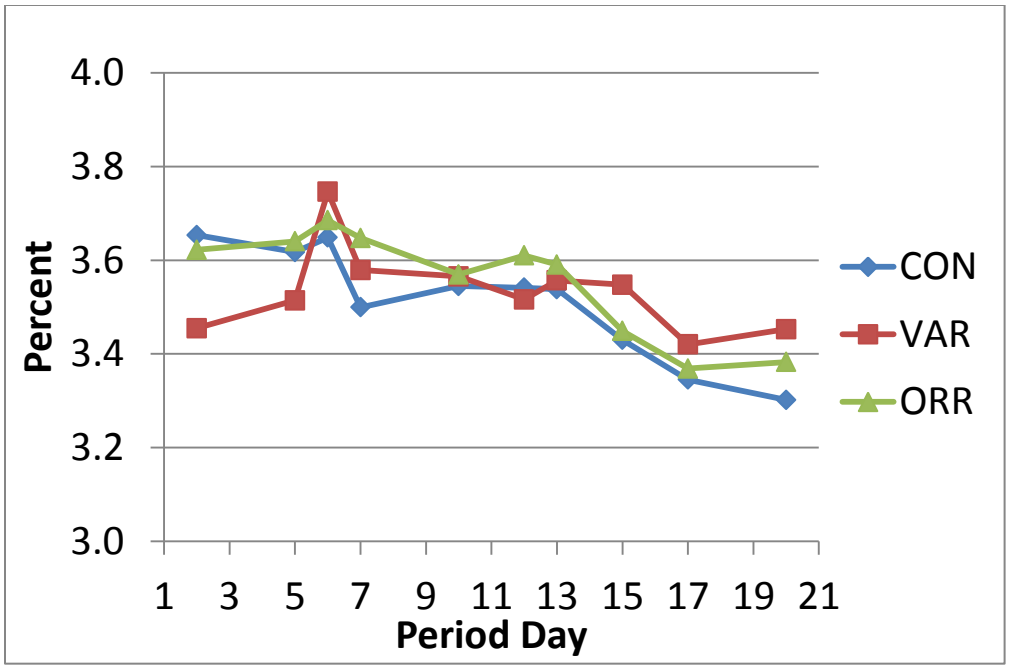

B.



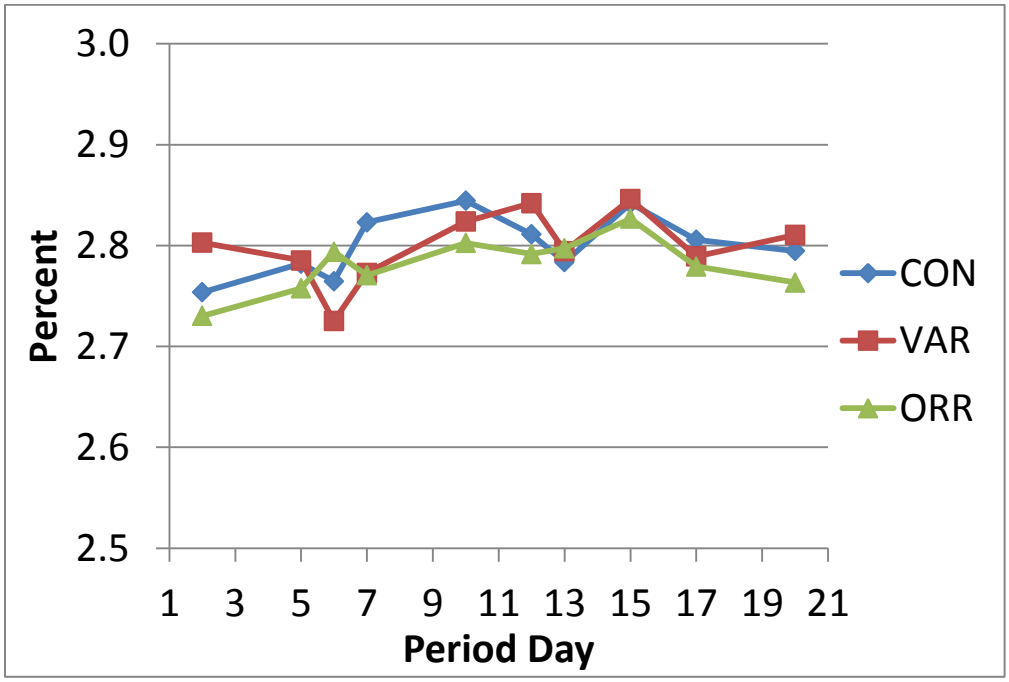

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR *P*<0.1; *b*= CON vs ORR treatments *P*<0.1. **SEM = 1.30 (A), 1.04 (B).**

**Figure 19** . Effect of treatment on daily milk production (A) and daily milk energy output (B).

A.

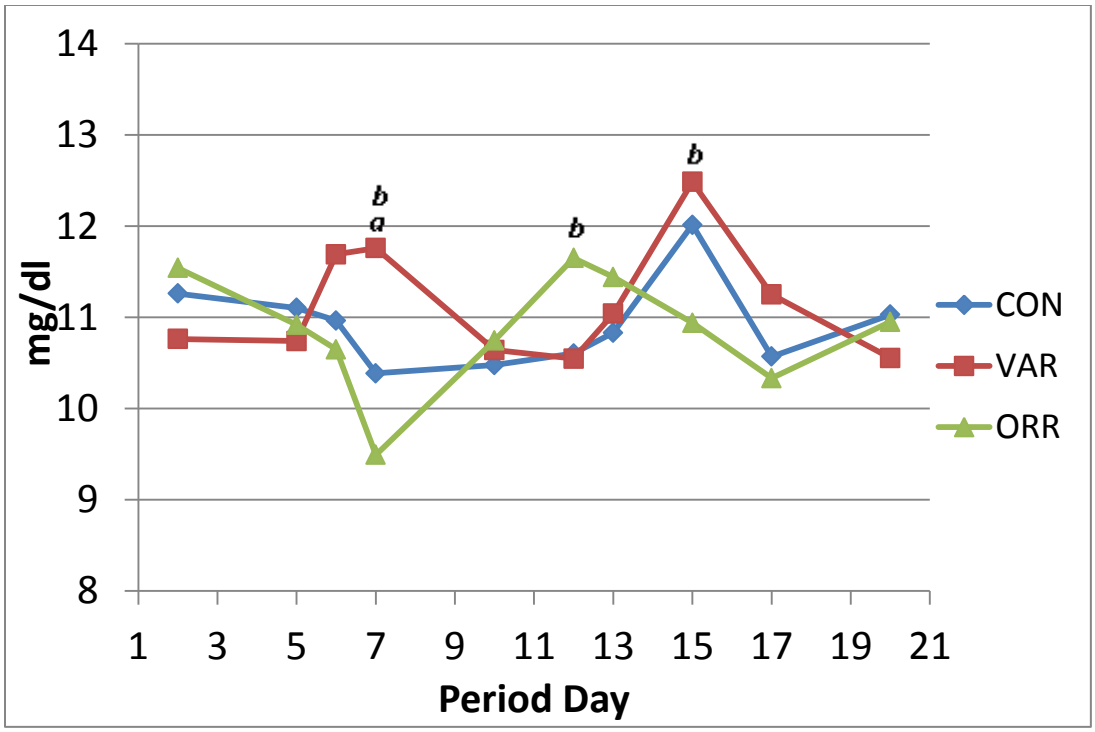



B.



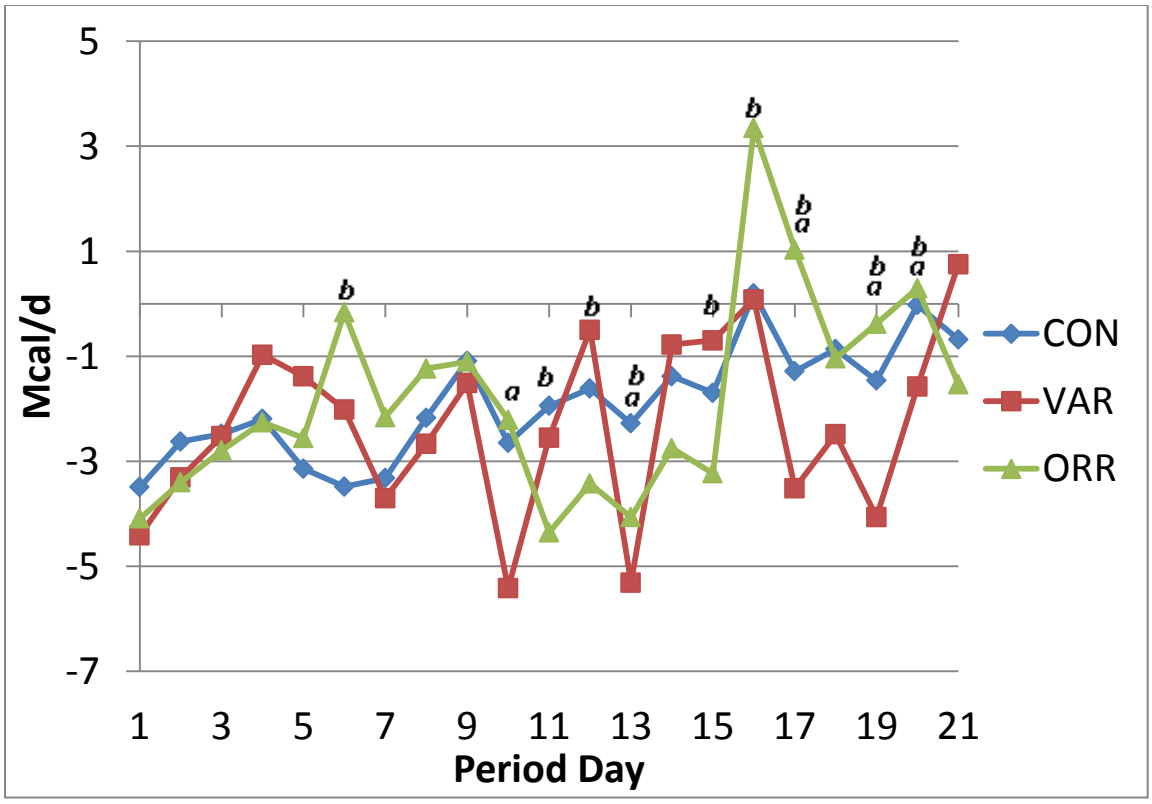

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. No treatment by day differences were observed. SEM = 0.20 (A), 0.06 (B)

**Figure 20**. Effect of treatment on daily milk fat percent (A) and daily milk protein percent (B).



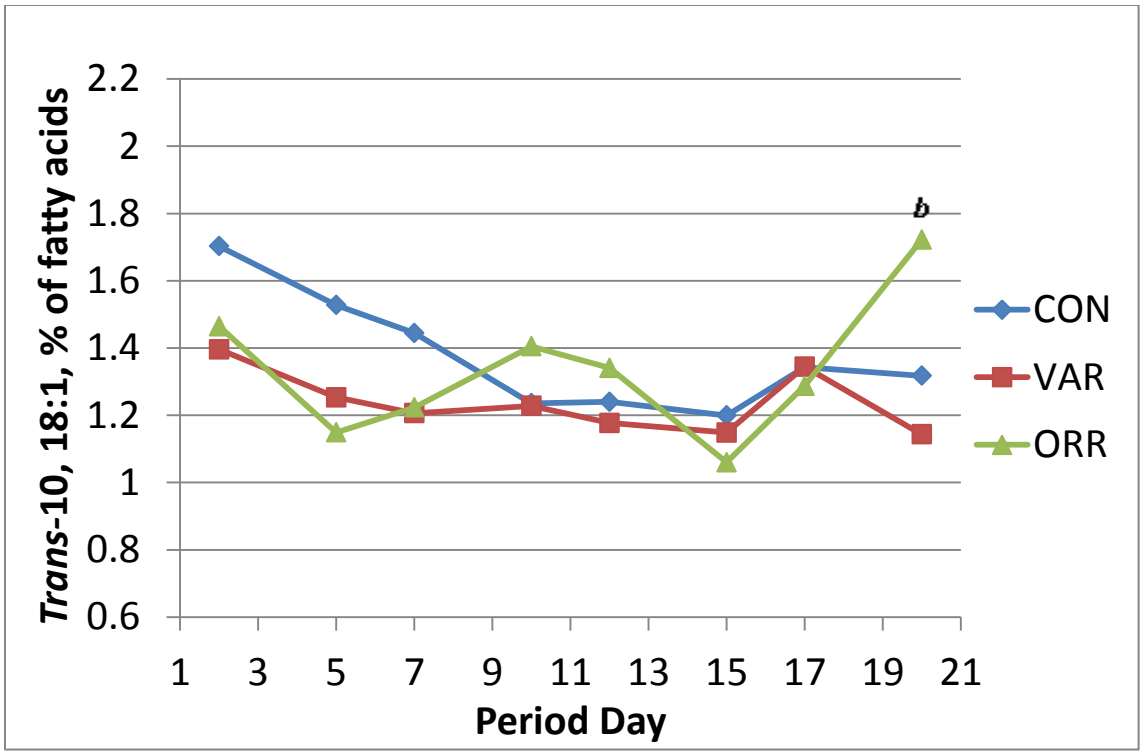

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 0.61.**

**Figure 21**. Effect of treatment on daily MUN concentrations.




Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR *P*<0.1; *b*= CON vs ORR treatments *P*<0.1. **SEM = 1.01**

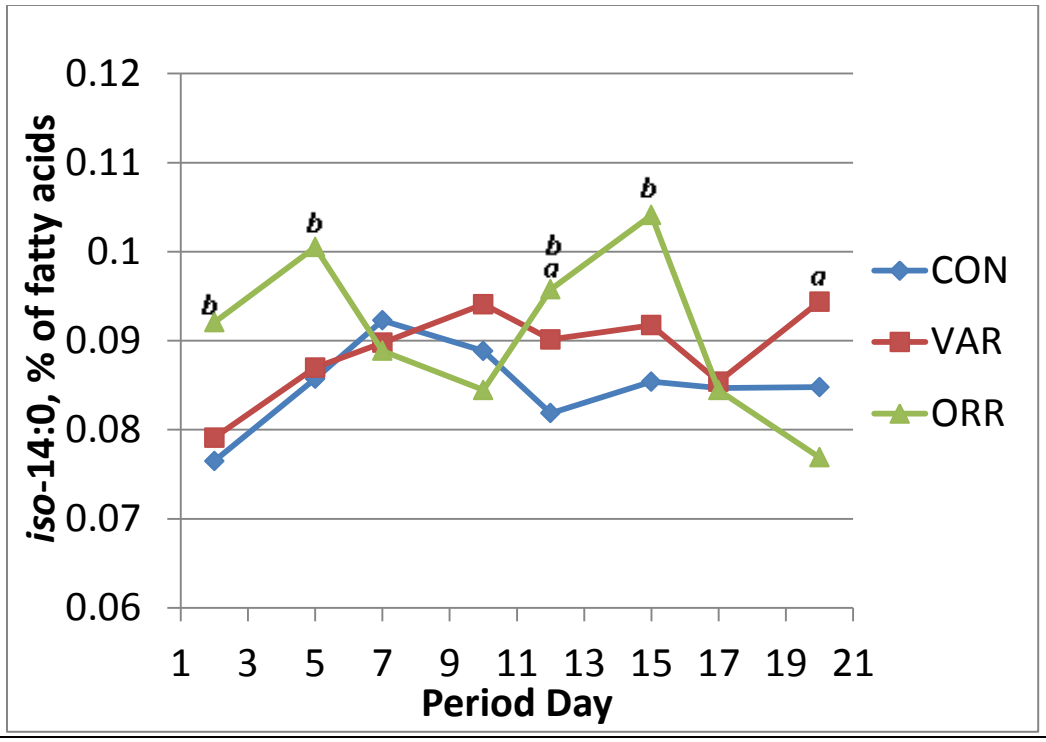
**Figure 22**. Effect of treatment on calculated daily net energy balance.



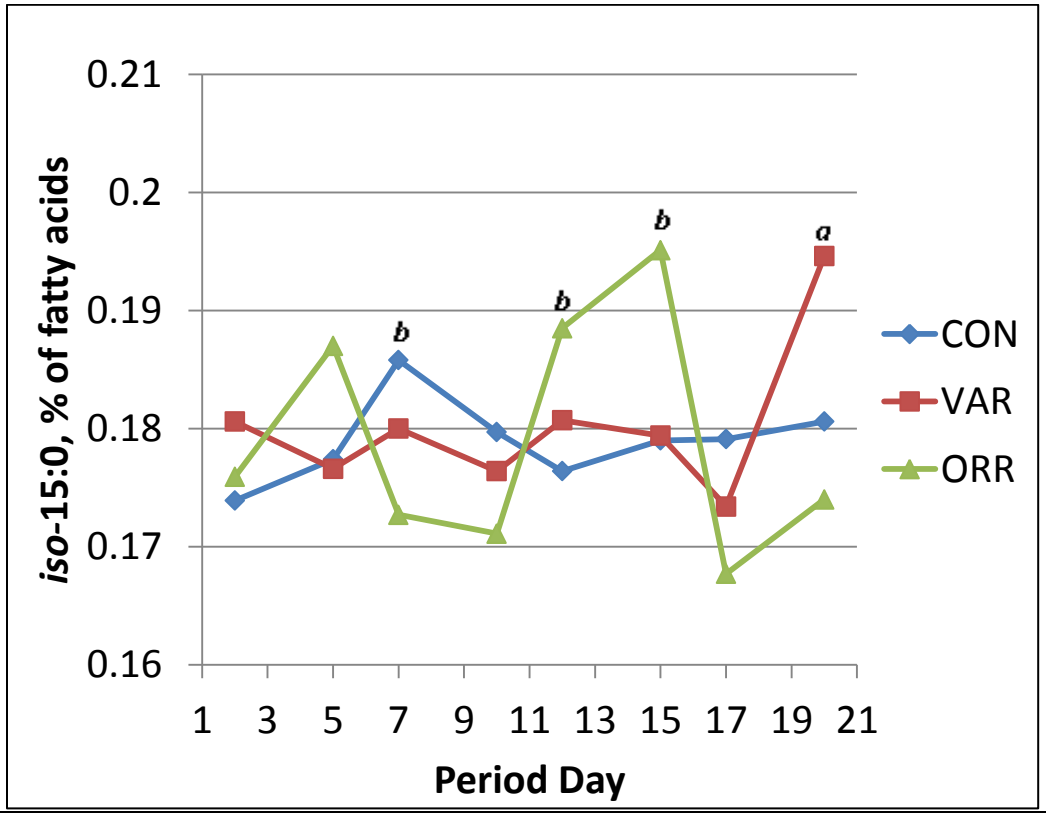

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 0.22**

**Figure 23**. Effect of treatment on milk fatty acid (biohydrogenation marker) *trans*-10, C18:1.

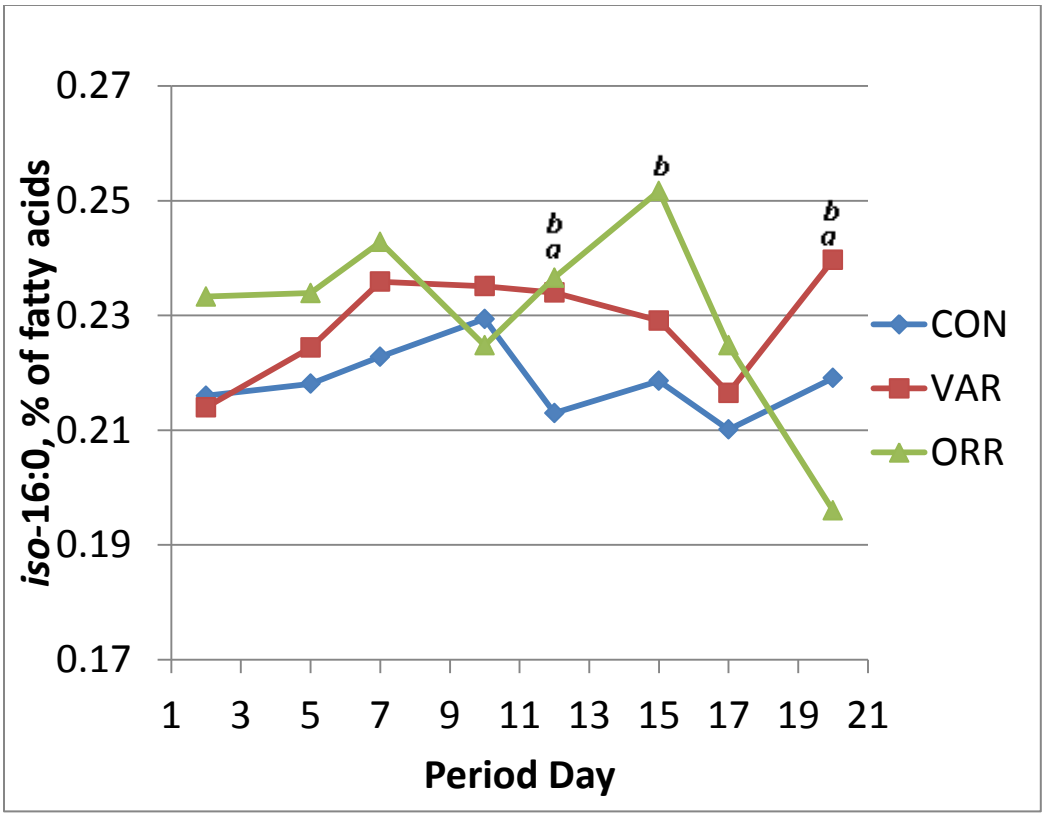
A.


0.12
0.11
0.1
0.09
0.08
0.07
0.06
iso-14:0, % of fatty acids
1 3 5 7 9 11 13 15 17 19 21
Period Day
CON
VAR
ORR
b
b
b
a
b
a

B.


0.21
0.2
0.19
0.18
0.17
0.16
iso-15:0, % of fatty acids
1 3 5 7 9 11 13 15 17 19 21
Period Day
CON
VAR
ORR
b
b
b
a

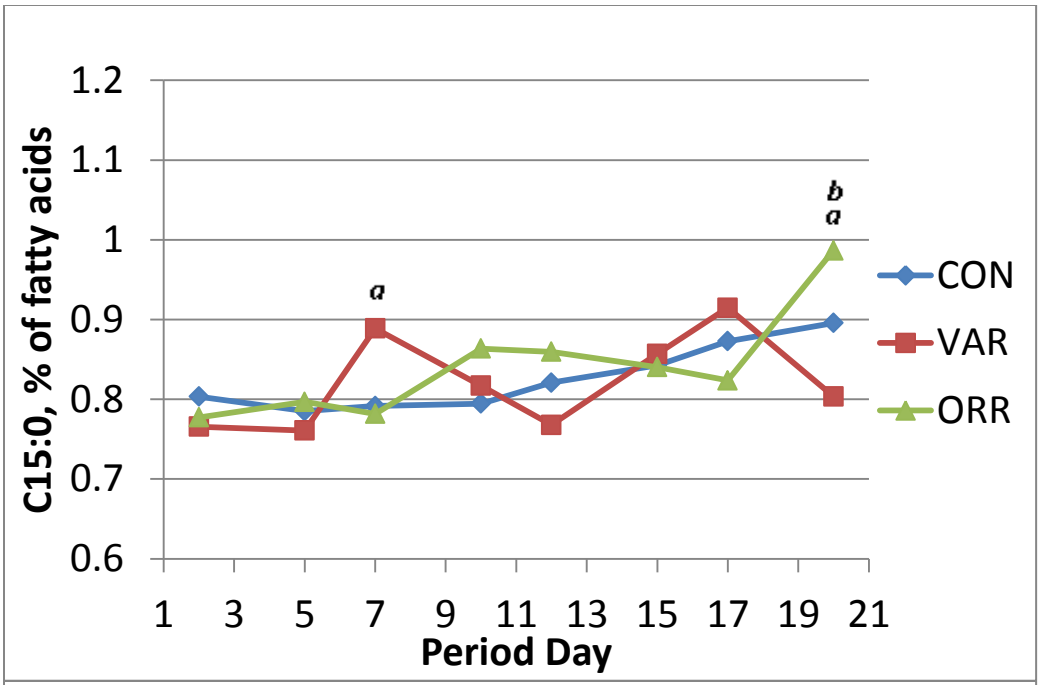
C.



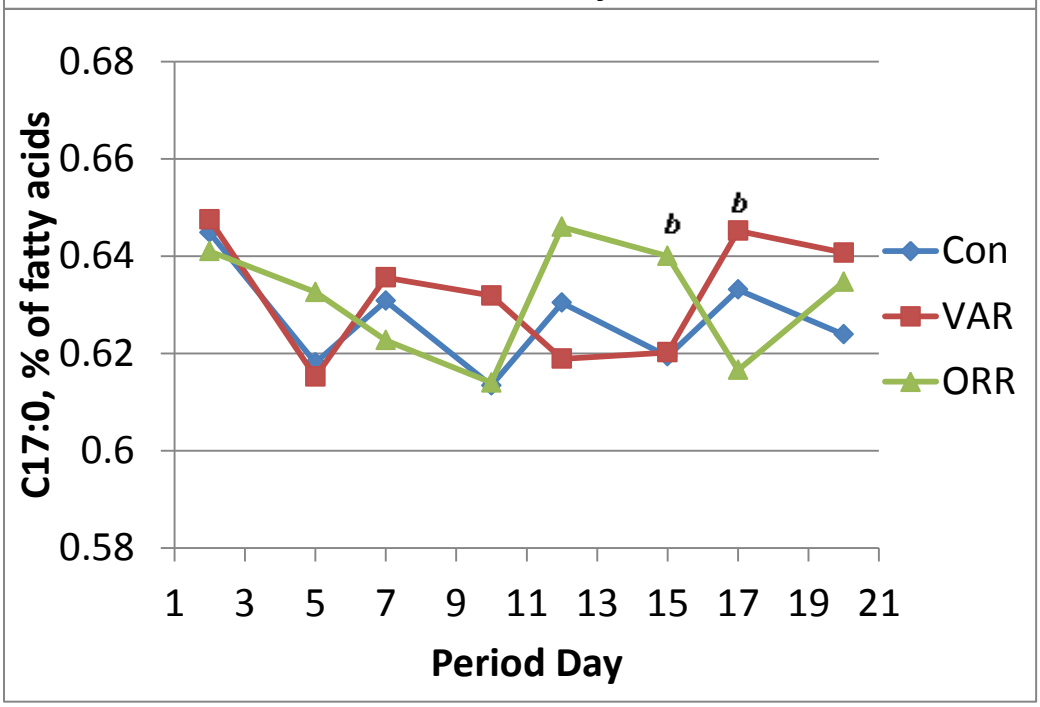

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR $P<0.1$; *b*= CON vs ORR treatments $P<0.1$. **SEM = 0.006 (A), 0.005 (B), 0.01 (C)**

**Figure 24**. Effect of treatment on milk fatty acids (cellulolytic markers), *iso*-14:0 (A), *iso*-15:0 (B), and *iso* 16:0 (C).

A.



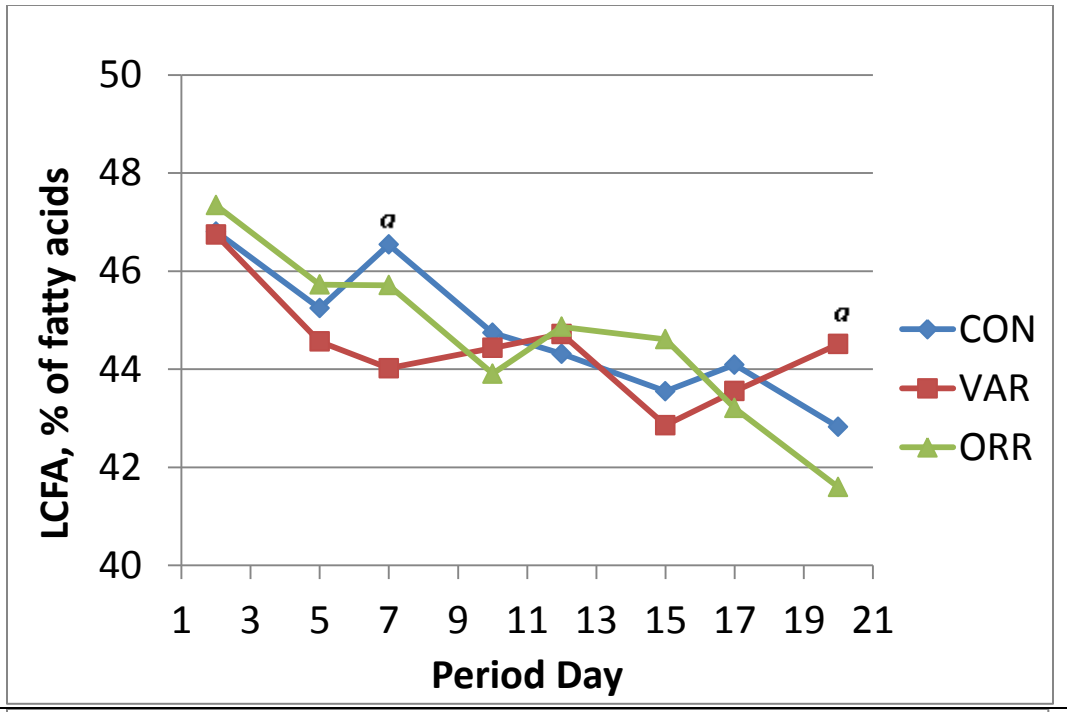

B.



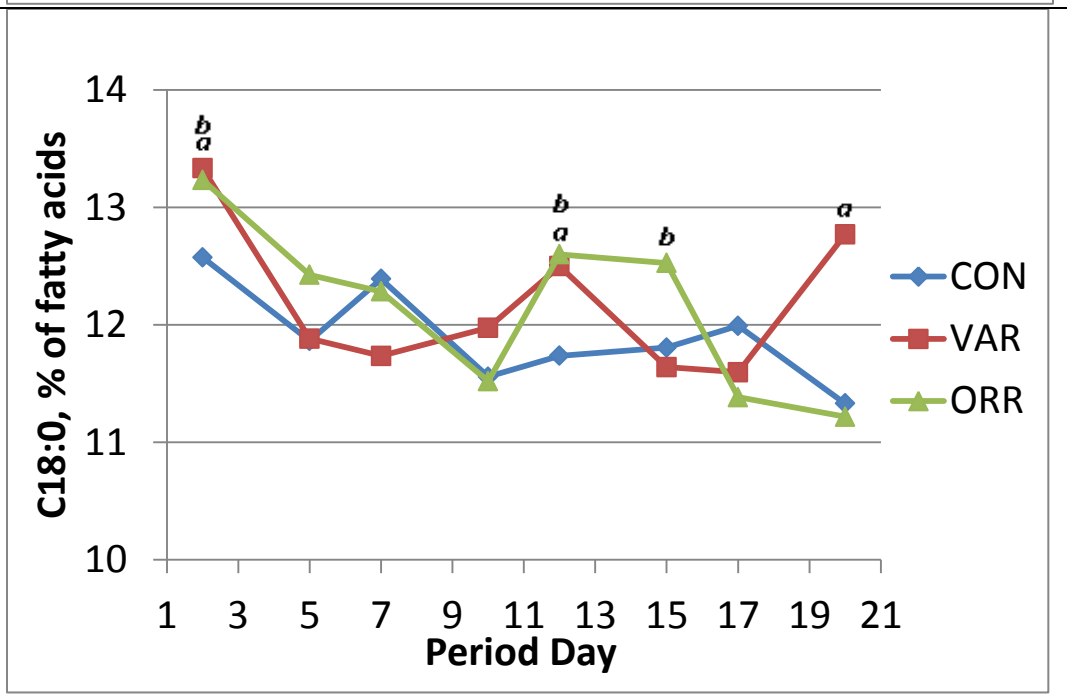

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR P<0.1; *b*= CON vs ORR treatments P<0.1. **SEM = 0.057 (A), 0.012 (B).**

**Figure 25**. Effect of treatment on milk fatty acids C15:0 (A) and C17:0 (B).

A.




B.




Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet. Treatment comparisons; *a*= CON vs. VAR P<0.1; *b*= CON vs ORR treatments P<0.1. **SEM = 1.20 (A), 0.43 (B).**

**Figure 26**. Effect of treatment milk long chain fatty acids (C ≥18) (A) and C18:0 (B).

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# Appendix

**Table 22**. Fatty acid composition of diet ingredients.

| **Fatty Acid, g/100g** [2] | **Alfalfa Silage A** | **Alfalfa Silage B** | **Grass Silage** | **Concentrate** |
|---|---|---|---|---|
| 10:0 | 1.38 | 0.86 | 0.06 | nd[1] |
| 12:0 | 2.18 | 2.71 | 1.46 | 0.26 |
| 14:0 | 0.765 | 1.21 | 1.79 | 0.49 |
| 14:1 | 0.61 | 0.68 | 0.65 | nd |
| 16:0 | 15.60 | 17.74 | 14.91 | 14.83 |
| 16:1 | 0.97 | 1.28 | 0.94 | 0.78 |
| 18:0 | 2.80 | 3.51 | 1.88 | 5.70 |
| Other *trans*, 18:1 | 0.36 | 0.42 | 0.43 | 0.20 |
| *trans*-9, 18:1 | 0.05 | nd | nd | 0.11 |
| *trans*-10, 18:1 | nd | nd | 0.10 | 0.27 |
| *trans*-11, 18:1 | nd | 0.13 | 0.34 | 0.16 |
| *trans*-12, 18:1 | 0.02 | 0.05 | 0.53 | 0.04 |
| *cis*-9, 18:1 | 2.67 | 2.39 | 5.89 | 26.05 |
| *cis*-11, 18:1 | 2.24 | 1.79 | 1.45 | 1.30 |
| 18:2 | 14.66 | 11.28 | 12.95 | 42.70 |
| 20:0 | 1.04 | 1.11 | 0.94 | 0.41 |
| 18:3 | 23.85 | 22.26 | 20.85 | 2.17 |
| 22:0 | 1.34 | 1.49 | 1.22 | 0.26 |
| 20:5 | 0.69 | 2.50 | 0.81 | 0.09 |
| Other FA | 28.81 | 28.61 | 32.84 | 4.20 |

[1]nd = fatty acid <0.01g/100g.

[2]Number of carbons: number of double bonds.

**Table 23**. Average fatty acid composition of the diets.

| | Diet | | |
|---|---|---|---|
| **Fatty Acid, g/100g[1]** | **CON** | **VAR** | **ORR** |
| 10:0 | 0.40 | 0.40 | 0.40 |
| 12:0 | 1.13 | 1.12 | 1.13 |
| 14:0 | 0.84 | 0.83 | 0.84 |
| 14:1 | 0.31 | 0.31 | 0.31 |
| 16:0 | 15.34 | 15.29 | 15.32 |
| 16:1 | 0.90 | 0.89 | 0.90 |
| 18:0 | 4.23 | 4.23 | 4.22 |
| Other *trans* 18:1 | 0.29 | 0.29 | 0.29 |
| *trans*-9, 18:1 | 0.07 | 0.07 | 0.07 |
| *trans*-10, 18:1 | 0.16 | 0.16 | 0.16 |
| *trans*-11, 18:1 | 0.15 | 0.15 | 0.15 |
| *trans*-12, 18:1 | 0.12 | 0.12 | 0.12 |
| *cis*-9, 18:1 | 15.27 | 15.31 | 15.23 |
| *cis*-11, 18:1 | 1.58 | 1.58 | 1.58 |
| 18:2 | 28.55 | 28.61 | 28.49 |
| 20:0 | 0.71 | 0.70 | 0.71 |
| 18:3 | 11.99 | 11.91 | 12.01 |
| 22:0 | 0.78 | 0.77 | 0.78 |
| 20:5 | 0.60 | 0.58 | 0.59 |
| Other FA | 16.72 | 16.61 | 16.74 |

Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[1]Number of carbons: number of double bonds.

**Table 24**. Effects of treatment on transcription mRNA abundance (LSM Ct values) in adipose tissue using reference genes as a covariate.

| | Treatment[1] | | | | P-value[2] | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene[3,4]** | **CON** | **VAR** | **ORR** | **SEM** | **Trt** | **Cow** | **Period** | **Period*Trt** |
| LPL | 28.5 | 27.4 | 27.8 | 0.97 | 0.67 | 0.25 | 0.08 | 0.57 |
| FASN | 29.4 | 29.3 | 28.9 | 0.86 | 0.90 | 0.06 | 0.06 | 0.12 |
| SCD | 27.7 | 27.0 | 26.1 | 1.28 | 0.77 | 0.36 | 0.06 | 0.62 |
| S14 | 24.9 | 25.2 | 23.8 | 1.03 | 0.60 | 0.09 | 0.04 | 0.17 |
| ATGL | 28.2 | 28.0 | 28.0 | 0.53 | 0.94 | 0.11 | 0.39 | 0.20 |
| ABHD5 | 28.6 | 28.2 | 28.5 | 0.38 | 0.66 | 0.25 | 0.07 | 0.48 |
| HSL | 30.7 | 30.0 | 31.3 | 1.06 | 0.63 | 0.23 | 0.36 | 0.36 |
| FIAF | 30.4 | 30.2 | 30.4 | 0.27 | 0.72 | 0.16 | 0.22 | 0.06 |

[1]Treatments: CON=consistent FNDF diet; VAR = daily variation FNDF diet; ORR= 5-d variation FNDF diet.
[2]Fixed effect of treatment, cow, period, and period by treatment interaction.
[3] Number of cows per treatment; 8, 8, and 7 CON, VAR, and ORR cows for LPL, FASN, S14, ATGL, and FIAF; 7, 8, and 7 CON, VAR, and ORR cows for SCD; 7, 7, and 6 CON, VAR, and ORR cows for ABHD5 and HSL.
[4]Lipoprotein lipase (LPL), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), thyroid hormone responsive SPOT 14 (S14), adipose triglyceride lipase (ATGL), abhydrolase domain containing 5 (ABHD5), hormone-sensitive lipase (HSL), and fasting-induced adipose factor (FIAF).

**Table 25**. Effect of period on transcription mRNA abundance (LSM Ct values) in adipose tissue using reference genes as a covariate

| | Period[1] | | | | P-value[2] | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene** | **Period 1** | **Period 2** | **Period 3** | **SEM** | **Period** | **1*2** | **1*3** | **2*3** |
| LPL[3,4,5,6] | 29.8 | 28.1 | 25.9 | 1.00 | 0.08 | 0.23 | 0.03 | 0.14 |
| FASN | 31.2 | 28.6 | 27.7 | 0.88 | 0.06 | 0.06 | 0.03 | 0.46 |
| SCD | 29.7 | 27.0 | 24.2 | 1.28 | 0.06 | 0.14 | 0.03 | 0.16 |
| S14 | 27.4 | 24.2 | 22.3 | 1.06 | 0.04 | 0.05 | 0.01 | 0.22 |
| ATGL | 28.5 | 27.5 | 28.2 | 0.54 | 0.39 | 0.20 | 0.74 | 0.37 |
| ABHD5 | 29.4 | 28.4 | 27.6 | 0.41 | 0.07 | 0.07 | 0.03 | 0.16 |
| HSL | 31.8 | 30.5 | 29.7 | 1.13 | 0.36 | 0.27 | 0.22 | 0.57 |
| FIAF | 30.8 | 30.3 | 30.1 | 0.28 | 0.22 | 0.20 | 0.10 | 0.53 |

[1]Cows were sampled during 3 different stages in lactation; Period 1 = 99 DIM, Period 2 = 120 DIM, and Period 3 = 141 DIM.
[2]Treatment comparisons, Period 1 vs. Period 2 (1 vs. 2), Period 1 vs. Period 3 (1 vs. 3), and Period 2 vs. Period 3 (2 vs. 3) and the overall effect of period.
[3]Number of cows for Period 1 are 8 cows for all genes.
[4]Number of cows for Period 2 are 8 cows for all genes.
[5]Number of cows for Period 3 are 7 cows for LPL, FASN, S14, and FIAF; 6 cows for SCD; and 4 cows for ABHD5, and HSL.
[6]Lipoprotein lipase (LPL), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), thyroid hormone responsive SPOT 14 (S14), adipose triglyceride lipase (ATGL), abhydrolase domain containing 5 (ABHD5), hormone-sensitive lipase (HSL), and fasting-induced adipose factor (FIAF).

**Table 26**. PCR product sequencing results

| Gene | Sequence[1] | Match[2] |
|---|---|---|
| ABHD5 | GTAATTTACCGTCTGGGACGCAGAGTAAGGAATCTGAC ACGAGAACCAGGCAGTC | 100 % |
| ACACA[3] | CAGCAACC_GGTCGTGAGGATGGCAGCTCTGGAGGTGT ATGTTCGAAGGG | 98% |
| ADSL[3] | AGCGGCACCCTATGCGCTCAGA_CGGTGCTGCAGCCTG GCCCGGCACCTGATGGCCCTTG | 98% |
| ATGL | ATCTGCCCGCAGGACAGCTCCACCAACATCCACGAGCT CCGAGTCACCAACACCAGCATC | 100% |
| FASN[3] | CACCGTCAACCTGGA_AGCTCGCTTTC_GACCTTGGCCT CGACTCA | 95% |
| FIAF | CCCCCAAGGCGAGTTCTGGCTGGGCCTGGAGAAGGTGC ATCACATCC | 100% |
| HSL[3] | TTCTGGCAAGCTTTCTGGA_TATC_CCGAGTATCCAGGT GCTATCGTCTCTAGCAAACAT | 94% |
| KEAP1 | GTGGTCGCCCTGCGCCTCCATGAGCGTACCCCGCAACC GAATTGGGGTGGGCGTCATCGA | 100% |
| LEP | GACACCAAAACCCTCATCAAGACAATTGTCACCAGGAT CAATGACATCTCACACACGCAG | 100% |
| LPL | CAGCAGCAAAACCTTTGTGGTGATCCATGGC | 100% |
| RPS9[3] | AGGCTGCCCGGGA_CTGCTGACGCTGGATGAGAAAGAC CCGCGGCGTCTGTTCGAAGGTA | 98% |
| SCD[3] | GTATCTGTGGGA_GAAACGTTTCAAA_CAGCCTGTTTTT TGCC_CCTTATTCCGTTATGC | 95% |
| S14[3] | ACTGCGTC_CCGTTAGGCTGCCTGCTGCTGTTC_CAACTC CTCACTCCTCTTACTAGCTT | 98% |

[1]Sequencing results of PCR products, 5’ to 3’ direction.

[2]NCBI *bos taurus* nucleotide sequences compared to the PCR product sequence (National Center for Biotechnology Information, Bethsada, MD), Algorithm (Altschul et al., 1997).
[3] NCBI *bos taurus* nucleotide sequence differences; ACACA (a), ADSL (a g), FASN (c a), HSL (a a ‘ _’), RPS9 (g), SCD (t a a), S14 (a a).

**Table 27**. Correlation between nutrients in CON diet

| | **CON Diet[1]** n= 462 | | | | | | |
|---|---|---|---|---|---|---|---|
| Variables[2] | FNDF | NDF | CP | Ash | Lignin | NEL_3X | NEL_disc |
| FNDF | 1 | | | | | | |
| NDF | 0.96** | 1 | | | | | |
| CP | 0.34** | 0.34** | 1 | | | | |
| Ash | 0.42** | 0.54** | 0.34** | 1 | | | |
| Lignin | 0.70** | 0.59** | 0.18** | -0.11** | 1 | | |
| NEL_3X | -0.60** | -0.77** | -0.17** | -0.75** | -0.22** | 1 | |
| NEL_disc[3] | -0.45** | -0.57** | -0.08 | -0.54** | -0.20** | 0.71** | 1 |

[1]CON= Control treatment, consistent diet FNDF.
[2]Peason correlations, *p = <0.05, **p = <0.01.
[3]NEL_disc = diet NEL adjusted for level of intake.

**Table 28**. Correlation between nutrients in VAR diet.

| | **VAR Diet[1]** n= 483 | | | | | | |
|---|---|---|---|---|---|---|---|
| Variables | FNDF | NDF | CP | Ash | Lignin | NEL_3X | NEL_disc |
| FNDF | 1 | | | | | | |
| NDF | 0.99** | 1 | | | | | |
| CP | -0.67** | -0.71** | 1 | | | | |
| Ash | -0.19** | -0.19** | 0.64** | 1 | | | |
| Lignin | -0.27** | -0.36** | 0.66** | 0.31** | 1 | | |
| NEL_3X | -0.62** | -0.68** | 0.24** | -0.39** | 0.06 | 1 | |
| NEL_disc[3] | -0.31** | -0.34** | -0.04 | -0.36** | -0.14** | 0.68** | 1 |

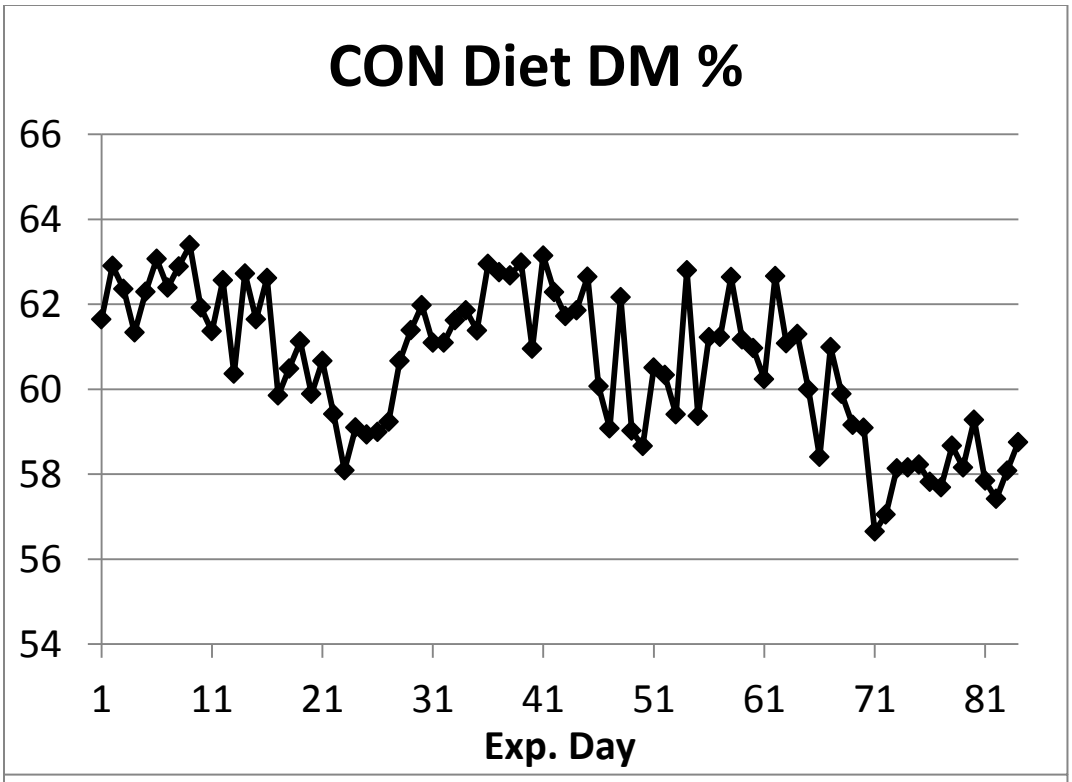
[1]VAR= Variable treatment, daily variation of FNDF
[2]Peason correlations, *p = <0.05, **p = <0.01
[3]NEL_disc = diet NEL adjusted for level of intake.

**Table 29**. Correlation between nutrients in ORR diet.

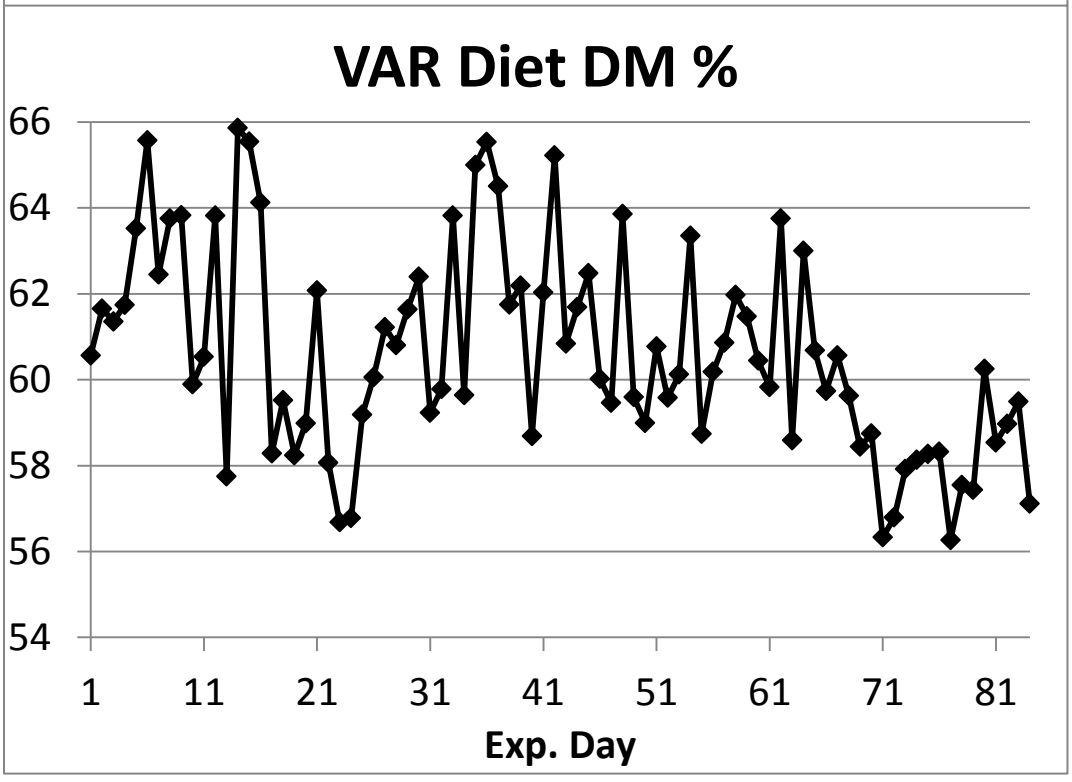
| | **ORR Diet[1]** n= 483 | | | | | | |
|---|---|---|---|---|---|---|---|
| Variables | FNDF | NDF | CP | Ash | Lignin | NEL_3X | NEL_disc |
| FNDF | 1 | | | | | | |
| NDF | 0.99** | 1 | | | | | |
| CP | 0.10* | 0.12** | 1 | | | | |
| Ash | 0.61** | 0.67** | 0.30** | 1 | | | |
| Lignin | 0.85** | 0.81** | 0.05 | 0.27** | 1 | | |
| NEL_3X | -0.86** | -0.91** | -0.06 | 0.79** | -0.64** | 1 | |
| NEL_disc[3] | -0.67** | -0.72** | -0.16** | -0.57** | -0.55** | 0.76** | 1 |

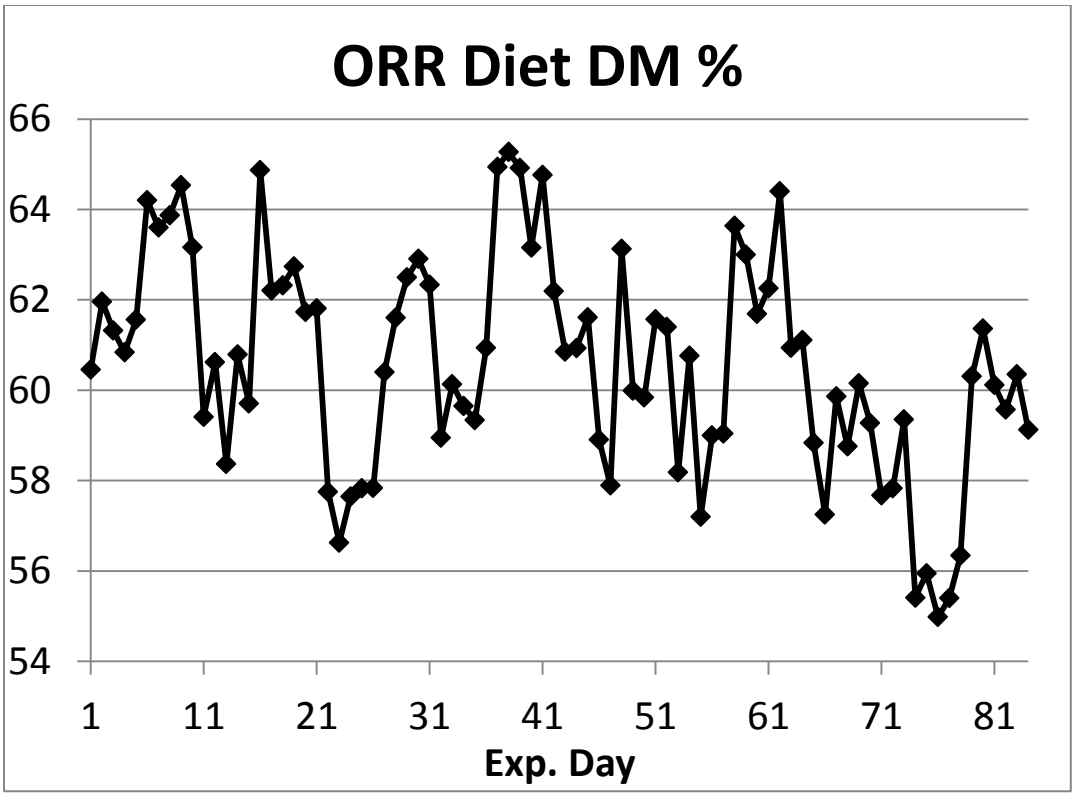
[1]ORR= Overreacting treatment, 5-d variation of FNDF
[2]Peason correlations, *p = <0.05, **p = <0.01
[3]NEL_disc = diet NEL adjusted for level of intake.

A.


CON Diet DM %
66
64
62
60
58
56
54
1
11
21
31
41
51
61
71
81
Exp. Day

B.

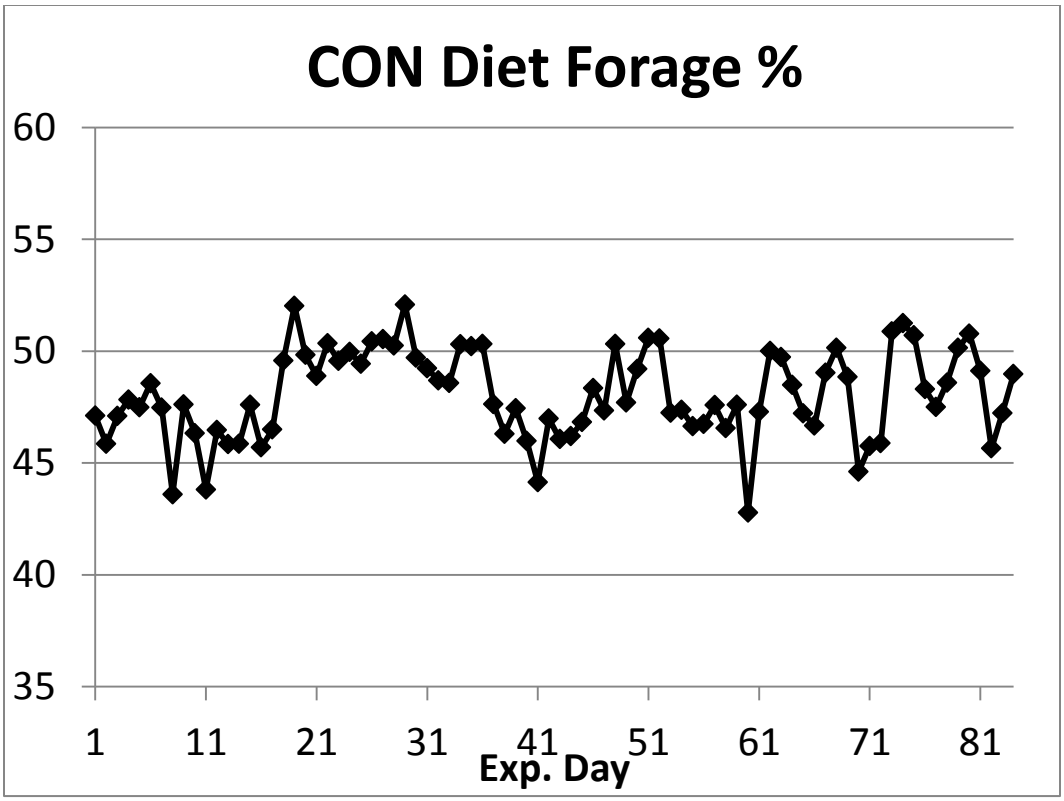

VAR Diet DM %
66
64
62
60
58
56
54
1
11
21
31
41
51
61
71
81
Exp. Day

C. 

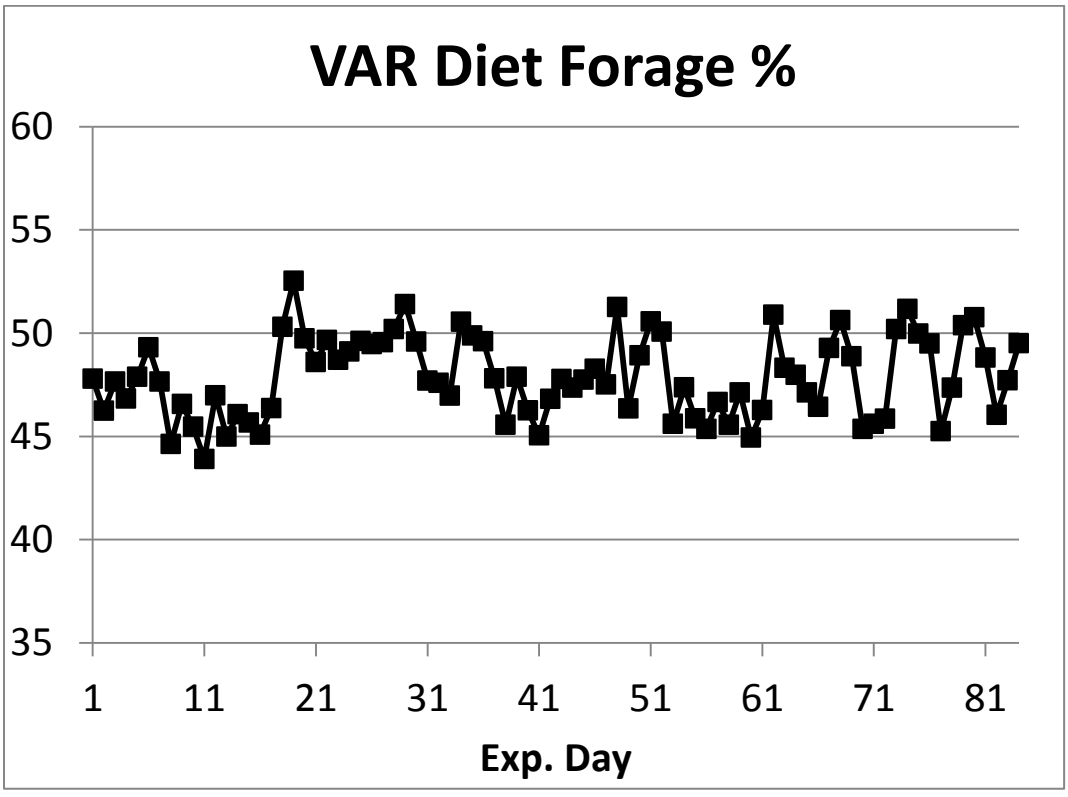

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

**Figure 27**. Changes in diet DM concentrations over the entire experiment for the CON (A), VAR (B), and ORR (C).

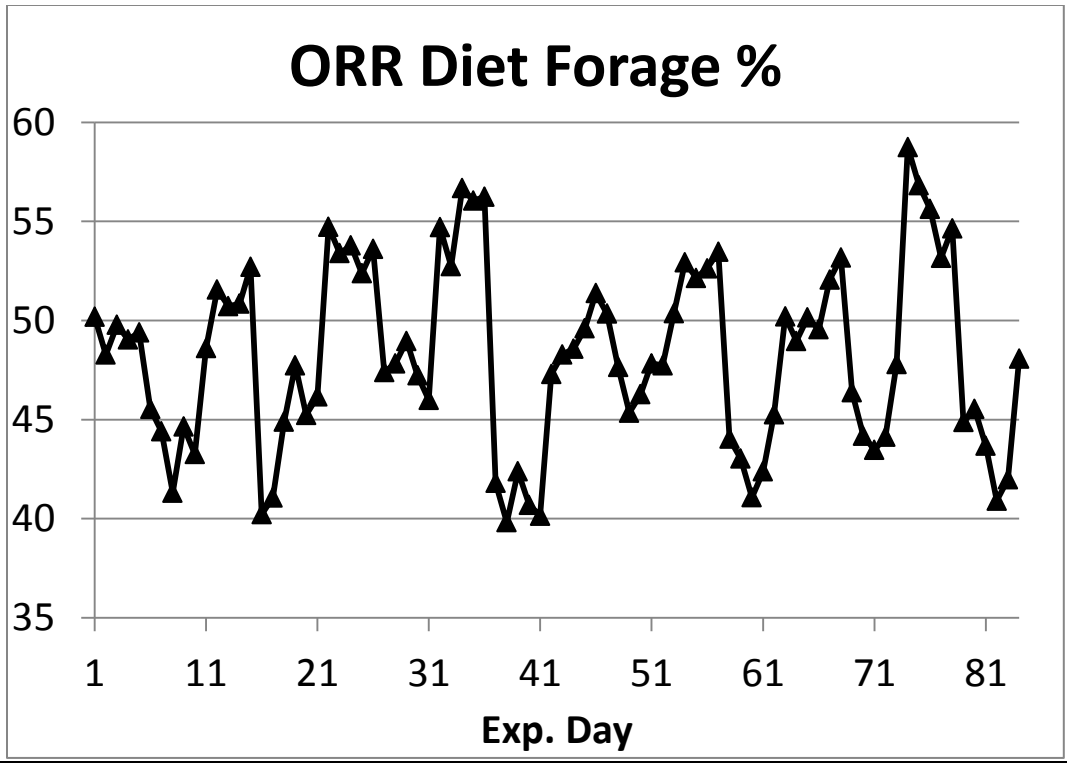
A.


CON Diet Forage %
60
55
50
45
40
35
1
11
21
31
41
51
61
71
81
Exp. Day

B.


VAR Diet Forage %
60
55
50
45
40
35
1
11
21
31
41
51
61
71
81
Exp. Day

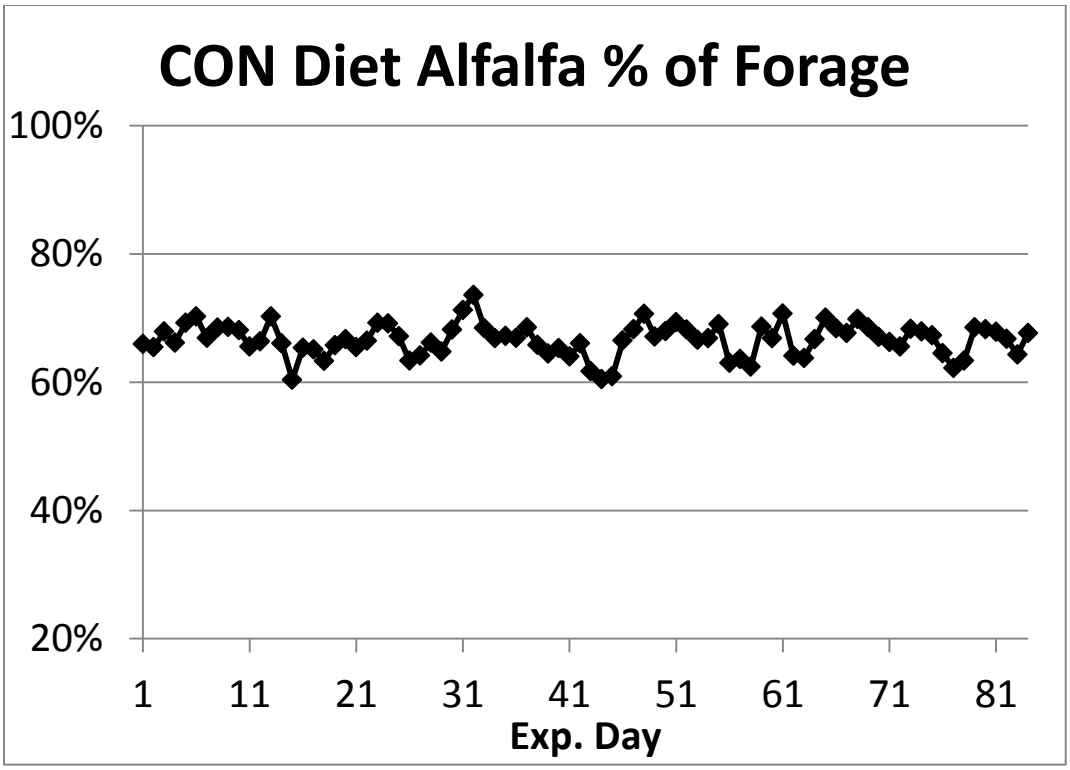
C.



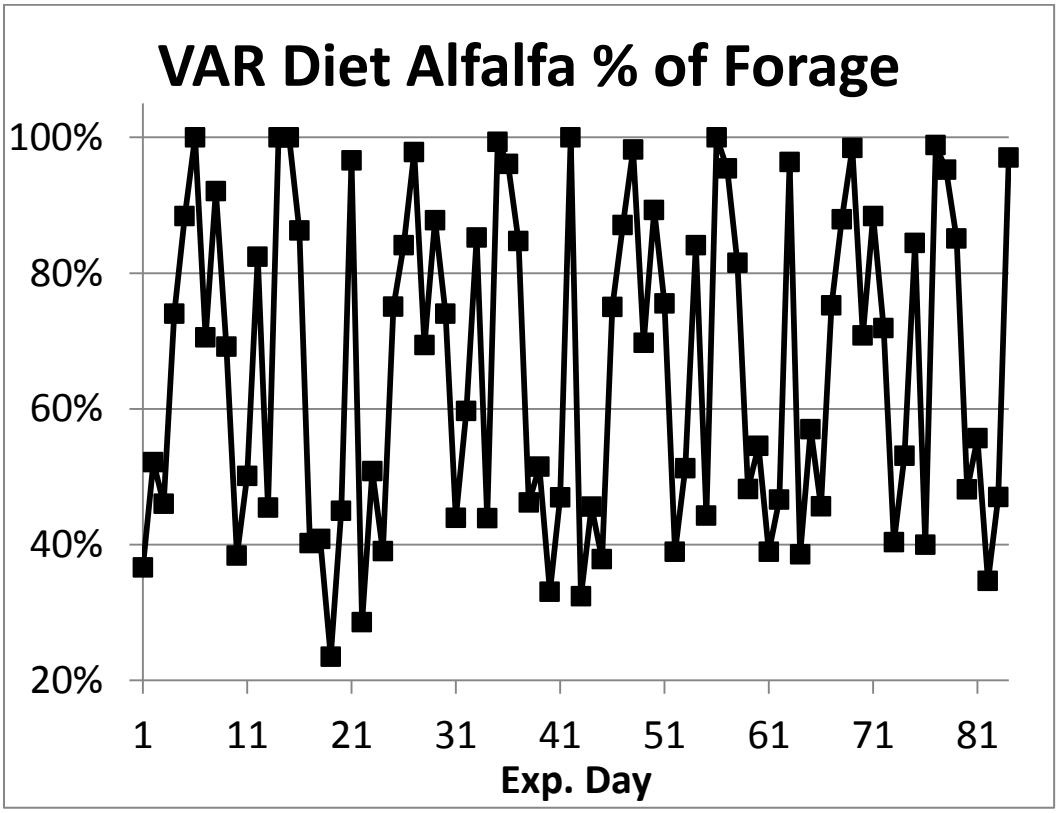

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

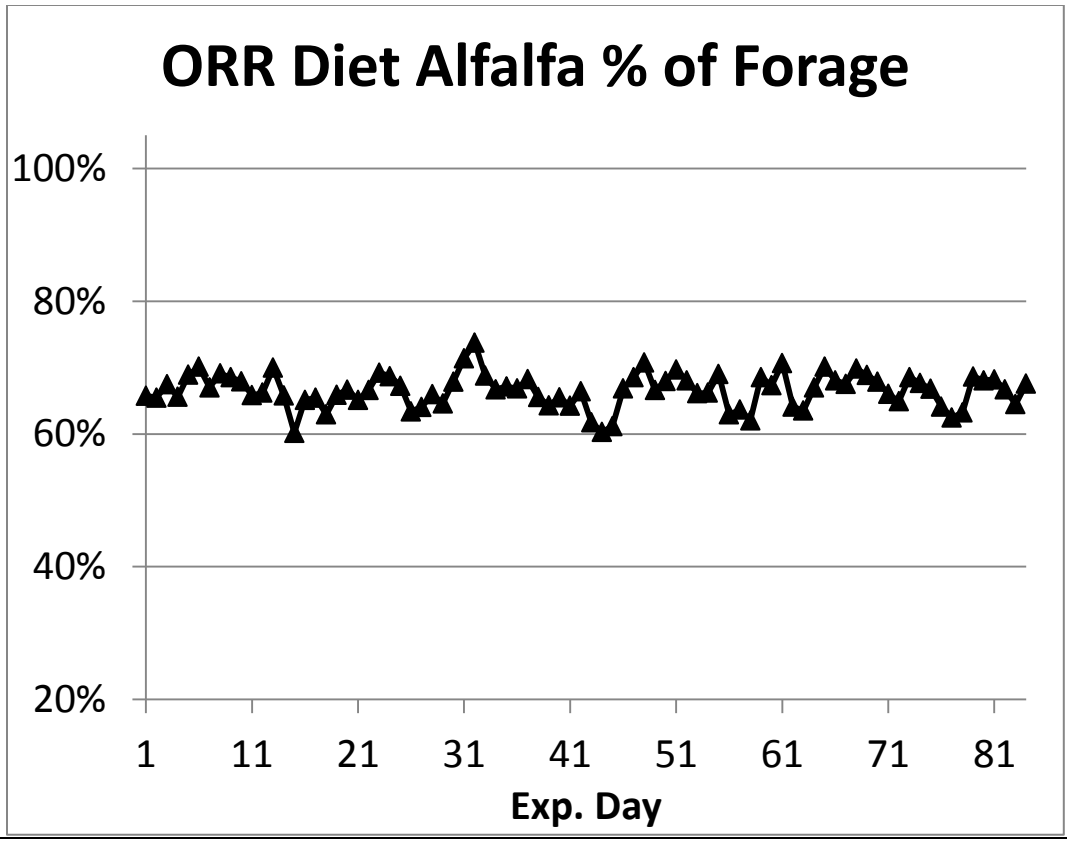
**Figure 28**. Changes in diet forage concentrations over the entire experiment for the CON (A), VAR (B), and ORR (C).

A.


CON Diet Alfalfa % of Forage
100%
80%
60%
40%
20%
1
11
21
31
41
51
61
71
81
Exp. Day

B.

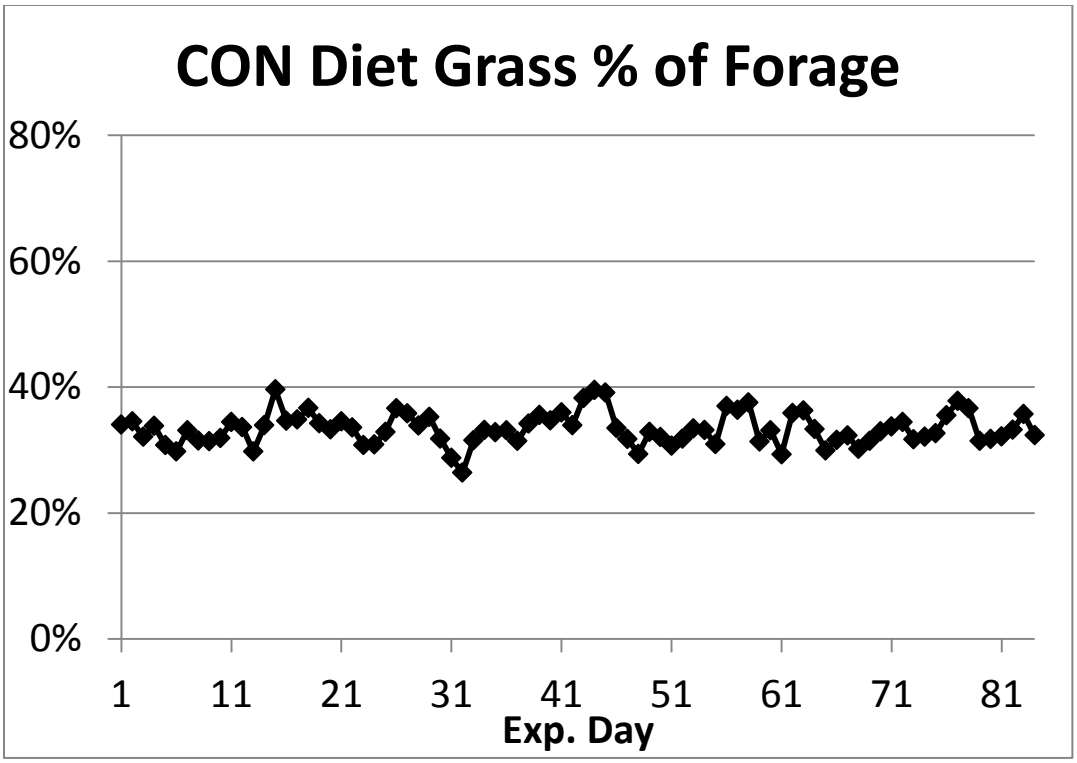

VAR Diet Alfalfa % of Forage
100%
80%
60%
40%
20%
1
11
21
31
41
51
61
71
81
Exp. Day

C. 

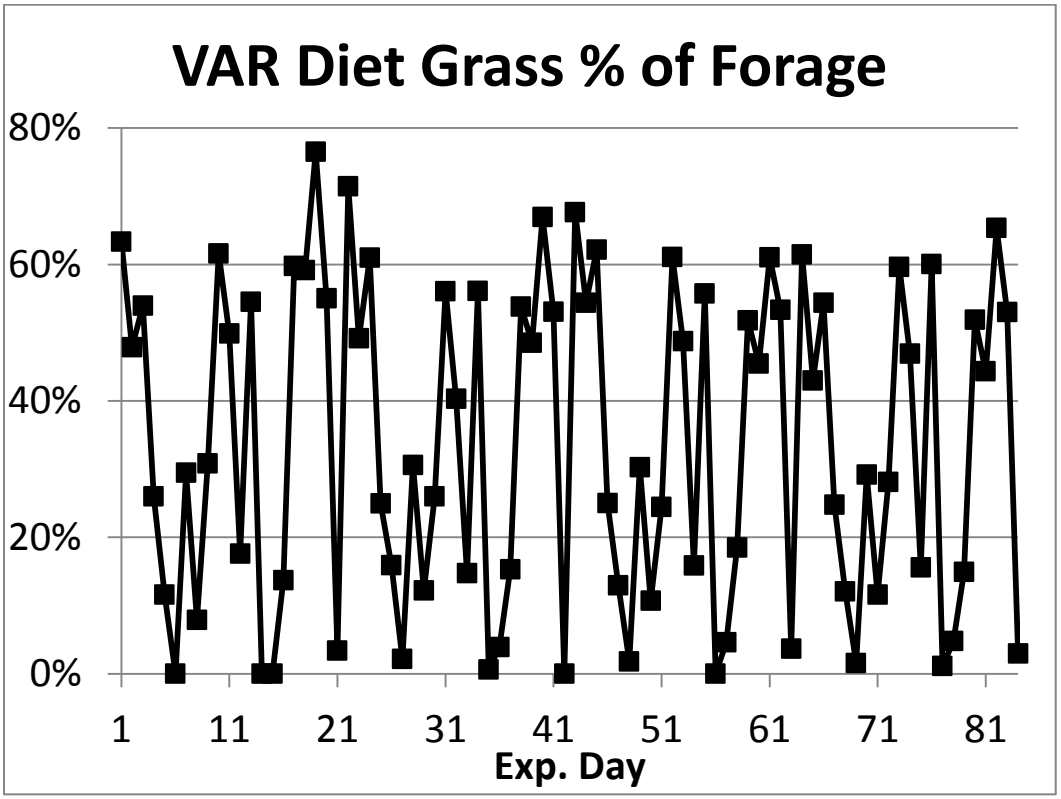

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

**Figure 29**. Changes in total alfalfa % of diet forage over the entire experiment for the Control (A), Variable (B), and Overreacting (C).

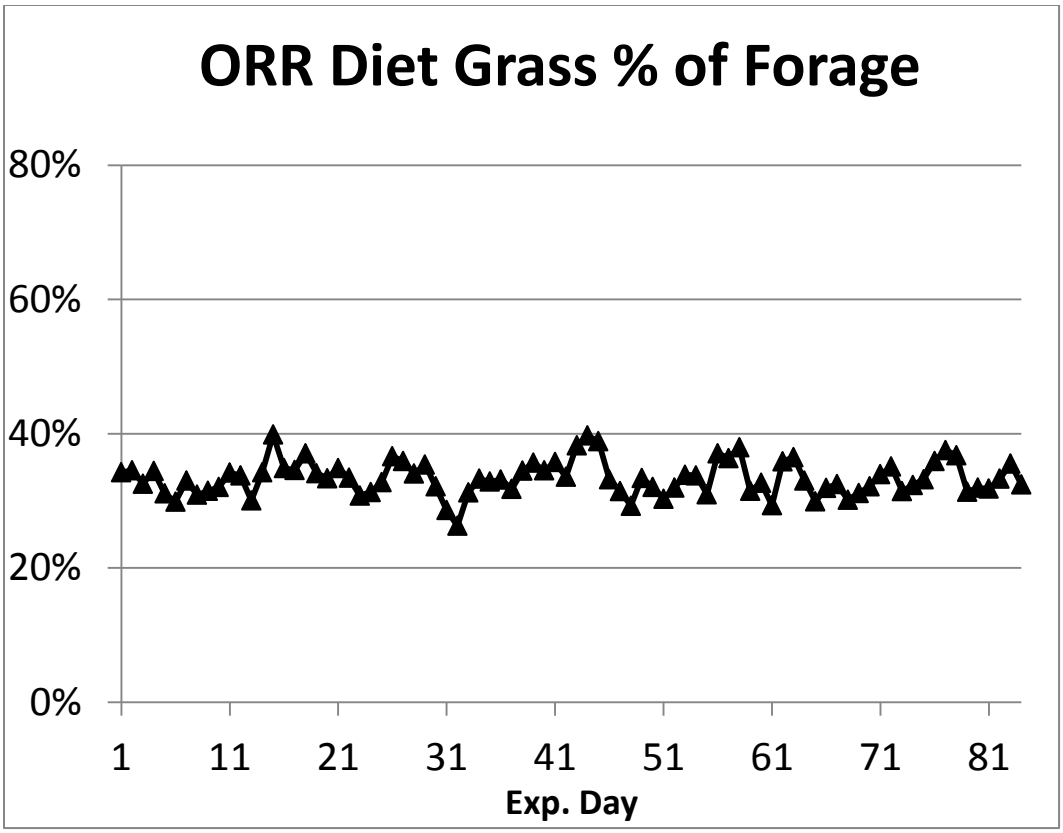
A.


CON Diet Grass % of Forage
80%
60%
40%
20%
0%
1
11
21
31
41
51
61
71
81
Exp. Day

B.


VAR Diet Grass % of Forage
80%
60%
40%
20%
0%
1
11
21
31
41
51
61
71
81
Exp. Day

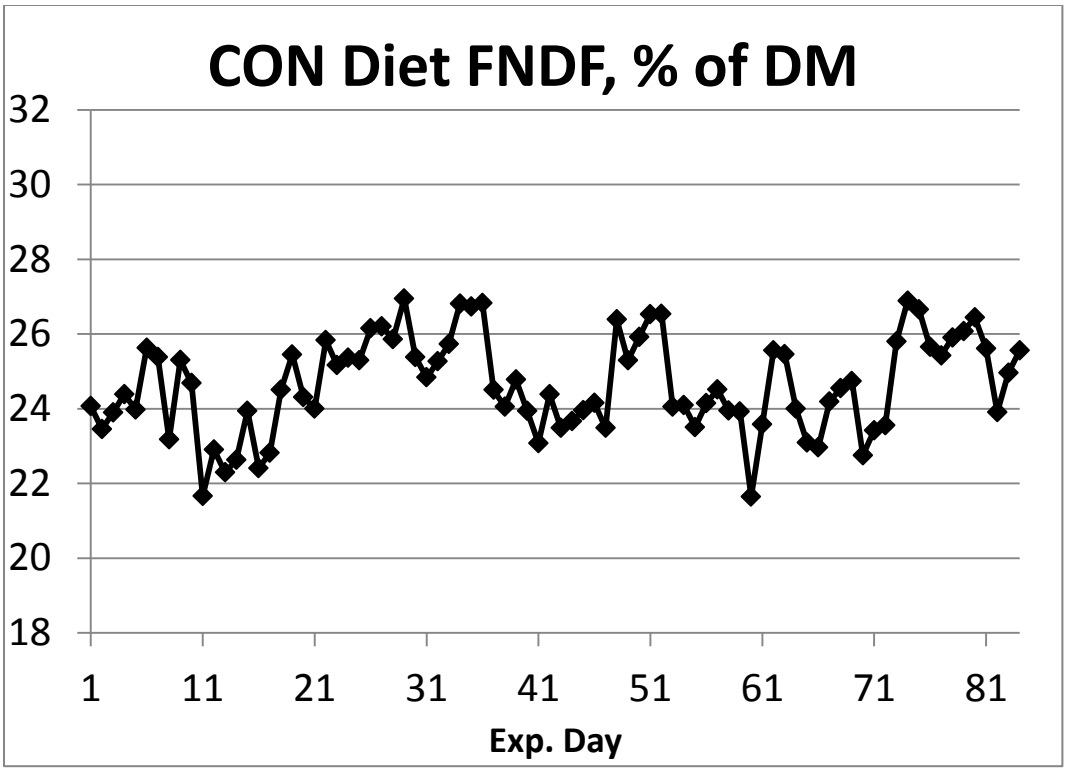
C.



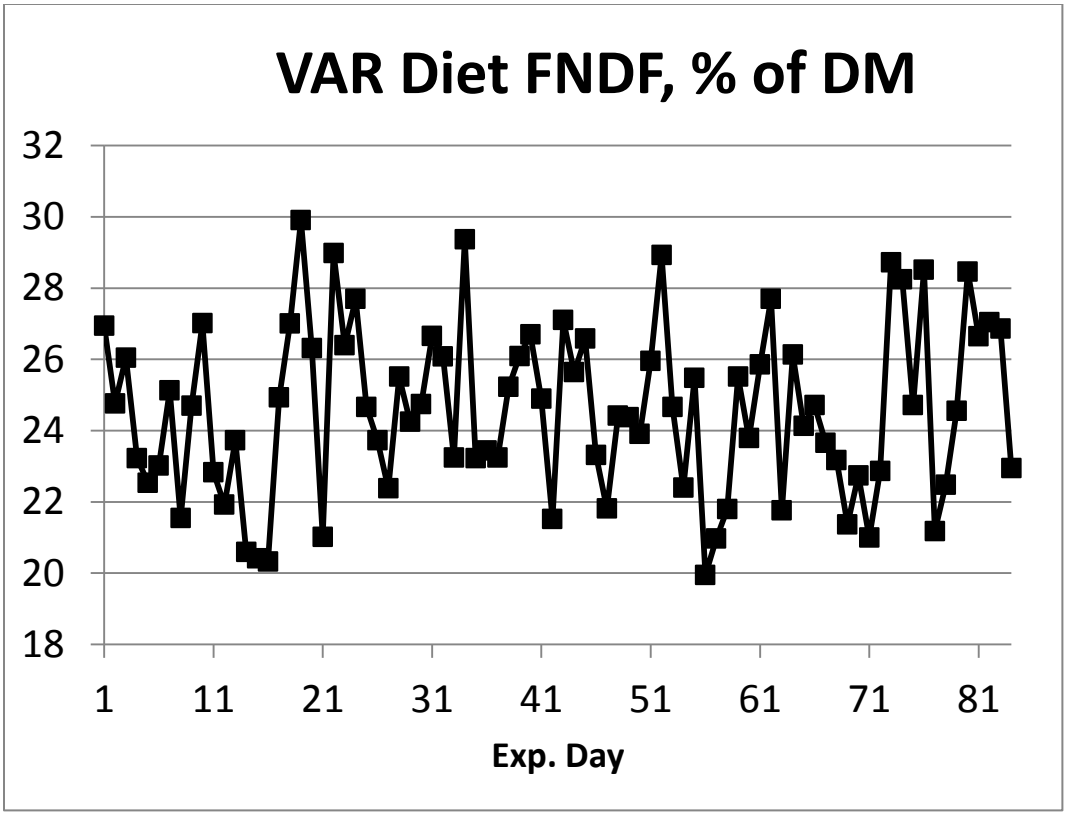

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

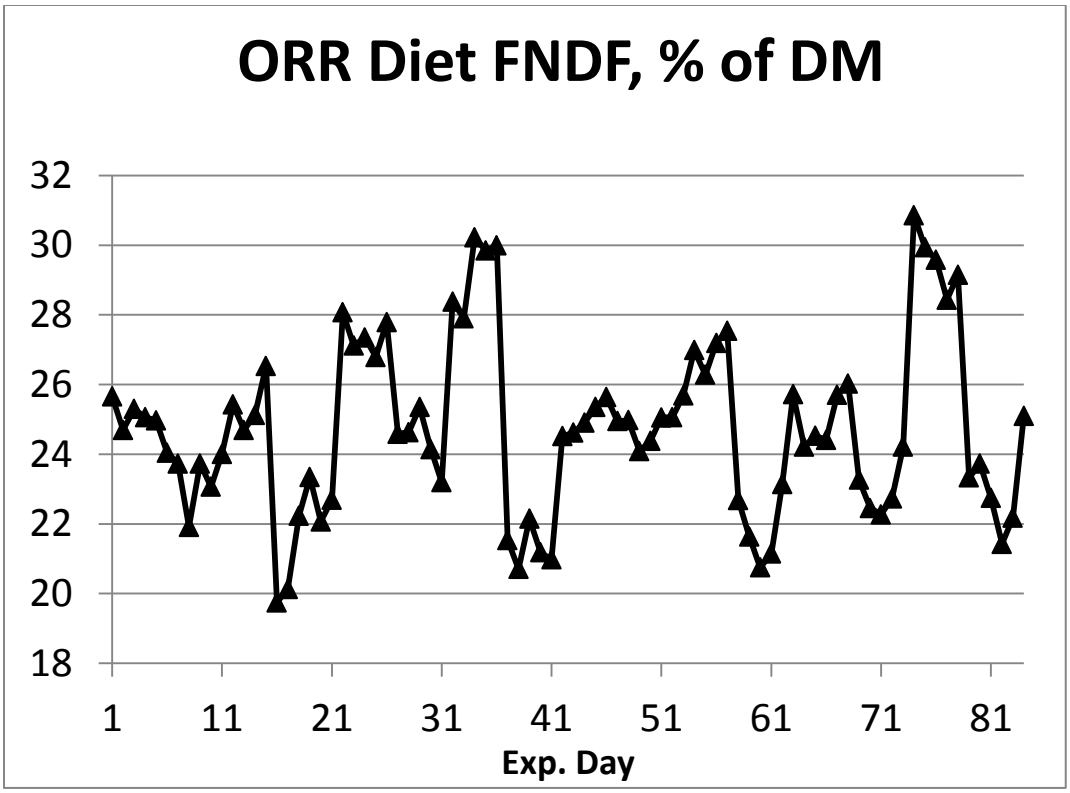
**Figure 30**. Changing grass concentrations over the entire experiment for the CON (A), VAR (B), and ORR (C).

A.


CON Diet FNDF, % of DM
32
30
28
26
24
22
20
18
1
11
21
31
41
51
61
71
81
Exp. Day

B.

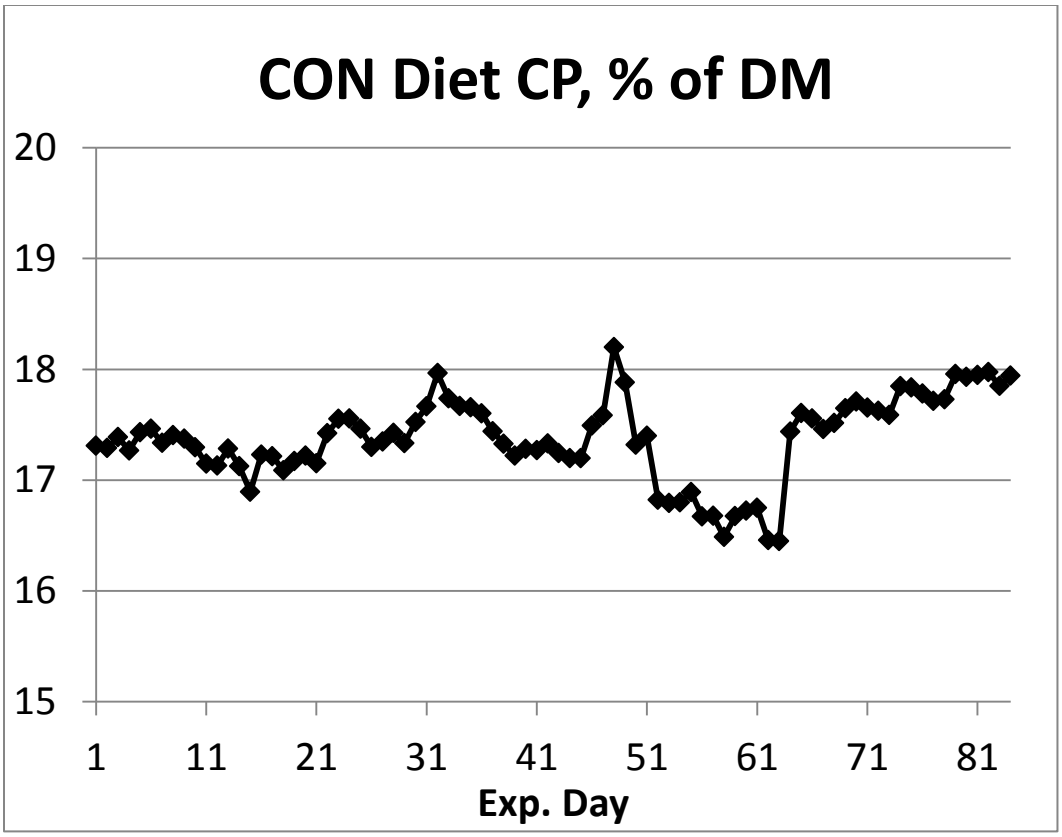

VAR Diet FNDF, % of DM
32
30
28
26
24
22
20
18
1
11
21
31
41
51
61
71
81
Exp. Day

C. 

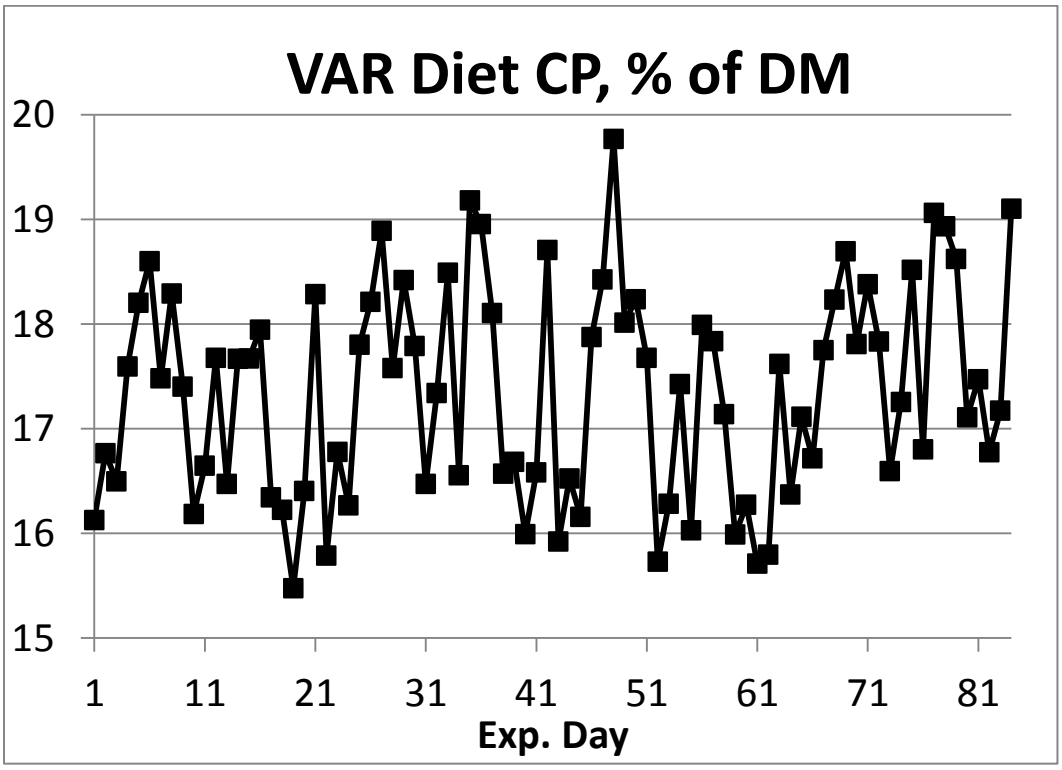

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

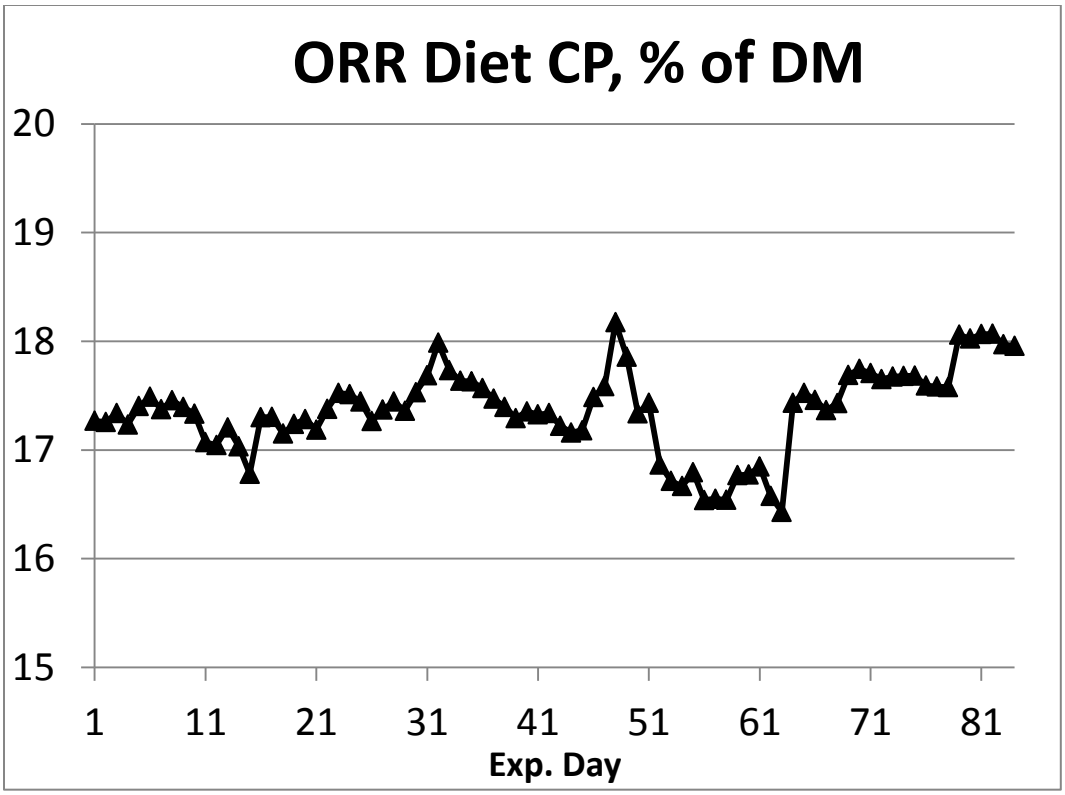
**Figure 31**. Changing diet FNDF, % of DM, over the entire experiment for the CON (A), VAR (B), and ORR (C).

A.


CON Diet CP, % of DM
20
19
18
17
16
15
1
11
21
31
41
51
61
71
81
Exp. Day

B.

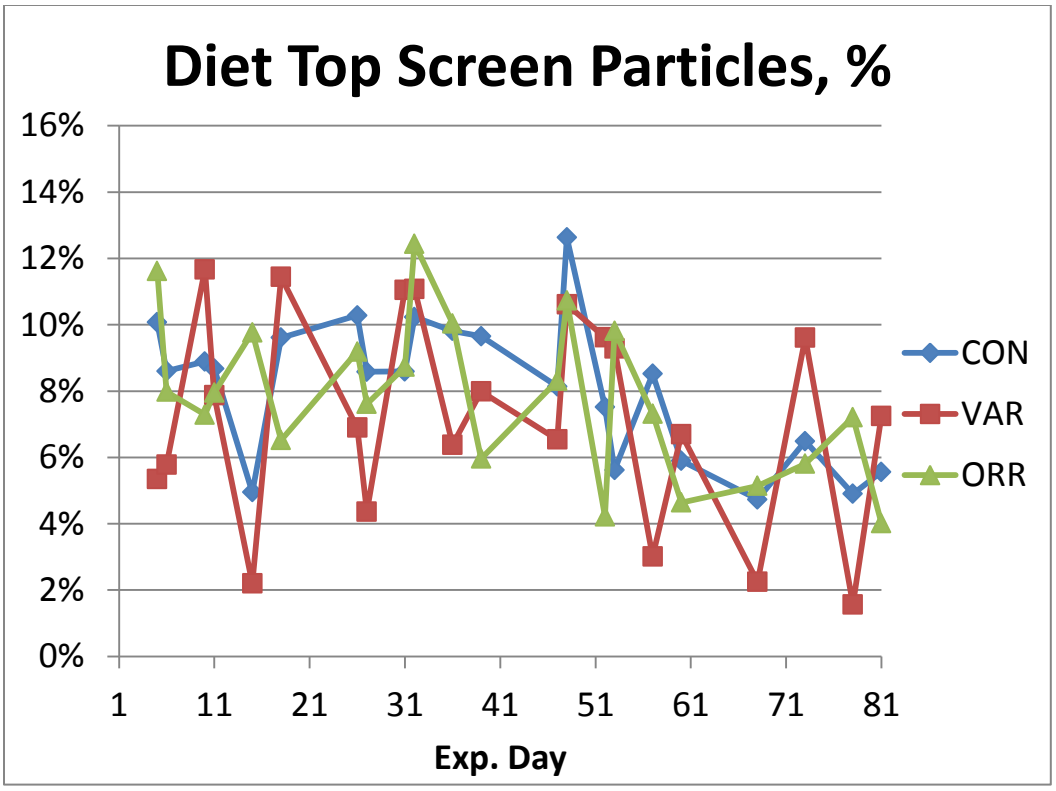

VAR Diet CP, % of DM
20
19
18
17
16
15
1
11
21
31
41
51
61
71
81
Exp. Day

C. 

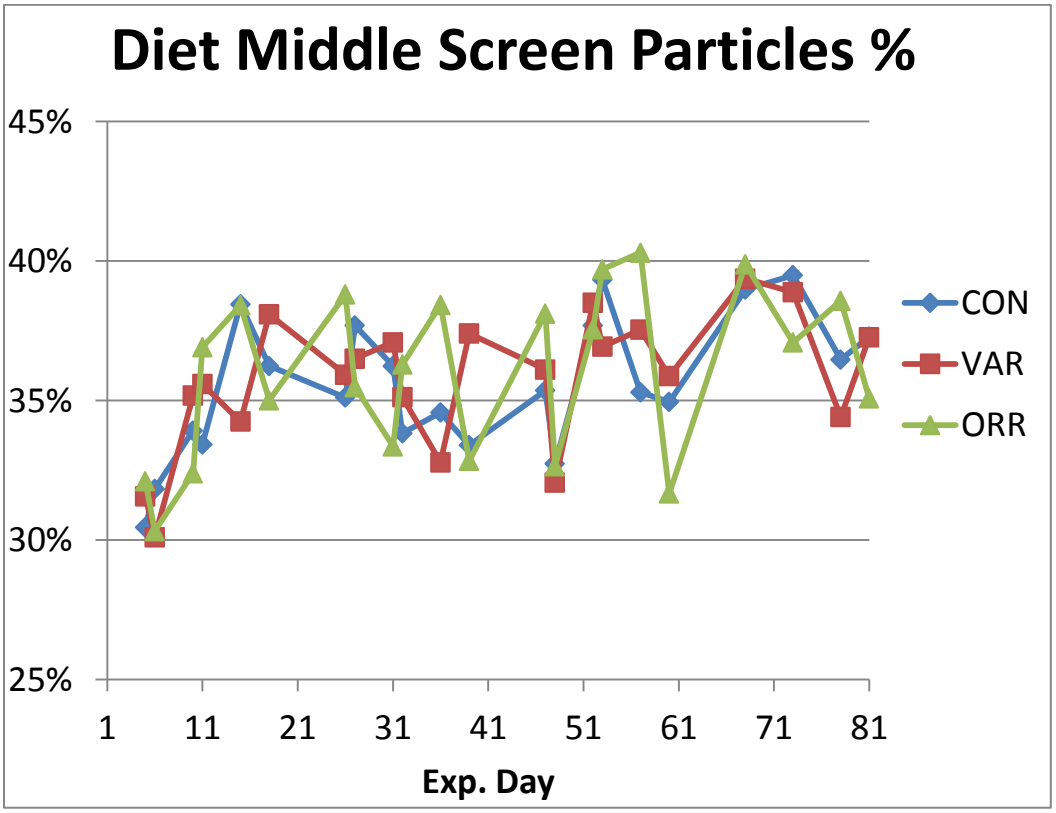

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

**Figure 32**. Changing diet CP, % of DM, over the entire experiment for the CON (A), VAR (B), and ORR (C).

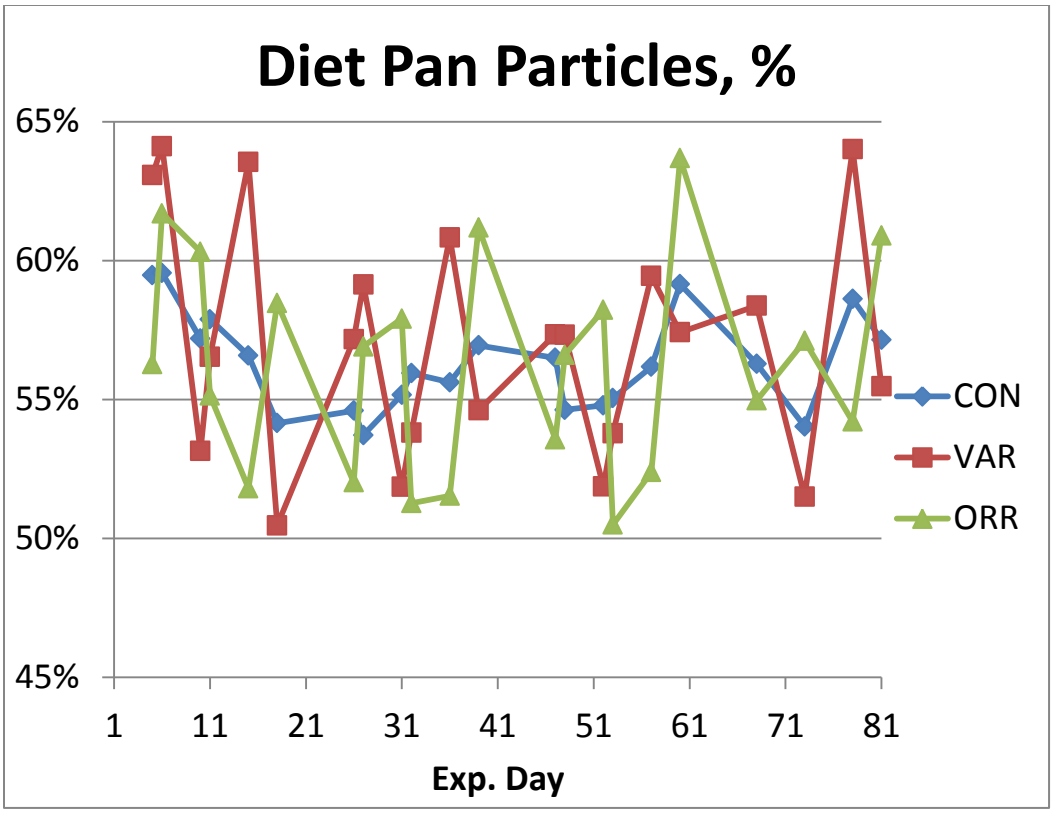
A.


Diet Top Screen Particles, %
16%
14%
12%
10%
8%
6%
4%
2%
0%
1
11
21
31
41
51
61
71
81
Exp. Day
CON
VAR
ORR

B.


Diet Middle Screen Particles %
45%
40%
35%
30%
25%
1
11
21
31
41
51
61
71
81
Exp. Day
CON
VAR
ORR

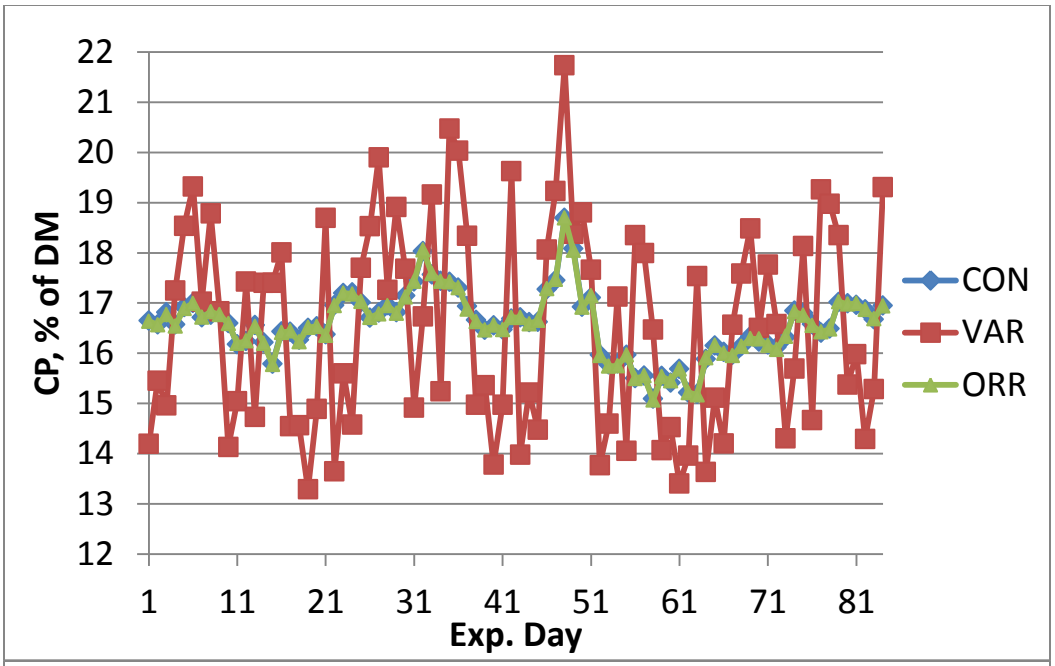
C.



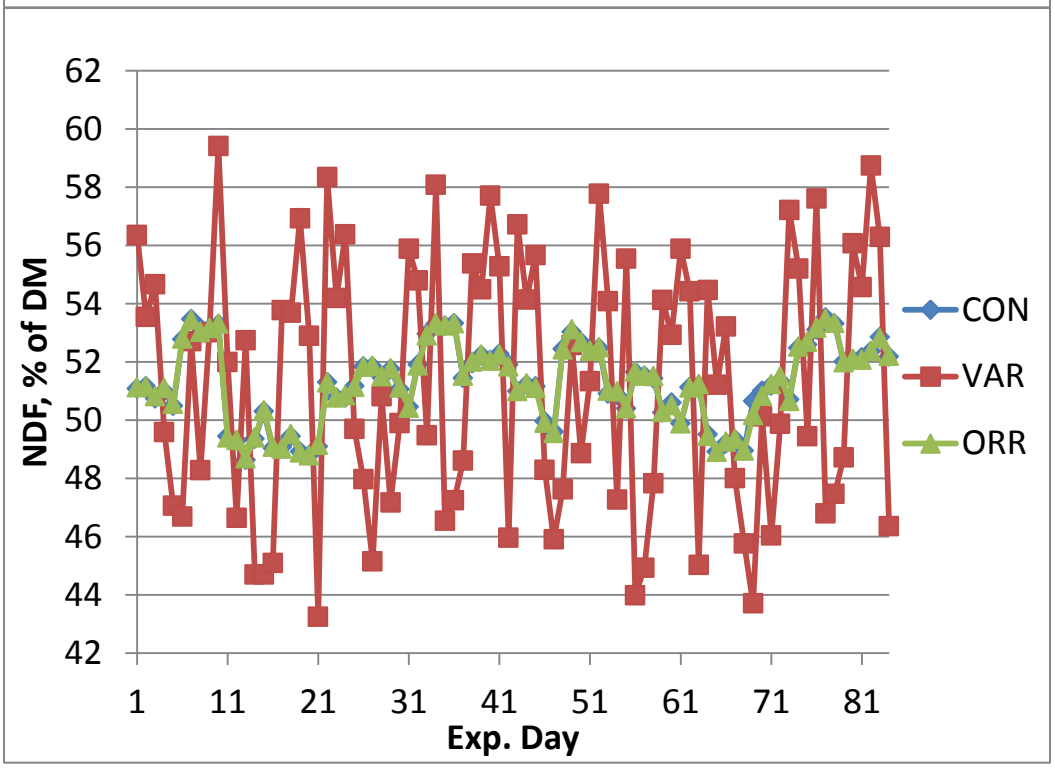

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF. Particle size was determined with the Penn State Particle Separator (Lammers et al., 1996).

**Figure 33**. Changes in diet as-fed particle size over the entire experiment for the top screen (A), middle screen (B), and pan (C), as assayed on a Penn State Particle Separator.

A.



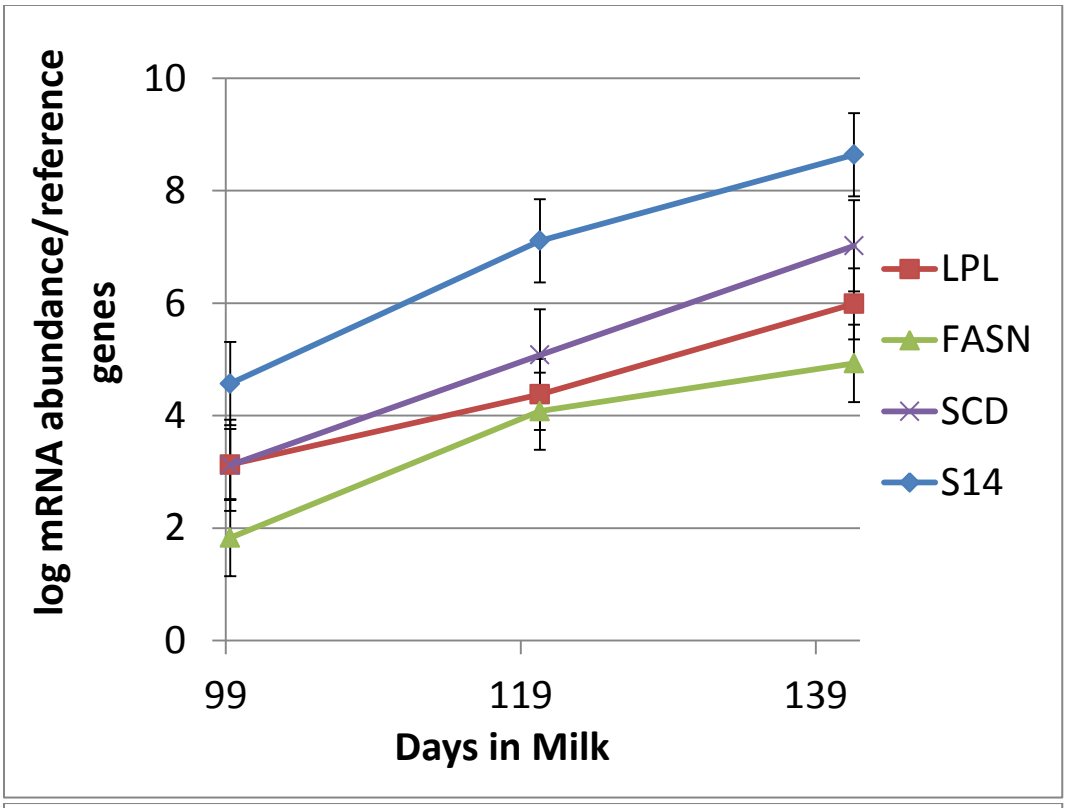

B.



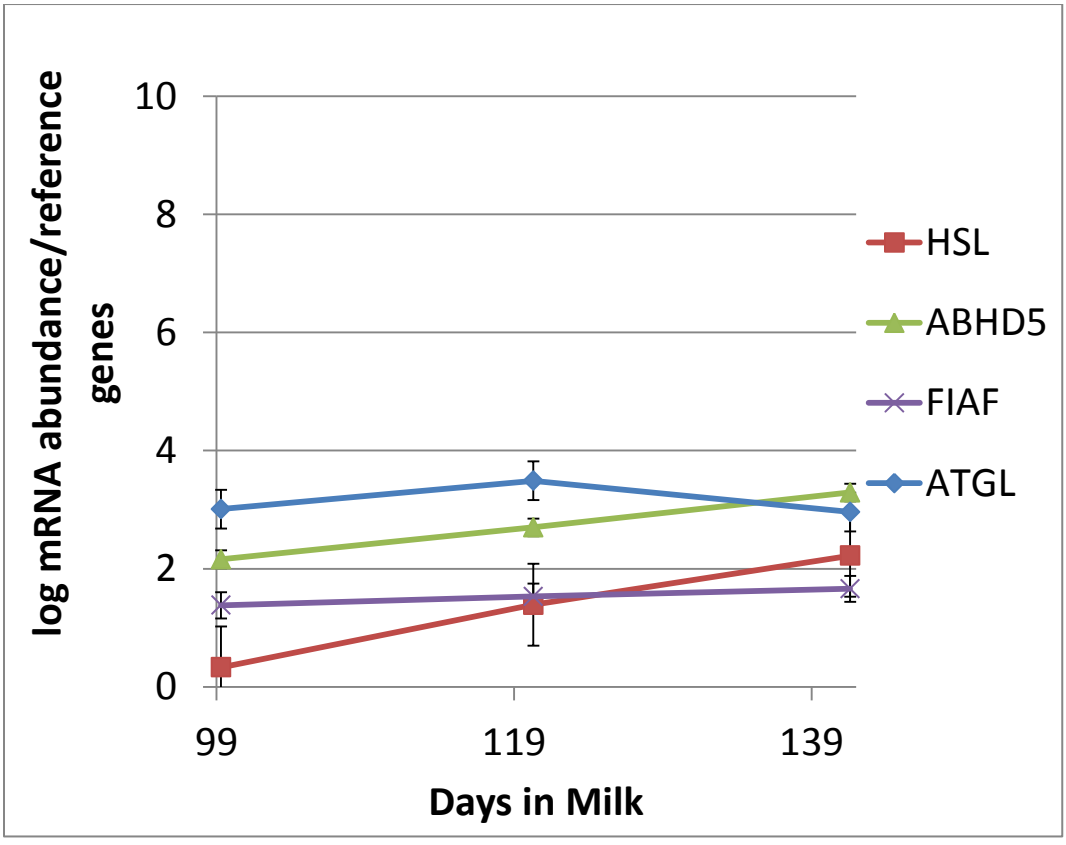

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF.

**Figure 34**. Changes in forage mix nutrient composition over the entire experiment for CP, % of DM, (A), and NDF, % of DM, (B).

A.

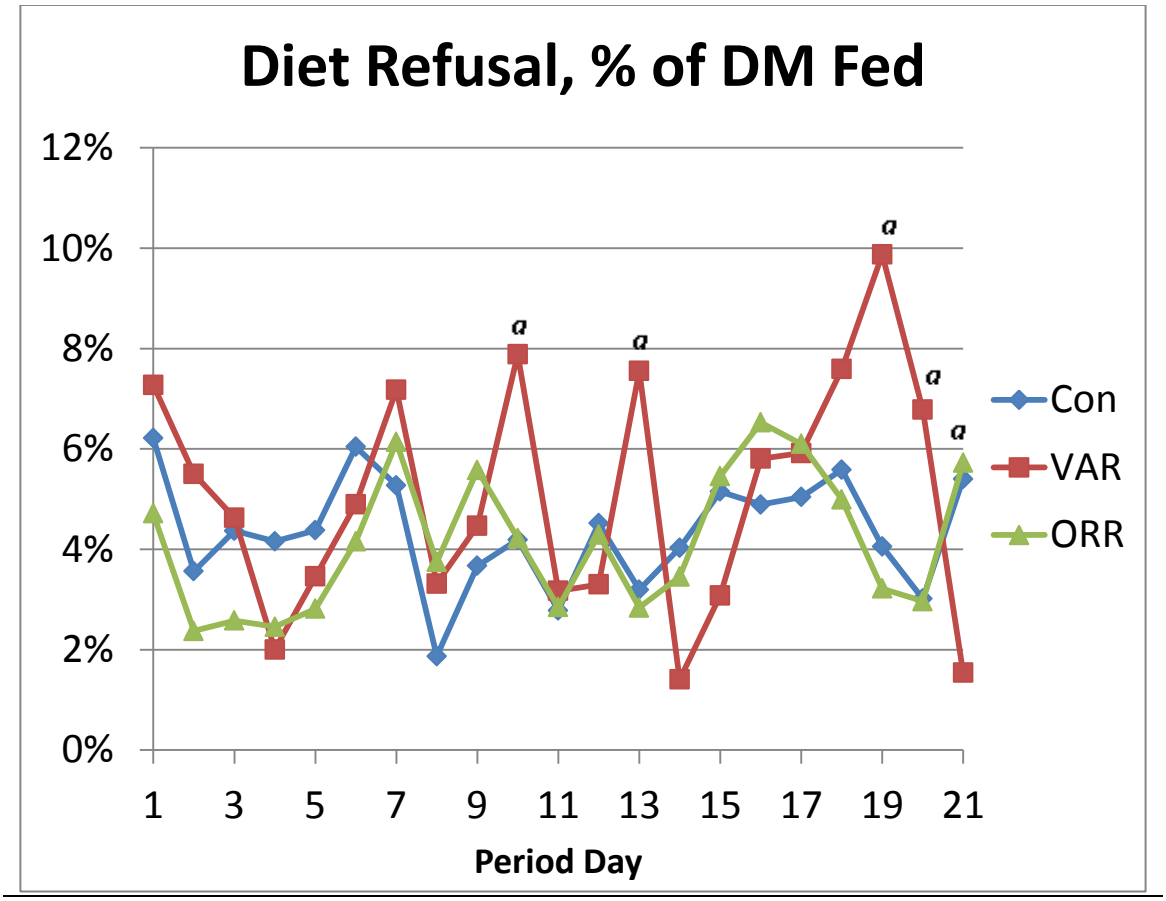

log mRNA abundance/reference genes
10
8
6
4
2
0
99
119
139
Days in Milk
LPL
FASN
SCD
S14

B.


log mRNA abundance/reference genes
10
8
6
4
2
0
99
119
139
Days in Milk
HSL
ABHD5
FIAF
ATGL

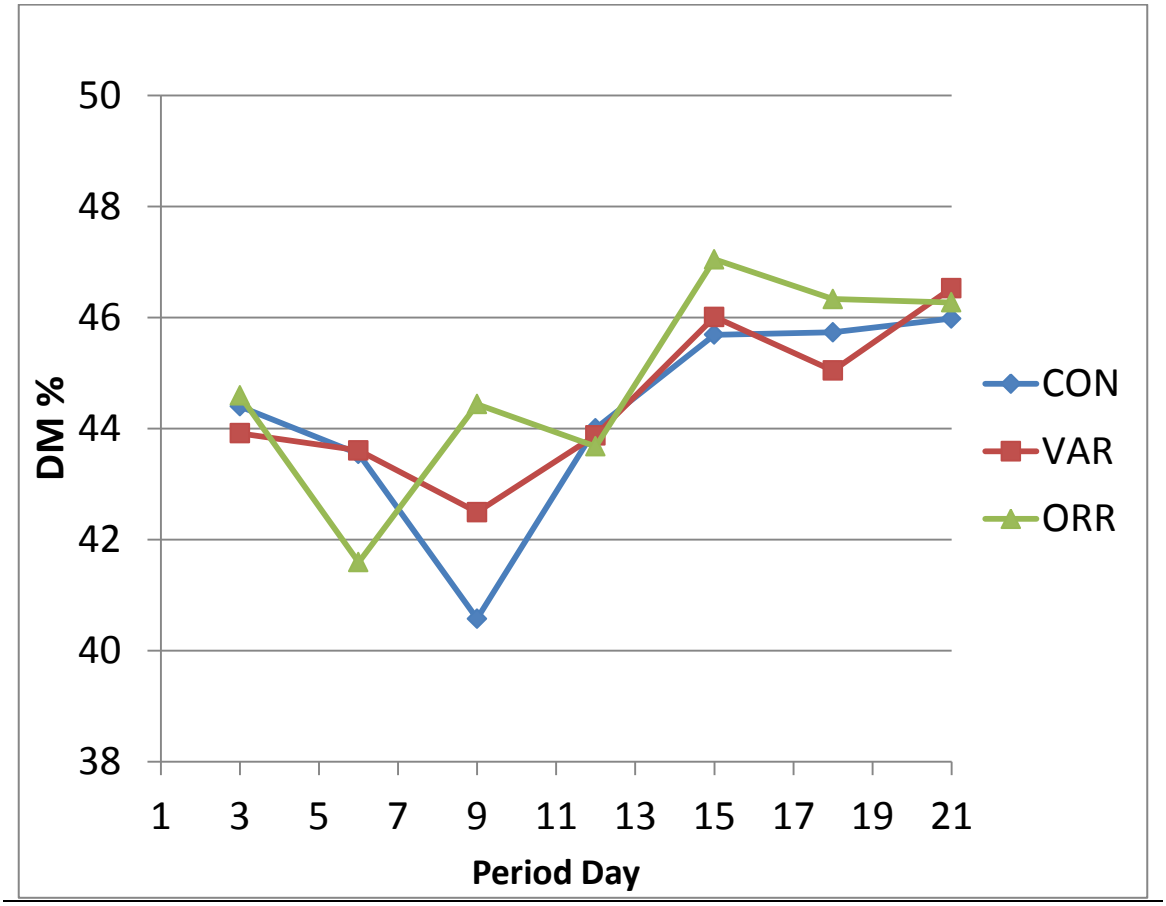
Cows were sampled during 3 different stages in lactation; Period 1 = 99 DIM, Period 2 = 120 DIM, and Period 3 = 141 DIM.

**Figure 35**. Adipose mRNA abundance over the 3 experimental periods for lipogenic related enzymes (A) and lipolytic related enzymes (B).



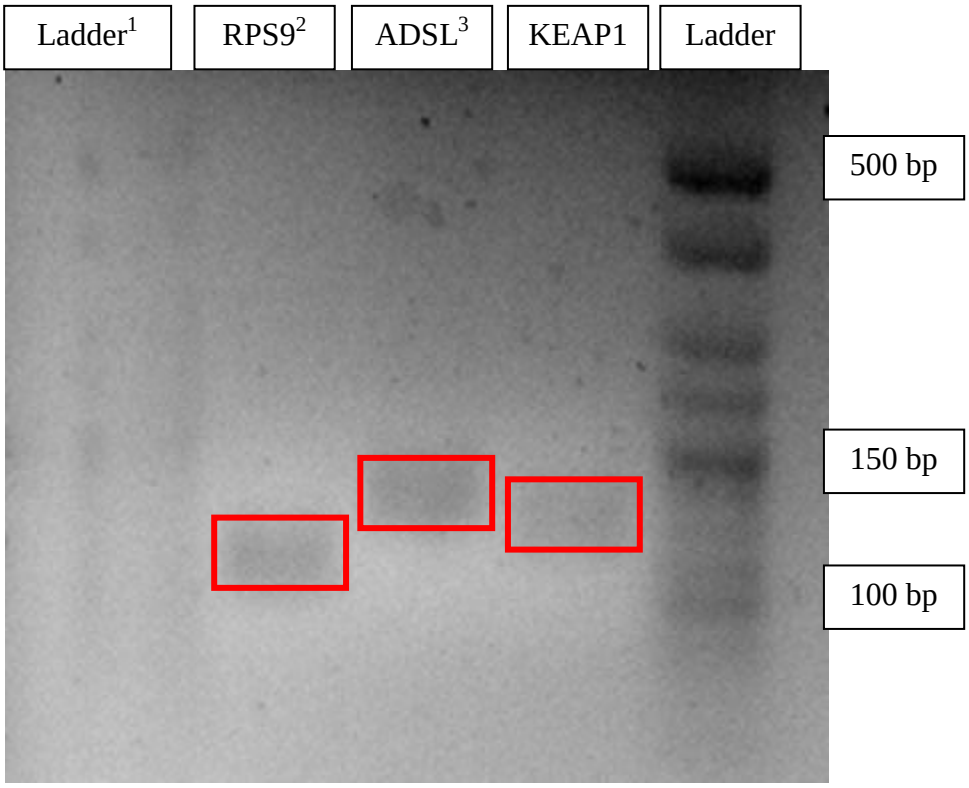

CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF. Treatment comparisons; *a*= CON vs. VAR treatments P<0.1; *b*= CON vs ORR treatments P<0.1. **SEM = 0.97**.

**Figure 36**. Changes in daily diet refusal as percent of daily DM fed.




CON = Control, consistent diet FNDF; VAR = Variable, daily variation in diet FNDF; ORR= Overreacting, 5-d variation in diet FNDF. No treatment day interactions were present. SEM = 1.66.

**Figure 37**. Effect of treatment on assayed DM concentration of diet refusal.



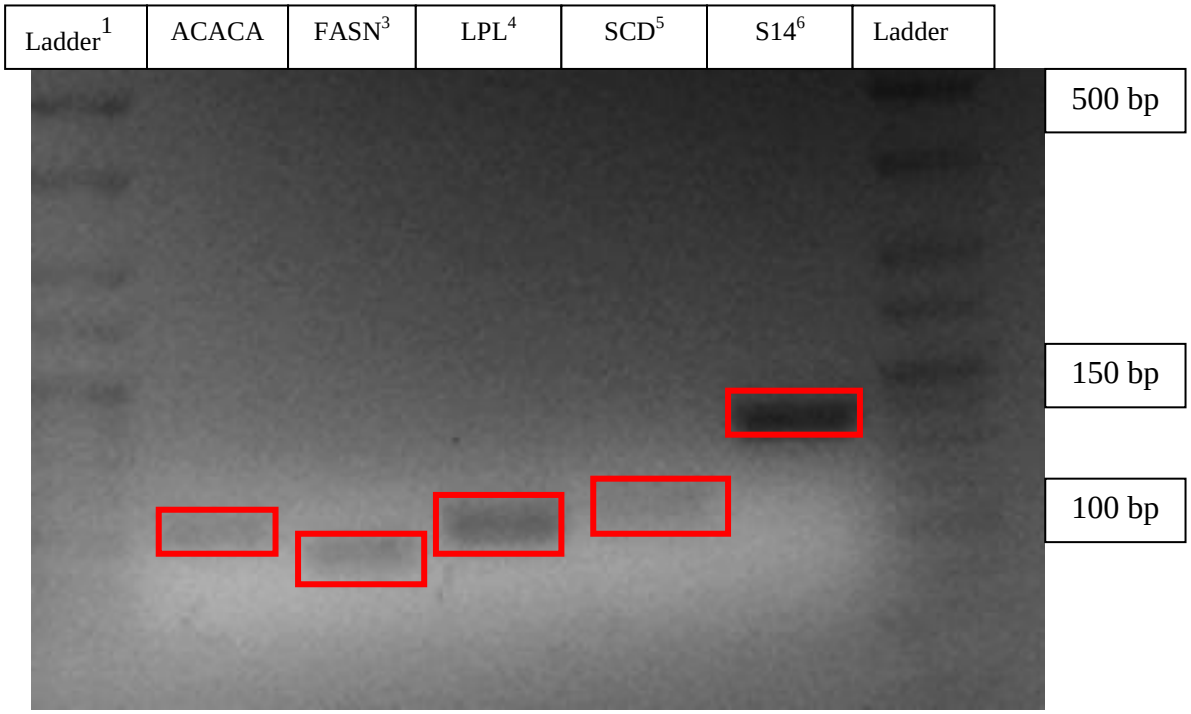

[1]Samples were electrophoresed through a 2% agarose gel electrophoresis and 25 to 500 bp ladder was used.

[2]Ribosomal protein S9 (RPS9; 108 bp).

[3]Adenylosucciante lyase (ADSL; 142 bp).

[4]Kelch-like ECH-associated protein 1 (KEAP1; 129 bp).

**Figure 38**. Verification of PCR product size of reference genes.




[1]Samples were electrophoresed through a 2% agarose gel electrophoresis and 25 to 500 bp ladder was used.

[2]Acetyl-coenzyme A carboxylase alpha (ACACA; 101 bp).

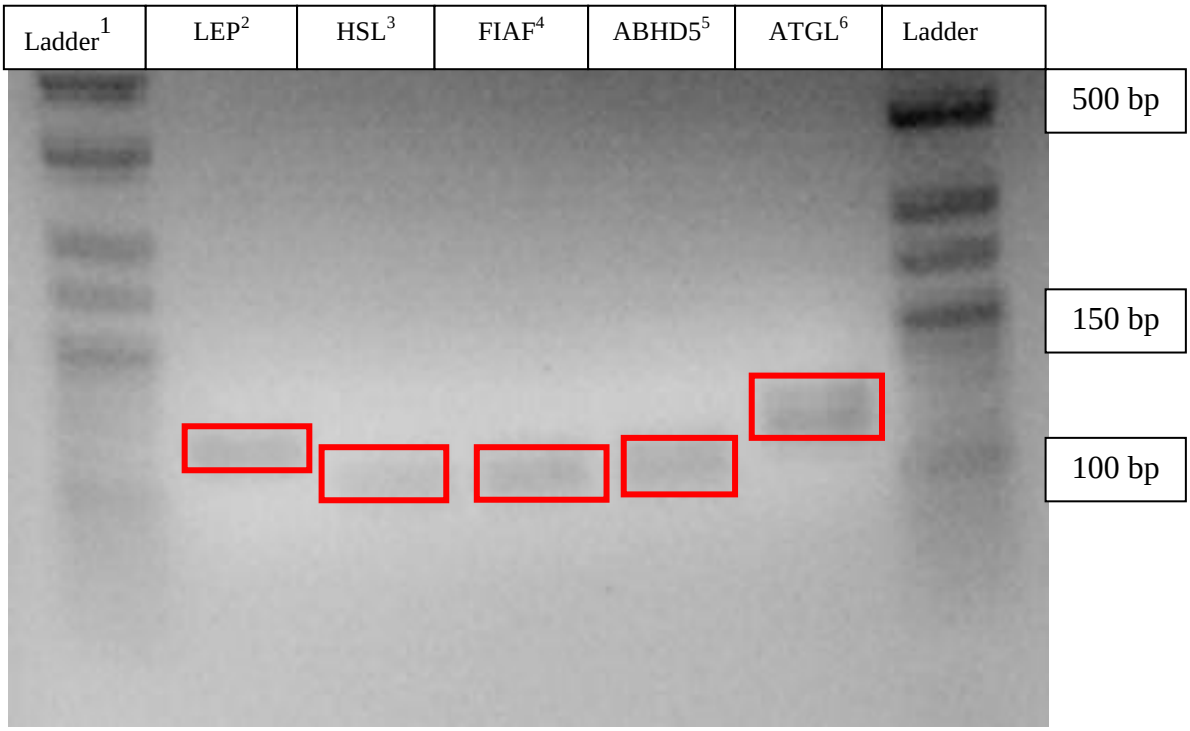
[3]Fatty acid synthase (FASN; 92 bp).

[4]Lipoprotein lipase (LPL; 101 bp).

[5]Stearoyl-CoA desaturase (SCD; 101 bp).

[6]Thyroid hormone responsive spot 14 (S14; 151 bp).

**Figure 39**. Verification of PCR product size of lipogenic genes.




[1]Samples were electrophoresed through a 2% agarose gel electrophoresis and 25 to 500 bp ladder was used.

[2]Leptin (LEP; 124 bp).

[3]Hormone-sensitive lipase (HSL; 101 bp).

[4]Fasting-induced adipose factor (FIAF; 102 bp).

[5]Abhydrolase domain containing 5 (ABHD5; 100 bp).

[6]Adipose triglyceride lipase (ATGL; 121 bp).

**Figure 40**. Verification of PCR product size of lipolytic genes