

University of Nevada, Reno

**Water requirements, fertility, and characterization of liver epigenetic markers
during periods of negative energy balance and subsequent compensatory growth
in beef cattle**

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Abstract

Thirteen crossbred Angus × Hereford bulls [29.6 ± 3.4 mo.; initial body weight (BW) = 715 ± 36.1 kg] were group-housed in a feedlot equipped with an automated water system for 30 d (adaption) + 168 d (trial). Data was collected and analyzed following a pre-post repeated measure design. Three dietary regimes were offered: BW maintenance (Phase 1: prior to BW loss and Phase 3: post BW loss); BW loss (Phase 2); and BW gain (Phase 4: compensatory gain). In order to induce a metabolic stress and a full recovery to initial state, each animal acted as its own control. Animals were fed Beardless wheat (*Triticum aestivum*) hay. Statistical analyses were performed using SAS 9.4 (SAS Inst., Cary, NC). In the first study, the objective was to evaluate how drastic changes in net energy requirements and body composition of breeding bulls could influence their water intake (WI). Environmental variables, individual WI, and BW were recorded daily. Principal component (PC) analyses were used to account for the relationship between environmental variables, animal requirements, body composition changes, and animal performance on WI. The first two PC presented lower discriminatory power among WI, performance, energy requirements, and environmental variables during phases 1 and 3 of BW maintenance. During phases 2 and 4, the first two PC had a high discriminatory power among WI, animal requirements, body composition, and environmental variables. Lower BW ($P = 0.006$), metabolic BW ($P = 0.004$), and net energy for maintenance ($P = 0.004$) observed in bulls at Phase 2, were followed by a drastic reduction in WI compared to Phase 4. In Phase 4, bulls had a high average daily gain which followed the tendency of lower WI ($P = 0.085$) relative to what was observed for Phase 2, indicating a change in efficiency of water utilization per unit of BW. Water intake for Phases 2

and 4 was primarily driven by animal requirements and secondarily by environmental factors. For animals undergoing BW loss and compensatory growth, WI is primarily driven by animal requirements and secondarily by environmental variables, particularly in animals undergoing nutritional and metabolic stress. Because of this, caution should be taken to avoid oversimplification of water requirements using the factorial approach. In the second study, the objective was to characterize the effects of nutritional status on epigenetic markers, such as DNA methylation and m⁶A RNA methylation, of bovine sperm. Higher levels of RNA m⁶A ($P = 0.004$) and DNA methylation ($P = 0.007$) of spermatic cells were observed at Phase 2 compared with Phase 1. In Phase 3, sperm RNA m⁶A methylation levels continued to be higher ($P = 0.004$), whereas the DNA of sperm cells was similar ($P = 0.426$) compared with the Phase 1. Growing bulls had a tendency ($P = 0.109$) of higher RNA m⁶A methylation levels than mature bulls. Phase 2 altered scrotal circumference ($P < 0.001$), sperm volume ($P = 0.007$), sperm total motility ($P = 0.004$), sperm progressive motility ($P = 0.004$), total sperm count ($P = 0.049$), normal sperm ($P < 0.001$), abnormal sperm ($P < 0.001$), primary sperm defects ($P = 0.039$), and secondary sperm defects ($P < 0.001$). In Phase 3, bulls had scrotal circumference, sperm volume, sperm motility, sperm progressive motility, total sperm count, normal and abnormal spermatozoa, and primary and secondary spermatozoa defects similar to Phase 1 ($P > 0.05$). Serum concentrations of insulin-like growth factor-1 (IGF-1) and leptin decreased during Phase 2 ($P = 0.010$) while no differences ($P > 0.05$) were detected between Phase 3 and 1; growing bulls tended ($P = 0.102$) to present higher leptin levels than mature bulls. Specific for mature bulls, DNA methylation was positively correlated with leptin concentration (0.569, $P = 0.021$). Whereas for young bulls, DNA methylation was positively correlated with abnormal spermatozoa (0.824, $P = 0.006$), primary

spermatozoa defect (0.711, $P = 0.032$), secondary spermatozoa defect (0.661, $P = 0.052$), and negatively correlated with normal spermatozoa (-0.824 , $P = 0.006$), total sperm count (-0.702 , $P = 0.035$), and sperm concentration (-0.846 , $P = 0.004$).

There was no significant correlation ($P > 0.05$) between RNA m⁶A and hormones and semen traits. In conclusion, the nutritional status of breeding bulls alters epigenetic markers, such as DNA methylation and RNA m⁶A methylation, in sperm, and the impact of change seems to be age-dependent. These markers may serve as biomarkers of sperm quality and fertility of bulls in the future. Detrimental effects on sperm production and seminal quality are observed at periods and places when and where environmental and nutritional limitations are a year-round reality and may carry hidden players that may influence a lifetime of underperformance. In the third study, the objective was to characterize the effects of dietary restriction and subsequent *ad libitum* feeding on body composition and hepatic gene expression of epigenetic regulators of DNA methylation, RNA m⁶A methylation, and histone acetylation in beef breeding bulls. Bulls undergoing negative energy balance (NEB) decreased ($P < 0.001$) of empty body weight [EBW; 23.1% (-139.1 kg)], empty body fat [EBF; 39.8% (-85.4 kg)], and empty body protein [EBP; 14.9% (-13.5 kg)]. A full recovery to initial state of EBW, EBF, and EBP was observed at the end of the *ad libitum* feeding. Body fat changes accounted for 77.1% of daily changes in body energy status, whereas body protein changes accounted for only 22.9% ($P < 0.001$). Bulls undergoing NEB tended ($P \leq 0.097$) to have increased gene expression of epigenetic regulators of RNA m⁶A methylation (*METTL14*, *VIRMA*, and *WTAP*), increased ($P \leq 0.050$) gene expression of epigenetic regulators of DNA methylation (*DNMT3A*) and histone-acetylation (*SIRT3* and *SIRT7*). Growing bulls had a tendency ($P \leq 0.072$) of higher RNA m⁶A methylation, *VIRMA*, and *WTAP* than mature bulls. Effect of diet \times

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“We cannot impose our will on a system. We can listen to what the system tells us and discover how its properties and our values can work together to bring forth something much better than could ever be produced by our will alone.”

Donella H. Meadows

“Do what you can, with what you have, where you are.”

Theodore Roosevelt

I dedicate this work to God:

“For from him and through him and to him are all things.

To him be the glory forever. Amen”.

Romans 11:36

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

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List of Abbreviations

| Acronym | Units | Description |
|----------|----------------|----------------------------------------------------------------------------------------------|
| ADF | g/kg DM | acid detergent fiber |
| ADG | kg/d | average daily gain |
| AI | | artificial insemination |
| ALKBH5 | fold change | alkB homolog 5 |
| aNDFom | g/kg DM | neutral detergent fiber with the addition of amylase and sodium sulfite and exclusive of ash |
| AS | % | abnormal spermatozoa |
| ASMS | | automated scale monitoring system |
| BCS | scale (1 to 9) | body condition score |
| BEN | g/d | excess bacterial nitrogen |
| BFN | g/d | bacterial fecal N |
| BFT | mm | back-fat thickness |
| BNA | g/d | bacterial nucleic acids |
| BSE | | breeding soundness exam |
| BSk | | Köppen code: cold-semi arid climate |
| BW | kg | body weight |
| CP | g/kg DM | crude protein |
| dailyBE | Mcal/d | daily changes on body energy |
| dailyEBF | g/d | daily changes on body fat |
| dailyEBP | g/d | daily changes on body protein |
| CV | % | coefficient of variation |
| DE | Mcal/d | digestible energy |
| DM | g/kg | dry matter |

| | | |
|-----------------|--------------------|--------------------------------------------------|
| DMI | kg/d | dry matter intake |
| DMIperBW | g/kg | dry matter intake per unit of body weight |
| DNMT1 | fold change | DNA methyltransferase 1 |
| DNMT3A | fold change | DNA methyltransferase 3 alpha |
| DNMT3B | fold change | DNA methyltransferase 3 beta |
| DR | L/min | drinking rate |
| EBF | kg | empty body fat |
| EBP | kg | empty body protein |
| EBW | kg | empty body weight |
| EE | g/kg DM | ether extract |
| EQEBW | kg | equivalent empty body weight |
| EQSBW | kg | equivalent shrunk body weight |
| EMS | g micP/g TDN | efficiency of microbial protein synthesis |
| FCBACT | g/d | fiber carbohydrate bacterial N |
| FFN | g/d | fecal N from the indigestible feed |
| FN | g/d | fecal nitrogen |
| FTO | fold change | alpha-ketoglutarate dependent dioxygenase |
| HR | beats/min | heart rate |
| IGF-1 | ng/mL | insulin-like growth factor-1 |
| LEP | ng/mL | leptin |
| LRNS | | Large Ruminant Nutrition System |
| [ME] | Mcal/kg DM | metabolizable energy concentration |
| MBW | kg ^{0.75} | metabolic body weight |
| MEI | Mcal/d | metabolizable energy intake |
| ME _m | Mcal/d | metabolizable energy requirement for maintenance |

| | | |
|------------------|-------------|----------------------------------------------------------------|
| METTL14 | fold change | methyltransferase like 14 |
| METTL3 | fold change | methyltransferase like 3 |
| MFN | g/d | metabolic fecal N |
| m ⁶ A | ng | N6-methyladenosine methylation |
| micP | g | microbial protein synthesis |
| MNS | g/d | microbial nitrogen synthesis |
| MNSRNI | g/g | microbial nitrogen synthesis relative to nitrogen intake |
| N | | nitrogen |
| NDEVD | # equipment | number of different equipment visited per day |
| NEB | | negative energy balance |
| [NEg] | Mcal/kg DM | concentration of net energy for gain |
| NEg | Mcal/d | net energy requirements for gain |
| [NEm] | Mcal/kg DM | concentration of net energy for maintenance |
| NEm | Mcal/d | net energy requirements for maintenance |
| NEt | Mcal/d | total net energy requirements |
| NEU | g/d | metabolizable nitrogen supply subtracting the net nitrogen use |
| NFC | g/kg DM | non-fibrous carbohydrates |
| NFCBACT | g/d | non-fibrous carbohydrate bacterial N |
| NI | g/d | nitrogen intake |
| NR | g/d | nitrogen retention |
| NRRNI | g/g | nitrogen retention relative to nitrogen intake |
| NS | % | normal spermatozoa |
| PC | | principal component |

| | | |
|------------------|------------------|------------------------------------------------------|
| PC1 | | first principal component |
| PC2 | | second principal component |
| PCA | | principal component analysis |
| PCV | % | packed cell volume |
| peNDF | g/kg DM | physically effective neutral detergent fiber |
| PSD | % | primary spermatozoa defect |
| RA | cm ² | ribeye area |
| RBM15 | fold change | ribonucleic acid binding motif protein 15 |
| RE | Mcal/d | retained energy |
| REV | | reverse primer |
| RH | % | relative humidity |
| RNB | g/d | ruminal nitrogen balance |
| RNBRNI | g/g | ruminal nitrogen balance relative to nitrogen intake |
| RNH ₃ | g N/d | recycled ammonia nitrogen |
| [S] | units/mL | sperm concentration |
| SBW | kg | shrunk body weight |
| SC | cm | scrotal circumference |
| SIRTn | fold change | sirtuin (1 through 7) |
| SM | % | sperm motility |
| SPM | % | sperm progressive motility |
| SpV | mL | sperm volume |
| SR | W/m ² | solar radiation |
| SRW | kg | standard reference weight |
| SSD | % | secondary spermatozoa defect |
| SV | scale (1 to 5) | sperm vigor |

| | | |
|----------|-----------------------|--------------------------------------------------------|
| TCP | g/d | total daily intake of crude protein |
| TDN | g/kg DM | total digestible nutrients |
| Temp | °C | ambient temperature |
| TETn | fold change | methylcytosine dioxygenase (1 to 3) |
| THI | | temperature-humidity index |
| TN | g/d | degraded tissue N |
| TRRN | g/d | total rumen microbial N required |
| TSDW | min/d | time spent drinking water |
| TSPTZ | units $\times 10^6$ | total sperm count |
| TWVD | # events/d | total waterer visited per day |
| UN | g/d | urine nitrogen |
| USG | | urine specific gravity |
| VIRMA | fold change | vir like m ⁶ A methyltransferase associated |
| WI | L | water intake |
| WIperMBW | mL/kg ^{0.75} | water intake per unit of metabolic body weight |
| WMS | | water monitoring systems |
| WS | mph | wind speed |
| WTAP | fold change | WT1 associated protein |
| WVWDED | % | waterer visits with successful drinking event |
| 18s | fold change | eukaryotic 18S ribosomal |
| 5-mC | ng | 5-methyl cytosine |

CHAPTER I:

DESCRIPTION OF THE PROBLEM

1.1. Introduction

Rangelands in the Western U.S are the main sources of forage for livestock operations (Reeves et al., 2017). However, forage quantity and/or quality change throughout the year and can be inadequate to meet animals' nutrient requirements. Thus, cattle in rangeland conditions, may lose body weight (BW) intermittently. This is a well-known challenge faced by grazing cattle year-round in the Western U.S. (NASEM, 2016).

After an animal has gone through a period of significant nutritional restriction or some significant unfavorable environmental perturbation, it will grow faster than its unrestricted cohorts when both are to be under an adequate nutritional strategy once again. This phenomenon is described as compensatory gain (Bohman, 1955). It is commonly observed in the cattle present in the Western U.S., animals experiencing compensatory growth during the spring and summer, after the winter season (Bohman, 1955).

Given the increasing frequency of environmental issues faced by beef production systems (Tedeschi et al., 2017), a better understanding of how water is used by cattle in periods of nutritional restriction followed by a compensatory growth may help us to quantify the role of cattle production on water scarcity, climate change, and global warming. Furthermore, changes in breeding bulls' fertility in response to nutritional factors is still neglected (Taylor et al., 2018). Although,

breeding soundness examination is currently used to evaluate bull fertility (NASEM, 2016), changes to fertility in response to environmental and nutritional factors may go undetected by this exam. Thus, molecular biology analyses may be used for unveiling the impact of silent players that may affect breeding bulls' fertility (e.g., sperm traits). Moreover, during periods of metabolic perturbation, it is well-known that the liver plays a central role in providing nutrients to peripheral tissues (Geisler et al., 2016; Zhang et al., 2018). Still, the literature lacks studies that characterize changes in hepatic epigenetic markers that potentially change in response to metabolic perturbations in beef cattle.

1.2. Literature review

1.2.1. Worldwide water use for beef production

Water scarcity is a global issue for food production, human health, and economic development, which affects 1-2 billion people worldwide (MEA, 2005). It is predicted that by 2025, 64% of the world population will live in a water-deprived basis, compared to 38% identified in similar conditions in 2009 (Rosegrant et al., 2020). Thus, in addition to climate change, water may become the weak point in all livestock systems. For example, animals that are in hot environments, such as the ones inhabiting Nevada's rangelands, are expected to drink 2-3 times more water than animals in cooler climates (Nardone et al., 2010). Therefore, the effects of climate change on water availability could force the livestock sector to establish a new priority in production of animal products that require a more conscientious use of water resources (Nardone et al., 2010).

In 1993, it was estimated by Beckett and Oltjen (1993) that beef cattle in the U.S consume 760 billion liters of water per year. Considering the total water used for beef cattle production, its majority goes to crop production, which are consumed by beef cattle, and it comes from both precipitation and irrigation (Beckett and Oltjen, 1993; Palhares et al., 2017). Irrigation of crops accounted for 12,991 billion liters of water per year, and pasture accounted for 11,243 billion liters of water per year in their simulations (Beckett and Oltjen, 1993).

A considerable amount of water is also utilized in the carcass processing (Palhares et al., 2017). For example, a harvest facility in California (personal communication to Beckett and Oltjen (1993)) calculated the amount of water used in processing steps from slaughter to trimmed boneless beef is around 1,533 liters per carcass. Therefore, the 33 million head harvested in 2020 (USDA, 2020) would have required 51 billion liters of water for processing alone at the harvesting facilities. This is less than the estimate by provided by Beckett and Oltjen (1993), who increased the estimated water amount used at the processing plant by 50%, resulting in an estimated 78.5 billion liters of water required to process beef for that year. One should also note that the estimated number of cattle harvested per year, 28.4 million in 1993, is less than USDA's 2020 estimate of 33 million head. The calculations of Beckett and Oltjen (1993) concluded by estimating that each kilogram of boneless beef produced in the U.S. requires 3,682 L of water to be produced.

Conservative and oversimplified approaches have suggested that the amount of water used per kilogram of boneless beef produced is much higher than what was previously described (Kreith and Davis, 1991; Robbins, 1998). Robbins (1998) estimated that 20,864 liters of water would be required per kilogram of boneless beef produced. While, Kreith and Davis (1991) suggested that the actual cost is 20,559

liters of water per kilogram. These estimates are perhaps less reliable, considering that they have only accounted for one type of production system. Moreover, Mekonnen and Hoekstra (2012) estimated that beef cattle were responsible for 33% of the global water footprint of animal production and almost 10% of the global water footprint of total agricultural production. Taken altogether, green, blue, and grey water, had very small variations in its global averages' estimates, 15,415-15,497 liters per kilogram of beef (Ran et al., 2016). In contrast, the study by Mekonnen and Hoekstra (2012) presents relatively large variations between regions and production systems. Grazing systems, for example, have a range of 16,353–26,155 liters per kilogram of beef, mixed systems of 11,744–16,869 liters per kilogram of beef, and industrial systems of 3856–13,089 L/kg of beef.

Hitherto, there appears to be a large variation in the water footprint estimations reported in literature. For instance, Palhares et al. (2017) demonstrated that in Brazil, a tropical country, the total water footprint ranged from 1,935 to 9,673 liters per kilogram of meat. Comparing these values to other studies and reported averages would entail an overestimation of 93% in the liters of water per kilogram of meat between studies. Therefore, due to the wide diversity of beef cattle production systems, accurate and precise estimates of water use, not necessarily footprint, are hard to achieve. Hence, an oversimplification of water use of beef herd has been adopted for years. Despite these previous efforts to assess beef livestock water use, most of the methodologies are still based on static frameworks and neglect to capture the structure of the problem of livestock water use quantification. Only Menendez et al. (2020) and Menendez and Tedeschi (2020) have estimated a substantial variation in water use by cattle, ranging from 7,500 to 22,500 liters per kilogram of meat, by then using a dynamic framework methodology. However, most of previous studies,

specifically characterized production systems in which animals are in daily anabolic positive gain, also an oversimplification of a *de facto* system. Thus, studies addressing the consequences of BW daily dynamicity, annually observed in Western U.S rangeland conditions (e.g., BW loss and BW gain), on cattle water usage are needed.

1.2.2. Water requirements for cattle

Animals meet their water requirements by drinking free water, consuming fresh feedstuffs containing water, and using metabolic water produced by the animal's metabolism (Tedeschi and Fox, 2017). However, water intake (WI) is rarely considered when formulating and balancing rations for animals because it is anticipated that animals have unrestricted access to water. Thus, as water becomes scarcer, water availability will be a serious problem, and addressing an animals' water consumption is part of the solution (Tedeschi and Fox, 2017).

Breed, rate of gain, and body composition

Several factors affect water consumption in cattle, including breed, rate of gain, and body composition (NASEM, 2016; Menezes et al., 2020). For example, *Bos taurus* cattle have higher water consumption than *Bos indicus* breeds, all else constant, especially as temperature increases (Winchester and Morris, 1956). Brew et al. (2011) demonstrated that Charolais x Angus cross steers consumed more water (42.8 L) than Angus x Brangus (30.8 L), Brangus (30.8 L), Charolais x Brangus (29.7 L), Brangus x Romosinuano (24.1 L), and Charolais x Romosinuano (20.7 L). All the breed crosses that were examined, other than the Angus x Charolais and the Charolais x Romosinuano, had some percentage of *Bos indicus* germplasm, which had been shown by Winchester and Morris (1956) to consume less water. However, even

though the Romosinuano breed is classified as a taurine breed, they are known for their tropical adaptability (Riley et al., 2014). Some tropically-adapted cattle, such as Romosinuano, do not have zebu influence, which suggests that other *Bos taurus* breeds could be selected to become more adaptable or drink less water while still maintaining positive performance characteristics. However, when data from these experiments are adjusted to metabolic BW and dry matter intake, genotype differences become negligible. Therefore, there is conjecture over whether observed values are directly attributable to genotype due to sampling variance, differences in metabolic BW, or level of dry matter intake.

There is limited evidence evaluating water requirements for rate of gain in bovines, so some extrapolation from other species may be necessary. Additional required water in support of growth has been evaluated in various small ruminant species (NRC, 2007). Suggested water requirements for small ruminant nursing and small ruminant weaned animals are 8 to 13 mL/g BW gain and 7 to 8 mL/g BW gain, respectively (NRC, 2007). Incorporating these suggested water requirements for gain with maintenance, nursing animals gaining between 100 and 400 g/d have an estimated water requirement of 255 to 316 mL/kg BW^{0.75} (NRC, 2007). Whereas weaned animals gaining 200 to 400 g/d would have a water requirement of 244 to 348 mL/kg BW^{0.75} (NRC, 2007). However, the range in daily gain of a small ruminant is not consistent with observations in the growing bovine.

Nutritional factors

Feeds contain some water in their composition, and the oxidation of certain nutrients in feeds produces metabolic water, then not all water must be provided by drinking (NASEM, 2016). Usually, feeds such as silage, green chop, or growing

pasture forage have a very high moisture content, whereas grains, hays, and dormant pasture forage have low moisture content. Cattle water requirements can increase when a diet is high in protein, salt, minerals, or diuretic substances (NRC, 2007; NASEM, 2016). According to NASEM (2016), the relationship between WI and DMI appears to be low because the production systems are not only characterized by thermoneutral conditions. For example, WI generally increases and DMI generally decreases in warmer months of the year, with the opposite relationship occurring in the cooler months. In general, data (Winchester and Morris, 1956; Bond et al., 1976; Hicks et al., 1988) indicates that most of the time WI will be substantially affected by DM composition. Despite the scientific knowledge gained throughout the years, we still face a long and steep road ahead until we can securely make inferences about which level of DM constituents affects WI in beef cattle.

Physiological state

Few studies have been undertaken to fully document water use according to the physiological state of beef animals. Young calves generally have higher intakes of water per kilogram of DM consumed (5.0-7.0 L) than the 3.5 to 5.5 L recommended for older cattle (Pettyjohn et al., 1963; ARC, 1965). During the last 4 months of pregnancy, cows may consume 30% more water than when non-pregnant (ARC, 1965). Under barn feeding conditions the estimated intake of free water for lactating cows is 0.87 kg water/kg milk produced (Winchester and Morris, 1956). Ewes carrying twins consume over twice the amount of water of nonpregnant ewes, whereas those carrying single lambs consume 138% (NRC, 2007). When corrected for water content of milk, lactating ewes consume 100 to 164% more water than dry ewes (Forbes, 1968). Thus, there are many opportunities for studies that address water

requirements by cattle in different physiological stages such as BW loss or compensatory gain.

Metabolic water

Metabolic water is important to all animals, particularly those living in arid and semi-arid environments, such as particular ruminant species like the caribou (Barboza et al., 2009). The catabolism of 1 kilogram of fat, carbohydrate, and protein produces 1190, 560, and 450 g of water, respectively (Winchester and Morris, 1956). In general, high-energy feeds produce more metabolic water than low-energy feeds, which is a complication in the matter of assessment of a cattle's water requirements (NASEM, 2016). In periods of negative energy balance, when fat and protein are being catabolized, metabolic water is also important to support water requirements (Barboza et al., 2009). For example, wild ruminants are able to mobilize energy from fat while preserving preferential protein (Barboza et al., 2020). Thus, metabolic water production is a potential substantial source to meet water requirements in bovine reared in extreme conditions of the Western U.S, by the dynamic process of tissue turnover and exchange among tissues year-round.

Water losses

Water losses by animals are principally through: urine, feces, and evaporation from the body surface and respiratory tract (NASEM, 2016). In addition, under severe stress, cattle and other species may lose a significant amount of water through drooling (McDowell and Weldy, 1967; NASEM, 2016). Unless animals are on a water-restricted diet, urinary excretion rate can usually be reduced without impairing the ability of the kidneys to excrete body wastes (Church, 1979). Under conditions of restricted WI, the body reabsorbs a greater amount of water than usual, thereby

concentrating the urine (Burgos et al., 2001). Although this capacity of concentrating urine solutes is limited, it can decrease water requirements by a small amount (NASEM, 2016).

In ruminants, the loss of water through feces is substantial, approximately equal to urinary losses (NRC, 1981). The high-fiber nature of a ruminant animal's diet requires proportionately more water to carry the ingesta through the gastrointestinal tract than for non-ruminants. Level of fiber is not, however, a sufficient reason to explain the level of water in the fecal matter. Cattle feces contain 75-85% water, while sheep and goat feces have 60-65% water (NRC, 1981). The ability to reabsorb water in the large intestine and excrete drier fecal pellets instead of wet and loose feces is presumably one mechanism of water conservation (NRC, 1981).

Water loss through the respiratory tract is extremely variable, depending upon relative humidity and respiration rate. It is commonly observed that expired air is over 90% saturated; hence, under conditions of low relative humidity, respiratory losses are high (NRC, 1981). Conversely, losses are low when inspired air is near saturation (e.g., temperate and tropical coastal environments; NRC (2007)). When respiration rate increases in response to high temperatures or other behavioral stimulus (e.g., physical activity increases by unintended consequences in a search for water/feed), the rate of respiratory water losses is increased (e.g., cattle may lose 23 mL/m²/h at 27°C and up to 50 mL/m²/h under severe heat stress; NRC (1985)).

Cutaneous evaporation of water is the major means of heat loss in cattle and sheep at high temperatures (Robertshaw, 1966; McDowell and Weldy, 1967). There are large differences among species in the importance of sweating with domestic livestock ranked in the descending order of horses, donkeys, cattle, buffaloes, goats, sheep, and swine (McDowell, 1972). The threshold skin temperature for sweating

varies among species, but cattle sweat reacting at about 25°C (McDowell et al., 1954).

Water availability, quality, and environment

The availability and quality of drinking water is important to ensure cattle water requirements (NASEM, 2016). Thus, cattle water requirements varies according to environmental conditions in which the animals are raised (Tedeschi and Fox, 2017). For instance, water contaminants such as nitrates, salt, and sulfates are known to depress WI (NASEM, 2016).

Utilizing records from seven separate feedlot experiments across several years and seasons to examine the impact of environmental factors on daily WI of finishing cattle, Arias and Mader (2011) concluded that mean ambient temperature, minimum temperature, and temperature-humidity index were the primary factors that influenced daily WI. Similar results were reported by Sexson et al. (2012). These researchers summarized water intake data from four separate beef cattle feedlot experiments conducted in the High Plain region of the United States over several years. They demonstrated that humidity and sea level pressure were negatively related to water intake, whereas temperature of the previous day, daily temperature, change in temperature from the previous day, average wind speed, and the temperature-humidity index, were positively related to daily water intake.

Other factors that can influence water requirements of cattle are water temperature, physical access to water, and intake of water from environmental precipitation (rain and snow; NASEM (2016)). Heating water to prevent freezing during winter helps to maintain drinking water availability (NASEM, 2016). Cattle prefer liquid water, but they can consume snow and ice when it is not available in

adequate amounts (NRC, 1981). Cattle under thermal stress can tolerate rapid ingestion of snow and ice by drawing from stored body heat and an immediate increase in metabolic rate. Thus, it will compensate for the heat required to melt frozen water and bring it to body temperature (Degen and Young, 1984).

Interacting factors

The wide diversity of production systems in which beef cattle are raised and the interaction of several components (e.g.,: environmental factors, diet, breed, body weight, composition of gain) complicates accurate estimates of WI requirements for beef cattle (Tedeschi and Fox, 2017). Earlier research by Winchester and Morris (1956) suggested a constant relationship between WI and DMI for cattle at thermal neutral conditions. However, WI generally increases and DMI generally decreases in warmer months of the year, with the opposite relationship occurring in the cooler months of the year. Bond et al (1976) deprived cattle of feed (for 48 h) and found a 13% increase in WI for cattle receiving a diet containing no forage (high-concentrate diet). Whereas cattle consuming a diet composed of almost all forage (88%) decreased WI by 92%. Thus, the assessment of WI from DMI is not consistent. Likewise, for cattle that have a BW less than 500 kg, water consumption increases from 22 to 38 liters per animal per day as body weight increases. However, cattle that weigh more than 500 kg have decreased WI as body weight increases (Sexson et al., 2012). Thus, the decline in WI associated with animals larger than 500 kg is a function of the change in composition of gain: as the proportion of fat increases, the proportion of protein and water gain decreases (NRC, 2000). Therefore, wide diversity of production systems in which beef cattle are raised and the interaction of

several components make it more challenging to determine the daily water requirement for beef cattle.

1.2.3. Breeding Systems of beef cattle in the U.S

The most commonly utilized breeding system in the U.S among cow-calf producers is natural service, which is roughly 93% of U.S beef operations (NAHMS, 2017). When this breeding method is used, herd sires are placed on pastures with females for a designated period of time (a breeding season) or indefinitely. More than 50% of U.S. beef operations do not have a set breeding season, meaning that bulls stay with females 365 days a year (NAHMS, 2017). Thus, at least 50% of the sires are exposed to annual nutritional or environmental stress. Furthermore, it is estimated that only about 10% of beef bulls in the U.S undergo a bull breeding soundness evaluation (BBSE, 2021). The remaining herds have 1 to 2 breeding seasons, with 34% having a single breeding season, generally during spring until mid-summer (NAHMS, 2017).

Thus, failure to properly evaluate bulls for natural breeding service (breeding season or 365 days a year) can result in negative economic profitability of a cow-calf operation, and thus producers may experience short- and long-term consequences. In the short-term, a low number of cows may get pregnant due to the sire's poor sperm production and/or quality, and increased number of culled females due to being open at the end of the breeding season (Kastelic and Thundathil, 2008). In the long-term, these bulls will take longer than fertile bulls to impregnate a group of cows; meaning that their offspring will be born later and are therefore younger and lighter at weaning. Furthermore, changes to a bulls' fertility in response to environmental and nutritional factors may go undetected by the breeding soundness examination. Therefore, there

are opportunities for studies that use molecular biology analyses for unveiling the impact of the environment on sperm traits, allowing the identification of potential biomarkers of fertility.

1.2.4. Artificial Insemination (AI) industry: Beyond the beef operations

The U.S. beef herd mating system is characterized by only 7% use of artificial insemination (AI) (NAHMS, 2017). However, on dairy cattle operations, the mating is characterized by 70% use of AI (Hall, 2019). Thus, the use of AI should not be neglected, since AI allows spreading of preferable genomes not only within of the U.S., but worldwide. According to the National Association of Animal Breeders (2020), more than 25 million doses of semen were commercialized within the U.S. and more than 36 million doses were exported in 2020. From the total, considering both beef and dairy AI's industry, 23.1% were semen doses of beef sires, from which 50.3% were commercialized within the U.S. whereas 49.7% were exported in 2020 alone. Therefore, producers and industry partners alike must drive huge attention for the management of breeding bulls, since they have substantial importance in determining the productive outcomes of the bovine herds not only in the U.S, but worldwide.

1.2.5. Epigenetic inheritance and fertility

Bulls have a pivotal role in determining performance in cattle herds due to their larger opportunity for propagating genes (Dekkers, 2004). One single bull can mate, or produce semen to inseminate, hundreds or even thousands of cows (Vishwanath, 2003). It was recently discovered that paternal nutrition affects

offspring metabolism and health (Fullston et al., 2012; Lambrot et al., 2013). Furthermore, studies in male mice showed that those under non-ideal nutritional regimes, promoting either excessive weight gain or loss, had DNA methylome changes in their sperm, which caused metabolic dysfunction in their offspring (Radford et al., 2014; McPherson et al., 2016; Schagdarsurengin and Steger, 2016).

The mode of action of DNA methylome pattern involves epigenetic control (i.e.: not related to alteration in DNA sequence) of gene expression. Thus, epigenetic inheritance very likely plays a role in determining traits related to metabolism and performance in cattle as well. If this is true, malnourished bulls could be propagating conditions related to poor health and performance to their offspring. This later assertive represents the variation in metabolic status of sires, as a result of non-ideal nutritional management (commonly leading to excess of fat deposition) or weight loss (tissue catabolism) due to adverse environmental conditions, or due to excessive activity during a breeding season (NASEM, 2016). Given the possibility of individual bulls to spread inheritable traits to herds locally and globally, overseeing transmission of undesirable metabolic conditions may negatively impact cattle industries in the U.S and around the world. Conversely, understanding how nutrition can affect the sperm DNA methylome gives an opportunity to improve beef cattle herds performance by providing bulls ideal nutritional conditions. Furthermore, epigenetic modifications of DNA (global DNA methylation, 5-mC) and transcripts (RNA methylation, N6-methyladenosine) have been attracting attention of scientists for their potential and promising biomarkers of fertility (Houshdaran et al., 2007; Lambrot et al., 2013; Yang et al., 2016). Thus, studies aiming to understand what factors can change these modifications in the sperm epigenome, including nutritional factors, are warranted.

1.2.6. Hepatic response to fasting and subsequent refeeding

The liver is a major metabolic organ, accounting for approximately 24% of whole body energy use (McBride and Kelly, 1990; Caton et al., 2000). This energy requirement arises from activities associated with absorption and transportation of nutrients for subsequent use by other tissues (Johnson et al., 1990). In addition, a large portion of this energy is used for maintenance of tissue integrity and mass (Baldwin et al., 2004). Several studies have demonstrated a reduction in the weight and metabolic activity of this organ during dietary restriction, which facilitates efficient coping with restricted nutrient availability, primarily through a reduction in its basal metabolic rate (Yambayamba et al., 1996b; Keogh et al., 2015a). During subsequent re-alimentation under a compensatory growth, the liver has been revealed to be one of the most responsive tissues, recovering faster than other organs and tissues (Yambayamba et al., 1996a; Keogh et al., 2015b). However, the reduced metabolic rate of the liver may continue into the initial stages of re-alimentation and thus facilitate the compensatory growth process (Drouillard et al., 1991).

A previous microarray-based examination of hepatic gene expression during feed restriction, followed by early re-alimentation phase of cattle, was reported by Connor et al. (2010). The authors noted alterations in the expression of genes associated with cellular division and mitochondrial function. Within those genes, the authors did not report any results about histones deacetylase gene expression, RNA methylation genes, or other genes associated to modulation of the liver epigenome. Nevertheless, epigenetic biomarker changes, such as RNA m⁶A methylation, DNA methylation, and histone post-translational modifications, likely serve a central role in the liver function; for example, providing nutrients to peripheral tissues during

metabolic perturbations in cattle as reported by the literature in other species (He et al., 2017; Zhong et al., 2018). Thus, studies that aim to characterize the effects of nutritional status on epigenetic markers in hepatic tissue of bovine are needed.

DNA Methylation

It is well known that the phenotype of animals can be modified by nutrition through epigenetic mechanisms (Zhang, 2015; Triantaphyllopoulos et al., 2016). As a key and central component of epigenetic networks, DNA methylation is labile in response to nutritional influences (Zhang, 2015). Alterations in DNA methylation profiles can lead to changes in gene expression, resulting in diverse phenotypes with the potential for impaired growth and health (Triantaphyllopoulos et al., 2016). It was recently discovered that maternal nutrition can affect an offspring's hepatic metabolism, and consequently feed efficiency (Devos et al., 2021). This later assertive was explained associating the variation in DNA methylation between animals of divergent residual feed intake (RFI; Devos et al. (2021)). According to the authors, low RFI steers had higher levels of methylation than high RFI steers. Potentially methylation allow more efficient sequestration of available resources towards growth (Devos et al., 2021). Furthermore, groups of cows who experienced a restricted diet during gestation were significantly more methylated when compared with a moderate diet (Devos et al., 2021). Given that forage production in the Western U.S rangelands is largely variable year-round (Van Soest, 2018), epigenetic markers may mediate nutrient use in animals raised in these conditions. Consequently, bovine may have presence of specific adaptations (e.g., DNA methylation) to handle the nutrient variation throughout the year. Thus, expression of genes that modulate DNA methylation is a potential target (Zhang, 2015). DNA methylation is mediated by

DNA methyltransferases (DNMTs) to yield 5-methylcytosine (5-mC), primarily at cytosine-phosphate-guanine dinucleoside sites (Day and Sweatt, 2010, 2011; Day et al., 2015). There are three DNMTs with different functions. DNMT3A and DNMT3B are responsible for addition of methyl groups, whereas DNMT1 is responsible for maintenance of DNA methylation patterns during cell replication. In addition, DNMT1 copy methylation marks on the parental strand to the daughter strand after replication. DNMT3A and DNMT3B complete the methylation process and correct errors left by DNMT1 (Jones and Liang, 2009). DNA methylation is reversible as 5-mC can be oxidized by the α -ketoglutarate dependent dioxygenases TET (ten-eleven translocation) family of dioxygenases to yield 5-hydroxymethylcytosine (Tahiliani et al., 2009; Day et al., 2015).

Histone modifications and Histone deacetylases

Histones have crucial functions in the regulation of gene expression (Roth et al., 2001). In eukaryotes, histone modification is a post-translational modification to histone proteins (Biterge and Schneider, 2014). Histone modifications can affect interactions of histones with DNA and adjacent proteins (Biterge and Schneider, 2014), and can regulate gene expression by altering chromatin structure or recruiting histone modifiers (Gelato and Fischle, 2008). Deacetylation of histones is mediated by histone deacetylases (HDACs) enzymes (Kuo and Allis, 1998) which catalyze the removal of acetyl groups from the acetyl lysine residue (Kuo and Allis, 1998). This process brings back the positive charge of the N-terminal tails of histones and increases their affinity for DNA, which leads to a repressive chromatin structure, displacing transcription factors from gene promoters and repressing transcription (Kuo and Allis, 1998). The sirtuins (SIRT) comprise one of the two known families of HDACs. At first, research focused on their role as repressors of gene expression.

However, Schwer et al. (2002) found that SIRT6 also deacetylates non-histone proteins, inducing the transcription of several genes involved in metabolism. Thus, SIRT6 appears to be an important metabolic regulator, especially of gluconeogenesis and lipid oxidation.

The expression profiles of sirtuins in bovines are quite different compared with what has been reported for other species (Frye, 1999; Cohen et al., 2004; Shi et al., 2005; Chen et al., 2008; Shan et al., 2009; Ghinis-Hozumi et al., 2011). The main reason for this can be explained by the primary metabolic differences between non-ruminant and ruminant species. Ruminants maintain their blood glucose concentrations in the fed state mainly through active gluconeogenesis, whereas in non-ruminant species, gluconeogenesis is activated only during energy deficit (Nafikov and Beitz, 2007). In ruminants, the main sources for gluconeogenesis are propionate, which comes from the rumen, and glycerol, that comes from the adipose tissue. While, amino acids and lactate provided by the muscle are the main substrates in non-ruminant animals (Lehninger et al., 2008). In the case of ruminants, the question is whether gluconeogenesis promotes the expression of sirtuins, or the reverse, whether expression of sirtuins favors gluconeogenesis (Ghinis-Hozumi et al., 2011). Animals undergoing BW loss followed by a compensatory gain are good models to clarify this question. Also, they may help us understand how a greater gluconeogenic capacity in the liver by ruminants is responsive to nutrient availability. One nutritional and management implication that we may get from this study is: in case of absence of responsive change in level of SIRT6 expression, it may be possible to clarify the onset of metabolic disorders (e.g., liver abscesses) in beef cattle from systems of aggressive grain-feeding programs, because of the low sensitivity of genes involved on gluconeogenesis metabolism.

N6-methyladenine (m⁶A) RNA methylation

As a novel epitranscriptomic marker, the dynamic and reversible chemical N6-methyladenine (m⁶A) is the most prevalent type of internal RNA methylation in eukaryotic mRNA (Tong et al., 2018; Zhong et al., 2018; Liu et al., 2019). It plays critical roles in regulating gene expression for fundamental cellular processes and diverse physiological functions (Jiang et al., 2021). The m⁶A is involved in many important biological processes through the post-transcriptional regulation of gene expression, including mRNA export, the processing of pri-miRNA, alternative splicing, mRNA degradation, and translation (Yang et al., 2018). Recent evidence indicates that m⁶A methylation regulates physiology and metabolism, such as lipid accumulation (Kang et al., 2018) and adipogenesis (Wang et al., 2015; Wu et al., 2018). The dysregulation of m⁶A patterns causes abnormal gene expression and functions, leading to the occurrence of certain inflammatory states, impairment of growth development, and metabolic diseases (Tong et al., 2018; Zhong et al., 2018; Liu et al., 2019). Therefore, the effects of m⁶A RNA methylation on physiology and metabolism have become a research hotspot in the RNA methylation field in recent years.

Emerging evidence shows that m⁶A modification plays a critical role in adipose hepatic glucose and lipid metabolism (Poritsanos et al., 2010; Lu et al., 2018). During the shifts of fasting-feeding periods, the liver is the central organ that maintains energy homeostasis, and hepatic gluconeogenesis and lipid oxidation is extremely important in ruminants in these conditions (Nafikov and Beitz, 2007). For example, recent evidence indicates that FTO-dependent m⁶A modification participates in glucose metabolism via hepatic gluconeogenesis (Peng et al., 2019; Yang et al., 2019). Peng et al. (2019) showed that hepatic knockdown of FTO (Fat mass and

obesity-associated protein) upregulates m⁶A abundance on forkhead box protein O1 (FOXO1) mRNA, which is an important transcription factor that regulates hepatic gluconeogenesis via glucose-6-phosphate. It was demonstrated that m⁶A can affect fatty oxidation during metabolic stress by selectively increasing the translation of enzymes associated to lipid oxidation (Lieberman et al., 2020). Thus, RNA m⁶A methylation may help us to unveil what occurs with an animal undergoing periods of feed restriction followed by compensatory growth.

Collectively, all these aforementioned studies show that m⁶A is a promising biomarker to look into animals undergoing different production levels. Understanding the patterns of hepatic epigenetics markers on animals at different production levels may also leverage the development of decision making systems for better management of forage resources while optimizing animal performance. Animal efficiency and productivity have vastly improved throughout the years. Still, we face a long and steep road ahead to be able to strategically manage the environmental stressors that impair forage production in the U.S western rangelands. Thus, the beef cattle industry needs to know more about epigenetic markers' behavior patterns on animals at different production levels, not only for the short-term assessment of livestock operations, but for the success of the whole industry that will require embracing precision livestock systems for optimizing decision making in the near future.

1.3. Conclusion

Cattle raised in rangeland conditions, intermittently lose BW when forage quantity or quality is inadequate to meet the animal's nutrient requirement throughout

the year. This is a common challenge for grazing cattle in the Western U.S, which means that grazing cattle, at some point in their lifetime, may experience BW loss and compensatory gain within or throughout the years. Thus, the first goal of this study is to characterize the water requirements for cattle at different metabolically-challenged periods at varying production levels. The outcomes from this study may leverage the development of decision making systems for better management of water resources while optimizing animal performance. The second goal is to characterize cattle sperm epigenetic markers, such as DNA methylation and RNA methylation, in animals undergoing nutritional challenges. The expected outcomes of this second goal are to characterize potential biomarkers of fertility and create adjustments for ideal nutritional management of breeding bulls. The third goal is to characterize how the metabolic status affects epigenetic markers that modulate gene transcription and translation in the liver of bovines. The expected outcome of this third goal is to characterize potential biomarkers involved in the regulation of the of molecular mechanisms in the liver, which is the central organ maintaining metabolic homeostasis during periods of nutritional and metabolic stress.

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CHAPTER II:

**WATER REQUIREMENTS OF BREEDING BULLS IS DIFFERENTLY
AFFECTED DURING PERIODS OF BODY WEIGHT LOSSES AND
COMPENSATORY GROWTH**

2.1. Abstract

The aim of this study was to evaluate how drastic changes in net energy requirements and body composition of breeding bulls could influence their water intake (WI) requirements. Thirteen crossbred Angus × Hereford bulls [29.6 ± 3.4 mo.; initial body weight (BW) = 715 ± 36.1 kg] were group-housed in a feedlot equipped with an automated water system for 30 d (adaption) + 168 d (trial). Data was collected and analyzed following a pre-post repeated measure design. Three dietary regimes were offered: BW maintenance (Phase 1: prior to BW loss and Phase 3: post BW loss); BW loss (Phase 2); and BW gain (Phase 4: compensatory gain). In order to induce a metabolic stress and a full recovery to initial state, each animal acted as its own control. Animals were fed Beardless wheat (*Triticum aestivum*) hay. Environmental variables, individual WI, and BW were recorded daily. Principal component (PC) analyses were used to account for the relationship between environmental variables, animal requirements, body composition changes, and animal performance on WI. Statistical analyses were performed using SAS 9.4 (SAS Inst., Cary, NC). The first two PC presented lower discriminatory power among WI, performance, energy requirements, and environmental variables during phases 1 and 3 of BW maintenance. During phases 2 and 4, the first two PC had a high discriminatory power among WI, animal requirements, body composition, and environmental variables. Lower BW ($P = 0.006$), metabolic BW ($P = 0.004$), and net energy for maintenance ($P = 0.004$) observed in bulls at Phase 2, were followed by a drastic reduction in WI compared to Phase 4. In Phase 4, bulls had a high average daily gain which followed the tendency of lower WI ($P = 0.085$) relative to what was observed for Phase 2, indicating a change in efficiency of water utilization per unit of BW. Water intake for Phases 2

and 4 was primarily driven by animal requirements and secondarily by environmental factors. This work provides the basis to assert that we cannot accurately and precisely estimate water usage footprint by beef cattle as it currently has been since most biological assumptions of current models heavily rely on environmental variables that do not share the same behavior nor interactions across beef cattle production systems. Ultimately, greater knowledge of the variables that dictate water requirements are exquisite, but of utmost importance since they will drive water usage, mitigation strategies, and natural resource management.

Key words: body composition, energy requirements, intake, nitrogen balance, metabolic water

2.2. Introduction

The wide diversity of production systems in which beef cattle are raised complicates accurate estimates of water intake (WI) requirements (Schlink et al., 2010), hence, an oversimplification of modeling WI has been adopted for years. Variables related to diet, environment, and relative animal body weight (BW) (Arias and Mader, 2011; Sexson et al., 2012; NASEM, 2016) have been considered the most effective estimators in diverse nutritional systems. However, the complexity of the biology driving water requirements, associated with changes in the environment wherein animals are embedded, need to be properly accounted for in order to accurately and precisely estimate appropriate footprints from the cattle industry. Numerous variables may interact with one another differently depending upon what physiological changes are occurring and the production cycle the animals are in, including but not limited to: body composition (Menezes et al., 2020), physiological

state (Bond et al., 1976), nitrogen balance (Meyer et al., 1955), metabolic water production (Tedeschi and Fox, 2017), and any water losses [e.g., respiration, NASEM (2016)].

A better understanding of how water is used by cattle in various regions around the world may help us to understand and adequately quantify the role of cattle production on water scarcity, climate change, and global warming. Furthermore, strategic research is needed to properly quantify water required and used by cattle. Conservative and oversimplified approaches have estimated that 20,864 L of water are required to produce 1 kg of meat (Robbins, 1998); or 20,559 L of water are required to produce 1 kg of boneless beef (Kreith and Davis, 1991); which if generalized, could potentially reflect an overestimation of water required for beef production (Beckett and Oltjen, 1993; Schlink et al., 2010). Understanding the patterns of WI at different production levels (e.g., BW maintenance, BW loss, and BW gain periods) may also leverage the development of decision support systems for better management of water resources while optimizing animal performance.

There is limited evidences of studies evaluating relationships between changes in energy requirements with WI in ruminants undergoing periods of substantial changes in BW and body composition. Therefore, this study aimed at characterizing the effects of changes in net energy requirements and body composition on factors that drive WI in cattle. We hypothesized that changes in net energy requirements, as periods of BW maintenance, BW losses, and compensatory growth, disproportionately affect WI requirements in cattle, more specifically of breeding bulls, thus bringing attention to necessary changes of current nutritional models.

2.3. Material and Methods

The animals used in this experiment were cared for according to guidelines approved by the Institutional Animal Care and Use Committee University of Nevada, Reno (protocol #00738).

Animal, treatment, and experimental area

The dataset was obtained from thirteen Angus × Hereford crossbred breeding bulls [29.6 ± 3.4 mo.; shrunk body weight (SBW) 611 ± 36.1 kg] over a period of 198 d (30 d adaption + 168 d trial), approximately five times more than the minimum test duration suggested by Ahlberg et al. (2018) necessary for collection of accurate WI phenotypes. Animals were housed at the Main Station Research Feedlot Facility at the Nevada Agricultural Experiment Station in Reno, NV. Bulls had free access to water and trace mineral salt during the whole trial. Beardless wheat (*Triticum aestivum*) hay was delivered once daily at approximately 0930h.

Three dietary regimes were offered to the bulls throughout the experimental period. Bulls coming from the breeding herd pastures were fed targeting for: no BW changes for 12 d (Phase 1: BW maintenance adjustment preceding a BW loss); gradual BW maintenance adjustment for 66 d (Phase 2: BW loss targeted for 0.6 kg/d); no BW changes for 12 d post BW loss period (Phase 3: BW maintenance post BW loss); and lastly, a recovery period with BW gain targeted for 1 kg/d following the BW loss period during the additional 78 d (Phase 4: compensatory growth). In order to induce a metabolic stress and a full recovery to initial state, each animal acted as its own control. The feeding protocol requisites were set as follows: animals should undergo the same stressors at the same time (e.g., nutritional and environmental),

animals should be at similar conditions in the beginning and at end of the trial (e.g., similar BW), no food restriction (e.g., food deprivation for longer than 24 hours), no more than 30% of overall BW should be lost, and no more than 20% of BW should be lost over an acute time period. The BW loss mimics an assumed scenario for a breeding season in the rangelands of the Western U.S.A. (e.g., roughly 162 kg of BW loss for a 725 kg breeding Bull in Nevada); variables of BW, WI, and feed intake were monitored daily to guarantee assurance of the protocol.

The animals were randomly assigned to one of two pens (15 × 28 m). Each pen contained 30 m² of shaded area and four automated water monitoring systems (WMS; Model WD-1000 Master, Intergado Ltd, Contagem, Minas Gerais, Brazil). Prior to the beginning of the trial, each animal was fitted with an electronic identification ear tag (FDX-ISO 11784/11785; Allflex, Joinville, Santa Catarina, Brazil).

Water Monitoring System

The hardware used for the WMS has been previously described and evaluated for monitoring WI and drinking behavior (Oliveira et al., 2018). In brief, the WMS is composed of a series of load cells for weighing the water (± 0.050 kg), as well as the BW of the animals while drinking (± 0.500 kg). For each voluntary visit to the waterer, the system records the animal identification, time, water consumed, and initial and final BW of the animal. The water troughs (0.30 x 0.37 x 0.20 m) were programmed to maintain the water temperature at 25 °C and were automatically filled (115 L) following each drinking event. Due to the dimensions of the scale/waterer, only one bull was allowed at a time into one individual water trough. These data were continuously recorded, transferred, and stored in the cloud for further analysis.

Waterers were manually cleaned once a week to ensure sanitation. In addition, the WMS scales were calibrated once weekly (using appropriate block calibration weights provided by the manufacturer) to ensure data accuracy.

Environmental variables

Environmental variables, including ambient temperature (Temp; °C), relative humidity (RH; %), solar radiation (SR; W/m²), and wind speed (WS; mph), were recorded daily throughout the entire trial (October through March) at one min intervals using a HOBO data logger (HOBO H8 Pro Series, Onset Computer Corp, Bourne, MA). The climate of the area is classified as BSk (cold-semi arid climate) according to Köppen-Geiger (Kottek et al., 2006), and is located at 39° 32' 38.87" N and 119° 48' 57.76" W. The annual average temperature, rainfall, snowfall, and frost-free period are 10.1 °C, 18.6 mm, 228.6 mm, and 8.25 months, respectively.

Feed chemical analysis

Representative feed samples were taken every 15 days during each dietary period. Feeds were oven dried at 55 °C for 72 h, then ground to pass through a 2-mm screen using a Wiley mill #4 (Thomas Scientific, Swedesboro, NJ). A 50-g subsample of each composite was shipped to Cumberland Valley Analytical Services (CVAS, Waynesboro, PA) for chemical analysis of dry matter (DM; method 930.15; AOAC (2000)), neutral detergent fiber with the addition of amylase and sodium sulfite and exclusive of ash (aNDFom; Van Soest et al. (1991)), acid detergent fiber exclusive of ash (ADF; Method # 973.18; AOAC (2000)), crude protein (CP; Method # 990.03; AOAC (2000)) using a Leco FP-528 Nitrogen Combustion Analyzer (Leco Corporation, St. Joseph, MO), soluble CP (Krishnamoorthy et al., 1982), a complete

mineral panel (Method # 985.01; AOAC (2000); using a Perkin Elmer 5300 DV ICP, Perkin Elmer, Shelton, CT), ether extract (EE; Method # 2003.05; AOAC (2000)), and non-fibrous carbohydrates (NFC) as shown in Supplementary Table 2. Sugar was analyzed according to Dubois et al. (1956) and starch according to Hall (2009). The Large Ruminant Nutrition System (LRNS; <http://www.nutritionmodels.com/lrns.html>; Tedeschi and Fox (2017)) was used to calculate the dietary concentration of metabolizable energy ([ME]), dietary concentration of net energy for maintenance ([NE_m]) and dietary concentration of net energy for gain ([NE_g]).

Water chemical analysis

Representative water samples were taken every 15 days during each dietary period. A 50-mL subsample of water was shipped to CVAS for chemical analysis of pH, hardness, total dissolved solids, calcium, phosphorus, sodium, magnesium, potassium, sulfate, manganese, copper, iron, zinc, nitrate as nitrogen, and nitrate were conducted according to Rice et al. (2017).

Animal data assessment

Data on ribeye area (RA, cm²) and back-fat thickness (BFT, mm) over the *Longissimus dorsi* (between the 12th and the 13th ribs) were recorded with an ultrasound (Aloka SSD 500; 3.5 MHz linear probe; Aloka Co. Ltd., Wallingford, CT). Images were analyzed using Image J® software (National Institutes of Health, Bethesda, MD, USA). The body condition score (BCS) was measured using the NU Beef BCS App (University of Nebraska-Lincoln, Nebraska, USA) utilizing a scale ranging from 1 to 9, as recommended by NASEM (2016). The ultrasound and BCS measurements were performed on d 0, 45, 90, 135, and 168 of the trial. The average

daily gain (ADG, kg/d) was measured daily by the WMS. To estimate the RA, BFT, and BCS daily changes, a first order model was fitted for each animal, regressing the d and the variables measured throughout the experimental periods.

Dry matter intake (DMI, kg/d) for each animal was estimated based on the data-integrated model using ADG measured by the WMS, and applied to the equation proposed by Minson and McDonald (1987), and suggested by Gonzalez et al. (2018). The metabolizable energy intake (MEI, Mcal/d) was calculated by multiplying the DMI by the digestible energy (DE, Mcal/kg) of the Beardless wheat (2.12 Mcal/kg; Supplementary Table 2), and then by 0.82. The 0.82 represents the conversion of DE to ME as proposed by NASEM (2016).

The metabolic BW (MBW, kg^{0.75}) was calculated as shrunk body weight (SBW) elevated to the 0.75 power, where SBW is considered a fixed factor (0.96) of BW.

The daily net energy requirement for maintenance (NE_m) was calculated as 0.077 Mcal/kg^{0.75}/d with a 15% increase for breeding bulls NASEM (2016).

The daily net energy requirement for gain (NE_g, Mcal/d) was calculated as $0.0635 \times \text{EQEBW}^{0.75} \times \text{EBW}^{1.097}$ according to NASEM (2016); where EQEBW is the equivalent empty BW in kg (calculated as $0.891 \times \text{equivalent SBW}$; EQSBW); EBW is empty BW in kg (calculated as $0.956 \times \text{ADG}$). To estimate EQSBW [$\text{SBW/d} \times (\text{SRW} \div \text{mature SBW})$], we used 800 kg and 900 kg, respectively, as the standard reference weight (SRW) and the mature BW for breeding bulls according to NASEM (2016).

The daily total net energy requirement (NE_t, Mcal/d) was calculated using the factorial approach by summing NE_m and NE_g (NASEM, 2016).

Body composition, empty body fat (EBF, kg), empty body protein (EBP, kg), and retained energy (RE, Mcal) contents were estimated according to Tedeschi (2019).

Physiological traits assessment

Blood samples, heart rate (HR, beats/min), and urine specific gravity (USG) were collected at 0700 h for d 0, 45, 90, 135, and 168 d. Upon 15 min of acclimatization to the squeeze chute, the HR was determined using a stethoscope (3M Littmann Master Classic; 3M Health Care, St Paul, Minnesota) by means of counting the number of HR in 15 s and then multiplying by four to convert to beats/min. Blood was collected via jugular venipuncture using vacutainer luer-lok access device (20-gauge × 1 in.; Airtite Product Co. Inc., Virginia Beach, VA), and a 10-mL heparinized plastic blood collection tube (BD Vacutainer, BD, Franklin Lakes, NJ). The heparinized tube was shipped to IDEXX laboratory (IDEXX Laboratories Inc., Westbrook, ME) for biochemistry analysis of packed cell volume (PCV, %). The USG was measured in duplicate using an automatic temperature-calibrated optical refractometer (MASTER-SUR/N α , Atago Co Ltd, Bellevue, WA 98005 U.S.A) that had a measurement range of 1.000 to 1.060 in increments of 0.001. All measurements were made in triplicate for each animal and the mean of the 3 measurements was used as the measured value.

Nitrogen metabolism

Urinary nitrogen (UN, g/d), fecal nitrogen (FN, g/d), ruminal N balance (RNB, g/d), recycled NH_3 (RNH_3 , g N/d), and microbial N synthesis (MNS, g/d) were predicted using the LRNS (Tedeschi and Fox 2017). The equation used for UN was: $\text{UN} = \text{BEN} + \text{BNA} + \text{NEU} + \text{TN}$; where BEN (g/d) is excess bacterial nitrogen, BNA (g/d) is bacterial nucleic acids, NEU (g/d) is the metabolizable nitrogen supply subtracting the net nitrogen (N) use (i.e., the inefficiency of use), and TN (g/d) is degraded tissue N. The equation used for FN was: $\text{FN} = \text{FFN} + \text{BFN} + \text{MFN}$; where FFN (g/d) is fecal N from the indigestible feed, BFN (g/d) is bacterial fecal N, and MFN (g/d) is metabolic fecal N. The nitrogen retention in the body (NR, g/d) was estimated as the difference between the N intake (NI, g/d) and urinary and fecal N losses. The RNB was estimated as following: $\text{RNB} = \text{NI} + \text{RNH}_3 - \text{TRRN}$; where TRRN is total rumen microbial N required. The equation used for RNH_3 was: $\text{RNH}_3 = [(121.7 - 12.01 \times \text{CP} + 0.3235 \div \text{CP}^2) \div 100] \times (\text{TCP} \div 6.25)$; where CP (%) is percentage of crude protein in the diet and TCP (g/d) is the total intake of CP. The MNS was estimated as follows: $\text{MNS} = \text{FCBACT} + \text{NFCBACT}$; where FCBACT (g/d) is the fiber carbohydrate bacterial N and NFCBACT (g/d) is the non-fibrous carbohydrate bacterial N.

To minimize the interference due to differences in the intake and size of the animals, the variables associated with the efficiency of nitrogen utilization were expressed as ratios following recommendations of Detmann et al. (2014). The efficiency of N retention (NRRNI, g/g) was estimated as: $\text{NRRNI} = \text{NR} \div \text{NI}$. The efficiency of RNB (RNBRNI, g/g) was estimated as: $\text{RNBRNI} = \text{RNB} \div \text{NI}$. The efficiency of microbial N synthesis (MNSRNI, g/g) was estimated as: $\text{MNSRNI} =$

MNS \div NI. Lastly, for the efficiency of microbial protein synthesis (EMS, g microbial crude protein/g TDN) was estimated as: EMS = (MNS \times 6.25) \div TDN.

Statistical analyses

Data was collected and analysed following a pre-post repeated measure design (Burgos et al., 2001). The statistical model used is shown below:

$$Y_{ij} = \mu + T_i + b_j + \epsilon_{ij}$$

Where Y_{ij} is the observation taken on the j^{th} experimental unit for the pre-post i^{th} treatment, μ is the overall mean, T_i is the effect of the i^{th} treatment, b_j is the effect of the j^{th} experimental unit, ϵ_{ij} is the unobservable random error on the j^{th} experimental unit associated with each i^{th} pre-post treatment.

The data were analyzed in three comparison steps: first, Phase 1 against Phase 2 (BW maintenance x BW loss), then Phase 3 against Phase 4 (BW maintenance x compensatory growth), and lastly, Phase 2 against Phase 4 (BW loss x compensatory growth). Because the non-equidistant time points during the BW gain phase (compensatory growth) could represent potential nonlinear patterns, the ante-dependence covariance structure was used (Kenward, 1987; Patel, 1991). Mean values were calculated when more than one value was obtained for a given parameter during the data collection period (e.g., WI and BW). Statistical analyses were performed using SAS (ver. 9.4, SAS Inst. Inc., Cary, NC) via the paired t-test using PROC TTEST. Descriptive statistics were obtained with PROC MEANS and Pearson correlation coefficients among variables were obtained with PROC CORR. Linear regressions were obtained with PROC REG. Statistical differences among slopes were

considered indicative of water requirement changes on any physiological conditions (maintenance, BW loss, and BW gain phases). Principal Component (PC) analysis (PCA) was performed with PROC PRINCOMP computed from the square symmetric correlation matrix. Correlation coefficients between the most prolific components, first (PC1) and second (PC2) principal components, were computed with each parameter studied. The parameters were: WI, BW, ADG, DMI, DMI per unit of BW (DMIperBW, g/kg), MBW, WI per unit of MBW (WIperMBW, L/kg^{0.75}), NEm, NEg, NEt, BCS, BFT, RA, Temp, RH, SR, and WS. Categorical behavioral WI data (percentage of waterer visits with successful drinking events (WVWDED)) were analyzed with a chi-square test using PROC FREQ. To investigate the association of the temporal changes of physiological traits (HR, USG, and PCV) with WI, these parameters were graphically analyzed plotting their standardized and mean values. Standardized values were obtained with PROC STANDARD. The PCA Biplot was plotted using R Studio (RStudio, 2020) performed by ggplot2 package. All other figures were generated by GraphPad Prism (ver. 9.0, GraphPad Inc., San Diego, CA). Outliers were tested by plotting the Studentized residuals and data points were removed if the Studentized residual was outside the range of -2.5 to 2.5. Normality assumption was tested using Shapiro-Wilk's test and homogeneity of variance was evaluated through Levene's test. Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$.

2.4. Results

Water and diet composition

The composition of water (Supplementary Table 2-1), mineral salt and Beardless wheat hay (Supplementary Table 2-2) are presented in the supplementary data.

Descriptive statistics and PCA

Descriptive statistics for environmental variables, animal performance, animal requirements, and body composition traits are shown in the supplementary data: Table 2-3, Table 2-4, and Table 2-5. The PC1 and PC2 explained 67.3% of the total variance from the environmental variables, animal performance, animal requirements, and body composition parameters on phases of maintenance (Phase 1 and Phase 3) (Supplementary Fig. 2-1). For Phase 2 (BW loss) and Phase 4 (compensatory growth), PC1 and PC2 explained 64.8% of the total variance from the environmental variables, animal performance, animal requirements, and body composition parameters (Supplementary Fig. 2-2). Those variances explained by PC1 and PC2 were considered sufficient to capture most of the variation through all phases of this study (Johnson and Wichern, 2002).

Both PC1 and PC2 presented lower discriminatory power among WI, animal performance, animal requirements, and environmental variables during phases of maintenance (Phase 1 and Phase 3; Table 2-1 and Table 2-2). Linear correlation coefficients revealed that PC1 was strongly associated with WI (0.85), WIperMBW (0.72), animal requirements (BW: 0.83, MBW: 0.83, and NEM: 0.83), body composition (BCS: 0.67, BFT: 0.85, and RA: 0.84), and environmental variables (Temp: 0.66, RH: -0.69, and SR: 0.71). Whereas PC2 was only strongly associated with animal performance (ADG: -0.94, DMI: -0.76, and DMIperBW: -0.90), and

animal requirements (NEg: -0.89 and NEt: -0.70). During Phase 2 and Phase 4, the two PC had a high discriminatory power among WI, animal requirements, body composition, and environmental variables. Water intake (0.77), WIperMBW (0.66), ADG (0.77), DMI (0.82), NEg (0.79), and NEt (0.83) were strongly and positively correlated with PC1, whereas DMIperBW (-0.72), BCS (0.66), and RA (0.66) were strongly correlated with PC2. Environmental variables were weakly correlated with both PC as the absolute values of the coefficient of linear correlation ranged from 0.01 to 0.33.

Animal performance, animal requirements, and body composition

Performance data is presented in Table 2-3, Table 2-4, and Table 2-5. During Phase 2, bulls decreased ($P < 0.001$) BW, MBW, ADG, DMI, DMIperBW, WIperMBW, NEm, NEg, NEt, BCS, BFT, and RA compared to Phase 1. Bulls undergoing Phase 4 increased ($P \leq 0.001$) BW, MBW, ADG, DMI, DMIperBW, WIperMBW, NEm, NEg, NEt, and RA; also, there was an increase on body fat depots (BFT, $P = 0.001$; BCS, $P = 0.055$) compared to Phase 3. There were no differences ($P \geq 0.109$) in BW, MBW, NEm, BCS, and BFT between Phase 2 and Phase 4. Moreover, we observed an increase ($P < 0.001$) in ADG, DMI, DMIperBW, WIperMBW, NEg, and NEt, as well as a tendency for increased overall muscle depots in the carcass upon full BW recovery (RA, $P = 0.069$).

Water requirements

Linear regression models were used to evaluate associations of BW, ADG, DMI, DMIperBW, MBW, WIperMBW, NEm, NEg, NEt, BCS, BFT, and RA with WI under the different physiological conditions (Table 2-6, Table 2-7, and Table 2-8).

Slope differences between phases indicate changes in WI requirements under the different physiological conditions. When comparing the slopes between Phase 1 and Phase 2, a tendency ($P \leq 0.070$) of lower WI was observed followed by a reduction in BW, MBW, and NEM during Phase 2. A lower WI was noted by a reduction in NEt ($P < 0.001$). During Phase 4 a higher ($P \leq 0.034$) WI was noted with an increase in BW, MBW, and NEM compared to Phase 3. A tendency ($P = 0.084$) of lower WI was observed in Phase 4. When comparing Phase 2 and Phase 4, a higher ($P \leq 0.006$) WI was followed by an increase of BW, MBW, and NEM observed during Phase 4. During Phase 4, a lower WI was observed ($P \leq 0.013$) followed by an increase in DMIperBW, NEt, BFT, and RA. In addition, under positive ADG as observed in Phase 4, bulls tended ($P = 0.085$) to have lower WI compared to Phase 2.

The pattern over time of BW changes is presented in Supplementary Fig. 2-3 and Supplementary Fig. 2-4. We observed a strong relationship between WI requirements and BW changes during all phases of this study (Fig. 2-1: $r = -0.73$, Fig. 2-2: $r = 0.74$, Table 2-6, Table 2-7, and Table 2-8). Water intake relative to metabolizable energy for maintenance (MEM) is presented in Fig. 2-3. The WI relative to MEM requirements decreased from the initial day of Phase 1 until the end of Phase 2. On Phase 3 through Phase 4, the WI relative to MEM requirements increased.

The relationship between WI and RE seems to be of a nonlinear nature (Fig. 2-4). The lowest levels of RE in the EBW seem to coincide with the lowest WI observed during Phase 2 and Phase 3. Moreover, as animals lose energy in the EBW (Phase 2), the WI decreased, whereas during Phase 4, the opposite occurred. As the animals recovered their body energy status during, we observed an increase in WI per

kg of fat and kg of protein in the EBW (Fig. 2-5). During periods of negative body protein turnover (Phase 2), WI also decreased. Conversely, WI increased when bulls were in a positive body protein turnover (Phase 4). When animals are losing weight, a WI seems to follow the adjustment pattern observed for NEM, whereas during Phase 4, WI appears to be more closely related to the surplus of energy above maintenance (Supplementary Fig. 2-5).

Water intake and drinking behavior

Drinking behavior and WI are presented in Table 2-9. Bulls undergoing Phase 2 decreased ($P < 0.001$) their WI by 36.6% compared to Phase 1. Moreover, a decrease ($P \leq 0.001$) of 28.3%, 28.6%, 3.5%, and 23.5% was observed respectively in time spent drinking water (TSDW), total waterer visited per day (TWVD), percentage of waterer visits with successful drinking event (WVWDED), and number of different equipment visited per day (NDEVD). Lastly, a tendency of 9.5% slower drinking rate (DR, $P = 0.088$) was found during Phase 2.

During Phase 4 bulls increased their WI, TSDW, DR, TWVD, WVWDED, and NDEVD ($P \leq 0.028$) by 216.8%, 28.3%, 40.5%, 107.1%, 4.8%, and 41.7%, respectively, compared to Phase 3. Lastly, when comparing Phase 4 to Phase 2, bulls undergoing Phase 4 increased ($P \leq 0.005$) their WI, TSDW, DR, TWVD, WVWDED, and NDEVD by 99.1%, 86.0%, 36.8%, 93.3%, 3.0%, and 30.8%, respectively.

Physiological traits and water intake

When animals experience BW drastic changes, PCV and USG have a negative correlation with WI whereas HR has a positive correlation (Fig. 2-6).

Nitrogen metabolism

Traits of nitrogen metabolism are presented in Table 2-10 and Table 2-11.

Through Phase 2 bulls presented a tendency ($P \leq 0.090$) to decrease their NI, FN, UN, RNH_3 , and MNS, respectively by 18.7%, 19.6%, 18.6%, 20%, and 19.0% compared to Phase 1. Still during Phase 2, bulls tended to increase ($P \leq 0.081$) their NR, RNB, and RNBRNI by 20.8%, 16.3%, and 2.5%, respectively. An increase ($P = 0.012$) in NRRNI of 2.5% was observed. There were no differences in MNSRNI ($P = 0.188$) and EMS ($P = 0.184$).

During Phase 4, bulls increased ($P < 0.001$) their NI, FN, UN, NRRNI, RNBRNI, RNH_3 , and MNS by 73.4%, 66.5%, 58.1%, 5.0%, 5.0%, 61.6%, and 60.7%, respectively compared to the previous phase, but decreased ($P < 0.001$) their NR, RNB, and MNSRNI by 55.2%, 53.0%, and 1.0%, respectively. There were no statistically significant differences observed for EMS ($P = 0.215$).

Lastly, during Phase 4 bulls increased ($P < 0.001$) their NI, FN, UN, NRRNI, RNBRNI, RNH_3 , and MNS by 118.3%, 118.5%, 112.8%, 2.6%, 9.7%, 114.6%, and 113.6%, respectively compared with Phase 2; while decreasing ($P < 0.001$) their NR, RNB, MNSRNI, and EMS by 110.8%, 99.2%, 1.9%, and 4.4%, respectively.

We observed a strong negative correlation between WI and RNB considering all evaluated phases of this study (Fig. 2-7: $r = -0.83$, $P < 0.001$).

2.5. Discussion

PCA

During periods of nutritional and metabolic stress, a low ($r \leq |0.336|$) concordance between both PC and environmental factors was observed. Such concordance in both PC suggests that environmental variables (e.g.: temperature, relative humidity, solar radiation, and wind speed) may not be the primary factors influencing daily WI for animals that are losing BW or are in periods of compensatory growth. On the other hand, during phases of BW maintenance (Phase 1 and Phase 3), at least one PC presented a high concordance ($r \geq |0.664|$) related to environmental variables. These results suggest that environmental variables may not be the primary factors influencing daily WI for animals that are losing BW or are in periods of compensatory growth. Arias and Mader (2011), using data across multiple years and seasons, observed similar results in cattle that had been previously nutrient-restricted and later introduced to a high-energy diet (1.43 Mcal/kg of NEg). The authors found small coefficients of determination ($r^2 < 0.25$) between environmental variables and daily WI. Different to our study, the authors observed this effect during summer and winter. Since WI, animal BW changes (BW, MBW, and ADG), and energy requirements (NEm, NEg, and NEt) had medium ($r = |0.500|$) to high correlation ($r = |0.830|$) coefficients with the two PC, biological (e.g., BW) and physiological traits [e.g., negative energy balance (NEB)], appear to play a large role in explaining WI. Similar results have been reported by Sexson et al. (2012), which concluded that BW was strongly related to WI. Therefore, WI seems to be primarily driven by animal requirements and secondarily by environmental factors, particularly in animals undergoing nutritional and metabolic stress.

Performance

Animals intermittently losing BW when undergoing fluctuations of feed quantity or quality may suffer from an inadequate supply of nutrients to meet their nutritional needs. This loss of BW leads to a decreased energy requirements until a period of re-alimentation is introduced, and then homeostasis is achieved (Drouillard et al., 1991). Body weight changes can be intrinsically associated with changes in WI, with the linear relationship between BW and WI becoming stronger as the animal loses BW. In our data, we observed that animals at a high level of performance would require less water than animals that are losing BW. Before BW losses, during the maintenance period, animals in homeostasis would require a BW loss of 29.19 kg/day to attain a WI equal to zero (no need for water or water balance equals to zero), all else equal. For animals losing BW, water balance tends to be more unfavourable. An animal would need to lose an additional 130.2 kg of BW to reach a null WI, all else equal. As animals lose BW and enter a more intense catabolic state, it appears that more, not less, drinkable water is required. An unintended consequence for an animal grazing in extensive rangeland systems could be that, in search for water, there is an increase in physical activity and overall requirements would be observed, hence leading the animal to a reinforcing cycle of tissue mobilization and negative water balance, for the already nutrient-deprived grazing animal. Given that range grazing animals are often in NEB (Arias and Mader, 2011; Sexson et al., 2012; NASEM, 2016), they would need to forage and migrate more to meet their drinkable water requirements.

During the phase of BW loss, we observed a decrease in the linear relationship between ADG and WI, which makes ADG a less reliable linear predictor of WI for animals undergoing BW losses rather than during maintenance. Nonetheless, the rate

at which animals lose BW does not seem to disproportionately affect WI, regardless if animals are in maintenance or losing BW. Even though the linear relationship between ADG and WI fades, a decrease in ADG seems to promote a decrease in WI with zero intakes to be achieved at daily weight losses of 6.95 and 5.71 kg for maintenance and BW loss periods, respectively, all else equal.

The DMI appears to follow the same trend as previously described. At lower levels of intake and performance, as expected for animals losing or maintaining BW, the rate at which animals eat does not affect WI. Nonetheless, DMI would be able to explain a greater deal of daily variations on WI for animals at maintenance than it would have for animals during BW loss.

The MBW will affect WI differently during maintenance or BW loss periods. Even though MBW has a slightly stronger linear relationship with WI during periods of BW loss, there is evidence that the animal's requirements are playing different roles in these different phases. During periods of BW loss and maintenance, animals lose metabolically active tissue in order to adapt to energy restrictions (NASEM, 2016). When comparing animals at compensatory growth with animals losing BW, we observed that the latter would need to metabolize 2.6 times as much metabolically active tissue in order to reach a WI of zero, all else equal. During respective phases, the compensatory growth animals consumed 199 mL/kg MBW, and during periods of BW loss animals consumed 401 mL/kg MBW. It is apparent that body tissue turnover is a costly trade-off for cattle to maintain its hydration status. The implications for the rangelands production systems, such as those of Nevada, are that for animals losing metabolically active body mass, a more aggressive search for water would be needed in order to maintain the water balance within their body. Considering that there is a

decay pattern on the mechanism of tissue catabolism, the more weight an animal loses, the less time the animal would be able to rely on tissue turnover to maintain its water balance. This would mean a need for grazing closer to water sources to ensure that their water requirements are fulfilled. Physical activity would further change an animal's NEm, likely creating a negative vicious cycle, which would be fairly detrimental to the animal (NASEM, 2016). Whether the animal's decision is driven by physiological mechanisms or social interaction within its group, it seems that the outcomes would have been the same. An increase in 1 kg of MBW at maintenance would have a greater effect on the increase of WI than the same change during BW loss. Simulating a MBW daily gain of $0.84 \text{ kg}^{0.75}$ (equivalent to an ADG of 0.8 kg), the response in WI would have been 3.6 times larger for animals at maintenance compared to while losing BW.

For maintenance and BW loss periods, a slight change in NEt promoted a disproportionate influence on WI. The slope of the BW loss period was much larger than at maintenance (4.5 times larger). This means that during BW loss periods, it is expected that NEt requirements would play a more influential role in controlling WI than it would have for animals at maintenance. Theoretically, animals could use BW loss as a strategy to decrease their requirements (CSIRO, 2007; NASEM, 2016) when needed, or even using the year-round shifts in body composition as a result of different planes of nutrition (Ferrell et al., 1986; Ferrell and Koong, 1987) to achieve a desirable homeostatic outcome; though the latter would not be an optimal strategy.

For variables that indicate body composition pools (BCS, BFT, and RA), or for the influence of their possible turnover on WI, we observed that, as animals lost BW, these variables tended to strengthen their linear relationship with WI. Such behavior

indicates that a mobilization of fat during BW loss, would need to be proportionately higher (19.4% more) compared to mobilization at maintenance in controlling water balance. This might not be a suitable physiological strategy for the animal because that animal would need to mobilize 0.67 units of BCS while at maintenance to reach a null WI (water balance), all else equal. Conversely, the same animal would need to mobilize 0.8 BCS units if it were losing BW. Wild ruminants have been reported to be able to mobilize energy from fat while preserving preferential protein depots (Barboza et al., 2020) which seem to be in agreement to what we found in our work. It also seems that rate at which body proteins are changed are more important than the size of the pool that is changed (Barboza et al., 2009).

When animals were at their lowest body energy levels, or RE, most of the variables analysed presented a low r^2 . A potential mechanism for saving water as animals enter a decreased RE state could be associated with an increase in serum urea concentrations which are reflective of body mechanisms that attempt to minimize body protein losses. This is achievable by increasing the recycling of urea-N, hence, saving water. As animals begin improving their body energy levels and moving toward a positive energy balance, the r^2 increased dramatically. Moreover, the dynamicity of body tissues inherent to the compensatory growth phase is positively correlated with the increase in WI. That linear behavior is also extended to BW, MBW and NEm. During compensatory growth, a great deal of the variation in WI was driven by the increase in BW and pools of more metabolically active tissues. These findings support the idea that there is an important process happening at the gastrointestinal level shown by an increase in water demand to supply visceral tissues undergoing increased turnover.

Utilizing records from four finishing experiments involving beef cattle, various authors found that DMI had a minimal impact on WI (Arias and Mader, 2011). Early research by Bond et al. (1976), observed a 13% increase in WI by European beef steers when depriving cattle of feed. Teixeira et al. (2006) reported an increase of 336% in WI per kg of ingested food for kid goats that were food restricted at 60% of ad libitum intake. In our study, during BW loss, bulls consumed approximately 1.2 L of water per kg of DMI more than during the compensatory. Collectively, these findings suggest that DMI is not the primary driver of WI during BW loss or compensatory growth periods. Rather, WI may be driven by a shift in physiological or metabolic responses (e.g., metabolic water production or exacerbated water losses due to environmental stressors) during changes in body composition that are yet to be properly quantified. Because of this, the importance of those metabolic processes (e.g., metabolic water) could have been overlooked for cattle, especially for cattle adapted to semi-arid and arid environments.

Water requirements

In terms of the efficiency of water utilization, it appears that during periods of compensatory growth and BW losses the WI per unit of MBW are similar (slope of $|2.77|$). However, a more detailed analysis showed that the following events are rather dynamic. Bulls losing BW use water 40% more efficiently compared to periods of compensatory growth (changing in slopes of MBW: $1.87 \div 1.33$, and NEm: $19.41 \div 13.74$). The same pattern is observed for BW changes. For instance, a decrease in 1 kg of BW during BW loss, reduces the WI by 5.01 L whereas a decrease of 1 kg of BW during compensatory growth, increases WI by 0.16 L. These results indicate that animals undergoing BW loss use water more efficiently than during

compensatory growth. Animals under compensatory growth decreased their WI per unit of ingested food (g/kg BW). In corroboration with previous results, there is a clear different pattern of WI for animals losing BW compared to periods of compensatory growth.

Water consumption in cattle may be influenced by body composition (Menezes et al., 2020). In this study, the water required per unit of body protein or body fat appears to be nonlinear. Slopes from the univariate analyses of BFT and RA indicated that once the lean-to-fat ratio decreases, as observed in animals at BW loss, a decrease in WI would be observed, should compensatory growth be the baseline. In fasting animals, because of protein and fat catabolism, oxidation water can be formed and this very same metabolic water pool, may eventually affect WI.

Water intake and drinking behavior

We observed that the amount of water ingested by bulls, from the higher to the lower, was compensatory > maintenance > BW loss. These differences in WI may be associated with changes in body composition over time (Morris et al., 1962; Aganga et al., 1989). These studies reported that WI is linked to metabolic water production and may be correlated to body composition, given the extent of the turnover ratio of body protein and fat depots. Due to its molecular structure and biochemical composition, protein molecules have a high affinity to water molecules (Listrat et al., 2016) and therefore requires adequate water to properly function. Furthermore, the WI requirements for animals in a catabolic state (e.g., BW loss) may be lower than animals at maintenance or in an anabolic state. If we consider the standard values of metabolic water produced per g of oxidized body protein (0.40) and body fat (1.07) as

proposed by Brody (1974), preferentially losing fat depots may be more advantageous than protein depots because not only is there a higher metabolic water production associated to it, but there is also an additional pool of water saved during the oxidative metabolism of N (Barboza et al., 2009). The greatest metabolic water production during the fasting period may be attributed to low WI requirements (Schmidt-Nielsen, 1965).

Differences observed in WI were reflected in the drinking behavior. One main factor that may influence TSDW, DR, TWVD, WWDED, and NDEVD among maintenance, BW loss, and compensatory growth periods is the higher number of drinking events readily available when feed is ad libitum (Forbes et al., 1991; Rossi et al., 1999). Furthermore, the lower TSDW, DR, TWVD, WWDED, and NDEVD during maintenance and BW loss compared to the compensatory growth periods, may also be associated with the greater pool of water present in the rumen as well as the massive saliva secretion during feeding events (Bailey, 1961). Thus, saliva secretion during fasting periods can easily buffer osmotic challenges of ingested food (Rossi et al., 1999), which results in a less potent stimulus for drinking.

Physiological traits and water intake

Our results suggest that during BW loss bulls increase PCV and USG, while simultaneously decreasing WI. This may be a physiological adaptation to reduce the need to excrete water to remove nitrogenous waste from protein turnover during periods of BW loss; whereas, during compensatory growth, the opposite occurred, indicating that animals may use redistribution of water through the more demanding sites as some nutrients become unavailable to them throughout the year. In addition,

our data suggest that HR changes are a reflex effect of energy expenditure changes during nutritional downturns (periods of BW maintenance and losses) and upturns (compensatory growth).

Nitrogen metabolism

Under poor nutritional regimes, it has been reported that a high RNB is needed to support microbial growth in the rumen (NRC, 1985; Detmann et al., 2014; Batista et al., 2017). Thus, because animals were fed with a medium-quality forage (NASEM, 2016), a RNB deficiency was observed throughout the entire trial with the worst period being the compensatory period. Most likely, this lower RNB is a product of a diminished N recycling, which is corroborated by a much higher RNH_3 and doubled MNS observed for animals under compensatory growth. Furthermore, we observed that RNB had a strong negative association with WI. Collectively, these findings suggest that RNB is negatively associated with mechanisms that preserve water, a mechanism much needed at periods and places when and where environmental and nutritional limitations are a year-round reality. These findings may also indicate that nutrient synchronization could affect WI requirements in cattle, and future research is needed to optimize WI with nutrient synchronization for improved performance.

2.6. Conclusion

For animals undergoing BW loss and compensatory growth, WI is primarily driven by animal requirements and secondarily by environmental variables, particularly in animals undergoing nutritional and metabolic stress. Because of this, caution should be taken to avoid oversimplification of water requirements using the factorial approach. Ultimately, the knowledge of water requirements and factors

influencing its regulation (e.g., body composition dynamics, WI behavior, etc.) can lead us to the development of more comprehensive mechanistic models that would improve our decision making on how to effectively manage our natural resources, improve grazing distribution in extensive production systems, optimize water mitigation strategies, and provide predictive scenarios to properly evaluate how to sustainably produce a wholesome meat product. Though still incipient, a thorough understanding and quantification of water requirements for beef cattle, to the same degree as other nutrients, would greatly benefit the cattle industry in addressing sustainable use of our natural resources and proactively address changes needed in the future to come.

2.7. Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.8. Author contributions

Conceptualization, M. A. F.; methodology, M. A. F. and F. H. M.; facilities and formal experiment, F. H. M., A. M. F., G. M. M., I. M. B., and A. E. M. S.; investigation, M.A.F. and F. H. M.; resources, M.A.F.; data curation, F. H. M.; writing original draft preparation, F. H. M.; writing review and editing, M. A. F., F. H. M., and A. B. N.; supervision, M. A. F. and A. B. N.; project administration, M. A. F. All authors have read and agreed to the published version of the manuscript.

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2.11. Tables

Table 2-1: Correlation coefficients between principal components and animal performance, energy requirements, body composition, and environmental variables affecting crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Principal Component (PC) | |
|---------------------------------|---------------------------------------------|---------------------------------------------|
| | First PC | |
| | Phase 1 ² & Phase 3 ² | Phase 2 ² & Phase 4 ² |
| WI, L/d | 0.853 | 0.773 |
| BW, kg | 0.831 | 0.620 |
| ADG, kg/d | 0.223 | 0.772 |
| DMI, kg/d | 0.595 | 0.824 |
| DMIperBW, g/kg | -0.179 | 0.635 |
| MBW, kg ^{0.75} | 0.830 | 0.620 |
| WIperMBW, mL/kg ^{0.75} | 0.722 | 0.665 |
| NEm, Mcal/d | 0.830 | 0.620 |
| NEg, Mcal/d | 0.311 | 0.791 |
| NEt, Mcal/d | 0.619 | 0.830 |
| BCS, 1 - 9 | 0.672 | 0.333 |
| BFT, mm | 0.852 | 0.112 |
| RA, cm ² | 0.836 | 0.552 |
| Temp, °C | 0.664 | -0.011 |
| RH, % | -0.688 | -0.183 |
| SR, W/m ² | 0.706 | 0.282 |
| WS, mph | 0.086 | 0.219 |

¹WI = water intake, BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WIperMBW = WI per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area, Temp = ambient temperature, RH = relative humidity, SR = solar radiation, WS = wind speed

²Phase 1 = BW maintenance, Phase 2 = BW loss, Phase 3 = BW maintenance after BW loss, Phase 4 = compensatory growth

Table 2-2: Correlation coefficients between principal components and animal performance, energy requirements, body composition, and environmental variables affecting crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Principal Component (PC) | |
|-------------------------------|---------------------------------------------|---------------------------------------------|
| | Second PC | |
| | Phase 1 ² & Phase 3 ² | Phase 2 ² & Phase 4 ² |
| WI, L/d | 0.038 | 0.273 |
| BW, kg | 0.142 | 0.595 |
| ADG, kg/d | -0.938 | -0.568 |
| DMI, kg/d | -0.758 | -0.540 |
| DMIperBW, g/kg | 0.903 | -0.719 |
| MBW, kg ^{0.75} | 0.139 | 0.591 |
| WIpMBW, mL/kg ^{0.75} | 0.004 | 0.104 |
| NEm, Mcal/d | 0.139 | 0.591 |
| NEg, Mcal/d | -0.887 | -0.559 |
| NEt, Mcal/d | -0.698 | -0.494 |
| BCS, 1 - 9 | 0.227 | 0.661 |
| BFT, mm | 0.237 | 0.568 |
| RA, cm ² | 0.208 | 0.665 |
| Temp, °C | 0.268 | 0.336 |
| RH, % | -0.118 | -0.306 |
| SR, W/m ² | 0.017 | 0.253 |
| WS, mph | 0.277 | -0.091 |

¹WI = water intake, BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WIpMBW = WI per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area, Temp = ambient temperature, RH = relative humidity, SR = solar radiation, WS = wind speed

²Phase 1 = BW maintenance, Phase 2 = BW loss, Phase 3 = BW maintenance after BW loss, Phase 4 = compensatory growth

Table 2-3: Performance, energy requirements, and body composition of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Treatment ² | | | | Mean Paired Difference | SEM ³ ± | P-value |
|---------------------------------|------------------------|--------------------|-------|--------------------|------------------------|-----------------------|---------|
| | Mean | SEM ³ ± | Mean | SEM ³ ± | | | |
| | Phase 1 vs. Phase 2 | | | | | | |
| BW, kg | 676.1 | 38.03 | 635.2 | 36.92 | 40.9 | 2.70 | <0.001 |
| ADG, kg/d | -0.005 | 0.010 | -2.3 | 0.18 | 2.3 | 0.18 | <0.001 |
| DMI, kg/d | 9.2 | 0.23 | 6.2 | 0.35 | 3.0 | 0.23 | <0.001 |
| DMIperBW, g/kg | 14 | 0.4 | 10 | 0.4 | 4 | 0.4 | <0.001 |
| MBW, kg ^{0.75} | 128.5 | 4.22 | 122.6 | 4.10 | 5.9 | 0.33 | <0.001 |
| WlperMBW, mL/kg ^{0.75} | 302 | 17.8 | 199 | 9.2 | 102 | 10.7 | <0.001 |
| NEm, Mcal/d | 11.35 | 0.478 | 10.82 | 0.471 | 0.53 | 0.032 | <0.001 |
| NEg, Mcal/d | 2.59 | 0.187 | 0.72 | 0.350 | 1.86 | 0.328 | <0.001 |
| NEt, Mcal/d | 13.93 | 0.653 | 11.54 | 0.716 | 2.39 | 0.353 | <0.001 |
| BCS, 1 - 9 | 5.68 | 0.194 | 5.12 | 0.160 | 0.56 | 0.078 | <0.001 |
| BFT, mm | 4.95 | 0.287 | 3.67 | 0.157 | 1.28 | 0.162 | <0.001 |
| RA, cm ² | 82.11 | 5.676 | 67.83 | 4.836 | 14.28 | 1.512 | <0.001 |

¹BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WlperMBW = water intake per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area

²Phase 1 = BW maintenance, Phase 2 = BW loss

³SEM = standard error of the mean

Table 2-4: Performance, energy requirements, and body composition of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Treatment ² | | | | Mean Paired Difference | SEM ³ ± | P-value |
|---------------------------------|------------------------|--------------------|-------|--------------------|------------------------|--------------------|---------|
| | Mean | SEM ³ ± | Mean | SEM ³ ± | | | |
| | Phase 3 vs. Phase 4 | | | | | | |
| BW, kg | 550.7 | 32.99 | 630.1 | 38.85 | -79.4 | 9.14 | <0.001 |
| ADG, kg/d | -0.004 | 0.010 | 3.6 | 0.41 | -3.6 | 0.41 | <0.001 |
| DMI, kg/d | 8.3 | 0.30 | 18.5 | 1.64 | -10.2 | 1.46 | <0.001 |
| DMIperBW, g/kg | 15 | 0.3 | 30 | 1.8 | -14 | 1.9 | <0.001 |
| MBW, kg ^{0.75} | 110.1 | 3.66 | 121.9 | 4.31 | -11.8 | 1.16 | <0.001 |
| WlperMBW, mL/kg ^{0.75} | 142 | 7.4 | 401 | 16.4 | -259 | 14.7 | <0.001 |
| NEm, Mcal/d | 9.72 | 0.437 | 10.75 | 0.498 | -1.03 | 0.112 | <0.001 |
| NEg, Mcal/d | 2.17 | 0.141 | 27.00 | 3.727 | -24.83 | 3.657 | <0.001 |
| NEt, Mcal/d | 11.89 | 0.566 | 37.75 | 4.126 | -25.86 | 3.726 | <0.001 |
| BCS, 1 - 9 | 4.66 | 0.166 | 4.86 | 0.227 | -0.20 | 0.096 | 0.055 |
| BFT, mm | 2.84 | 0.130 | 3.74 | 0.291 | -0.90 | 0.200 | 0.001 |
| RA, cm ² | 59.43 | 4.443 | 72.78 | 5.283 | -13.35 | 1.845 | <0.001 |

¹BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WlperMBW = water intake per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area

²Phase 3 = BW maintenance after BW loss, Phase 4 = compensatory growth

³SEM = standard error of the mean

Table 2-5: Performance, energy requirements, and body composition of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Treatment ² | | | | Mean Paired Difference | SEM ³ ± | P-value |
|---------------------------------|------------------------|-----------------------|-------|-----------------------|------------------------|-----------------------|---------|
| | Mean | SEM ³ ± | Mean | SEM ³ ± | | | |
| | Phase 2 vs. Phase 4 | | | | | | |
| BW, kg | 635.2 | 36.92 | 630.1 | 38.85 | 5.1 | 7.66 | 0.521 |
| ADG, kg/d | -2.3 | 0.18 | 3.6 | 0.41 | -5.9 | 0.47 | <0.001 |
| DMI, kg/d | 6.2 | 0.35 | 18.5 | 1.64 | -12.3 | 1.52 | <0.001 |
| DMIperBW, g/kg | 10 | 0.4 | 30 | 1.8 | -20 | 1.9 | <0.001 |
| MBW, kg ^{0.75} | 122.6 | 4.10 | 121.9 | 4.31 | 0.7 | 1.03 | 0.531 |
| WlperMBW, mL/kg ^{0.75} | 199 | 9.2 | 401 | 16.4 | -202 | 12.0 | <0.001 |
| NEm, Mcal/d | 10.82 | 0.471 | 10.75 | 0.498 | 0.07 | 0.099 | 0.507 |
| NEg, Mcal/d | 0.72 | 0.350 | 27.00 | 3.727 | -26.28 | 3.669 | <0.001 |
| NEt, Mcal/d | 11.54 | 0.716 | 37.75 | 4.126 | -26.21 | 3.704 | <0.001 |
| BCS, 1 - 9 | 5.12 | 0.160 | 4.86 | 0.227 | 0.26 | 0.148 | 0.109 |
| BFT, mm | 3.67 | 0.157 | 3.74 | 0.291 | -0.07 | 0.269 | 0.786 |
| RA, cm ² | 67.83 | 4.836 | 72.78 | 5.283 | -4.95 | 2.478 | 0.069 |

¹BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WlperMBW = water intake per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area

²Phase 2 = BW loss, Phase 4 = compensatory growth

³SEM = standard error of the mean

Table 2-6: Univariate regression analysis of each variable used for estimating water intake requirements (L/d) of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Phase ² | Intercept | | Slope | | r ² | P-value | | |
|--------------------------|--------------------|-----------|-------------------|-------|-------------------|----------------|-----------|--------|--------------------------|
| | | alpha | SE ³ ± | beta | SE ³ ± | | Intercept | Slope | Interaction ⁴ |
| BW, kg | 1 | -355.8 | 134.78 | 0.55 | 0.199 | 0.510 | 0.016 | 0.009 | 0.069 |
| | 2 | -90.9 | 12.08 | 0.18 | 0.019 | 0.623 | <0.001 | <0.001 | |
| ADG, kg/d | 1 | 37.2 | 1.34 | 5.35 | 1.346 | 0.612 | <0.001 | 0.003 | 0.893 |
| | 2 | 28.6 | 1.95 | 5.01 | 1.057 | 0.323 | <0.001 | <0.001 | |
| DMI, kg/d | 1 | 5.8 | 7.40 | 3.33 | 0.809 | 0.629 | 0.454 | 0.002 | 0.513 |
| | 2 | 6.6 | 3.48 | 2.34 | 0.514 | 0.293 | 0.064 | <0.001 | |
| DMIperBW, g/kg | 1 | 5.6 | 7.84 | 2.24 | 0.573 | 0.604 | 0.491 | 0.003 | 0.377 |
| | 2 | 9.0 | 4.26 | 1.23 | 0.399 | 0.159 | 0.040 | 0.003 | |
| MBW, kg ^{0.75} | 1 | -455.0 | 153.80 | 4.18 | 1.309 | 0.505 | 0.014 | 0.010 | 0.070 |
| | 2 | -126.4 | 16.54 | 1.33 | 0.148 | 0.616 | <0.001 | <0.001 | |
| NE _m , Mcal/d | 1 | -455.4 | 153.83 | 43.29 | 13.557 | 0.505 | 0.014 | 0.010 | 0.069 |
| | 2 | -126.5 | 16.54 | 13.74 | 1.534 | 0.616 | <0.001 | <0.001 | |
| NE _g , Mcal/d | 1 | 29.7 | 1.85 | 2.35 | 0.540 | 0.656 | <0.001 | 0.001 | - |
| | 2 | 17.3 | 1.56 | 0.00 | - | 0.000 | <0.001 | - | |
| NE _t , Mcal/d | 1 | 4.2 | 7.30 | 2.27 | 0.517 | 0.658 | 0.580 | 0.001 | <0.001 |
| | 2 | -91.7 | 21.53 | 10.23 | 2.007 | 0.433 | <0.001 | <0.001 | |
| BCS, 1 - 9 | 1 | -261.8 | 103.07 | 52.25 | 18.096 | 0.455 | 0.029 | 0.016 | 0.292 |
| | 2 | -126.3 | 18.11 | 29.24 | 3.576 | 0.577 | <0.001 | <0.001 | |
| BFT, mm | 1 | -70.4 | 37.92 | 21.24 | 7.576 | 0.440 | 0.093 | 0.019 | 0.422 |
| | 2 | -26.7 | 6.49 | 8.67 | 0.722 | 0.655 | <0.001 | <0.001 | |
| RA, cm ² | 1 | -116.4 | 52.71 | 5.50 | 1.906 | 0.455 | 0.052 | 0.016 | 0.451 |
| | 2 | -58.0 | 12.09 | 1.81 | 0.144 | 0.744 | 0.744 | <0.001 | |

¹BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WiperMBW = water intake per unit of MBW, NE_m = net energy for maintenance, NE_g = net energy for gain, NE_t = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area. ²Phase 1 = BW maintenance, Phase 2 = BW loss. ³SE = standard error ⁴Interaction between the slope and phase

Table 2-7: Univariate regression analysis of each variable used for estimating water intake requirements (L/d) of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Phase ² | Intercept | | Slope | | r ² | P-value | | |
|--------------------------|--------------------|-----------|-------------------|-------|-------------------|----------------|-----------|--------|--------------------------|
| | | alpha | SE ³ ± | beta | SE ³ ± | | Intercept | Slope | Interaction ⁴ |
| BW, kg | 3 | 3.5 | 34.55 | 0.02 | 0.062 | 0.013 | 0.921 | 0.742 | 0.034 |
| | 4 | -111.7 | 9.81 | 0.25 | 0.015 | 0.780 | <0.001 | <0.001 | |
| ADG, kg/d | 3 | 14.9 | 2.16 | -1.86 | 10.071 | 0.007 | 0.001 | 0.861 | 0.952 |
| | 4 | 52.1 | 7.05 | -0.16 | 3.012 | 0.000 | <0.001 | 0.958 | |
| DMI, kg/d | 3 | 27.6 | 45.02 | -1.55 | 5.599 | 0.015 | 0.567 | 0.793 | 0.647 |
| | 4 | -18.8 | 59.88 | 5.36 | 4.335 | 0.036 | 0.756 | 0.223 | |
| DMIperBW, g/kg | 3 | 12.1 | 11.45 | 0.21 | 0.753 | 0.010 | 0.322 | 0.788 | 0.084 |
| | 4 | 125.2 | 14.89 | -3.44 | 0.696 | 0.300 | <0.001 | <0.001 | |
| MBW, kg ^{0.75} | 3 | -0.3 | 45.52 | 0.15 | 0.450 | 0.013 | 0.995 | 0.741 | 0.026 |
| | 4 | -165.4 | 13.15 | 1.87 | 0.115 | 0.778 | <0.001 | <0.001 | |
| NE _m , Mcal/d | 3 | -0.3 | 45.53 | 1.59 | 4.659 | 0.013 | 0.995 | 0.741 | 0.026 |
| | 4 | -165.4 | 13.15 | 19.41 | 1.189 | 0.778 | <0.001 | <0.001 | |
| NE _g , Mcal/d | 3 | 15.3 | 1.83 | -0.01 | 0.662 | 0.000 | <0.001 | 0.991 | 0.406 |
| | 4 | 24.3 | 14.84 | 1.83 | 0.934 | 0.072 | 0.108 | 0.056 | |
| NE _t , Mcal/d | 3 | 15.1 | 8.08 | 0.02 | 0.668 | 0.000 | 0.100 | 0.980 | 0.627 |
| | 4 | 23.1 | 18.68 | 1.06 | 0.675 | 0.043 | 0.221 | 0.124 | |
| BCS, 1 - 9 | 3 | 118.0 | 105.17 | -22.0 | 22.54 | 0.096 | 0.291 | 0.354 | 0.359 |
| | 4 | -65.7 | 9.53 | 22.58 | 1.869 | 0.658 | <0.001 | <0.001 | |
| BFT, mm | 3 | -57.4 | 124.66 | 25.98 | 44.241 | 0.047 | 0.659 | 0.576 | 0.851 |
| | 4 | 13.1 | 3.09 | 13.65 | 1.813 | 0.537 | <0.001 | <0.001 | |
| RA, cm ² | 3 | 7.1 | 34.01 | 0.41 | 1.698 | 0.006 | 0.840 | 0.816 | 0.681 |
| | 4 | 1.3 | 3.89 | 3.58 | 0.541 | 0.472 | <0.001 | <0.001 | |

¹BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WiperMBW = water intake per unit of MBW, NE_m = net energy for maintenance, NE_g = net energy for gain, NE_t = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area ²Phase 3 = BW maintenance after BW loss, Phase 4 = compensatory growth ³SE = standard error ⁴Interaction between the slope from univariate regression analysis and phase

Table 2-8: Univariate regression analysis of each variable used for estimating water intake requirements (L/d) of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Phase ² | Intercept | | Slope | | r ² | P-value | | |
|--------------------------|--------------------|-----------|-------------------|-------|-------------------|----------------|-----------|--------|--------------------------|
| | | alpha | SE ³ ± | beta | SE ³ ± | | Intercept | Slope | Interaction ⁴ |
| BW, kg | 2 | -90.9 | 12.08 | 0.18 | 0.019 | 0.623 | <0.001 | <0.001 | 0.006 |
| | 4 | -111.7 | 9.81 | 0.25 | 0.015 | 0.780 | <0.001 | <0.001 | |
| ADG, kg/d | 2 | 28.6 | 1.95 | 5.01 | 1.057 | 0.323 | <0.001 | <0.001 | 0.085 |
| | 4 | 52.1 | 7.05 | -0.16 | 3.012 | 0.000 | <0.001 | 0.958 | |
| DMI, kg/d | 2 | 6.6 | 3.48 | 2.34 | 0.514 | 0.293 | 0.064 | <0.001 | 0.437 |
| | 4 | -18.8 | 59.88 | 5.36 | 4.335 | 0.036 | 0.756 | 0.223 | |
| DMIperBW, g/kg | 2 | 9.0 | 4.26 | 1.23 | 0.399 | 0.159 | 0.040 | 0.003 | <0.001 |
| | 4 | 125.2 | 14.89 | -3.44 | 0.696 | 0.300 | <0.001 | <0.001 | |
| MBW, kg ^{0.75} | 2 | -126.4 | 16.54 | 1.33 | 0.148 | 0.616 | <0.001 | <0.001 | 0.004 |
| | 4 | -165.4 | 13.15 | 1.87 | 0.115 | 0.778 | <0.001 | <0.001 | |
| NE _m , Mcal/d | 2 | -126.5 | 16.54 | 13.74 | 1.534 | 0.616 | <0.001 | <0.001 | 0.004 |
| | 4 | -165.4 | 13.15 | 19.41 | 1.189 | 0.778 | <0.001 | <0.001 | |
| NE _g , Mcal/d | 2 | 17.3 | 1.56 | 0.00 | - | 0.000 | <0.001 | - | - |
| | 4 | 24.3 | 14.84 | 1.83 | 0.934 | 0.072 | 0.108 | 0.056 | |
| NE _t , Mcal/d | 2 | -91.7 | 21.53 | 10.23 | 2.007 | 0.433 | <0.001 | <0.001 | 0.004 |
| | 4 | 23.1 | 18.68 | 1.06 | 0.675 | 0.043 | 0.221 | 0.124 | |
| BCS, 1 - 9 | 2 | -126.3 | 18.11 | 29.24 | 3.576 | 0.577 | <0.001 | <0.001 | 0.113 |
| | 4 | -65.7 | 9.53 | 22.58 | 1.869 | 0.658 | <0.001 | <0.001 | |
| BFT, mm | 2 | -26.7 | 6.49 | 8.67 | 0.722 | 0.655 | <0.001 | <0.001 | 0.013 |
| | 4 | 13.1 | 3.09 | 13.65 | 1.813 | 0.537 | <0.001 | <0.001 | |
| RA, cm ² | 2 | -58.0 | 12.09 | 1.81 | 0.144 | 0.744 | 0.744 | <0.001 | 0.001 |
| | 4 | 1.3 | 3.89 | 3.58 | 0.541 | 0.472 | <0.001 | <0.001 | |

¹BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WIperMBW = water intake per unit of MBW, NE_m = net energy for maintenance, NE_g = net energy for gain, NE_t = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area ²Phase 2 = BW loss, Phase 4 = compensatory growth ³SE = standard error ⁴Interaction between the slope from univariate regression analysis and phase

Table 2-9: Water intake and drinking behavior of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Treatment ² | | | | Mean Paired Difference | SEM ³ (±) | P-value |
|---------------------|------------------------|--------------------|------|--------------------|------------------------|----------------------|---------|
| | Mean | SEM ³ ± | Mean | SEM ³ ± | | | |
| Phase 1 vs. Phase 2 | | | | | | | |
| WI, L/d | 35.8 | 3.21 | 22.8 | 1.73 | 13.0 | 1.63 | <0.001 |
| TSDW, min/d | 6.0 | 0.15 | 4.3 | 0.11 | 1.7 | 0.26 | <0.001 |
| DR, L/min | 6.3 | 0.16 | 5.7 | 0.16 | 0.6 | 0.33 | 0.088 |
| TWVD, # events/d | 2.1 | 0.04 | 1.5 | 0.03 | 0.6 | 0.11 | <0.001 |
| WVWDED, % | 99.0 | - | 95.5 | - | - | - | 0.005 |
| NDEVD, # equipment | 1.7 | 0.03 | 1.3 | 0.02 | 0.4 | 0.09 | 0.001 |
| Phase 3 vs. Phase 4 | | | | | | | |
| WI, L/d | 14.3 | 1.07 | 45.4 | 3.63 | -31.0 | 2.96 | <0.001 |
| TSDW, min/d | 3.6 | 0.10 | 8.0 | 0.16 | -4.4 | 0.46 | <0.001 |
| DR, L/min | 4.2 | 0.09 | 5.9 | 0.15 | -1.7 | 0.68 | 0.028 |
| TWVD, # events/d | 1.4 | 0.03 | 2.9 | 0.04 | -1.5 | 0.11 | <0.001 |
| WVWDED, % | 93.7 | - | 98.5 | - | - | - | <0.001 |
| NDEVD, # equipment | 1.2 | 0.02 | 1.7 | 0.02 | -0.5 | 0.09 | <0.001 |
| Phase 2 vs. Phase 4 | | | | | | | |
| WI, L/d | 22.8 | 1.73 | 45.4 | 3.63 | -22.6 | 2.31 | <0.001 |
| TSDW, min/d | 4.3 | 0.11 | 8.0 | 0.16 | -3.7 | 0.31 | <0.001 |
| DR, L/min | 5.7 | 0.16 | 5.9 | 0.15 | 0.2 | 0.62 | 0.005 |
| TWVD, # events/d | 1.5 | 0.03 | 2.9 | 0.04 | -1.4 | 0.10 | <0.001 |
| WVWDED, % | 95.5 | - | 98.5 | - | - | - | <0.001 |
| NDEVD, # equipment | 1.3 | 0.02 | 1.7 | 0.02 | -0.4 | 0.08 | 0.001 |

¹WI = water intake, TSDW = time spent drinking water, DR = drinking rate, TWVD = total waterer visited per day, WVWDED = percentage of waterer visits with successful drinking event, NDEVD = number of different equipment visited per day

²Phase 1 = BW maintenance, Phase 2 = BW loss, Phase 3 = BW maintenance after BW loss, Phase 4 = compensatory growth

³SEM = standard error of the mean

Table 2-10: Nitrogen intake, fecal and urine excretion, nitrogen retention, ruminal nitrogen balance, recycled ammonia, and microbial nitrogen synthesis on crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Treatment | | | | Mean Paired Difference | SEM ² ± | P-value |
|--------------------------|-----------|--------------------|-------|--------------------|------------------------|--------------------|---------|
| | Mean | SEM ² ± | Mean | SEM ² ± | | | |
| Phase 1 vs. Phase 2 | | | | | | | |
| NI, g/d | 110.3 | 10.88 | 89.8 | 4.57 | 20.6 | 5.46 | 0.086 |
| FN, g/d | 92.3 | 9.02 | 74.2 | 3.79 | 18.1 | 4.57 | 0.072 |
| UN, g/d | 62.3 | 6.16 | 50.7 | 2.59 | 11.6 | 3.12 | 0.090 |
| NR, g/d | -44.3 | 4.31 | -35.2 | 1.81 | -9.2 | 2.19 | 0.058 |
| NRRNI, g/g | -0.40 | 0.004 | -0.39 | 0.002 | -0.01 | 0.002 | 0.012 |
| RNB, g/d | -44.2 | 3.48 | -37.1 | 1.69 | -7.2 | 2.03 | 0.071 |
| RNBRNI, g/g | -0.40 | 0.004 | -0.41 | 0.002 | 0.01 | 0.002 | 0.081 |
| RNH ₃ , g N/d | 47.0 | 4.45 | 37.7 | 1.87 | 9.4 | 2.25 | 0.060 |
| MNS, g/d | 117.3 | 11.20 | 95.0 | 4.71 | 22.3 | 5.68 | 0.074 |
| MNSRNI, g/g | 1.06 | 0.002 | 1.05 | 0.001 | 0.004 | 0.002 | 0.188 |
| EMS, g micP/g TDN | 180.8 | 0.80 | 179.6 | 0.42 | 1.2 | 0.50 | 0.184 |
| Phase 3 vs. Phase 4 | | | | | | | |
| NI, g/d | 113.0 | 10.77 | 195.9 | 4.28 | -82.9 | 5.16 | <0.001 |
| FN, g/d | 99.8 | 7.81 | 162.2 | 3.58 | -62.4 | 4.32 | <0.001 |
| UN, g/d | 68.3 | 5.33 | 107.9 | 2.45 | -39.7 | 2.95 | <0.001 |
| NR, g/d | -47.8 | 3.74 | -74.2 | 1.71 | 26.4 | 2.06 | <0.001 |
| NRRNI, g/g | -0.40 | 0.004 | -0.38 | 0.002 | -0.02 | 0.002 | <0.001 |
| RNB, g/d | -48.3 | 3.48 | -73.9 | 1.60 | 25.6 | 1.92 | <0.001 |
| RNBRNI, g/g | -0.40 | 0.004 | -0.38 | 0.002 | -0.02 | 0.002 | <0.001 |
| RNH ₃ , g N/d | 50.0 | 3.85 | 80.8 | 1.77 | -30.8 | 2.130 | <0.001 |
| MNS, g/d | 126.3 | 9.70 | 202.9 | 4.45 | -76.7 | 5.365 | <0.001 |
| MNSRNI, g/g | 1.05 | 0.003 | 1.04 | 0.001 | 0.01 | 0.001 | <0.001 |
| EMS, g micP/g TDN | 172.8 | 1.04 | 171.7 | 0.43 | 1.1 | 0.40 | 0.215 |

¹NI = N intake, FN = fecal N, UN = urine N, NR = N retention, NRRNI = N retention relative to NI, RNB = ruminal N balance, RNBRNI = RNB relative to NI, RNH₃ = recycled NH₃, MNS = microbial N synthesis, MNSRNI = MNS relative to NI, EMS = efficiency of microbial N synthesis, micP = microbial protein, TDN = total digestible nutrients

²SEM = standard error of the mean

Table 2-11: Nitrogen intake, fecal and urine excretion, nitrogen retention, ruminal nitrogen balance, recycled ammonia, and microbial nitrogen synthesis on crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Treatment | | | | Mean Paired Difference | SEM ² ± | P-value |
|--------------------------|---------------------|--------------------|-------|--------------------|------------------------|--------------------|---------|
| | Mean | SEM ² ± | Mean | SEM ² ± | | | |
| | Phase 2 vs. Phase 4 | | | | | | |
| NI, g/d | 89.8 | 4.57 | 195.9 | 4.28 | -106.2 | 4.39 | <0.001 |
| FN, g/d | 74.2 | 3.79 | 162.2 | 3.58 | -87.9 | 3.68 | <0.001 |
| UN, g/d | 50.7 | 2.59 | 107.9 | 2.45 | -57.2 | 2.51 | <0.001 |
| NR, g/d | -35.2 | 1.81 | -74.2 | 1.71 | 39.0 | 1.76 | <0.001 |
| NRRNI, g/g | -0.39 | 0.002 | -0.38 | 0.002 | -0.01 | 0.002 | <0.001 |
| RNB, g/d | -37.1 | 1.69 | -73.9 | 1.60 | 36.8 | 1.64 | <0.001 |
| RNBRNI, g/g | -0.41 | 0.002 | -0.38 | 0.002 | -0.04 | 0.002 | <0.001 |
| RNH ₃ , g N/d | 37.7 | 1.87 | 80.8 | 1.77 | -43.2 | 1.81 | <0.001 |
| MNS, g/d | 95.0 | 4.71 | 202.9 | 4.45 | -107.9 | 4.572 | <0.001 |
| MNSRNI, g/g | 1.06 | 0.001 | 1.04 | 0.001 | 0.02 | 0.001 | <0.001 |
| EMS, g micP/g TDN | 179.6 | 0.42 | 171.7 | 0.43 | 7.9 | 0.377 | <0.001 |

¹NI = N intake, FN = fecal N, UN = urine N, NR = N retention, NRRNI = N retention relative to NI, RNB = ruminal N balance, RNBRNI = RNB relative to NI, RNH₃ = recycled NH₃, MNS = microbial N synthesis, MNSRNI = MNS relative to NI, EMS = efficiency of microbial N synthesis, micP = microbial protein, TDN = total digestible nutrients

²SEM = standard error of the mean

2.12. Figures

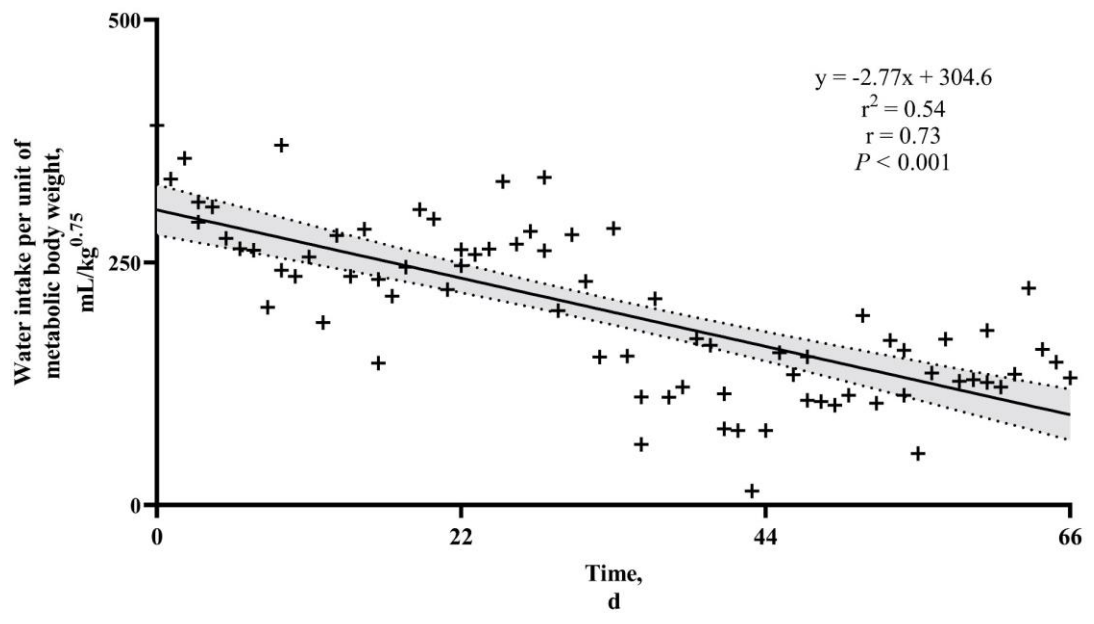


Figure 2- 1: Water intake per unit of metabolic body weight (mL/kg^{0.75}, +) on crossbred Angus × Hereford breeding bulls undergoing periods of body weight loss. Shaded area indicates 95% confidence interval.

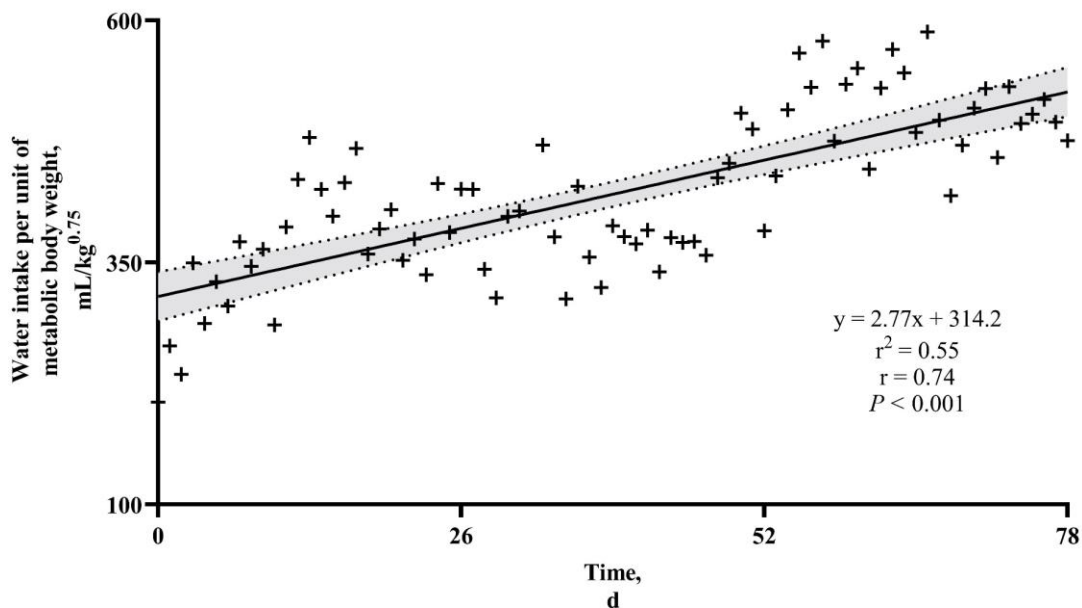


Figure 2-2: Water intake per unit of metabolic body weight ($\text{mL}/\text{kg}^{0.75}$, $+$) on crossbred Angus \times Hereford breeding bulls undergoing periods of compensatory growth. Shaded area indicates 95% confidence interval.

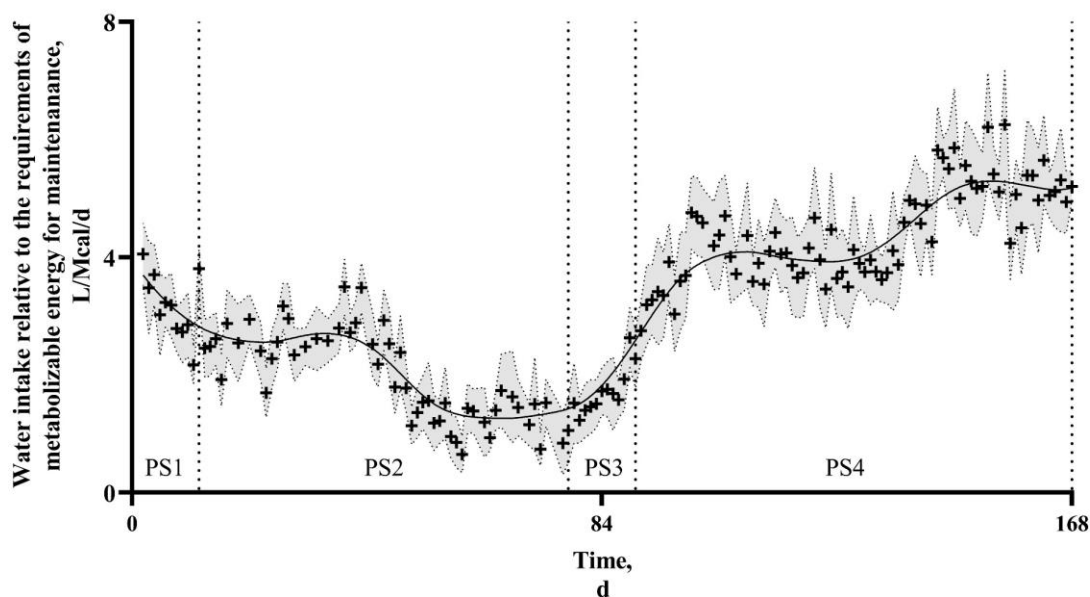


Figure 2-3: Water intake relative to the requirements of metabolizable energy for maintenance ($\text{L}/\text{Mcal}/\text{d}$, $+$) on crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Shaded area indicates 95% confidence interval.

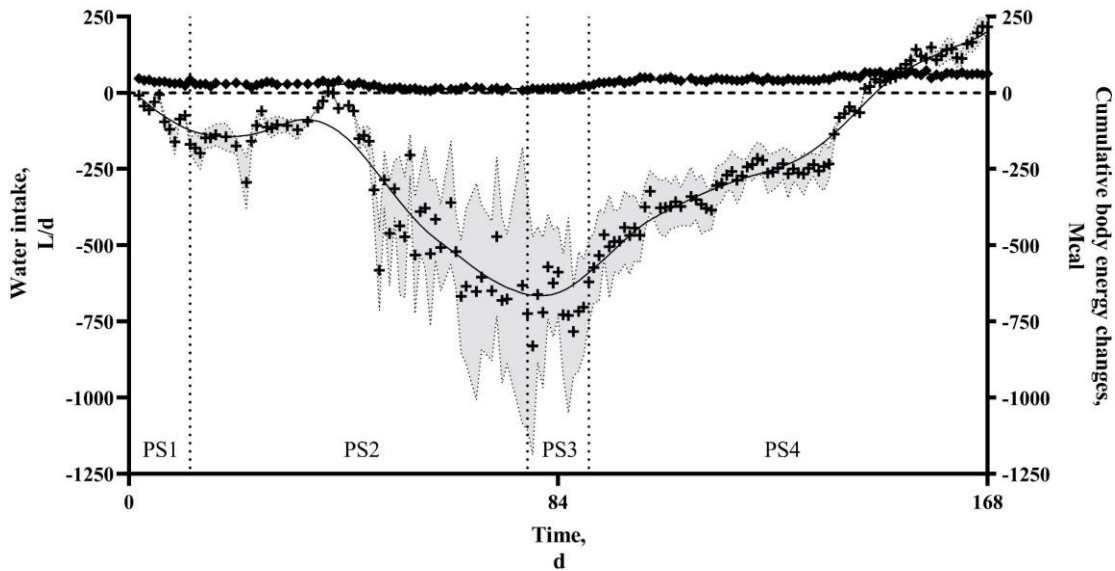


Figure 2-4: Water intake (L/d, \blacklozenge) and cumulative body energy changes (Mcal, \blackplus) on crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Light and dark shaded area indicates 95% confidence interval.

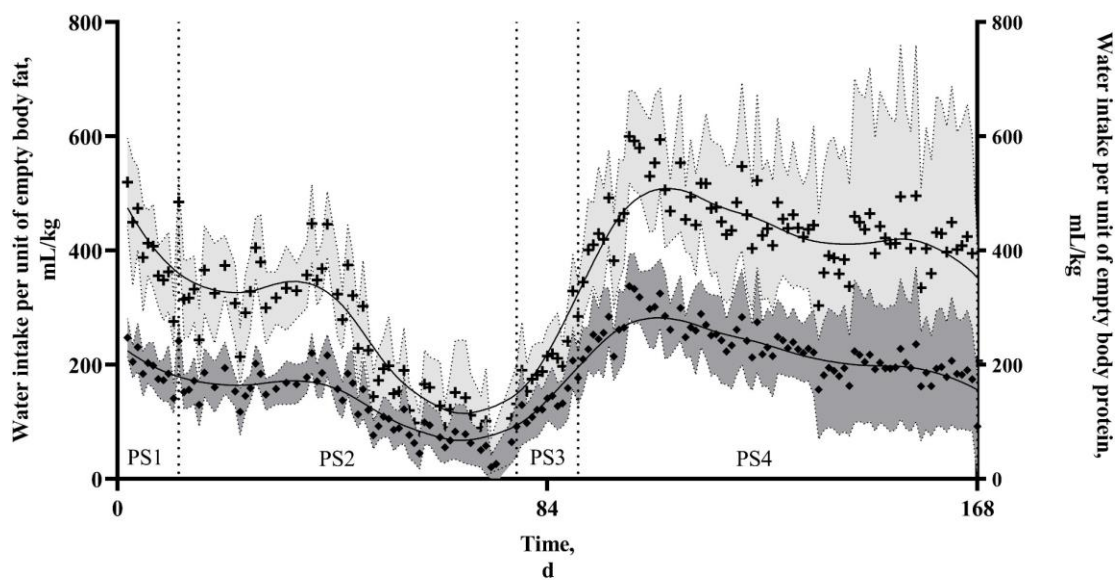


Figure 2-5: Water intake per unit of empty body fat (mL/kg, +) and per unit of empty body protein (mL/kg, ◆) on crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Light and dark shaded area indicates 95% confidence interval.

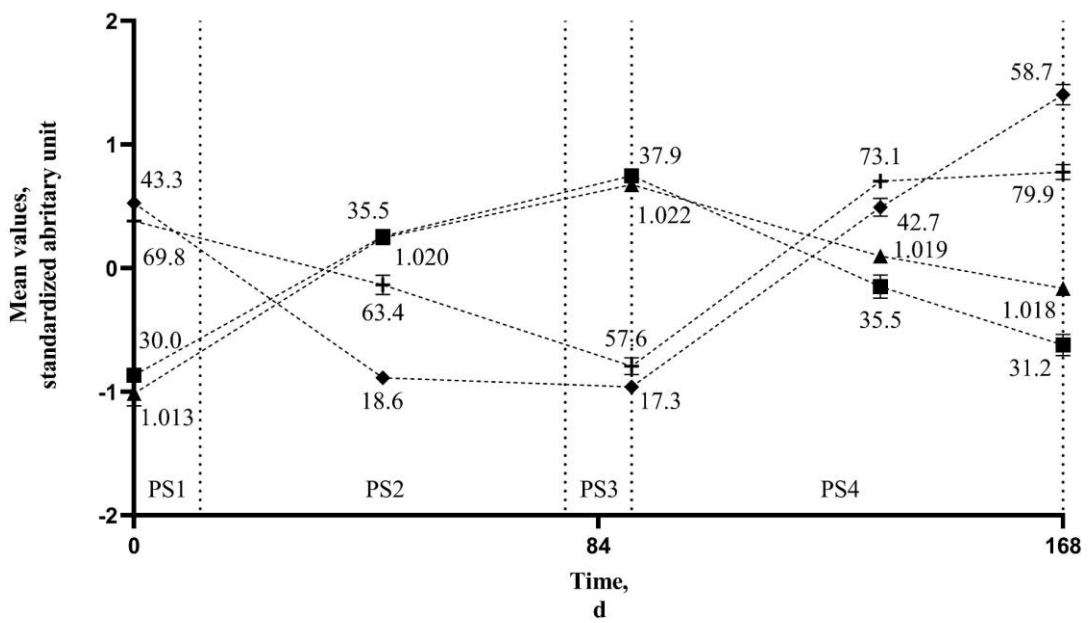


Figure 2-6: Behavior over time of water intake (L/d, ◆), package cell volume (%), ■), urine specific gravity (arbitrary unit, ▲) and heart rate (beats/min, +) on crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Standardized arbitrary values followed by their mean value.

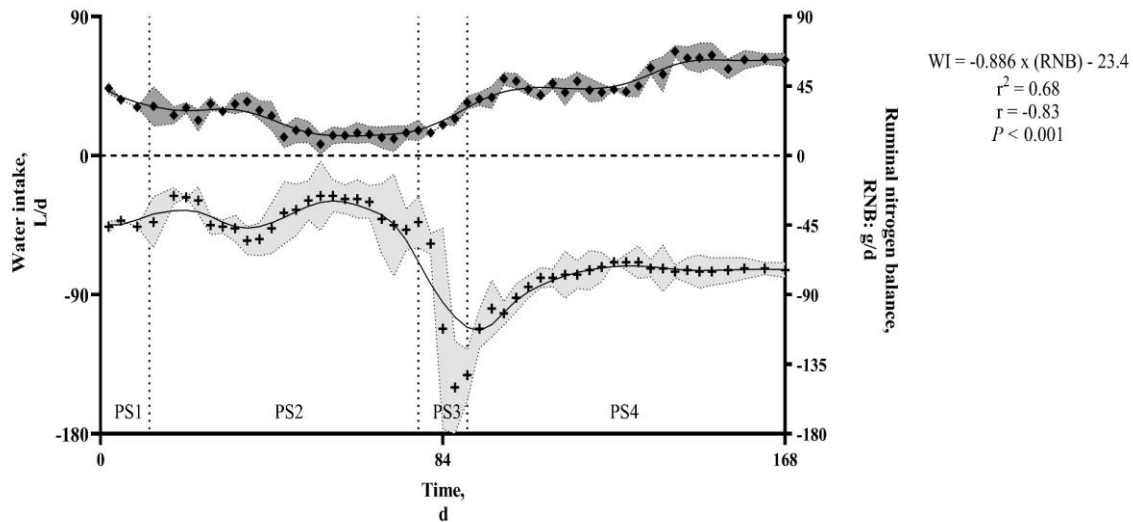


Figure 2-7: Water intake (L/d, \blacklozenge) and ruminal nitrogen balance (RNB, g/d, \blackplus) on crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Light and dark shaded area indicates 95% confidence interval.

2.13. Supplementary material

Supplementary tables

Supplementary Table 2-1: Water chemical composition offered to the crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | |
|--------------------------------------|-------|
| pH | 7.61 |
| Hardness as CaCO ₃ , mg/L | 214 |
| Total dissolved solids, mg/L | 600 |
| Calcium, g/L | 0.05 |
| Phosphorus, g/L | 0.001 |
| Sodium, g/L | 0.12 |
| Magnesium, g/L | 0.22 |
| Potassium, g/L | 0.03 |
| Sulfate, g/L | 0.06 |
| Manganese, mg/L | 0.05 |
| Copper, mg/L | 0.01 |
| Iron, mg/L | 0.05 |
| Zinc, mg/L | 0.01 |
| Nitrate as N, mg/L | <0.50 |
| Nitrate as NO ₃ , mg/L | <2.20 |

¹Cumberland Valley Analytical Services Laboratory, CaCO₃ = calcium carbonate, N = nitrogen, NO₃ = nitrate

Supplementary Table 2-2: Beardless wheat hay and mineral salt chemical composition fed to crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item | <i>Triticum aestivum</i> hay | Mineral salt |
|-------------------------------------|------------------------------|--------------|
| Dry matter ¹ , g/kg | 945.0 | - |
| TDN ² , g/kg DM | 579.0 | - |
| [ME] ² , Mcal/kg DM | 1.92 | - |
| [NEm] ² , Mcal/kg DM | 1.07 | - |
| [NEg] ² , Mcal/kg DM | 0.52 | - |
| CP ¹ , g/kg DM | 86.0 | - |
| Soluble protein ¹ , % CP | 40.9 | - |
| aNDFom ¹ , g/kg DM | 522.0 | - |
| peNDF ¹ , g/kg DM | 510.0 | - |
| ADF ¹ , g/kg DM | 387.0 | - |
| Lignin ¹ , g/kg DM | 51.4 | - |
| NFC ¹ , g/kg DM | 355.9 | - |
| Starch ¹ , g/kg DM | 26.0 | - |
| Sugars ¹ , g/kg DM | 100.0 | - |
| EE ¹ , g/kg DM | 25.0 | - |
| Ash ¹ , g/kg DM | 11.1 | - |
| Calcium ¹ , g/kg DM | 2.3 | 180.0 |
| Phosphorus ¹ , g/kg DM | 2.8 | 60.0 |
| Magnesium ¹ , g/kg DM | 2.6 | 40.0 |
| Potassium ¹ , g/kg DM | 25.1 | 5.0 |
| Sulfur ¹ , g/kg DM | 1.6 | - |
| Sodium ¹ , g/kg DM | 0.6 | 180.0 |
| Chloride ¹ , g/kg DM | 3.9 | - |
| Iron ¹ , mg/kg DM | 138.0 | - |
| Manganese ¹ , mg/kg DM | 33.0 | 3600.0 |
| Zinc ¹ , mg/kg DM | 22.0 | 3600.0 |
| Copper ¹ , mg/kg DM | 9.0 | 1200.0 |
| Cobalt, mg/kg | - | 12.0 |
| Iodine, mg/kg | - | 60.0 |
| Selenium, mg/kg DM | - | 27.0 |
| Vitamin A, IU/kg | - | 330695 |
| Vitamin D ₃ , IU/kg | - | 33070 |
| Vitamin E, IU/kg | - | 331 |

¹Chemical composition from Cumberland Valley Analytical, Services Laboratory, TDN = total digestible nutrients, CP = crude protein, aNDFom = neutral detergent fiber with the addition of amylase and sodium sulfite and exclusive of ash, peNDF = physically effective neutral detergent fiber, ADF = acid detergent fiber, EE = ether extract, NFC = non-fibrous carbohydrates = [100 – (%CP + %aNDFom + %EE + %ash)]

²Estimated by the Large Ruminant Nutrition System, [ME] = metabolizable energy concentration, [NEm] = net energy concentration for maintenance, [NEg] = net energy concentration for gain

Supplementary Table 2-3: Descriptive statistics analysis from weather conditions during periods of body weight maintenance, body weight loss, and compensatory growth of crossbred Angus × Hereford breeding bulls

| Item ¹ | Phase ² | Mean | SD ³ | Minimum | Maximum |
|----------------------|--------------------|--------|-----------------|---------|---------|
| Temp, °C | 1 | 9.71 | 2.33 | 3.54 | 12.67 |
| | 2 | 5.69 | 3.92 | -4.24 | 12.63 |
| | 3 | 2.26 | 2.57 | -2.64 | 8.33 |
| | 4 | 4.42 | 3.20 | -4.33 | 13.68 |
| RH, % | 1 | 40.80 | 10.09 | 21.23 | 56.46 |
| | 2 | 54.94 | 21.69 | 23.44 | 96.16 |
| | 3 | 69.22 | 9.12 | 44.19 | 90.86 |
| | 4 | 54.12 | 10.94 | 27.90 | 79.71 |
| SR, W/m ² | 1 | 180.05 | 46.53 | 94.55 | 274.85 |
| | 2 | 115.02 | 44.21 | 19.79 | 189.77 |
| | 3 | 88.63 | 18.96 | 40.19 | 117.57 |
| | 4 | 134.83 | 46.70 | 4.17 | 229.13 |
| WS, kph | 1 | 2.95 | 2.57 | 0.42 | 9.95 |
| | 2 | 2.12 | 2.08 | 0.40 | 9.88 |
| | 3 | 2.16 | 1.83 | 0.35 | 9.19 |
| | 4 | 4.23 | 3.49 | 0.39 | 20.10 |

¹Temp = ambient temperature, RH = relative humidity, SR = solar radiation, WS = wind speed

²Phase: 1 = Body weight maintenance (12 days, October); 2 = Body weight loss (66 days, mid-October to mid-December); 3 = Body weight maintenance after body weight loss (12 days, December); 4 = Compensatory growth (78 days, January to March)

³SD = Standard deviation

Supplementary Table 2-4: Descriptive statistics analysis from animal performance, energy requirements, body composition, and environmental variables affecting crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

| Item ¹ | Mean | SD ² | Minimum | Maximum |
|---------------------------------|--------|-----------------|---------|---------|
| <i>Phase 1³</i> | | | | |
| WI, L/d | 35.81 | 14.50 | 9.53 | 94.30 |
| BW, kg | 676.10 | 132.50 | 503.20 | 921.10 |
| ADG, kg/d | -0.005 | 0.96 | -2.31 | 2.06 |
| DMI, kg/d | 9.23 | 2.03 | 4.89 | 14.39 |
| DMIperBW, g/kg | 13.90 | 3.04 | 7.66 | 21.57 |
| MBW, kg | 128.50 | 18.88 | 103.10 | 162.20 |
| WIperMBW, mL/kg ^{0.75} | 301.53 | 96.07 | 87.52 | 634.11 |
| NEm, Mcal/d | 11.35 | 1.66 | 9.13 | 14.36 |
| NEg, Mcal/d | 2.59 | 3.87 | 0.00 | 16.03 |
| NEt, Mcal/d | 13.93 | 4.53 | 9.13 | 29.13 |
| BCS, 1 – 9 | 5.68 | 0.69 | 4.40 | 7.27 |
| BFT, mm | 4.95 | 1.04 | 3.20 | 7.53 |
| RA, cm ² | 82.11 | 20.19 | 57.75 | 116.17 |
| <i>Phase 2³</i> | | | | |
| WI, L/d | 22.90 | 13.47 | 0.05 | 81.25 |
| BW, kg | 635.20 | 134.28 | 404.00 | 934.70 |
| ADG, kg/d | -2.23 | 3.36 | -30.57 | 3.07 |
| DMI, kg/d | 6.33 | 3.31 | 0.004 | 41.75 |
| DMIperBW, g/kg | 9.88 | 4.26 | 0.006 | 48.38 |
| MBW, kg | 122.60 | 18.39 | 87.40 | 164.30 |
| WIperMBW, mL/kg ^{0.75} | 199.06 | 102.50 | 0.42 | 541.37 |
| NEm, Mcal/d | 10.88 | 1.71 | 7.74 | 14.52 |
| NEg, Mcal/d | 0.78 | 2.89 | 0.00 | 25.28 |
| NEt, Mcal/d | 11.66 | 3.80 | 7.74 | 38.16 |
| BCS, 1 – 9 | 5.11 | 0.63 | 3.42 | 6.88 |
| BFT, mm | 3.65 | 0.79 | 1.97 | 6.34 |
| RA, cm ² | 68.10 | 18.03 | 39.75 | 105.22 |

¹WI = water intake, BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area

²SD = Standard deviation

³Phase 1 = BW maintenance, Phase 2 = BW loss

Supplementary Table 2- 5: Descriptive statistics analysis from animal performance, energy requirements, body composition, and environmental factors studied on crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance, body weight loss, and compensatory growth

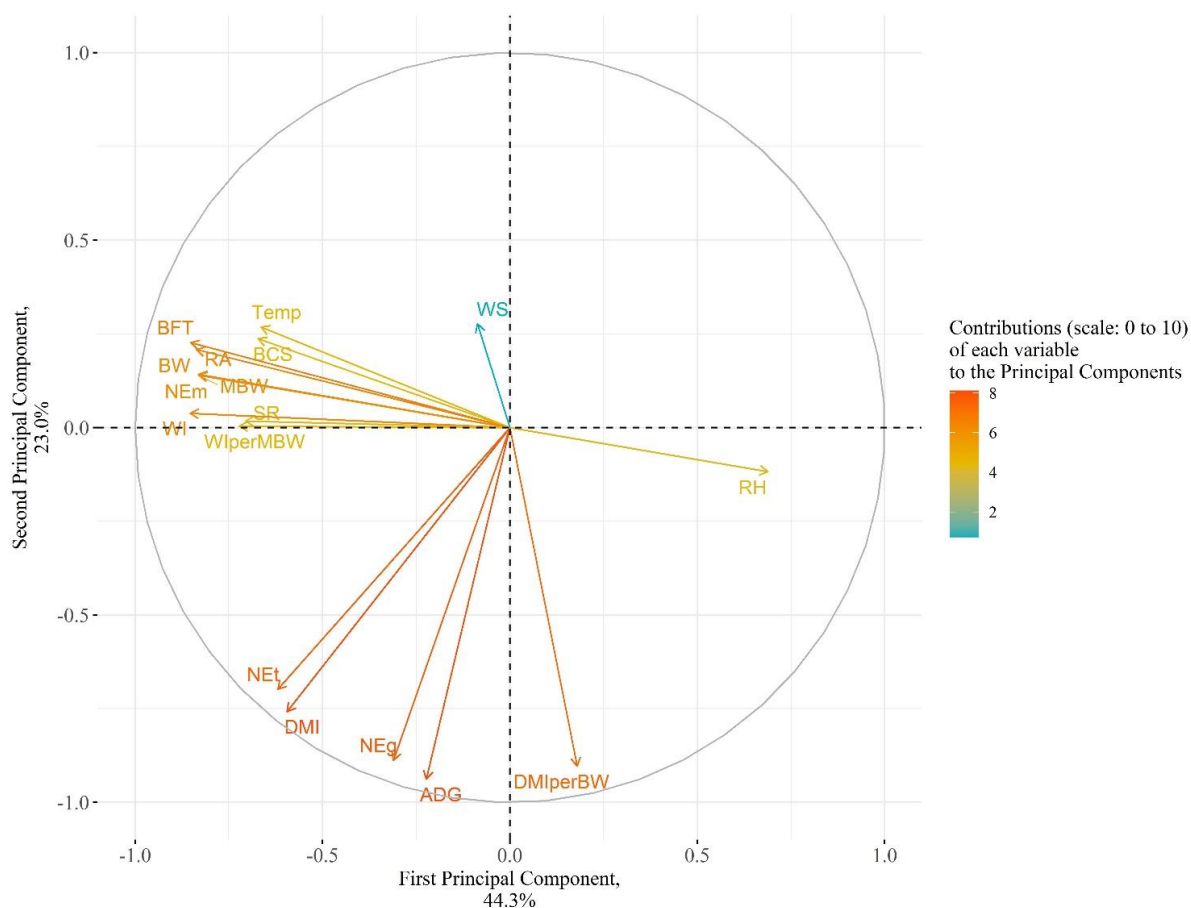
| Item ¹ | Mean | SD ² | Minimum | Maximum |
|---------------------------------|--------|-----------------|---------|---------|
| <i>Phase 3³</i> | | | | |
| WI, L/d | 13.35 | 8.59 | 0.03 | 50.43 |
| BW, kg | 550.70 | 115.00 | 382.50 | 767.50 |
| ADG, kg/d | -0.004 | 0.95 | -2.20 | 2.47 |
| DMI, kg/d | 8.32 | 2.09 | 3.53 | 15.33 |
| DMIperBW, g/kg | 15.31 | 3.37 | 8.58 | 24.79 |
| MBW, kg | 110.10 | 17.62 | 83.90 | 141.40 |
| WIperMBW, mL/kg ^{0.75} | 142.42 | 81.37 | 0.30 | 398.95 |
| NEm, Mcal/d | 9.72 | 1.52 | 7.43 | 12.52 |
| NEg, Mcal/d | 2.17 | 3.54 | 0.00 | 19.33 |
| NEt, Mcal/d | 11.89 | 4.10 | 7.43 | 31.85 |
| BCS, 1 – 9 | 4.66 | 0.58 | 3.45 | 5.81 |
| BFT, mm | 2.84 | 0.46 | 1.98 | 3.43 |
| RA, cm ² | 59.43 | 15.45 | 39.09 | 76.70 |
| <i>Phase 4³</i> | | | | |
| WI, L/d | 47.06 | 19.73 | 7.15 | 133.73 |
| BW, kg | 630.10 | 139.60 | 410.20 | 951.00 |
| ADG, kg/d | 3.66 | 3.97 | -13.15 | 62.94 |
| DMI, kg/d | 18.72 | 21.89 | 0.88 | 528.07 |
| DMIperBW, g/kg | 29.57 | 30.64 | 1.21 | 661.54 |
| MBW, kg | 121.90 | 22.76 | 88.40 | 166.10 |
| WIperMBW, mL/kg ^{0.75} | 408.25 | 131.67 | 63.29 | 938.96 |
| NEm, Mcal/d | 10.95 | 1.78 | 7.83 | 14.71 |
| NEg, Mcal/d | 27.54 | 36.15 | 0.00 | 695.25 |
| NEt, Mcal/d | 38.48 | 36.48 | 7.83 | 708.15 |
| BCS, 1 – 9 | 5.00 | 0.82 | 3.38 | 7.55 |
| BFT, mm | 3.93 | 1.38 | 1.97 | 9.13 |
| RA, cm ² | 76.14 | 21.99 | 39.90 | 110.07 |

¹WI = water intake, BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back fat thickness, RA = ribeye area

²SD = Standard deviation

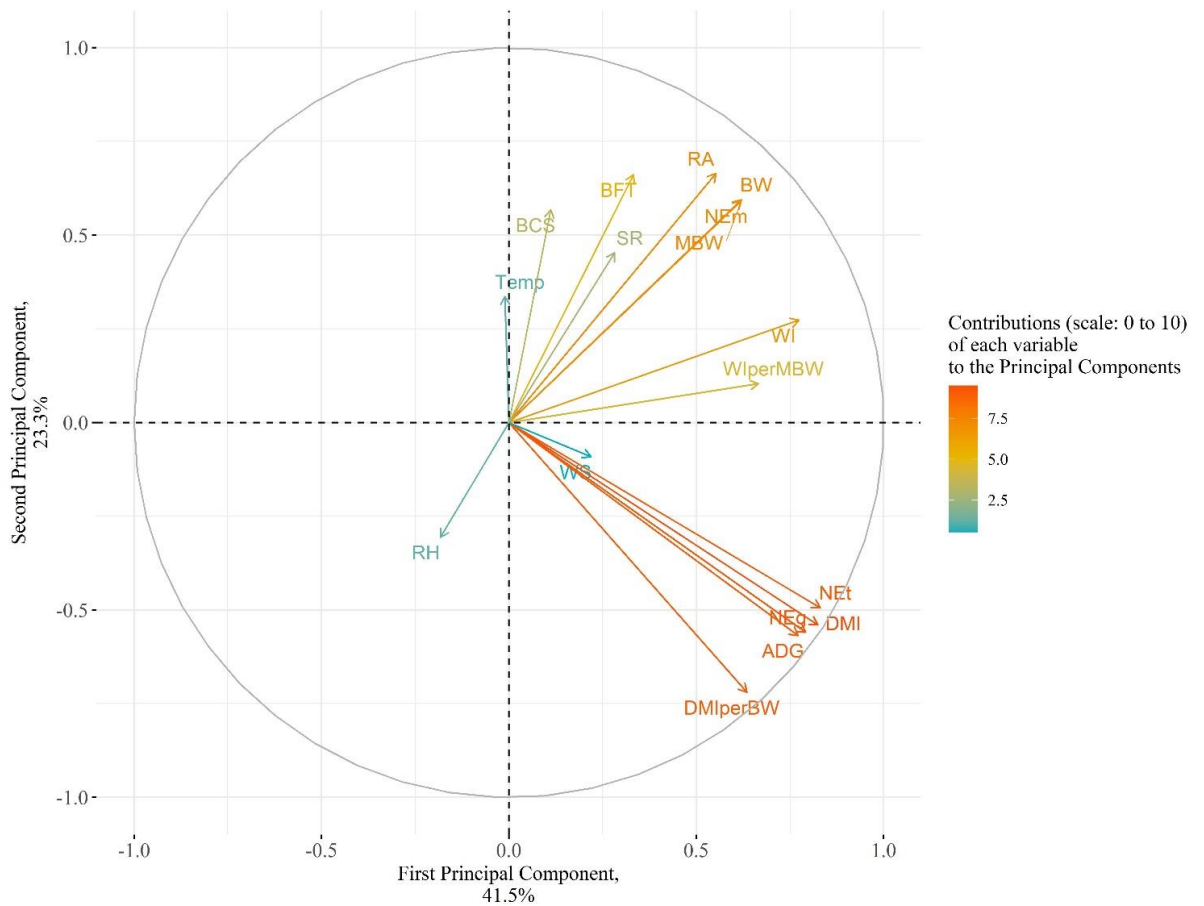
³Phase 3 = BW maintenance after BW loss, Phase 4 = compensatory growth

Supplementary figures



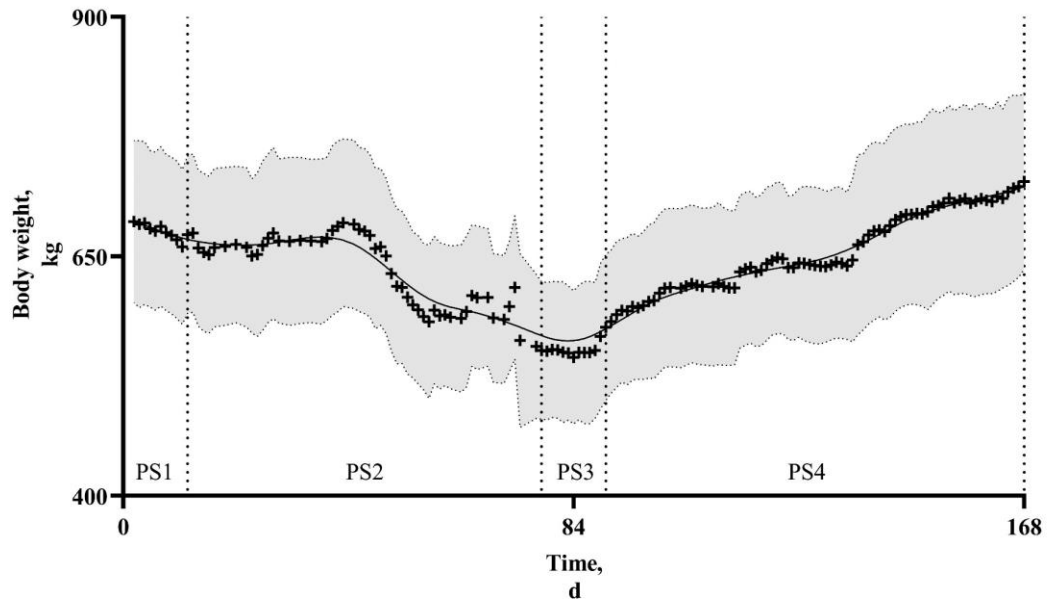
Supplementary Figure 2-1: Biplot Principal Component Analysis of variables that explaining water intake during periods of body weight maintenance of crossbred Angus \times Hereford breeding bulls

WI = water intake, BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WIperMBW = WI per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back-fat thickness, RA = ribeye area, Temp = ambient temperature, RH = relative humidity, SR = solar radiation, WS = wind speed

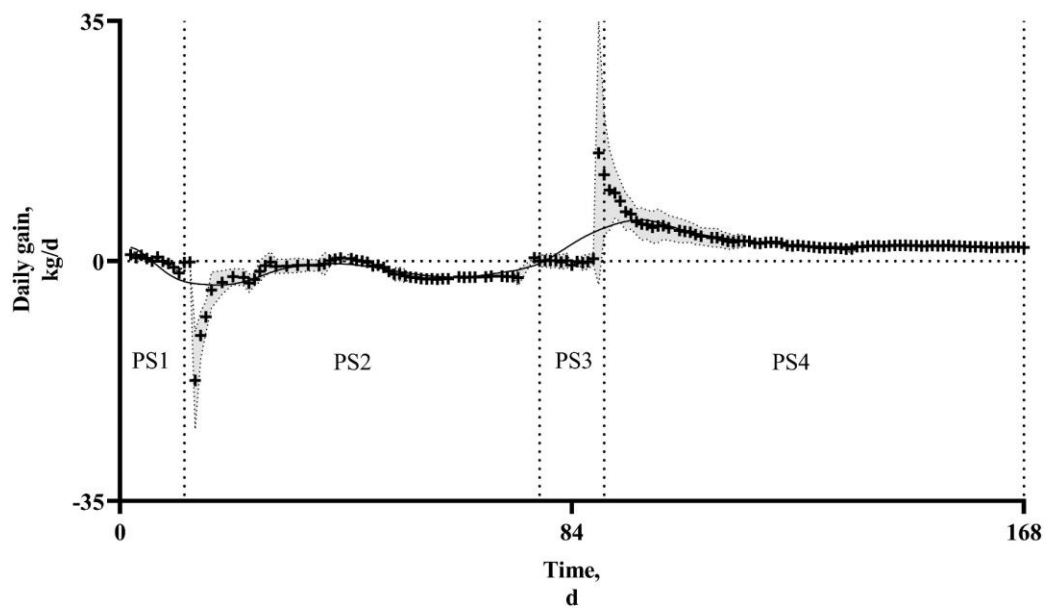


Supplementary Figure 2-2: Biplot Principal Component Analysis of variables that explaining water intake during periods of body weight loss and body weight gain of crossbred Angus \times Hereford breeding bulls

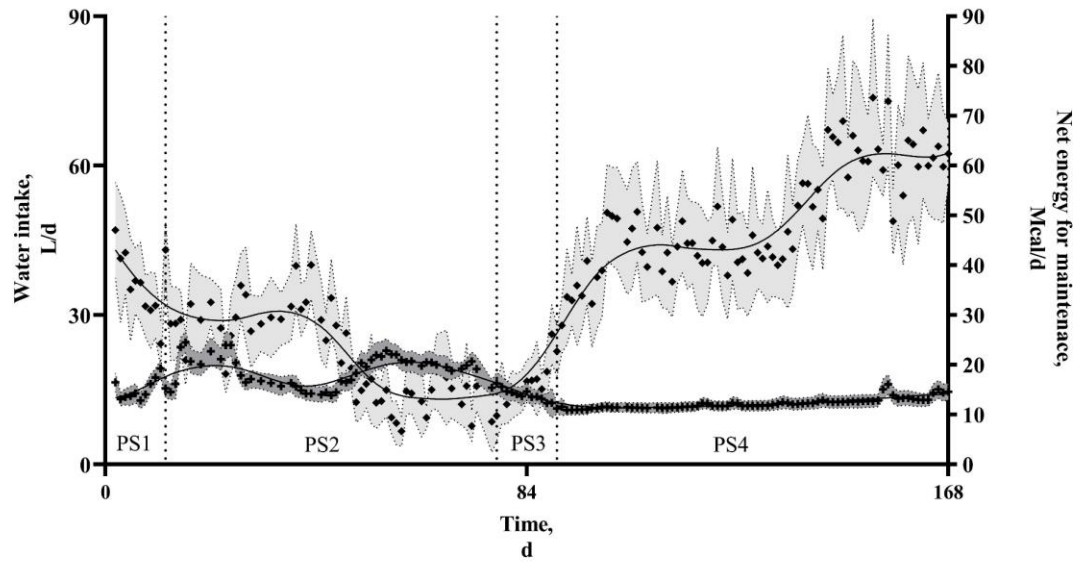
WI = water intake, BW = body weight, ADG = average daily gain, DMI = dry matter intake, DMIperBW = DMI per unit of BW, MBW = metabolic BW, WIperMBW = WI per unit of MBW, NEm = net energy for maintenance, NEg = net energy for gain, NEt = total net energy, BCS = body condition score, BFT = back-fat thickness, RA = ribeye area, Temp = ambient temperature, RH = relative humidity, SR = solar radiation, WS = wind speed



Supplementary Figure 2-3: Body weight (kg, \pm) changes of crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Shaded area indicates 95% confidence interval.



Supplementary Figure 2-4: Daily gain (kg/d, \pm) changes of crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Shaded area indicates 95% confidence interval.



Supplementary Figure 2-5: Water intake (L/d, ◆) and net energy for maintenance (Mcal/d, +) of crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (PS1 and PS3), body weight loss (PS2), and compensatory growth (PS4). Light and dark shaded area indicates 95% confidence interval.

CHAPTER III:

**SPERM DNA 5-MC AND RNA M⁶A METHYLATION ARE DIFFERENTLY
AFFECTED DURING PERIODS OF BODY WEIGHT LOSSES AND BODY
WEIGHT GAIN OF YOUNG AND MATURE BREEDING BULLS**

3.1. Abstract

Aiming to characterize the effects of nutritional status on epigenetic markers, such as DNA 5-mC methylation and RNA m⁶A methylation, of bovine sperm, twelve Angus × Hereford crossbred breeding bulls were submitted to nutritional changes for a period of 180 days: no change in BW (Phase 1 = 12 d); BW loss (Phase 2 = 78 d); and BW gain (Phase 3 = 90 d) in a repeated measures design. Animals were fed Beardless wheat (*Triticum aestivum*) hay and mineral mix. Statistical analyses were performed using SAS 9.4 (SAS Inst., Cary, NC). Higher levels of RNA m⁶A ($P = 0.004$) and DNA methylation ($P = 0.007$) of spermatic cells were observed at Phase 2 compared with Phase 1. In Phase 3, sperm RNA m⁶A methylation levels continued to be higher ($P = 0.004$), whereas the DNA of sperm cells was similar ($P = 0.426$) compared with the Phase 1. Growing bulls had a tendency ($P = 0.109$) of higher RNA m⁶A methylation levels than mature bulls. Phase 2 altered scrotal circumference ($P < 0.001$), sperm volume ($P = 0.007$), sperm total motility ($P = 0.004$), sperm progressive motility ($P = 0.004$), total sperm count ($P = 0.049$), normal sperm ($P < 0.001$), abnormal sperm ($P < 0.001$), primary sperm defects ($P = 0.039$), and secondary sperm defects ($P < 0.001$). In Phase 3, bulls had scrotal circumference, sperm volume, sperm motility, sperm progressive motility, total sperm count, normal and abnormal spermatozoa, and primary and secondary spermatozoa defects similar to Phase 1 ($P > 0.05$). Serum concentrations of insulin-like growth factor-1 (IGF-1) and leptin decreased during Phase 2 ($P = 0.010$) while no differences ($P > 0.05$) were detected between Phase 3 and 1; growing bulls tended ($P = 0.102$) to present higher leptin levels than mature bulls. Specific for mature bulls, DNA methylation was positively correlated with leptin concentration (0.569, $P = 0.021$). Whereas for young

bulls, DNA methylation was positively correlated with abnormal spermatozoa (0.824, $P = 0.006$), primary spermatozoa defect (0.711, $P = 0.032$), secondary spermatozoa defect (0.661, $P = 0.052$), and negatively correlated with normal spermatozoa (-0.824 , $P = 0.006$), total sperm count (-0.702 , $P = 0.035$), and sperm concentration (-0.846 , $P = 0.004$). There was no significant correlation ($P > 0.05$) between RNA m⁶A and hormones and semen traits. In conclusion, the nutritional status of breeding bulls alters epigenetic markers, such as DNA methylation and RNA m⁶A methylation, in sperm, and the impact of change seems to be age-dependent. These markers may serve as biomarkers of sperm quality and fertility of bulls in the future. Detrimental effects on sperm production and seminal quality are observed at periods and places when and where environmental and nutritional limitations are a year-round reality and may carry hidden players that may influence a lifetime of underperformance.

Key words: body composition, DNA methylation, IGF-1, leptin, RNA methylation, semen

3.2. Introduction

Bull fertility is critically important for cattle production systems, impacting the reproductive performance of herds through both natural breeding and artificial insemination (AI). In fact, as one bull has the potential to breed thousands of females by AI, the impact of bull fertility to the cattle industry is tremendous (Thundathil et al., 2016). Still, bulls' subfertility rates are estimated to reach up to 25% in the USA (Kennedy et al., 2002). Therefore, factors influencing fertility of bulls, including nutrition, health, and quality and production traits of the semen along with methods to

assess fertility should not be overlooked (DeJarnette et al., 2004; Kenny and Byrne, 2018; Butler et al., 2020).

Breeding soundness exam (BSE) is currently used to evaluate bull fertility. This exam includes general physical examination, inspection of genital organs, and assessment of sperm production and quality (Kastelic and Thundathil, 2008). Nevertheless, changes to fertility in response to environmental and nutritional factors may go undetected by this exam. Interestingly, transcriptome, metabolomic, and proteomic studies have revealed potential biomarkers in sperm and seminal plasma of farm animals, such as bulls and stallions (Novak et al., 2010; Das et al., 2013; Menezes et al., 2019). Therefore, advances in the field of molecular biology bring new possibilities to unveil the impact of the environment on semen quality, allowing the identification of potential biomarkers of fertility.

Among sperm biomarkers for fertility, epigenetic markers deserve attention for their potential to change in response to nutrition and environmental factors. Epigenetics refers to heritable changes in gene expression without changes to the DNA sequence itself (Egger et al., 2004). Well-established epigenetic mechanisms include factors such as DNA methylation, histone post-translational modifications, and non-coding RNAs (Tammen et al., 2013). Different patterns of sperm DNA methylation, one of the most widely studied epigenetic mechanisms, have been associated with sperm quality and male fertility (Schagdarsurengin and Steger, 2016; Kropp et al., 2017; Khezri et al., 2020; Marcho et al., 2020) and have been reported to change in response to environmental challenges, such as exposure to toxins (Acharya et al., 2020). Nutritional factors also have been reported to affect sperm DNA methylation in animal studies: in mice, studies have shown that different dietary

protein or folate levels alter sperm DNA methylation (Lambrot et al., 2013; Watkins et al., 2018); in sheep, dietary supplementation of rumen-protected methionine altered sperm DNA methylation of rams (Gross et al., 2020); in cattle, different planes of nutrition during the prepubertal period affects sperm DNA methylation of pubertal bulls (Perrier et al., 2020). Furthermore, it is now well-accepted that changes to the epigenome of sperm, including DNA methylation, in response to the environment can have an impact on the offspring health (Pembrey et al., 2014; Schagdarsurengin and Steger, 2016). Therefore, studies addressing the consequences of physiological and metabolic stressors on bull sperm DNA methylation are important for their impact on the cattle industry.

Another epigenetic modification that has been attracting the attention of scientists for its potential to regulate mRNA processing and translation is methylation of the N6 position of adenosine (m^6A), the most known internal modification of mammalian mRNA (Desrosiers et al., 1974; Meyer et al., 2012). Mature spermatid cells are considered transcriptionally inactive, but sperm carry RNA nonetheless (Casas and Vavouri, 2014). It is now well-accepted that small non-coding RNAs present in sperm are delivered to the oocyte at fertilization and are important to regulate early embryonic development (Liu et al., 2012) and to affect offspring phenotype (McPherson et al., 2015; Rodgers et al., 2015), but the majority of transcripts in sperm consists of fragments of longer transcripts, such as mRNA, and these have a potential to serve as biomarkers of fertility (Casas and Vavouri, 2014). Indeed, recent studies have shown that m^6A RNA methylation of sperm is critical for the regulation of spermatogenesis (Lin et al., 2017; Lin and Tong, 2019). Therefore, m^6A methylation of fragments of mRNA in the ejaculate can be seen as a remnant of

the process of spermatogenesis, making it a promising biomarker of sperm quality and fertility. Thus, studies aiming to understand what factors can change this modification in sperm, including nutritional factors, are required.

Animal experimental studies and human epidemiological observations have established the importance of parental nutrition in inducing sperm epigenetic modifications with consequences to offspring health and metabolism (Schagdarsurengin and Steger, 2016; Donkin and Barres, 2018). Nevertheless, studies focusing on sperm epigenetic markers and their correlation with sperm quality in farm animals in response to nutrition are limited. Thus, this study aimed to characterize the effects of nutritional status on epigenetic markers, such as DNA methylation and m⁶A RNA methylation, in sperm of postpubertal bulls. We hypothesized that nutritional stress, during periods of negative energy balance (NEB) and subsequent compensatory growth, affect sperm m⁶A RNA methylation and 5-mC DNA methylation, thus highlighting the importance of proper nutritional management of breeding bulls, including but not limited to the breeding season.

3.3. Material and Methods

The animals used in this experiment were cared for according to guidelines approved by the Institutional Animal Care and Use Committee University of Nevada, Reno (protocol #00738).

Animal, treatment, and experimental area

The dataset was obtained from twelve Angus × Hereford crossbred breeding bulls (n = 6, 23±0.55 months [young bulls], 558±6.1 kg; and n = 6, 47±1.2 months

[mature bulls], 740 ± 30.5 kg) over a period of 180 days. Animals were housed at the Main Station Research Feedlot Facility at the Nevada Agricultural Experiment Station in Reno, NV. Bulls had free access to water and trace mineral salt during the whole trial. Beardless wheat (*Triticum aestivum*; 945 g/kg dry matter, 579 g/kg total digestible nutrients; 86 g/kg crude protein) hay was delivered daily.

Three dietary regimes were offered to the bulls throughout the experimental period. Bulls coming from the breeding herd pastures were fed targeting for no BW changes for 12 d as baseline (Phase 1: BW maintenance adjustment preceding a BW loss); BW loss for 78 d (Phase 2: BW loss targeted for 0.6 kg/d); fed for BW gain targeted for 1 kg/d for 90 d (Phase 3: compensatory growth). Bulls were kept apart from cows on pasture and no semen was collected from the previous Spring breeding season to the beginning of this trial. In order to induce a metabolic stress and a full recovery to initial state, each animal acted as its own control in a repeated measures design. Furthermore, the feeding protocol requisites entailed simulating same conditions at the beginning (Phase 1) and at the end of the experimental period (end of Phase 3) per metrics of BW and body condition score (BCS).

The animals were randomly assigned to one of two pens (15×28 m). Each pen contained 30 m^2 of shaded area and four automated scales that recorded body weight changes daily (ASMS; Model WD-1000 Master, Intergado Ltd, Contagem, Minas Gerais, Brazil). Prior to the beginning the trial, each animal was fitted with an electronic identification ear tag (FDX-ISO 11784/11785; Allflex, Joinville, Santa Catarina, Brazil). Due to the dimensions of the scale, only one bull was allowed at the time into one individual scale when accessing the water troughs. The BW data were continuously recorded, transferred, and stored in the cloud for further analysis. The

ASMS scales were calibrated once weekly (using appropriate block calibration weights provided by the manufacturer) to ensure data accuracy.

Environmental conditions

Environmental conditions may trigger metabolic stress mechanisms that may change the capability of animals to perform or recover from a given stressful event. The climate of the area where the study was conducted is classified according to Köppen-Geiger (Kottek et al., 2006) as BSk (cold-semi arid climate) located at global positioning system of latitude 39° 32' 38.87" N and longitude of 119° 48' 57.76" W. The annual average temperature, rainfall, snowfall, and frost-free period are 10.1 °C, 18.6 mm, 228.6 mm, and 8.25 months, respectively. The temperature-humidity index (THI) adjusted for solar radiation and wind speed according to Mader et al. (2006) was used to characterize environmental conditions. Ambient temperature (°C), relative humidity (%), solar radiation (W/m²), and wind speed (mph), were recorded daily throughout the entire trial (October to March) at one min intervals using a HOBO data logger (HOBO H8 Pro Series, Onset Computer Corp, Bourne, MA).

Animal data assessment

The BCS was measured using the NU Beef BCS App (University of Nebraska-Lincoln, Nebraska, USA) utilizing a scale ranging from 1 to 9, as recommended by NASEM (2016). The BCS measurements were performed on 0, 45, 90, 135, and 180 d of the trial. The BW was measured daily by the ASMS. The average daily gain (ADG, kg/d) was measured daily by the ASMS.

Reproductive traits and sperm processing

At the initial day of trial, bulls were submitted to a BSE. All bulls met (Table 3-1) the physical exam and the requirements for the minimal scrotal circumference (≥ 34 cm), sperm motility ($\geq 30\%$), and normal sperm morphology ($\geq 70\%$) as recommended by the Society of Theriogenology (Chenoweth et al., 1993). Bulls were restrained individually in a squeeze chute and submitted to semen collection via electroejaculation (Palmer, 2005), performed on days 0, 90, and 180 of the trial. All bulls were cleaned to remove extra gonadal reserves of sperm on days 45 and 135 of the trial. Scrotal circumference was measured using a scrotal tape. Immediately after each semen collection, a sample was taken for immediate evaluation of sperm parameters (motility, progressive motility, and vigor) and the remaining ejaculate was taken to a nearby laboratory in the experimental station where it was subdivided for processing of RNA isolation, assessment of concentration and morphology, and cryopreservation.

For evaluation of motility, progressive motility, and vigor, a 10 μL sample of the ejaculate was placed into a microcentrifuge tube containing 10 μL of warm (37°C) PBS, homogenized, and placed in a warm (37°C) glass with a slide cover for analysis of motility, progressive motility, and vigor. These parameters were evaluated microscopically (Leica DM 500, 40x magnification; Wetzlar, Germany). For vigor, a scale from 0 to 5 representing the intensity of movement was used.

Processing of semen for isolation of RNA from spermatid cells was performed immediately post ejaculation as follows: fresh semen samples were centrifuged at 1000 x g for 10 min at 4°C for separation of sperm and seminal fluid; the cells pellet received 1 mL PBS followed by centrifugation and supernatant was discarded; the cells pellet then were incubated with lysis buffer (0.1% SDS, 0.5% Triton-X; 500 μL) for 10 min on ice and then once again centrifuged and supernatant was discarded;

finally, cell pellets were placed into sterile, RNase- and DNA-free, microcentrifuge tubes containing 500 μ L of Qiazol lysis reagent (Qiagen, Hilden, Germany) and samples were frozen at -80°C until further processing for RNA extraction.

Spermatozoa concentration was performed up to 30 min after semen collection using a hemocytometer. Briefly, fresh semen was added to formalin in a 1:200 dilutions. Then, 8 μ l of this mixture were placed in the two sides of the hemocytometer to allow a double count. The sperm count was performed microscopically (SKU: T690B-PCT200INF-PL, AmScope, CA, USA) using 40X objective. Five squares on the diagonal from each side of the hemocytometer were counted. The number of sperm cells were determined by the average from the two sides multiplied by a constant of 10 000 000 and the semen volume.

For morphology assessment of spermatic cells, slides were prepared within 30 min after semen collection. A semen drop was placed at the end of a glass slide followed by a drop of eosin-nigrosin stain-nigrosin stain (Hancock Stain, Animal Reproduction systems, CA, USA). The mixture was slowly spread on the slide with the edge of another glass slide at 30 degrees and left to dry. Slides were analyzed within 1 week microscopically (Leica DM 500 Microscope), using 100X objective lens with immersion oil (Cargille Laboratories, NJ, USA). A total of 100 cells were counted throughout the slide and the number of normal and abnormal sperm observed was recorded. Abnormalities were designated as defects involving the head (e.g., detached head, defects in size and shape), midpiece (e.g., distal mid-piece reflex, bowed mid-piece, proximal droplet), or tail (e.g., bent tail, coiled tail). Then, sperm abnormalities were divided into two categories called primary (underdeveloped, double forms, acrosome defects, crater-diadem defect, pear-shape head, small and free abnormal heads, proximal droplet, double bent and coiled tail, and accessory tail)

and secondary (giant and short broad heads, detached head, detached acrosome membranes, abaxial midpiece, distal droplet, simple bent tail, and terminal coiled tail) as recommended by Chenoweth et al., (1993).

For cryopreservation, semen samples were kept in a water bath at 37°C prior to spermatozoa concentration assessment. Following concentration assessment, samples received the necessary volume of semen extender to obtain 30 straws/bull in a concentration of 25×10^6 sperm cells/straw. A Tris egg yolk extender (Two-step extender, Continental Plastic Corp., WI, USA) containing Tris 12.10 g/L, citric acid 6.9 g/L, fructose 5.0 g/L, glycerol 70 ml/L, 20% egg yolk (v/v) and an antibiotic cocktail was used. Following manufacturer's instructions, fraction A of the extender was added at 37 °C and fraction B was added following cooling at the semen at 4°C for 4 hours. Semen straws were then filled by suction, closed with polyvinyl alcohol, placed in a floating straw rack (cat#: FSR-101-LC, ARS, CA, USA) for 10 min, and stored in liquid nitrogen for future isolation of DNA.

RNA extraction and N6-Methyladonesin assessment

Total RNA was extracted from fresh spermatozoa samples via miRNEasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples of RNA were solubilized in RNase- and DNase-free molecular grade water (Invitrogen, Carlsbad, CA, USA), quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and stored at -80°C for further analyses. The RNA m6A levels were colorimetrically quantified in RNA of spermatozoa cells samples via the Epiquik m⁶A RNA Methylation Quantification Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions using a microplate reader (SpectraMax M2e; Molecular devices, LLC,

San Jose, CA) and analyzed through the SoftMax Pro software (Molecular devices, LLC, San Jose, CA). Briefly, 100 ng of RNA samples (260/280 ratio > 2.0) were added to each well (2 replicates per sample), plus 2 replicates per negative (0 ng/uL), plus 2 replicates per positive controls (0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 ng/uL) and absorbance was read at 450 nm. The intra-assay coefficient of variation (CV) averaged 10.05%. The cutoff limit to ensure consistent results between replicate was an intra-assay CV of 12.5%.

Genomic DNA extraction and global DNA methylation

For genomic DNA extraction, frozen semen samples were thawed at 37°C for 30 seconds and spermatic cells pellets were washed three times in 10 mL PBS via centrifugation at $10,000 \times g$ for 10 min. After washing, cell pellets were resuspended in 500 μ L of PBS and transferred to a microcentrifuge tube to be stored at -80°C for further processing and analysis. Total genomic DNA was extracted from 50.0×10^6 spermatozoa cell samples using the Purelink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). Briefly, spermatic cells selective bound to Purelink Genomic DNA silica-based membrane (Invitrogen, Carlsbad, CA) in the presence of chaotropic salts. The cells were digested using Purelink Genomic proteinase K at 55 °C (Invitrogen, Carlsbad, CA). Any residual RNA was removed by digestion with Purelink Genomic RNase A (Invitrogen, Carlsbad, CA) prior to binding samples to the silica membrane. Then, samples of DNA were solubilized in 35 μ L of Purelink Genomic DNA Elution Buffer (Invitrogen, Carlsbad, CA), quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and stored at -20°C for further analyses. The 5-mC DNA methylation levels were determined using the colorimetric enzyme-linked immunosorbent assay, MethylFlash 5-mC DNA

methylation quantification kit (Epigentek Inc., Farmingdale, NY, USA) and a microplate reader (SpectraMax M2e; Molecular devices, LLC, San Jose, CA) and were analyzed through the SoftMax Pro software (Molecular devices, LLC, San Jose, CA) according to the manufacturer's instructions. Briefly, 100 ng of DNA samples (260/280 ratio > 1.8) were added to each well (2 replicates per sample), plus 2 replicates per negative (0 ng/uL), plus 2 replicates per positive controls (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ng/uL) and absorbance was read at 450 nm. The intra-assay CV averaged 5.09%. The cutoff limit to ensure consistent results between replicate was an intra-assay CV of 7.5%.

Physiological traits assessment

Blood samples were collected at 0700 hours at 0, 90, and 180 days. Blood was collected via jugular venipuncture using vacutainer luer-lok access device (20-gauge \times 1 in.; Airtite Product Co. Inc., Virginia Beach, VA), and a 10-mL heparinized plastic blood collection tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) to determine plasma concentrations of insulin-like growth factor-1 (IGF-1). Additional blood samples (10-mL) were collected from all bulls, via jugular venipuncture into tubes containing no additives (Vacutainer, Becton Dickinson) to determine serum leptin concentration. All blood samples were immediately placed on ice following collection for transportation and then centrifuged at $1,200 \times g$ for 25 min at 4 °C in the laboratory. Serum and plasma samples were stored frozen at -20 °C until further laboratory analysis.

Plasma concentrations of IGF-1 were assessed using a human specific commercial ELISA kit (SG100; R&D Systems Inc., Minneapolis, MN) previously

validated for bovine samples (Moriel et al., 2012). Briefly, 50 uL of plasma samples were added to each well (2 replicates per sample), plus 2 replicates per negative (0 ng/mL), plus 2 replicates per positive controls (0.094, 0.188, 0.375, 0.75, 1.5, 3.0, and 6.0 ng/mL) and absorbance was read at 450 nm adjusted for wavelength at 540 nm. Intra-assay CV averaged 1.70%, and the inter-assay 6.30%. Commercial bovine ELISA kit was utilized to determine the serum concentration of leptin (EKU05589; Biomatik USA, LLC, Wilmington, DE). Briefly, 100 uL serum samples were added to each well (2 replicates per sample), plus 2 replicates per negative (0 ng/mL), plus 2 replicates per positive controls (0.156, 0.312, 0.625, 1.25, 2.5, 5.0, and 10.0 ng/mL) and absorbance was read at 450 nm. Intra-assay CV averaged 4.70%, and the inter-assay averaged 5.65%. For IGF-1 and leptin analyses, the cutoff limit to ensure consistent results between replicate was an intra-assay CV of 10.0%.

Statistical analyses

Data were collected and analysed following a pre-post repeated measure design (Burgos et al., 2001). The statistical model used is shown below:

$$Y_{ij} = \mu + T_i + A_j + b_j + T_i \times A_j + \varepsilon_{ij}$$

Where Y_{ij} is the observation taken (BW, BCS, ADG, DNA 5-mC methylation, RNA m⁶A methylation, hormones, reproductive, and semen traits) on the j_{th} experimental unit for the pre-post i_{th} treatment, μ is the overall mean, T_i is the effect of the i_{th} treatment (periods of BW maintenance, BW loss, and BW gain), b_j is the effect of the j_{th} experimental unit (each animal acted as its own control), A_j is the effect of the j_{th} age (young and mature bulls), $T_i \times A_j$ is the interaction effect of the i_{th} treatment and

the j_{th} age, and ε_{ij} is the unobservable random error on the j_{th} experimental unit associated with each i_{th} pre-post treatment.

Statistical analyses were performed using PROC MIXED procedure of SAS (ver. 9.4, SAS Inst. Inc., Cary, NC). Pearson correlation coefficients among variables were obtained with PROC CORR. All figures were generated by GraphPad Prism (ver. 9.0, GraphPad Inc., San Diego, CA). Outliers were tested by plotting the studentized residuals and data points were removed if the Studentized residual was outside the range of -2.5 to 2.5 . Normality assumption was tested using Shapiro-Wilk's test and homogeneity of variance was evaluated through Levene's test. Percentage data (sperm motility, sperm progressive motility, normal spermatozoa, abnormal spermatozoa, primary spermatozoa defect, secondary spermatozoa defect) were converted to a proportion, an arc sine transformation was done, and transformed data were analyzed (non-transformed data were reported). Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$ using Tukey's post hoc test. Data were evaluated as repeated measures over time (Kaps and Lamberson, 2004).

3.4. Results

Environmental conditions

Descriptive statistics analyses from adjusted THI were presented in Table 3-2. According to the Livestock Weather Safety Index (LCI, 1970), animals were in their thermal comfort zone during the whole trial.

Performance

When bulls experienced negative energy balance (NEB), growing bulls decreased 26.8% and 20.4% of BW and BCS, respectively, whereas mature bulls, decreased 25.7% and 26.8% (Figure 3-1A and Figure 3-2). A full recovery to initial state of BW and BCS was observed for growing and mature bulls at the end of the compensatory growth (phase 3) as planned. The ADG experienced by growing bulls were similar (Figure 3-1B; $P \geq 0.14$) to mature bulls over time.

5-methyl cytosine (5-mC) DNA methylation

The abundance of DNA methylation of spermatic cells tended to be higher ($P = 0.07$; Figure 3-3A) during the phase of BW loss compared to the other phases. No differences in DNA methylation were detected between the periods of BW maintenance and compensatory growth. Effects of age ($P = 0.426$) or time \times age ($P = 0.791$) were not observed for DNA methylation of spermatic cells.

N6-methyladenosine (RNA m⁶A) methylation

The abundance of RNA m⁶A methylation in spermatic cells was greater ($P = 0.004$; Figure 3-3B) during periods of NEB and compensatory growth compared with the initial day of the trial (bulls coming from the breeding herd pastures). No differences ($P > 0.05$) were detected between NEB and compensatory growth periods for RNA m⁶A methylation. Young bulls ($0.13 \text{ ng} \pm 0.01$) had a tendency ($P = 0.109$) of higher RNA m⁶A methylation levels than mature bulls ($0.10 \text{ ng} \pm 0.009$). No significant ($P = 0.368$) time \times age interaction was observed for RNA m⁶A methylation of spermatic cells.

Reproductive and Sperm morphology traits

The bulls' reproductive parameters are presented in Figure 3-4 and Figure 3-5. Time influenced scrotal circumference ($P < 0.001$; Figure 3-4A), sperm motility ($P = 0.004$; Figure 3-4B), sperm progressive motility ($P = 0.004$; Figure 3-4C), sperm volume ($P = 0.007$; Figure 3-5A), and total sperm count ($P = 0.049$; Figure 3-5C). During the NEB, bulls decreased 8.4% of scrotal circumference, ejaculated 40.8% less sperm volume, decreased 34.0% of sperm motility, decreased 35.9% of sperm progressive motility, and decreased 50.0% of total sperm count compared to the initial day of the trial. By the end of the compensatory phase, bulls had similar ($P > 0.05$) scrotal circumference, sperm volume, sperm motility, sperm progressive motility, and total sperm count compared to the initial day of the trial. Young bulls had lower ($P = 0.005$) scrotal circumference ($33.9 \text{ cm} \pm 1.34$) than mature bulls ($40.6 \text{ cm} \pm 0.95$). Effect of time \times age interaction was not detected for scrotal circumference ($P = 0.558$). There were no effects of age ($P \geq 0.168$) and time \times age interaction ($P \geq 0.438$) in sperm volume, sperm motility, sperm progressive motility, and total sperm count. Additionally, no effects of time ($P \geq 0.459$), age ($P \geq 0.598$), and time \times age interaction ($P \geq 0.406$) were detected in sperm vigor (Figure 3-4D) and sperm concentration (Figure 3-5B).

In terms of spermatozoa morphology, effect of time was detected for normal spermatozoa ($P < 0.001$; Figure 3-6A), abnormal spermatozoa ($P < 0.001$; Figure 3-6B), primary spermatozoa defect ($P = 0.039$; Figure 3-6C), and secondary spermatozoa defect ($P < 0.001$; Figure 3-6D). During the NEB period, bulls decreased 47.7% of normal spermatozoa, increased 205.9% abnormal spermatozoa, increased 106.4% of primary spermatozoa defect, and increased 321.7% of secondary spermatozoa defect compared to the initial day of the trial. By the end of the

compensatory growth period, bulls had similar ($P > 0.05$) normal and abnormal spermatozoa and primary and secondary spermatozoa defects to the initial day of the trial. In addition, there were no effects of age ($P \geq 0.342$) and time \times age interaction ($P \geq 0.206$) in normal and abnormal spermatozoa, primary and secondary spermatozoa defects.

Physiological traits

The IGF-1 and leptin concentrations are presented in Figure 3-7. Bulls decreased ($P = 0.010$; Figure 3-7A) IGF-1 concentration during the NEB. Whereas comparing the baseline with compensatory growth, bulls had similar ($P > 0.05$) IGF-1 concentrations. Effects of age ($P = 0.577$) and time \times age interaction ($P = 0.868$) were not detected for plasma concentrations of IGF-1.

Effect of time ($P = 0.155$) was not detected for serum concentrations of leptin (Figure 3-7B). A tendency was detected for the time \times age interaction ($P = 0.082$) for serum leptin concentrations. Young bulls tended ($P = 0.102$) to present greater leptin concentration than mature bulls during the overall evaluated sampling days of the trial, which was more evident under compensatory growth period.

Pearson correlation analyses

Pearson correlation among DNA methylation, RNA m⁶A methylation, hormones, and semen traits is presented in Table 3-3 and Table 3-4. Specific for mature bulls, DNA methylation was positively correlated with leptin concentration (0.569, $P = 0.021$) and tended to be negatively correlated with sperm total motility (-0.446, $P = 0.083$). Whereas for young bulls, DNA methylation was positively correlated with abnormal spermatozoa (0.824, $P = 0.006$), primary spermatozoa

defect (0.711, $P = 0.032$), secondary spermatozoa defect (0.661, $P = 0.052$), and negatively correlated with normal spermatozoa (-0.824 , $P = 0.006$), total sperm count (-0.702 , $P = 0.035$), and sperm concentration (-0.846 , $P = 0.004$). Furthermore, in young bulls DNA methylation tended to be negatively correlated with IGF-1 serum levels (-0.578 , $P = 0.103$).

In mature bulls, IGF-1 serum levels were positively correlated with normal sperm (0.524, $P = 0.025$), sperm volume (0.542, $P = 0.019$), total sperm count (0.504, $P = 0.033$), and negatively correlated with abnormal spermatozoa (-0.498 , $P = 0.035$). There was a tendency for IGF-1 levels to be negatively correlated with sperm total motility (-0.414 , $P = 0.087$), sperm progressive motility (-0.402 , $P = 0.098$), and primary spermatozoa defect (-0.430 , $P = 0.075$). In young bulls, IGF-1 was positively correlated with normal sperm (0.824, $P = 0.006$), and negatively correlated with abnormal sperm (-0.839 , $P = 0.005$), primary spermatozoa defects (-0.705 , $P = 0.034$), and secondary spermatozoa defects (-0.667 , $P = 0.049$). RNA m⁶A methylation and leptin serum concentrations had no significant correlation ($P \geq 0.151$) with any parameter measured.

3.5. Discussion

Fertility of bulls is critically important for the success of cow-calf operations and the AI industry (Thundathil et al., 2016). Investigating the impact of management practices and nutritional and environmental factors on semen quality and fertility is therefore crucial. However, testing male fertility via mating or AI is considered time-consuming and expensive (Larsson and Rodrigues-Martinez, 2000). Sperm quality parameters individually evaluated have limited value for predicting fertility, but the more sperm parameters that can be tested, especially in combination, the better the

prediction of fertility (Farrell et al., 1998; Gillan et al., 2005; Morrell et al., 2017).

Thus, unveiling biomarkers in sperm of bulls is important to improve fertility predictions. The objective of this study was to investigate if periods of nutritional and metabolic changes affect two sperm epigenetic markers, DNA methylation and RNA m6A methylation, and their correlations with sperm quality parameters in postpubertal bulls.

Nutrition and energy balance are known to alter sperm quality parameters of ruminants (Martin et al., 2010; Ros-Santaella et al., 2019). Grazing breeding bulls in extensive production systems, such as the ones observed in the rangelands of Western U.S., often undergo NEB at least in some point of their year-round production cycle (NASEM, 2016). Indeed, in the present study, the rates of weight loss and weight gain successfully induced metabolic changes according to NASEM (2016), and a detrimental impact of NEB on reproductive and sperm quality parameters was evident. Specifically, the period of NEB decreased scrotal circumference, sperm volume, sperm motility, and normal spermatozoa count while increasing abnormal spermatozoa count and spermatozoa primary and secondary defects. Although a period of NEB did not decrease sperm concentration, it decreased the total sperm count in the ejaculate because of less ejaculate volume. These results may have important implications for the bovine AI industry and for beef operations in rangeland conditions. For example, difference in total sperm count per ejaculate does reduce the total number of insemination doses obtained from one ejaculate and bulls undergoing NEB in grazing conditions may produce a lower number of calves. Therefore, ranchers and the AI industry should pay attention to nutritional management of

breeding bulls, as nutritional strategy could likely result in large differences in total sperm output.

In the present study, nutritional management of bulls successfully induced a period of NEB and a subsequent return to their initial metabolic state. The adjusted THI supported our hypothesis that animals would undergo similar metabolic challenges simultaneously. Leptin and IGF-1, important regulators of energy homeostasis (Kawai and Rosen, 2010; Park and Ahima, 2015), were used in this study as metabolic parameters. IGF-1 is involved in the main metabolic pathways related to animal growth and distribution of nutrients to different tissues (Owens et al., 1993) whereas leptin has been positively associated with dry matter intake, average daily gain, and body fatness measures of beef steers and heifers (Foote et al., 2016). In the present study, IGF-1 serum concentrations were lower during the period of NEB whereas no changes were observed in circulating levels of leptin in response to diet. The return to positive energy status following NEB was characterized by a steep rise in circulating IGF-1 levels, which likely promoted an accelerated growth during the compensatory growth period shifting tissue deposition patterns towards fat deposition on depots where it could be more easily reassessed in case another NEB period was to come (Yang et al., 2019; Barboza et al., 2020). Interestingly, IGF-1 was positively correlated with ejaculate volume and several quality sperm parameters, including sperm motility and progressive motility, normal spermatozoa, and total sperm count in mature bulls while it was negatively correlated with abnormal spermatozoa irrespective of bulls age. This in agreement with a previous study that reported a positive correlation between serum IGF-1 levels and sperm motility and concentration

in buffalo bulls (Kumar et al., 2019), which indicates that IGF-1 may be a potential biomarker for fertility of bulls.

DNA methylation is one the most extensively studied epigenetic mechanisms and has been reported to be altered in various human tissues in response to changes in body composition (Aronica et al., 2017; Nishida et al., 2020). The impact of nutrition on this epigenetic mechanism has also been shown in studies that showed that BW changes affect the methylation pattern of several genes, including genes associated with obesity (Cordero et al., 2011), metabolism (Martin-Nunez et al., 2014), and fertility (Sujit et al., 2018; Liu et al., 2019). In sperm, several animal studies have unveiled the impact of nutrition on DNA methylation (Lambrot et al., 2013; Watkins et al., 2018; Gross et al., 2020; Perrier et al., 2020), but studies investigating how differential tissue utilization and metabolic states affect the sperm epigenome and its potential reversal by nutritional management are limited. In the present study, sperm DNA methylation tended to increase in bulls undergoing NEB and was negatively correlated with normal spermatozoa, total spermatozoa count, and ejaculate volume and was positively correlated with spermatozoa defects in young, but not mature, bulls, which indicates that DNA methylation may serve as a biomarker of semen quality in young bulls. Sperm DNA methylation has been associated with sperm abnormalities and poor sperm quality in infertile men (Houshdaran et al., 2007; Lambrot et al., 2013). Reasons for why young bulls were more susceptible to changes in sperm DNA methylation in response to nutrition and what mechanisms are involved in this process remain to be unveiled by future studies. Since no techniques were employed for the separation of live and dead sperm prior to sperm processing in the present study, it is possible that changes in DNA methylation were affected by

differences in sperm viability in response to diet, but this hypothesis requires further study. Furthermore, since DNA methylation can influence the transcription of small non-coding RNAs (Sato et al., 2011) and sperm microRNAs affect the offspring phenotype (McPherson et al., 2015; Rodgers et al., 2015), the possibility that a period of NEB regulates the sperm epigenome of bulls with effects on next generations impacting cattle productivity deserves further investigation. This possibility is strengthened by the recent mice studies reporting that paternal diet influences the sperm epigenome with consequences to reproductive parameters and health of the offspring (Fullston et al., 2012; Schagdarsurengin and Steger, 2016; Watkins et al., 2018).

The RNA m⁶A methylation is crucial for regulation of various biological processes, including spermatogenesis (Zheng et al., 2013). It is becoming clear that RNA m⁶A methylation is an important regulator of gene expression, involved in the regulation of mRNA processing, including actions related to mRNA splicing, stability, translation, and nuclear export, and decay (Liu and Jia, 2014; Chandola et al., 2015; Roignant and Soller, 2017). The regulation of RNA m⁶A methylation is complex and involves m⁶A writers (methyltransferases), erasers (demethylases), and readers (m⁶A-binding proteins) (Liu and Jia, 2014; Roignant and Soller, 2017). Various m⁶A readers have been identified to date and they are important to determine the fate of m⁶A-containing mRNA, such as enhancing translation or mRNA decay (Lee et al., 2020). In the present study, sperm m⁶A RNA methylation was greater in periods of NEB and compensatory weight gain in comparison to a period of BW maintenance. It is possible that different genes were methylated or different m⁶A readers were active during steroidogenesis in periods of NEB in comparison to

compensatory weight gain, but further research is required to confirm these hypotheses. In the present study, no correlation was observed between m⁶A RNA methylation and semen quality and sperm morphology, which may indicate that m⁶A RNA methylation is acting on regulation of genes not directly related to the traits measured herein. This finding highlights an important need to investigate major unforeseen events that have not been taken into consideration thus far, rightfully so because of the inherent limitations of not measuring metabolism at the cellular level while in field conditions, but nonetheless, can evidently impact the efficiency and sustainability of cattle production systems. Therefore, further studies are required to unveil what is the impact of changes in RNA m⁶A methylation in response to metabolic changes and whether m⁶A RNA methylation can be used as a biomarker of fertility.

In conclusion, the nutritional status of breeding bulls alters epigenetic markers, such as DNA methylation and RNA m⁶A methylation, in sperm and the impact of change seems to be age-dependent. These markers may serve as biomarkers of sperm quality and fertility of bulls in the future. The period of NEB during the breeding season of cattle may affect fertility of bulls and even have an impact in the health and performance of offspring through changes in sperm DNA methylation which may or may not be reversible. Detrimental effects on sperm production and seminal quality are observed at periods and places when and where environmental and nutritional limitations are a year-round reality and may carry hidden players that may influence a lifetime of underperformance.

3.6. Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.7. Author contributions

Conceptualization, M.A.F and L.F.S.; methodology, M.A.F. and L. F. S.; facilities and formal experiment, F. H. M., A. M. F., C. A. P. B., E. C. A., I. M. B., and A. E. M. S.; investigation, M.A.F. and F. H. M.; resources, M.A.F. and L. F. S.; data curation, F. H. M.; writing—original draft preparation, F. H. M.; writing—review and editing, M.A.F., F. H. M., and L. F. S.; supervision, M.A.F., L. F. S., and A. B. N.; project administration, M.A.F. All authors have read and agreed to the published version of the manuscript.

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3.10. Tables

Table 3-1: Descriptive statistics analysis of scoring criteria of breeding soundness exam (BSE)

| Item ^{1*} | Mean | SD ² | Min ² | Max ² | CV ² (%) | Amp ² |
|--------------------|------|-----------------|------------------|------------------|---------------------|------------------|
| SC | 38.4 | 3.35 | 34 | 43 | 8.7 | 9 |
| SM | 75.0 | 16.83 | 55 | 95 | 22.4 | 40 |
| NS | 80.3 | 8.06 | 70 | 92 | 10.0 | 22 |

¹SC = scrotal circumference, SM = sperm motility, NS = normal spermatozoa

²SD = Standard deviation, Min = Minimum, Max = Maximum, CV = Coefficient of variation, Amp = Amplitude

*Breeding soundness exam was performed as recommended by the Society of Theriogenology (Chenoweth et al., 1993). Bulls met the physical exam requirements overall internal and external reproductive tract.

Table 3-2: Descriptive statistics analysis for adjusted temperature-humidity index (THI) during periods of body weight maintenance, body weight loss, and compensatory growth of young (n=6) and mature (n=6) crossbred Angus × Hereford breeding bulls

| Phase ¹ | Mean (THI) | SD ² | Min ² | Max ² | CV ² (%) | Amp ² | % days THI ³ Normal* | % days THI Alert* | % days THI Danger* |
|--------------------|------------|-----------------|------------------|------------------|---------------------|------------------|---------------------------------|-------------------|--------------------|
| Phase 1 | 56.9 | 3.5 | 51.6 | 63.0 | 6.2 | 11.4 | 100.0 | 0 | 0 |
| Phase 2 | 48.9 | 7.5 | 34.8 | 60.1 | 15.4 | 25.2 | 100.0 | 0 | 0 |
| Phase 3 | 49.5 | 4.9 | 36.7 | 58.8 | 9.9 | 22.05 | 100.0 | 0 | 0 |

¹Phase: 1 = BW maintenance adjustment preceding BW loss (12 days, October); 2 = BW loss (78 days, mid-October to December); 3 = Compensatory growth (90 days, January to March)

²SD = Standard deviation, Min = Minimum, Max = Maximum, CV = Coefficient of variation, Amp = Amplitude

³THI = adjusted temperature-humidity index (unitless; Meyer et al., 2006)*Thresholds cut-offs point from Livestock Weather Safety Index (LCI, 1970), THI ≤ 74 = Normal, 74 > THI ≤ 79 = Alert, 79 > THI ≤ 84 = Danger, THI > 84 = Emergency

Table 3-3: Pearson correlation specific for age between DNA 5-mC, RNA m⁶A, hormones and semen traits during periods of body weight maintenance, body weight loss, and compensatory growth of young (n=6) crossbred Angus × Hereford breeding bulls

| Item ¹ | 5-mC | m ⁶ A | IGF-1 | LEP | NS | AS | PSD | SSD | SpV | SM | SPM | TSPTZ | SV | [S] |
|-------------------|-----------------|------------------|--------------------------------|-------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|-------------------|-------------------|--------------------------------|-------------------|--------------------------------|
| Young bulls | | | | | | | | | | | | | | |
| 5-mC | 1.000 (1.00) | 0.081 (0.837) | -0.578 (0.103) [§] | -0.107 (0.783) | -0.824 (0.006) [§] | 0.824 (0.006) [§] | 0.711 (0.032) [§] | 0.661 (0.052) [§] | -0.742 (0.021) [§] | -0.275 (0.474) | -0.469 (0.202) | -0.702 (0.035) [§] | -0.469 (0.202) | -0.846 (0.004) [§] |
| m ⁶ A | - | 1.000 (1.000) | 0.345 (0.362) | 0.520 (0.151) | 0.168 (0.665) | -0.165 (0.664) | -0.264 (0.492) | 0.002 (0.996) | -0.161 (0.678) | -0.100 (0.797) | -0.101 (0.799) | 0.004 (0.992) | 0.152 (0.697) | 0.216 (0.577) |
| IGF-1 | - | - | 1.000 (1.000) | 0.493 (0.178) | 0.824 (0.006) [§] | -0.839 (0.005) [§] | -0.705 (0.034) [§] | -0.667 (0.049) [§] | 0.351 (0.353) | 0.524 (0.147) | 0.528 (0.141) | 0.146 (0.708) | 0.493 (0.177) | -0.073 (0.851) |
| LEP | - | - | - | 1.000 (1.000) | 0.297 (0.436) | -0.297 (0.436) | -0.473 (0.198) | 0.011 (0.978) | -0.050 (0.898) | 0.431 (0.247) | 0.430 (0.248) | 0.152 (0.696) | 0.369 (0.408) | 0.363 (0.337) |

¹5-mC = DNA 5-mC methylation, m⁶A = N⁶-methyladenosine methylation, IGF-1 = insulin-like growth factor 1, LEP = leptin, NS = normal spermatozoa, AS = abnormal spermatozoa, PSD = primary spermatozoa defect, SSD = secondary spermatozoa defect, SpV = sperm volume, SM = sperm motility, SPM = sperm progressive motility, TSPTZ = total sperm count, SV = sperm vigor, [S] = sperm concentration

²Young bulls (n=6), mature bulls (n=6)

[§]Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$

Table 3-4: Pearson correlation specific for age between DNA 5-mC, RNA m⁶A, hormones and semen traits during periods of body weight maintenance, body weight loss, and compensatory growth of mature (n=6) crossbred Angus × Hereford breeding bulls

| Item ¹ | 5-mC | m ⁶ A | IGF-1 | LEP | NS | AS | PSD | SSD | SpV | SM | SPM | TSPTZ | SV | [S] |
|-------------------|-----------------|------------------|-------------------|-------------------------------|-------------------------------|--------------------------------|--------------------------------|-------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|-------------------|-------------------|
| Mature bulls | | | | | | | | | | | | | | |
| 5-mC | 1.000 (1.00) | 0.011 (0.968) | -0.316 (0.233) | 0.569 (0.021) [§] | -0.250 (0.349) | 0.225 (0.401) | 0.148 (0.584) | 0.071 (0.794) | -0.172 (0.523) | -0.446 (0.083) [§] | -0.172 (0.524) | -0.300 (0.218) | -0.146 (0.589) | 0.012 (0.963) |
| m ⁶ A | - | 1.000 (1.000) | -0.077 (0.761) | -0.211 (0.401) | -0.024 (0.923) | 0.046 (0.857) | -0.134 (0.595) | 0.163 (0.517) | -0.047 (0.850) | -0.220 (0.379) | -0.229 (0.362) | -0.110 (0.665) | 0.117 (0.644) | 0.121 (0.633) |
| IGF-1 | - | - | 1.000 (1.000) | -0.179 (0.476) | 0.524 (0.025) [§] | -0.498 (0.035) [§] | -0.430 (0.075) [§] | -0.306 (0.216) | 0.542 (0.019) [§] | 0.414 (0.087) [§] | 0.402 (0.098) [§] | 0.504 (0.033) [§] | -0.019 (0.937) | 0.261 (0.294) |
| LEP | - | - | - | 1.000 (1.000) | -0.291 (0.242) | 0.258 (0.300) | 0.195 (0.436) | 0.125 (0.621) | -0.216 (0.388) | -0.309 (0.211) | -0.289 (0.245) | -0.320 (0.195) | -0.335 (0.174) | -0.328 (0.183) |

¹5-mC = DNA 5-mC methylation, m⁶A = N⁶-methyladenosine methylation, IGF-1 = insulin-like growth factor 1, LEP = leptin, NS = normal spermatozoa, AS = abnormal spermatozoa, PSD = primary spermatozoa defect, SSD = secondary spermatozoa defect, SpV = sperm volume, SM = sperm motility, SPM = sperm progressive motility, TSPTZ = total sperm count, SV = sperm vigor, [S] = sperm concentration

²Young bulls (n=6), mature bulls (n=6)

[§]Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$

3.11. Figures

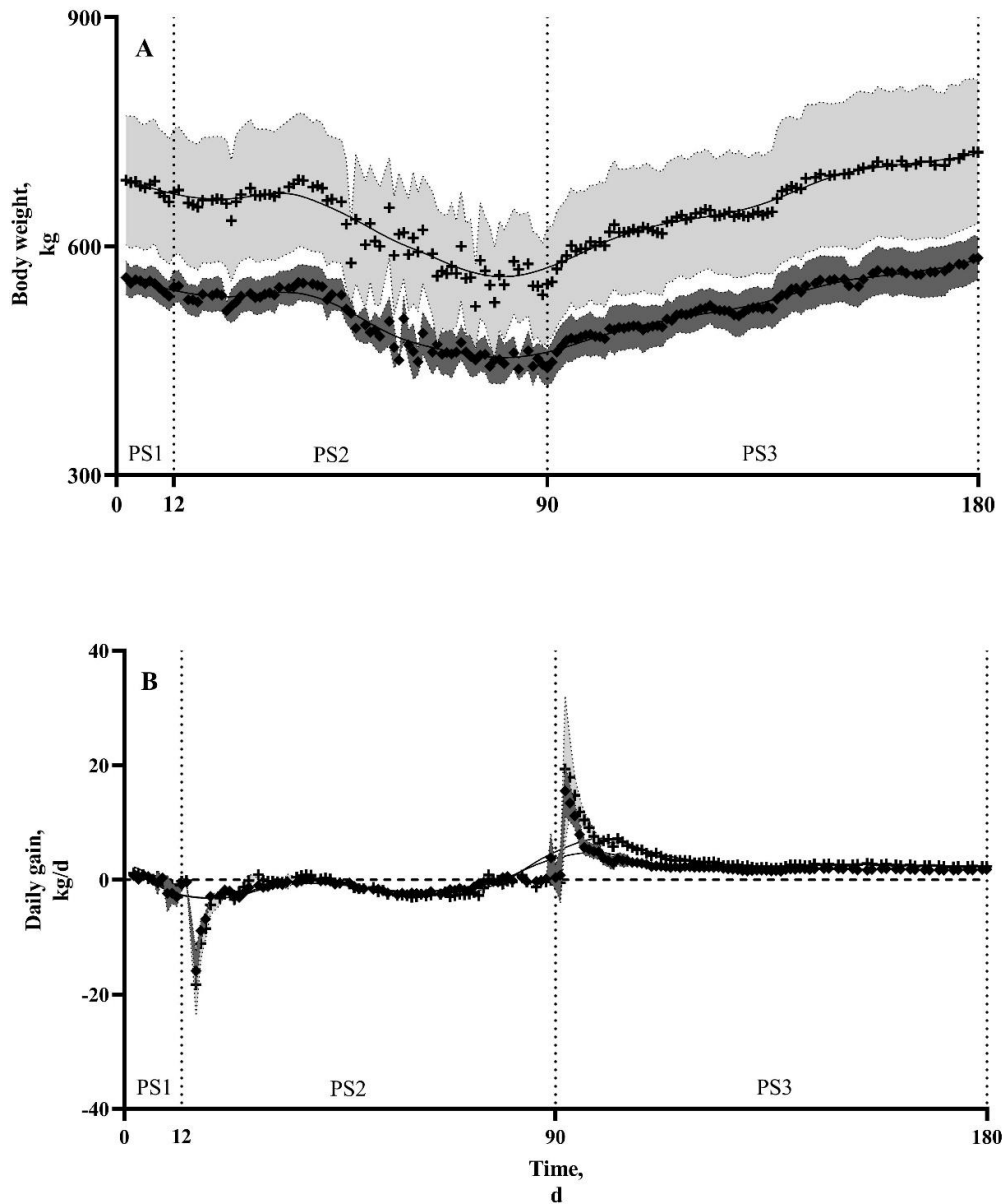


Figure 3-1: Body weight (kg, A) and daily gain (kg/d, B) changes of young ($n=6$, \blacklozenge) and mature ($n=6$, \blackplus) crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (PS1), body weight loss (PS2), and compensatory growth (PS3). Light and dark shaded area indicates 95% confidence interval.

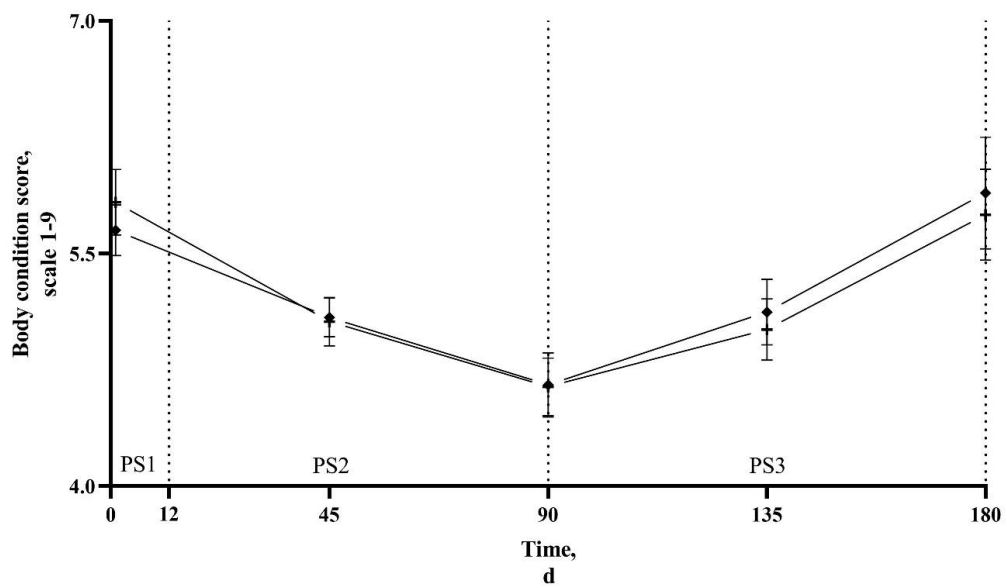


Figure 3-2: Body condition score (scale 1-9) changes of young (n=6, ◆) and mature (n=6, +) crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (PS1), body weight loss (PS2), and compensatory growth (PS3). Error bars show the standard error of the mean.

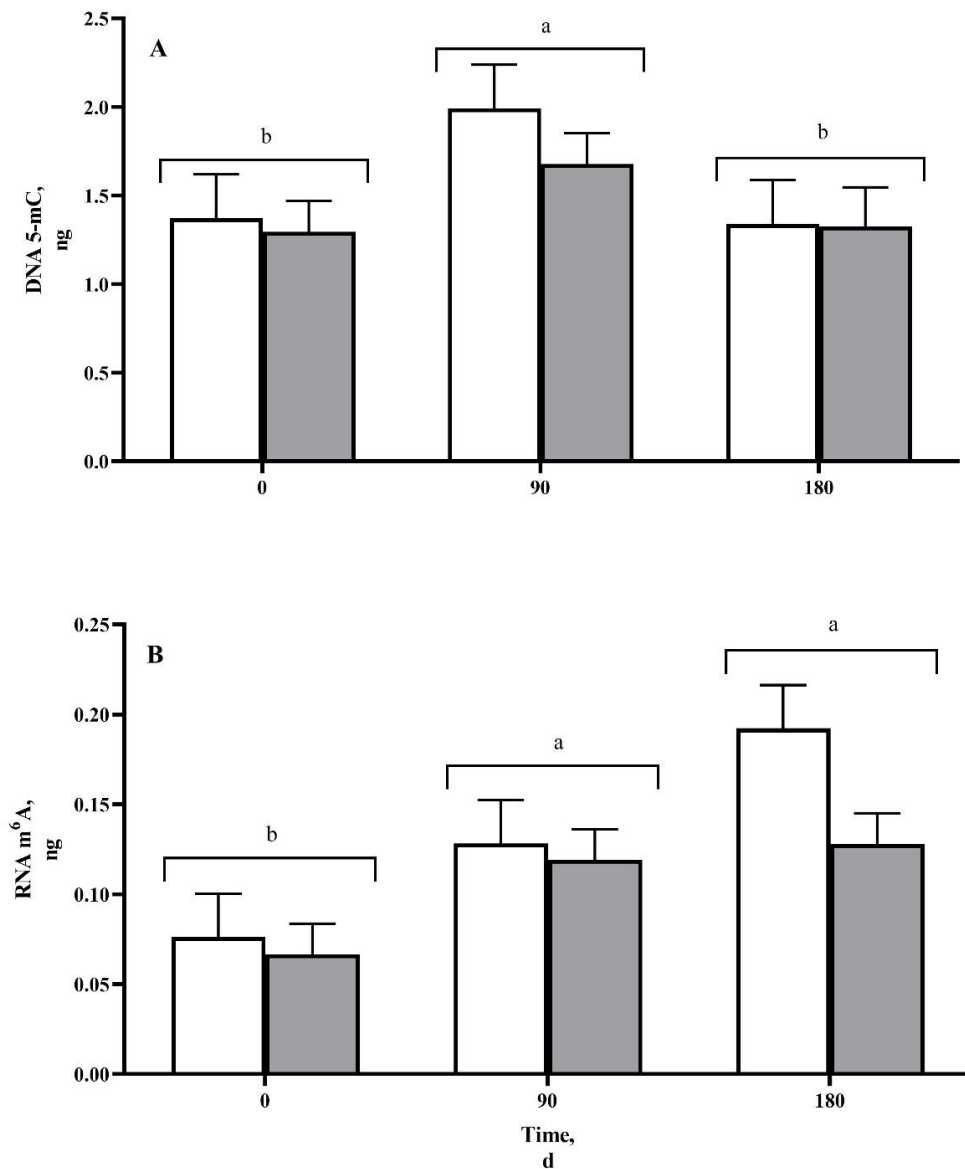


Figure 3-3: 5-methyl cytosine DNA methylation (ng, A) and N6-methyladenosine RNA methylation (ng, B) in sperm of young ($n=6$, unshaded, \square) and mature ($n=6$, shaded, \blacksquare) crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (Day = 0), body weight loss (Day = 90), and compensatory growth (Day = 180). Error bars show the standard error of the mean. Least square means followed by different letters are statistically different (RNA m^6A ; Tukey's test; $P < 0.05$) or statistical tendency (DNA 5-mC; Tukey's test; $0.05 < P \leq 0.10$).

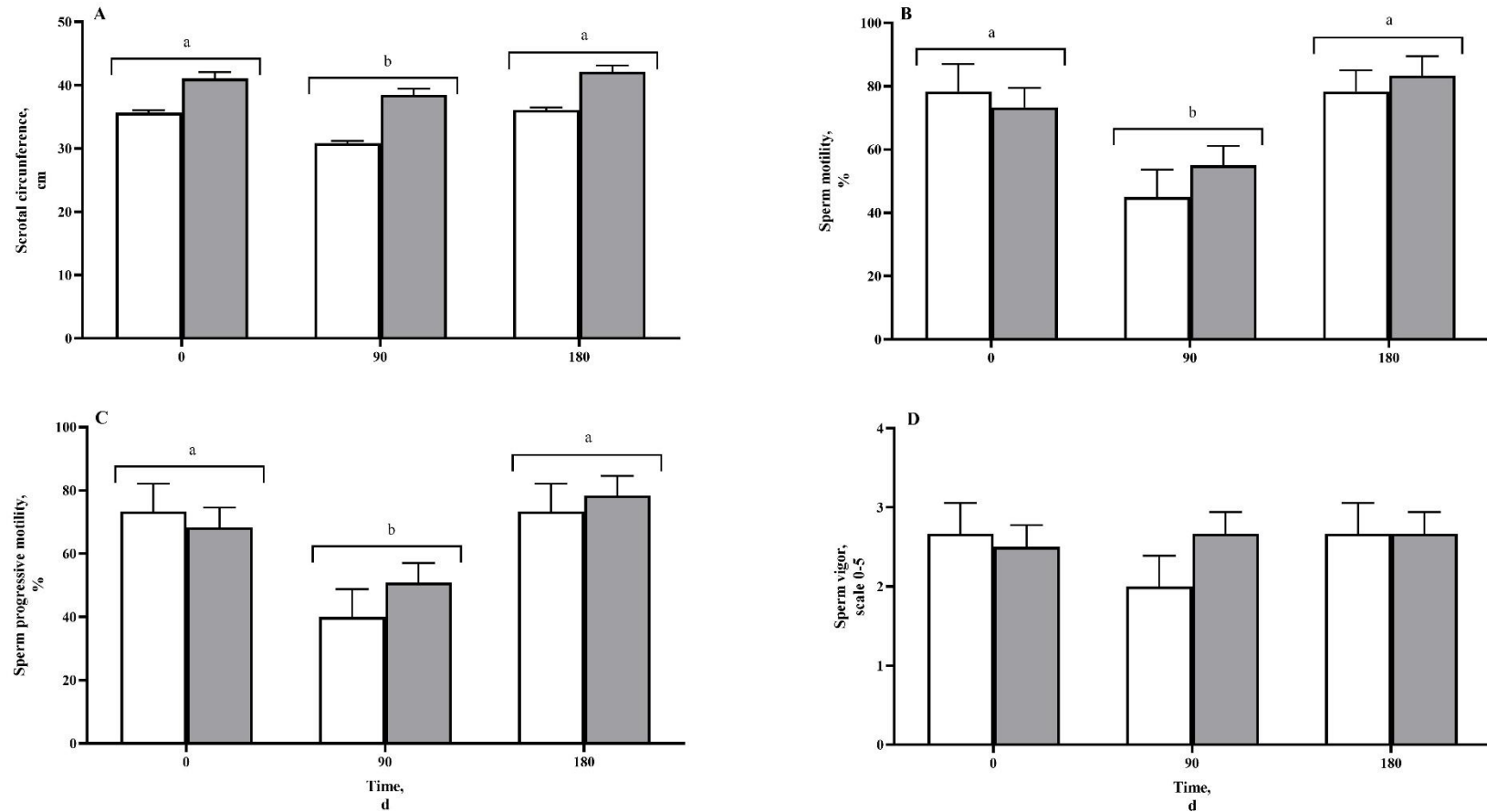


Figure 3-4: Scrotal circumference (cm, A), sperm motility (% , B), sperm progressive motility (% , C), and sperm vigor (scale 0-5, D) of young (n=6, unshaded, □) and mature (n=6, shaded, ■) crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (Day = 0), body weight loss (Day = 90), and compensatory growth (Day = 180). Error bars show the standard error of the mean. Least square means followed by different letters are statistically different (Tukey's test; $P < 0.05$).

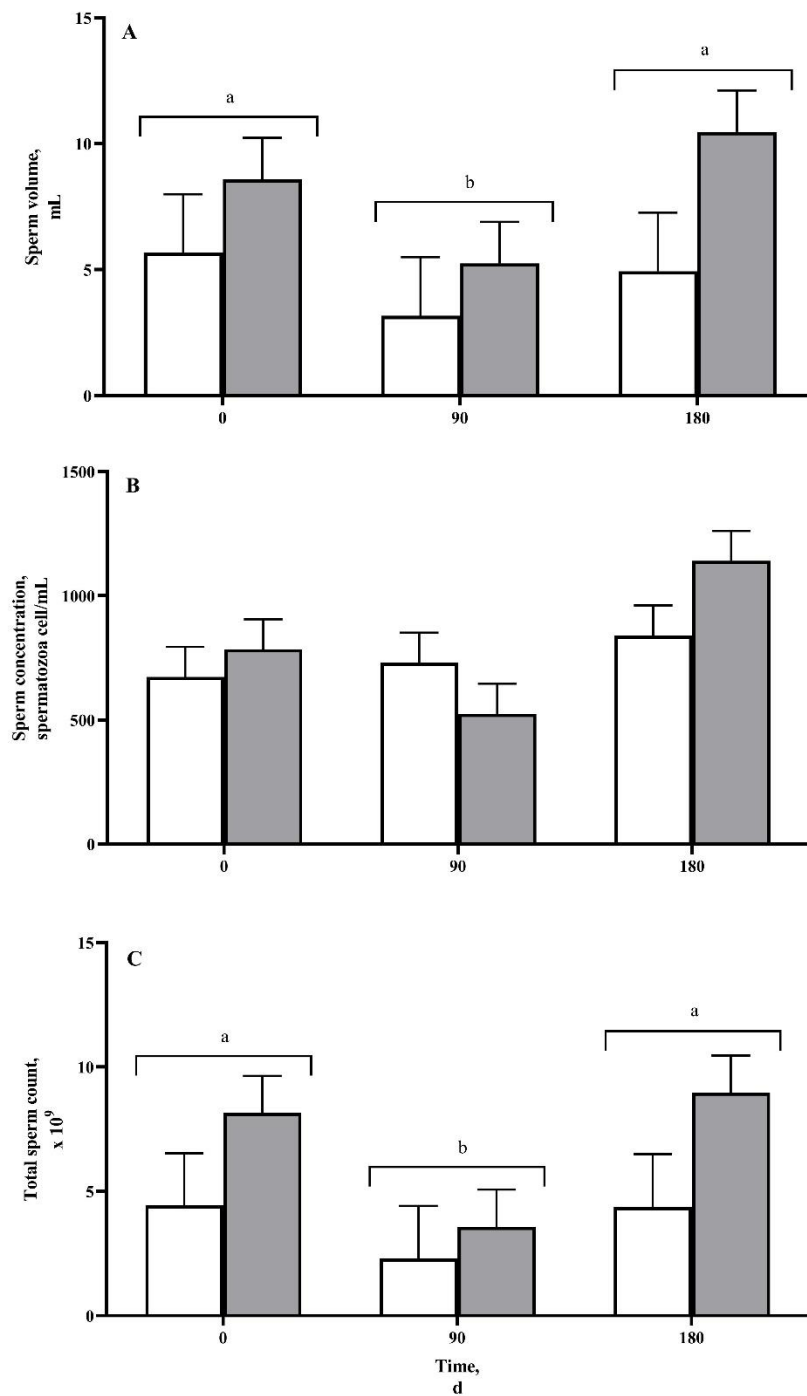


Figure 3-5: Sperm volume (mL, A), sperm concentration (spermatozoa cell/mL, B), and total sperm count ($\times 10^9$, C) of young ($n=6$, unshaded, \square) and mature ($n=6$, shaded, \blacksquare) crossbred Angus \times Hereford breeding bulls undergoing periods of body weight maintenance (Day = 0), body weight loss (Day = 90), and compensatory growth (Day = 180). Error bars show the standard error of the mean. Least square means followed by different letters are statistically different (Tukey's test; $P < 0.05$).

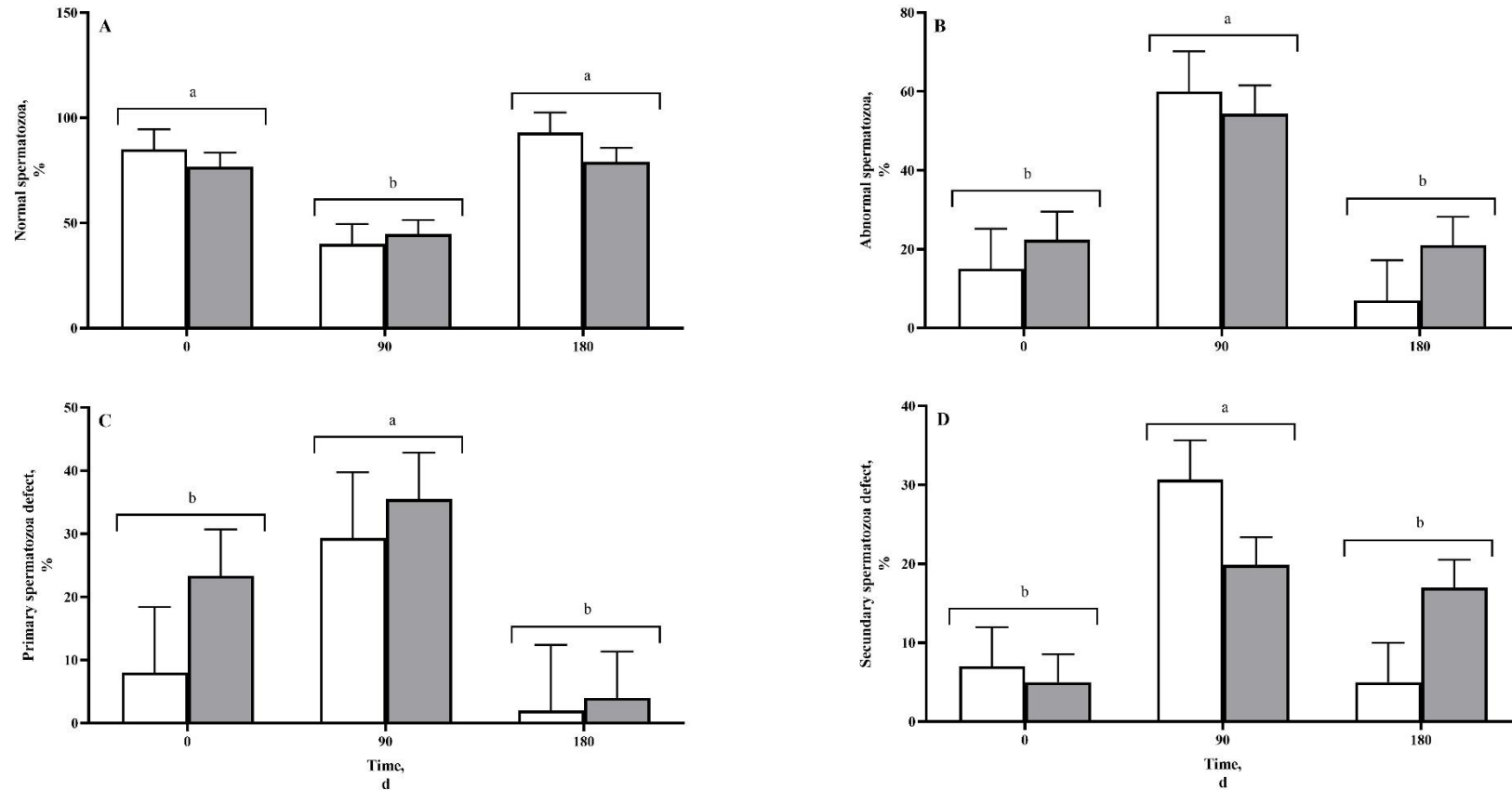


Figure 3-6: Normal spermatozoa (%), abnormal spermatozoa (%), primary spermatozoa defect (%), and secondary spermatozoa defect (%) of young (n=6, unshaded, □) and mature (n=6, shaded, ■) crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (Day = 0), body weight loss (Day = 90), and compensatory growth (Day = 180). Error bars show the standard error of the mean. Least square means followed by different letters are statistically different (Tukey's test; $P < 0.05$).

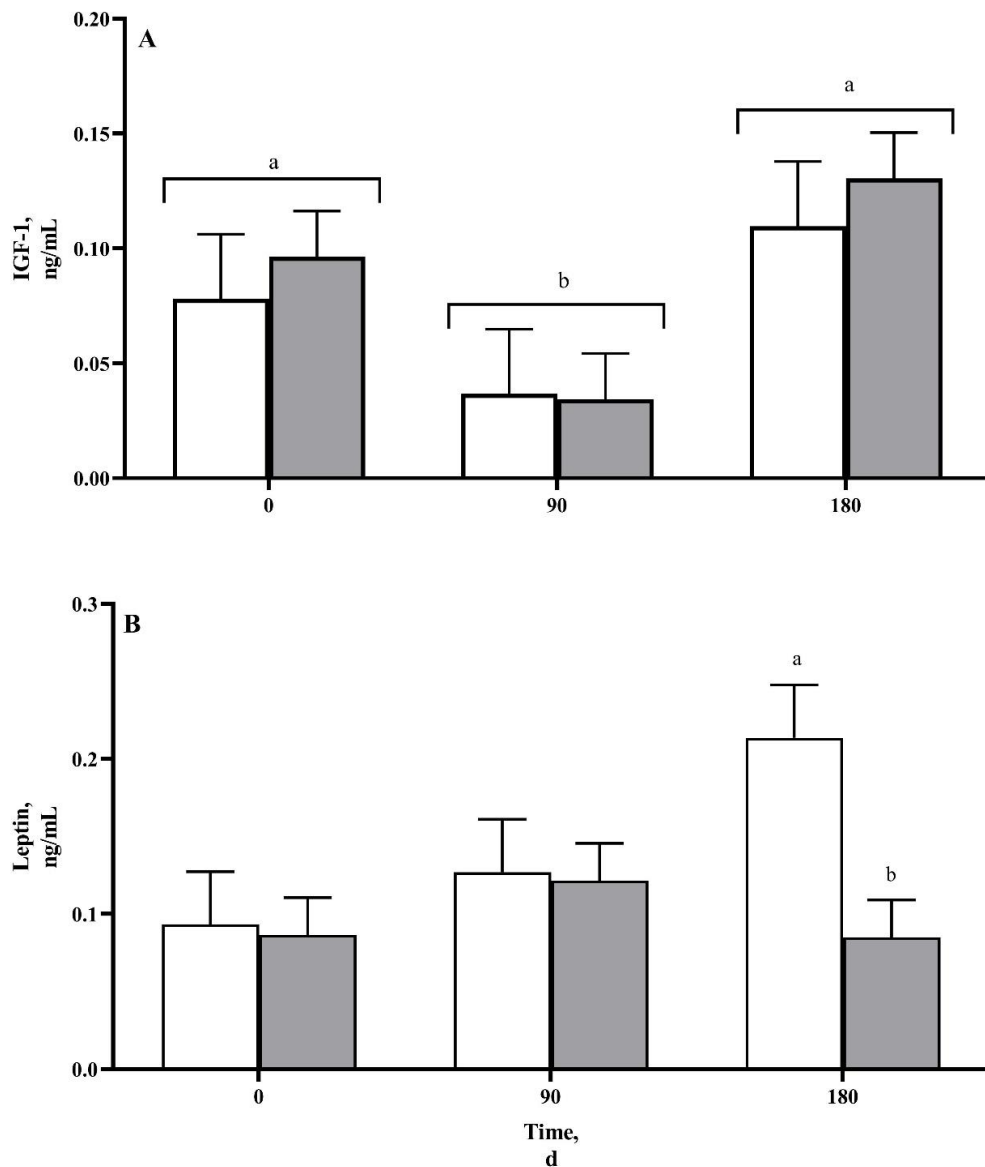


Figure 3-7: Insulin-like growth factor-1 (ng/mL, A) and leptin (ng/mL, B) concentration of young (n=6, unshaded, □) and mature (n=6, shaded, ■) crossbred Angus × Hereford breeding bulls undergoing periods of body weight maintenance (Day = 0), body weight loss (Day = 90), and compensatory growth (Day = 180) as function of the periods of sampling. Error bars show the standard error of the mean. Least square means followed by different letters are statistically different (Tukey's test; $P < 0.05$).

CHAPTER IV:**CHARACTERIZATION OF BODY COMPOSITION AND LIVER
EPIGENETIC MARKERS DURING PERIODS OF NEGATIVE ENERGY
BALANCE AND SUBSEQUENT COMPENSATORY GROWTH IN BEEF
BREEDING BULLS**

4.1. Abstract

Epigenetics is the study of heritable modifications in gene regulation without changes in the genome itself. Recently, epigenetics has been attracting attention for its association with physiology and metabolism mechanisms that can control both short and long-lasting metabolic fate. This study aimed to characterize the effects of dietary restriction and subsequent *ad libitum* feeding on body composition and hepatic gene expression of epigenetic regulators of DNA methylation, RNA m⁶A methylation, and histone acetylation in beef breeding bulls. Twelve Angus × Hereford crossbred bulls were submitted to two dietary regimes: net energy balance (NEB) for 90 days, and *ad libitum* feeding (90 days). In order to induce a metabolic stress and a full recovery to initial state, each animal acted as its own control, in a repeated measures design. Animals were fed Beardless wheat (*Triticum aestivum*) hay and mineral mix during the trial. Statistical analyses were performed using SAS 9.4 (SAS Inst., Cary, NC). Bulls undergoing negative energy balance (NEB) decreased ($P < 0.001$) of empty body weight [EBW; 23.1% (-139.1 kg)], empty body fat [EBF; 39.8% (-85.4 kg)], and empty body protein [EBP; 14.9% (-13.5 kg)]. A full recovery to initial state of EBW, EBF, and EBP was observed at the end of the *ad libitum* feeding. Body fat changes accounted for 77.1% of daily changes in body energy status, whereas body protein changes accounted for only 22.9% ($P < 0.001$). Bulls undergoing NEB tended ($P \leq 0.097$) to have increased gene expression of epigenetic regulators of RNA m⁶A methylation (*METTL14*, *VIRMA*, and *WTAP*), increased ($P \leq 0.050$) gene expression of epigenetic regulators of DNA methylation (*DNMT3A*) and histone-acetylation (*SIRT3* and *SIRT7*). Growing bulls had a tendency ($P \leq 0.072$) of higher RNA m⁶A methylation, *VIRMA*, and *WTAP* than mature bulls. Effect of diet × age interaction

was not detected ($P \geq 0.137$) for *METTL14*, *VIRMA*, *WTAP*, *DNMT3A*, *SIRT3* or *SIRT7*. Growing bulls tended to have greater RNA m⁶A methylation levels than mature bulls, indicating that, while contemporaneously fed the same diet during periods of undernourishment followed by compensatory growth, age has an impact on this epigenetic mechanism. In conclusion, metabolic status seems to carry a greater impact on regulating bovine hepatic epigenetic mechanisms that modulate gene transcription, such as DNA methylation and histone acetylation, than on epigenetic mechanisms that regulate gene translation, such as RNA m⁶A methylation. During periods of undernourishment followed by compensatory growth, body fat appears to have a greater impact on epigenetic markers that modulate hepatic gene transcription.

Key words: body composition, DNA methyltransferases, DNA demethylases, RNA m⁶A methylation, sirtuins

4.2. Introduction

Compensatory growth, an accelerated growth phenomenon caused by some extent of nutrient restriction and subsequent re-alimentation, is an important component of many beef production systems, particularly in pastoral and rangeland systems where animals are subjected to a seasonal pasture availability (Drouillard et al., 1991; Ashfield et al., 2013). Studies have shown that compensatory growth acts as a metabolic adaptation involved in the regulation of the major pathways of intermediary metabolism, particularly in the liver, which is the central organ maintaining metabolic homeostasis during the shifts of fasting-refeeding periods (Geisler et al., 2016). Thus, the liver is a well-accepted target tissue to study the

potential impact of metabolic status on epigenetic markers that modulate gene transcription and translation.

Epigenetics refers to heritable changes in gene regulation without changes in the DNA sequence itself (Wolffe and Matzke, 1999). Well-established epigenetic mechanisms include DNA methylation and histone post-translational modifications (Tammen et al., 2013). DNA methylation is known to regulate gene expression by affecting transcription rates and is regulated by DNA methyltransferases (*DNMTs*), which catalyze DNA methylation, and ten-eleven translocation enzymes (*TETs*), which catalyze DNA demethylation (Kohli and Zhang, 2013). Histone acetylation, regulated by histone acetyltransferases and histone deacetylases, is a histone modification that regulates gene transcription (Kuo and Allis, 1998). Recently, the methylation of the N6 position of adenosine (m⁶A), another epigenetic mechanism, has drawn attention for its potential to regulate several physiological processes (Liu and Jia, 2014; Chandola et al., 2015). The m⁶A methylation of RNA is proposed to alter several processes associated with mRNA processing, including mRNA stability, mRNA decay, mRNA nuclear export, and translation initiation (Schwartz et al., 2014; Wang et al., 2014; Wang et al., 2015). Regulators of m⁶A methylation of RNA include enzymes that are part of a methyltransferase complex and enzymes that catalyze m⁶A demethylation (Roignant and Soller, 2017). Interestingly, various epigenetic mechanisms have been correlated with development of diseases in the liver, including DNA methylation (Zhu, 2005; Bian et al., 2013), histone acetylation (Yuan et al., 2011), and RNA m⁶A methylation (Zhao et al., 2020; Li et al., 2021).

Recent literature has focused on understanding how the metabolic pathways of the liver may shift or how cellular adaptations allow animals to withstand physiological changes brought upon by periods of fasting-refeeding transition

(Ramalingam et al., 2017; Bideyan et al., 2021). Hitherto, the literature lacks studies that characterize changes in hepatic epigenetic markers during periods of negative energy balance (NEB) and compensatory growth in beef cattle. Therefore, this study aimed to characterize the effects of dietary restriction and subsequent *ad libitum* feeding on body composition and hepatic gene expression of epigenetic regulators of DNA methylation, RNA m⁶A methylation, and histone-acetylation proteins in beef breeding bulls.

4.3. Material and Methods

The animals used in this experiment were cared for according to guidelines approved by the Institutional Animal Care and Use Committee University of Nevada, Reno (protocol #00738).

Animal, treatment, and experimental area

The dataset was obtained from twelve Angus × Hereford crossbred breeding bulls [n = 6, 23±0.55 mo (growing bulls), 558±6.1 kg; and n = 6, 47±1.2 mo (mature bulls), 740±30.5 kg] over a period of 180 d. Animals were housed at the Main Station Research Feedlot Facility at the Nevada Agricultural Experiment Station in Reno, NV. Bulls had free access to water and trace mineral salt during the whole trial. Beardless wheat (*Triticum aestivum*; 945 g/kg dry matter, 579 g/kg total digestible nutrients; 86 g/kg crude protein) hay was offered daily.

Two dietary regimes were offered to the bulls during the experimental period. Bulls were fed targeting gradual body weight (BW) maintenance adjustment for 90 d (Phase 1: negative energy balance, NEB, with BW loss targeted for 0.6 kg/d); and subsequently, a recovery period for 90 d with BW gain targeted for 1 kg/d following

the BW loss period (Phase 2: *ad libitum* feeding, compensatory growth). In order to induce a metabolic stress and a full recovery to initial state, each animal acted as its own control, in a repeated measures design.

The animals were randomly assigned to one of two pens (15 × 28 m). Each pen contained 30 m² of shaded area and four automated scales that recorded body weight changes daily (ASMS; Model WD-1000 Master, Intergado Ltd, Contagem, Minas Gerais, Brazil). Prior to the beginning the trial, each animal was fitted with an electronic identification ear tag (FDX-ISO 11784/11785; Allflex, Joinville, Santa Catarina, Brazil). Due to the dimensions of the scale, only one bull was allowed at the time into one individual scale when accessing the water troughs. The BW data were continuously recorded, transferred, and stored in the cloud for further analysis. The empty body weight (EBW, kg) was calculated according to (NASEM, 2016). Body composition: empty body fat (EBF, kg), empty body protein (EBP, kg), daily changes on body energy (dailyBE, g/d), daily changes on body fat (dailyEBF, g/d), and daily changes on body protein (dailyEBP, g/d) contents were estimated according to (Tedeschi, 2019).

Hepatic tissue biopsy

Biopsies of hepatic tissues were performed at 90 and 180 d of the trial. Liver sampling was performed via needle biopsy (Tru-Cut biopsy needle; Care Fusion Corporation, San Diego, CA) 4 h before supplement feeding according to the procedures described by Molgaard et al. (2012). The incision was made between the 11th and 12th ribs for collection of samples from the right hepatic lobe (Miranda et al., 2010). Immediately, three liver samples (100 mg of tissue) were placed in cryotubes, frozen and stored in liquid nitrogen at -196°C until processing.

RNA extraction and N6-Methyladenosine assessment

Total RNA was extracted from hepatic samples via E.Z.N.A. micro RNA kit (Omega bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. Samples of RNA were solubilized in RNase- and DNase-free molecular grade water (Invitrogen, Carlsbad, CA, USA), quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and stored at -80°C for further analyses. Global RNA m⁶A methylation levels were colorimetrically quantified in RNA of liver tissue samples via the Epiquik m⁶A RNA Methylation Quantification Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions using a microplate reader (SpectraMax M2e; Molecular devices, LLC, San Jose, CA) and analyzed through the SoftMax Pro software (Molecular devices, LLC, San Jose, CA). Briefly, 200 ng of pure RNA samples (260/280 ratio > 2.0) were added to each well (2 replicates per sample), plus two replicates per negative control (0 ng/uL), plus two replicates per positive controls (0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 ng/uL) and absorbance was read at 450 nm. The intra-assay coefficient of variation averaged 7.59%. The cutoff limit to ensure consistent results between replicate was an intra-assay coefficient of variation of 10.0%.

Quantitative polymerase-chain reaction (qPCR)

For analysis of relative gene expression, 0.5 μg of RNA were used to generate single-stranded cDNA through High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), according to manufacturer's instructions. Relative mRNA abundance of target genes was quantified through qPCR with a

CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) using SYBR select Master Mix (Applied Biosystems, Waltham, MA). Target genes included: regulators of RNA m⁶A methylation, such as components of the methyltransferase complex: methyltransferase like 3 (*METTL3*), methyltransferase like 14 (*METTL14*), ribonucleic acid binding motif protein 15 (*RBM15*), vir like m⁶A methyltransferase associated (*VIRMA*), WT1 associated protein (*WTAP*); and demethylases: alpha-ketoglutarate dependent dioxygenase (*FTO*) and alkB homolog 5 (*ALKBH5*); regulators of DNA methylation, such as DNMTs [DNA methyltransferase 1 (*DNMT1*), DNA methyltransferase 3 alpha (*DNMT3A*), and DNA methyltransferase 3 beta (*DNMT3B*)] and TETs [methylcytosine dioxygenase 1 (*TET1*), methylcytosine dioxygenase 2 (*TET2*), and methylcytosine dioxygenase 3 (*TET3*)]; and sirtuins (*SIRT*s 1-7), which are class III histone deacetylases (Allis and Jenuwein, 2016). The thermocycling protocols were: 95°C for 3 minutes (1 cycle), 95°C for 10 seconds followed by 55°C for 30 seconds (40 cycles) for *METTL3*, *METTL14*, *RBM15*, *VIRMA*, *WTAP*, *ALKBH5*; and 95°C for 3 minutes (1 cycle), 95°C for 10 seconds followed by 60°C for 30 seconds (40 cycles) for *FTO*. Expression levels of target genes were normalized to expression levels of 18S ribosomal RNA (*18S*). Relative abundance of mRNA was calculated using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001) and expressed as fold change relative to growing bulls under *ad libitum* feeding. The primers for target genes were designed using the primer Express program (Applied Biosystems, Waltham, MA). The primers' sequences and amplicon sizes are provided in Table 4-1 and 4-2.

Statistical analyses

Data were collected and analyzed following a pre-post repeated measure design (Burgos et al., 2001). The statistical model used is shown below:

$$Y_{ij} = \mu + T_i + A_j + b_j + T_i \times A_j + \varepsilon_{ij}$$

Where Y_{ij} is the observation taken (EBW, EBF, EBP, dailyBE, dailyEBF, dailyEBP, RNA m⁶A methylation, and gene expression data) on the j_{th} experimental unit for the pre-post i_{th} treatment, μ is the overall mean, T_i is the effect of the i_{th} treatment, A_j is the effect of the j_{th} age, b_j is the effect of the j_{th} experimental unit, $T_i \times A_j$ is the interaction effect of the i_{th} treatment and the j_{th} age, and ε_{ij} is the unobservable random error on the j_{th} experimental unit associated with each i_{th} pre-post treatment.

Statistical analyses were performed using SAS (ver. 9.4, SAS Inst. Inc., Cary, NC). Data were analyzed using PROC MIXED procedures of SAS. Pearson correlation coefficients among variables were obtained with PROC CORR. All figures were generated by GraphPad Prism (ver. 9.0, GraphPad Inc., San Diego, CA). Outliers were tested by plotting the Studentized residuals and data points were removed if the Studentized residual was outside the range of -2.5 to 2.5 . Normality assumption was tested using Shapiro-Wilk's test and homogeneity of variance was evaluated through Levene's test. Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$ using Tukey's post hoc test. Because of negative and positive data, values for dailyBE, dailyEBF, and dailyEBP were transformed via log scale ($\log_{10}(y + 1 - \text{minimum}(y))$) for statistical analysis, and then means were back-transformed to the original scale for presentation.

4.4.Results

Body composition

The pattern of empty body fat and empty body protein over time change is shown in Figure 4-1. Body composition parameters: empty body weight (EBW), empty body fat (EBF), and empty body protein (EBP), are shown in Table 4-3 and Figure 4-2. When bulls experienced NEB, they decreased ($P < 0.001$) 23.1% (-139.1 kg), 39.8% (-85.4 kg), and 14.9% (-13.5 kg) of their EBW, EBF, and EBP, respectively. A full recovery to initial pool of EBW, EBF, and EBP was observed at the end of the *ad libitum* feeding phase. Young bulls had lower ($P \leq 0.008$) EBW, EBF, and EBP than mature bulls. No significant ($P \geq 0.112$) time \times age interaction was observed for EBW, EBF, nor EBP.

Changes on dailyBE, dailyEBF, and dailyEBP are shown in Table 4-3 and Figure 4-3. Bulls in NEB lost 2.0 Mcal/d of body energy, whereas under compensatory growth, they increased 1.6 Mcal of retained body energy per d ($P < 0.001$). Assuming heat of combustion of fat as 9.367 Mcal/kg and protein as 5.686 Mcal/kg (Garrett et al., 1959; Tedeschi et al., 2018), 77.1 % of daily body energy pool was changed via mobilization of fat (-161.1 g/d; $P < 0.001$) and only 22.9% (-78.9 g/d; $P < 0.001$) via mobilization of protein. The recovery in body energy pool during *ad libitum* feeding followed similar aforementioned pattern, which 77.6% was via body fat (135.9 g/d; $P < 0.001$) and 22.4% via body protein (64.7 g/d; $P < 0.001$). Effect of age was not detected ($P \geq 0.134$) for daily changes on body energy, body fat, and body protein. Effect of time \times age interaction was detected ($P \leq 0.030$) for daily changes on body energy and EBF. No significant ($P = 0.195$) time \times age interaction was observed for changes in EBP.

N6-methyladenosine (RNA m⁶A) methylation

Growing bulls had a tendency (Figure 4-4, $P = 0.072$) of higher RNA m⁶A methylation than mature bulls. There were no differences in RNA m⁶A methylation abundance in response to dietary level ($P = 0.683$) or for diet \times age interaction ($P = 0.902$).

Gene expression of components of the methyltransferase complex

Bulls undergoing NEB tended to have a greater gene expression of *METTL14* (Figure 4-5, $P = 0.097$), *VIRMA* ($P = 0.095$), and *WTAP* ($P = 0.094$). Furthermore, growing bulls had a tendency of higher gene expression of *VIRMA* ($P = 0.066$) and *WTAP* ($P = 0.066$) than mature bulls. In contrast, there were no effects of age on relative mRNA abundance of *METTL14* ($P = 0.747$). Effect of diet \times age interaction was not detected ($P \geq 0.137$) for *METTL14*, *VIRMA*, or *WTAP*. Lastly, there were no effects ($P \geq 0.335$) of diet, age, and diet \times age interaction on relative mRNA abundance of *METTL3* and *RBM15*.

Gene expression of RNA m⁶A demethylases

There were no differences (Figure 4-6) in gene expression of *ALKBH5* and *FTO* for dietary level ($P \geq 0.203$), age ($P \geq 0.257$) or for diet \times age interaction ($P \geq 0.129$).

Gene expression of sirtuins

Gene expression of sirtuins are shown in Figure 4-7 and Figure 4-8. Bulls in NEB increased gene expression of *SIRT3* ($P = 0.050$) and *SIRT7* ($P = 0.046$) by 1.50-fold change compared to compensatory growth period. Effects of age ($P \geq 0.113$) and diet \times age interaction ($P \geq 0.267$) were not detected for *SIRT3* and *SIRT7*. There were

no differences in gene expression of *SIRT1*, *SIRT2*, *SIRT4*, *SIRT5*, and *SIRT6* for dietary level ($P \geq 0.299$), age ($P \geq 0.197$) or for diet \times age ($P \geq 0.690$).

Gene expression of regulators of DNA methylation

Bulls in NEB tended to increase gene expression of *DNMT3A* (Figure 4-9; $P = 0.069$) by 2.0-fold change compared to compensatory period. Effects of age ($P = 0.761$) and diet \times age interaction ($P = 0.245$) were not detected on *DNMT3A*. There was no difference in gene expression of *DNMT1* for dietary level ($P \geq 0.782$), age ($P \geq 0.572$) or for diet \times age interaction ($P \geq 0.677$). In the current study, *DNMT3B* mRNA levels were below detectable in liver samples. There were no differences in gene expression of *TET1*, *TET2*, and *TET3* for dietary level (Figure 4-10; $P \geq 0.312$), age ($P \geq 0.294$) or for diet \times age interaction ($P \geq 0.167$).

Pearson correlation analyses

Pearson correlation specific for animal age is presented in Table 4-4 (young bulls) and Table 4-5 (mature bulls).

Specific for mature bulls, dailyBE tended to be negatively correlated with *SIRT3* (-0.652 , $P = 0.056$). For young bulls, dailyBE was positively correlated with *ALKBH5* (0.650 , $P = 0.042$), negatively correlated with *SIRT3* (-0.629 , $P = 0.050$) and *DNMT3A* (-0.669 , $P = 0.035$), and tended to be negatively correlated with *METTL14* (-0.626 , $P = 0.053$), *SIRT7* (-0.583 , $P = 0.077$), and *TET2* (-0.625 , $P = 0.053$).

Changes in dailyEBF tended to be negatively correlated with *SIRT3* (-0.567 , $P = 0.063$) for mature bulls. For young bulls, dailyEBF was positively correlated with *ALKBH5* (0.664 , $P = 0.036$), negatively correlated with *METTL14* (-0.631 , $P =$

0.050) and *DNMT3A* (-0.674 , $P = 0.033$), and tended to be negatively correlated with *SIRT3* (-0.624 , $P = 0.054$), *SIRT7* (-0.582 , $P = 0.078$), and *TET2* (-0.623 , $P = 0.055$).

Specific for young bulls, changes in dailyEBP were negatively correlated with *SIRT3* (-0.641 , $P = 0.046$) and *DNMT3A* (-0.652 , $P = 0.041$), and tended to be positively correlated with *ALKBH5* (0.611 , $P = 0.061$), and negatively correlated with *METTL14* (-0.612 , $P = 0.060$), *SIRT7* (-0.583 , $P = 0.077$) and *TET2* (-0.630 , $P = 0.051$). For mature bulls, dailyEBP had no significant correlation ($P \geq 0.05$) with any parameter measured.

4.5. Discussion

The liver plays a key-role in regulating blood glucose levels, processing of dietary nutrients, and regulating whole-body energy metabolism during periods of NEB and subsequent compensatory growth (Drouillard et al., 1991; Trefts et al., 2017; Bideyan et al., 2021). Transcriptional regulation is fundamental to the execution of each these physiological responses (Keogh et al., 2018). Nevertheless, information about the impact of these drastic dietary changes on the epigenome of bovine liver is limited. The present study aimed to unveil the effects of dietary restriction and subsequent *ad libitum* feeding on body composition and on gene expression of key epigenetic regulators in the bovine liver, in attempting to understand how animals would fully recover from periods of nutritionally-induced metabolic stress and whether or not these would carry future consequences to the liver as a metabolic power house.

As animals lose BW and enter a more intense catabolic state, it appears that body energy lost as fat, is three times more than as body protein. In part, the loss of

adipose tissue may be a result of the oxidation of fat to support maintenance requirements (NASEM, 2016). Furthermore, because fat has 1.65 times more energy than protein (9.367 vs. 5.686 Mcal/kg) our findings suggest that fat mobilization has preference over protein mobilization during feed restriction, which seem to be in agreement to what Barboza et al. (2020) found in their work. Notwithstanding, after a period of compensatory growth following the NEB, our animals achieved similar body composition to initial day of trial. It appears that during fasting-refeeding periods, the shifts in body tissue pool would have different epigenetic footprints. For instance, the shifts in body fat pool would have a greater impact on metabolic status of epigenetic markers that could modulate hepatic gene transcription and translation. The strong inverse correlation of *SIRT3* (age-related or not) with body changes in energy status and fat, support our previous hypothesis about shifts in body tissue pool would have different epigenetic footprints. Recent data indicate that hepatic *SIRT3* modulates intermediary metabolism and fatty-acid oxidation (Wang et al., 2019). Interestingly, we found that only for young bulls, changes to body protein pools were correlated with the evaluated epigenetic markers that modulate hepatic gene transcription. In part, this intrinsic difference may be attributed to a higher deposition of protein tissue in younger animals (Tedeschi, 2019), and then, as the animal grows, fat deposition will account for the majority of body energy pool overall.

The m⁶A methylation of RNA is proposed to alter several mechanisms associated with processing of transcripts to control gene expression (Schwartz et al., 2014; Wang et al., 2014; Wang et al., 2015). In our study, RNA m⁶A levels were similar between NEB and compensatory growth period, which indicates that m⁶A methylation of RNA may not be the main regulator of transcriptional response in cattle undergoing similar metabolic stress. Indeed, the role of this newly emerging

layer of gene expression control and behavior in response to metabolic stressors remains to be fully understood (Engel and Chen, 2018; Engel et al., 2018).

Interestingly, growing bulls tended to have greater RNA m⁶A methylation levels than mature bulls, indicating that age is more impactful to this epigenetic mechanism than dietary changes. Since the levels of RNA m⁶A methylation are regulated by a balance between the actions of methyltransferases and demethylases (Fu et al., 2014; Liu et al., 2014; Wang et al., 2015), our data suggest that *VIRMA* and *WTAP* are driving the tendency for differences observed herein. Recent studies using high-throughput RNA sequencing technology have revealed that the expression of mammalian mRNAs change extensively during the aging process (White et al., 2015; Baumgart et al., 2016). Thus, our results support the idea that the genome may change its ability to effectively regulate gene expression in the liver as animals age (Ono and Cluter, 1978; White et al., 2015). Both the former and the latter, may play an important role in adaptability to environmentally-challenged areas such as those in the Western United States, markedly used in cow/calf operations.

Sirtuins are NAD⁺-dependent enzymes, hence a potential target as a biosensor of nutritional status in many organisms (Etchegaray and Mostoslavsky, 2016). For instance, *SIRT3*, a mitochondrial enzyme, regulates multiple metabolic components of the citric acid cycle, urea cycle, fatty acid oxidation, and oxidative phosphorylation (Zhang et al., 2020). Our results revealed that bulls undergoing NEB increase gene expression of *SIRT3*. Thus, our findings suggest that *SIRT3* is an important histone deacetylase activated by undernourishment, shifting metabolic processes to achieve a desirable homeostatic outcome for the nutrient-deprived bovine. Since previous studies have demonstrated that *SIRT3* enhances hepatic urea cycle (Hallows et al., 2011; Keshet et al., 2018) and nitrogen recycling is the main biological mechanism

for ruminant survival (Van Soest, 2018), it is possible that undernourished animals may shift urea recycling to meet ruminal nitrogen needs through *SIRT3*. Likewise, findings of the present study showed that bulls undergoing NEB have higher gene expression of *SIRT7*, a nucleolar protein that modulates cellular processes for organism survival (Kiran et al., 2015). Additionally, *SIRT7* is known to play a major role in regulating ribosomal nucleic acid (rRNA) and protein synthesis during periods of low metabolic energy state by shutting down gene transcription (Chen et al., 2013; Kiran et al., 2015). These reductions in rRNA and protein synthesis may act as an important mechanism to conserve energy in nutrient-deprived animals (Chen et al., 2013; Kiran et al., 2015). Our findings indicate a potential mechanism through which *SIRT7* helps cattle maintain energy homeostasis under periods of nutrient restriction.

The nutritional effects on the epigenome, especially during long-term dietary transitions, have been thoroughly discussed in the scientific literature (Jimenez-Chillaron et al., 2012; Parrillo et al., 2019). Our results added to the scientific scope and revealed that bulls undergoing NEB tend to increase gene expression of *DNMT3A*. Given that *DNMT3A* catalyzes DNA methylation (Hervouet et al., 2009; Zhang et al., 2018; Kim et al., 2020), an important epigenetic mechanism that represses gene expression, our findings indicate that hepatic genes may have been repressed in bulls undergoing NEB. Such mechanisms indicate how cattle under NEB would be driving the hepatic transcriptional machinery to focus only on genes required for a given challenging physiological state. Taken together, our findings reveal an interplay between regulators of modifiers of chromatin and transcripts in response to drastic changes in nutrient requirements. While regulators of DNA methylation (e.g., *DNMT3A*) act to silence genes whose activity are not required under NEB, histone-acetylation proteins operate to shut down DNA transcription

(e.g., *SIRT7*) of genes that are not required (Chen et al., 2013) and to activate (e.g., *SIRT3*) mitochondrial enzymes involved in fatty acid β -oxidation, amino acid metabolism, and electron transport chain which are required during periods of energy restriction (Ahmad et al., 2021).

In conclusion, metabolic status seems to carry a greater impact on regulating bovine hepatic epigenetic mechanisms that modulate gene transcription, such as DNA methylation and histone acetylation, than on epigenetic mechanisms that regulate gene translation, such as RNA m⁶A methylation. During periods of undernourishment followed by compensatory growth, body fat appears to have a greater impact on epigenetic markers that modulate hepatic gene transcription.

4.6. Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.7. Autor contributions

Conceptualization, M.A.F and L.F.S.; methodology, M.A.F. and L. F. S.; facilities and formal experiment, F. H. M., A. M. F., C. A. P. B., E. C. A., I. M. B., and A. E. M. S.; investigation, M.A.F., L. F. S., and F. H. M.; resources, M.A.F., and L. F. S.; data curation, F. H. M.; writing—original draft preparation, F. H. M.; writing—review and editing, M.A.F., F. H. M., and L. F. S.; supervision, M.A.F., L. F. S., and A. B. N.; project administration, M.A.F. All authors have read and agreed to the published version of the manuscript.

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4.10. Tables

Table 4-1: Primer sequences for gene transcripts analyzed by quantitative real-time reverse transcription polymerase chain reaction (qPCR)

| Gene ¹ | NCBI reference sequence | Primer design ² | Primer sequence | TM ³ (°C) |
|----------------------------------------------|-------------------------|----------------------------|--------------------------------|----------------------|
| <i>Gene control</i> | | | | |
| <i>18S</i> | NM_001033614.2 | FWD | 5'-CACCAGACTTGCCCTCCA-3' | 55-60 |
| | | REV | 5'-AGAAACGGTTACCACATCCA-3' | |
| <i>m⁶A RNA methyltransferases</i> | | | | |
| <i>METTL3</i> | NM_001102238.2 | FWD | 5'-GCAACCCAACTGGATCACCC-3' | 55 |
| | | REV | 5'-TGAACCCGGCAACCACATCT-3' | |
| <i>METTL14</i> | NM_001083714.1 | FWD | 5'-TTGCAGGAGATCCGAGAGCG-3' | 55 |
| | | REV | 5'-CACTTTCAGCTCCCAACTGCTG-3' | |
| <i>RBM15</i> | XM_010803256.3 | FWD | 5'-CTGGCATGGGCACTTGTCCT-3' | 55 |
| | | REV | 5'-TTCTCCTTCCCTTTCCTTTGT-3' | |
| <i>VIRMA</i> | NM_001205426.1 | FWD | 5'-AAACATGGCGGTGGACTCGG-3' | 55 |
| | | REV | 5'-ATGAGAACTTTGCTCGGCGCT-3' | |
| <i>WTAP</i> | NM_001113254.2 | FWD | 5'-GGAGCAAGAAATGCAAGAGTGAC-3' | 55 |
| | | REV | 5'-CCACCATTGCTGATCTCAGTTG-3' | |
| <i>m⁶A RNA demethylases</i> | | | | |
| <i>ALKBH5</i> | NM_001205517.3 | FWD | 5'-TCAAGCCTATCCGGGTGTCG-3' | 55 |
| | | REV | 5'-CAGCAGCGTATCCACTGAGCA-3' | |
| <i>FTO</i> | NM_001098142.1 | FWD | 5'-GCACAGTCACACTCAAAGATCACA-3' | 60 |
| | | REV | 5'-TGCTGGAGCACCAGAGAAATG-3' | |

¹*18S* = eukaryotic 18S ribosomal; *METTL3* = methyltransferase like 3; *METTL14* = methyltransferase like 14; *RBM15* = RNA binding motif protein 15; *VIRMA* = vir like m⁶A methyltransferase associated; *WTAP* = WT1 associated protein; *ALKBH5* = alkB homolog 5; *FTO* = alpha-ketoglutarate dependent dioxygenase

²FWD = forward primer (anti-sense strand); REV = reverse primer (sense strand)

³Optimal temperature (Annealing, extension, and read fluorescence)

Table 4-2: Primer sequences for gene transcripts analyzed by quantitative real-time reverse transcription polymerase chain reaction (qPCR)

| Gene ¹ | NCBI reference sequence | Primer Design ² | Primer sequence | TM (°C) |
|-------------------------------|-------------------------|----------------------------|-----------------------------------|---------|
| <i>Sirtuins</i> | | | | |
| <i>SIRT1</i> | NM_001192980.3 | FWD | 5'-CAACAGCATCTTGCTGATTTG-3' | 60 |
| | | REV | 5'-TTTCATGATAGCAAGCGGTTCA-3' | |
| <i>SIRT2</i> | NM_001113531.1 | FWD | 5'-CGCAGGGTCATCTGTTTGGT-3' | 65 |
| | | REV | 5'-GGCATAGAGGCCCGTGTTT-3' | |
| <i>SIRT3</i> | NM_001206669.1 | FWD | 5'-TGGGCTGACGTTGCTGTTT-3' | 65 |
| | | REV | 5'-TGATTCAAGAGATTCCGCATCA-3' | |
| <i>SIRT4</i> | NM_001075785.1 | FWD | 5'-CAGGTCAGAAAAGGTGGACTTT-3' | 60 |
| | | REV | 5'-GTACAAAATCCCATGCTGGAT-3' | |
| <i>SIRT5</i> | NM_001034295.2 | FWD | 5'-TGGCCGAATTCAACATGGA-3' | 60 |
| | | REV | 5'-ACGGCCCCTGGAAATGA-3' | |
| <i>SIRT6</i> | NM_001098084.1 | FWD | 5'-TCGCCTGGTCATCGTCAA-3' | 65 |
| | | REV | 5'-CATCAACATAACCGTGGATTTCG-3' | |
| <i>SIRT7</i> | NM_001075217.1 | FWD | 5'-GCTCCACGGGAACATGTACA-3' | 65 |
| | | REV | 5'-CATCAAACACCCGCACATATTC-3' | |
| <i>DNA methyltransferases</i> | | | | |
| <i>DNMT1</i> | NM_182651.2 | FWD | 5'-AGGAGGCTGCCAAGGACTAGT-3' | 60 |
| | | REV | 5'-TCTGAACTGACTGATTGACATGTGA-3' | |
| <i>DNMT3A</i> | XM_024998363.1 | FWD | 5'-GATCCGAAAGCATCACCTGAGT-3' | 60 |
| | | REV | 5'-GCGTATTCTAAGGAATTTTCGGTATTT-3' | |
| <i>DNMT3B</i> | NM_181813.2 | FWD | 5'-GGGAAGGAGTTTGGAAATAGGA-3' | 60 |
| | | REV | 5'-CGGAGAACTTCGCATCACC-3' | |
| <i>DNA demethylases</i> | | | | |
| <i>TET1</i> | XM_024986940.1 | FWD | 5'-ACTCACAGCTTACCGAGAGTCTCA-3' | 65 |
| | | REV | 5'-AGCTCCACAAGTCTCTGGATCAA-3' | |
| <i>TET2</i> | XM_005207682.4 | FWD | 5'-CGGCCCGGACTATGTGTCT-3' | 60 |
| | | REV | 5'-CTTGATGAAGCGCAGGTAAGTG-3' | |
| <i>TET3</i> | XM_024999367.1 | FWD | 5'-GAGATGCGGCCTCAACGAT-3' | 60 |
| | | REV | 5'-AGCATATTTGCAGCCATTGAAG-3' | |

¹*SIRT1* = sirtuin 1; *SIRT2* = sirtuin 2; *SIRT3* = sirtuin 3; *SIRT4* = sirtuin 4; *SIRT5* = sirtuin 5; *SIRT6* = sirtuin 6; *SIRT7* = sirtuin 7; *DNMT1* = DNA methyltransferase 1; *DNMT3A* = DNA methyltransferase 3 alpha; *DNMT3B* = DNA methyltransferase 3 beta; *TET1* = methylcytosine dioxygenase 1; *TET2* = methylcytosine dioxygenase 2; *TET3* = methylcytosine dioxygenase 3

²FWD = forward primer; REV = reverse primer

Table 4-3: Body composition changes during periods of negative energy balance and subsequent compensatory growth in beef breeding bulls

| Item ¹ | Treatment ² | | | SEM ³ | P-value ⁴ | | |
|-------------------|------------------------|---------------------|--------------------|------------------|----------------------|-------|---------|
| | Initial state | NEB | CG | | T | A | T vs. A |
| EBW, kg | 603.2 ^a | 464.1 ^b | 591.7 ^a | 25.01 | <0.001 | 0.005 | 0.417 |
| EBF, kg | 214.4 ^a | 129.0 ^b | 206.0 ^a | 17.06 | <0.001 | 0.008 | 0.112 |
| EBP, kg | 90.4 ^a | 76.9 ^b | 89.3 ^a | 2.18 | <0.001 | 0.003 | 0.131 |
| dailyBE, Mcal/d | - | -2.0 ^a | 1.6 ^b | 0.21 | <0.001 | 0.141 | 0.030 |
| dailyEBF g/d | - | -161.1 ^a | 135.9 ^b | 18.65 | <0.001 | 0.134 | 0.024 |
| dailyEBP g/d | - | -78.9 ^a | 64.7 ^b | 5.01 | <0.001 | 0.177 | 0.195 |

¹EBW = empty body weight, EBF = empty body fat, EBP = empty body protein, dailyBE = daily changes on body energy, dailyEBF = daily changes on body fat, dailyEBP = daily changes on body protein

²NEB = negative energy balance (dietary restriction), CG = compensatory growth (*ad libitum* feeding)

³SEM = standard error of the mean

⁴T = treatment effect, A = age effect, T vs. A = treatment × age interaction effect. Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$.

^{a,b,c} Means within row without common superscript are statistically different (Tukey's test).

Table 4-4: Pearson correlation between body composition and molecular biology data during periods of negative energy balance and subsequent compensatory growth in growing breeding bulls

| Item ¹ | dailyBE | dailyEBF | dailyEBP |
|-------------------|--------------------|--------------------|--------------------|
| m ⁶ A | 0.003 (0.993) | 0.012 (0.974) | -0.020 (0.956) |
| <i>ALKBH5</i> | 0.650 (0.042)* | 0.664 (0.036)* | 0.611 (0.061)* |
| <i>FTO</i> | 0.098 (0.788) | 0.100 (0.784) | 0.093 (0.799) |
| <i>METTL3</i> | 0.229 (0.525) | 0.236 (0.511) | 0.208 (0.565) |
| <i>METTL14</i> | -0.626 (0.053)* | -0.631 (0.050)* | -0.612 (0.060)* |
| <i>RBM15</i> | 0.199 (0.582) | 0.205 (0.569) | 0.179 (0.620) |
| <i>VIRMA</i> | -0.411 (0.239) | -0.405 (0.246) | -0.425 (0.221) |
| <i>WTAP</i> | -0.237 (0.571) | -0.240 (0.568) | -0.230 (0.584) |
| <i>SIRT1</i> | 0.234 (0.577) | 0.237 (0.572) | 0.225 (0.593) |
| <i>SIRT2</i> | -0.132 (0.716) | -0.132 (0.716) | -0.132 (0.716) |
| <i>SIRT3</i> | -0.629 (0.050)* | -0.624 (0.054)* | -0.641 (0.046)* |
| <i>SIRT4</i> | 0.286 (0.424) | 0.290 (0.416) | 0.273 (0.445) |
| <i>SIRT5</i> | 0.191 (0.597) | 0.185 (0.609) | 0.206 (0.568) |
| <i>SIRT6</i> | -0.132 (0.715) | -0.128 (0.724) | -0.142 (0.696) |
| <i>SIRT7</i> | -0.583 (0.077)* | -0.582 (0.078)* | -0.583 (0.077)* |
| <i>TET1</i> | 0.047 (0.897) | 0.038 (0.917) | 0.072 (0.843) |
| <i>TET2</i> | -0.625 (0.053)* | -0.623 (0.055)* | -0.630 (0.051)* |
| <i>TET3</i> | 0.103 (0.776) | 0.097 (0.791) | 0.122 (0.738) |

TABLE CONTINUE ON NEXT PAGE

Table 4-4 continued

| Item ¹ | dailyBE | dailyEBF | dailyEBP |
|-------------------|--------------------|--------------------|--------------------|
| <i>DNMT1</i> | -0.216 (0.549) | -0.209 (0.562) | -0.232 (0.520) |
| <i>DNMT3A</i> | -0.669 (0.035)* | -0.674 (0.033)* | -0.652 (0.041)* |

¹*ALKBH5* = alkB homolog 5; dailyEBF = daily changes on empty body fat; dailyEBP = daily changes on empty body protein; dailyBE = daily changes on body energy; *DNMT1* = DNA methyltransferase 1; *DNMT3A* = DNA methyltransferase 3 alpha; *FTO* = alpha-ketoglutarate dependent dioxygenase; m⁶A = N6-methyladenosine methylation; *METTL3* = methyltransferase like 3; *METTL14* = methyltransferase like 14; *RBM15* = RNA binding motif protein 15; *SIRT1* = sirtuin 1; *SIRT2* = sirtuin 2; *SIRT3* = sirtuin 3; *SIRT4* = sirtuin 4; *SIRT5* = sirtuin 5; *SIRT6* = sirtuin 6; *SIRT7* = sirtuin 7; *TET1* = methylcytosine dioxygenase 1; *TET2* = methylcytosine dioxygenase 2; *TET3* = methylcytosine dioxygenase 3; *VIRMA* = vir like m⁶A methyltransferase associated; *WTAP* = WT1 associated protein. *Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$

Table 4-5: Pearson correlation between body composition and molecular biology data during periods of negative energy balance and subsequent compensatory growth in mature breeding bulls

| Item ¹ | dailyBE | dailyEBF | dailyEBP |
|-------------------|--------------------|--------------------|-------------------|
| m ⁶ A | -0.032 (0.940) | -0.017 (0.968) | -0.110 (0.795) |
| <i>ALKBH5</i> | -0.231 (0.581) | -0.223 (0.596) | -0.274 (0.511) |
| <i>FTO</i> | 0.364 (0.422) | 0.360 (0.428) | 0.381 (0.399) |
| <i>METTL3</i> | 0.338 (0.459) | 0.337 (0.460) | 0.341 (0.455) |
| <i>METTL14</i> | 0.389 (0.388) | 0.378 (0.403) | 0.443 (0.320) |
| <i>RBM15</i> | -0.386 (0.345) | -0.394 (0.334) | -0.342 (0.408) |
| <i>VIRMA</i> | 0.047 (0.913) | 0.037 (0.932) | 0.098 (0.817) |
| <i>WTAP</i> | -0.301 (0.468) | -0.305 (0.463) | -0.279 (0.504) |
| <i>SIRT1</i> | 0.267 (0.609) | 0.256 (0.624) | 0.324 (0.531) |
| <i>SIRT2</i> | -0.237 (0.572) | -0.254 (0.545) | -0.146 (0.731) |
| <i>SIRT3</i> | -0.652 (0.056)* | -0.567 (0.063)* | -0.250 (0.588) |
| <i>SIRT4</i> | 0.564 (0.145) | 0.568 (0.142) | 0.541 (0.166) |
| <i>SIRT5</i> | 0.209 (0.620) | 0.206 (0.624) | 0.221 (0.600) |
| <i>SIRT6</i> | -0.078 (0.854) | -0.092 (0.829) | -0.004 (0.993) |
| <i>SIRT7</i> | -0.149 (0.750) | -0.151 (0.746) | -0.136 (0.771) |
| <i>TET1</i> | -0.367 (0.372) | -0.365 (0.374) | -0.374 (0.361) |
| <i>TET2</i> | -0.622 (0.136) | -0.624 (0.134) | -0.605 (0.150) |
| <i>TET3</i> | -0.406 (0.319) | -0.412 (0.311) | -0.368 (0.370) |

TABLE CONTINUE ON NEXT PAGE

Table 4-5 continued

| Item ¹ | dailyBE | dailyEBF | dailyEBP |
|-------------------|-------------------|-------------------|-------------------|
| <i>DNMT1</i> | 0.083 (0.846) | 0.079 (0.852) | 0.097 (0.819) |
| <i>DNMT3A</i> | -0.212 (0.615) | -0.213 (0.613) | -0.205 (0.625) |

¹*ALKBH5* = alkB homolog 5; dailyEBF = daily changes on empty body fat; dailyEBP = daily changes on empty body protein; dailyBE = daily changes on body energy; *DNMT1* = DNA methyltransferase 1; *DNMT3A* = DNA methyltransferase 3 alpha; *FTO* = alpha-ketoglutarate dependent dioxygenase; m⁶A = N6-methyladenosine methylation; *METTL3* = methyltransferase like 3; *METTL14* = methyltransferase like 14; *RBM15* = RNA binding motif protein 15; *SIRT1* = sirtuin 1; *SIRT2* = sirtuin 2; *SIRT3* = sirtuin 3; *SIRT4* = sirtuin 4; *SIRT5* = sirtuin 5; *SIRT6* = sirtuin 6; *SIRT7* = sirtuins 7; *TET1* = methylcytosine dioxygenase 1; *TET2* = methylcytosine dioxygenase 2;; *TET3* = methylcytosine dioxygenase 3; *VIRMA* = vir like m⁶A methyltransferase associated; *WTAP* = WT1 associated protein. *Statistical significance was declared at $P \leq 0.05$ and statistical tendency $0.05 < P \leq 0.10$

4.11. Figures

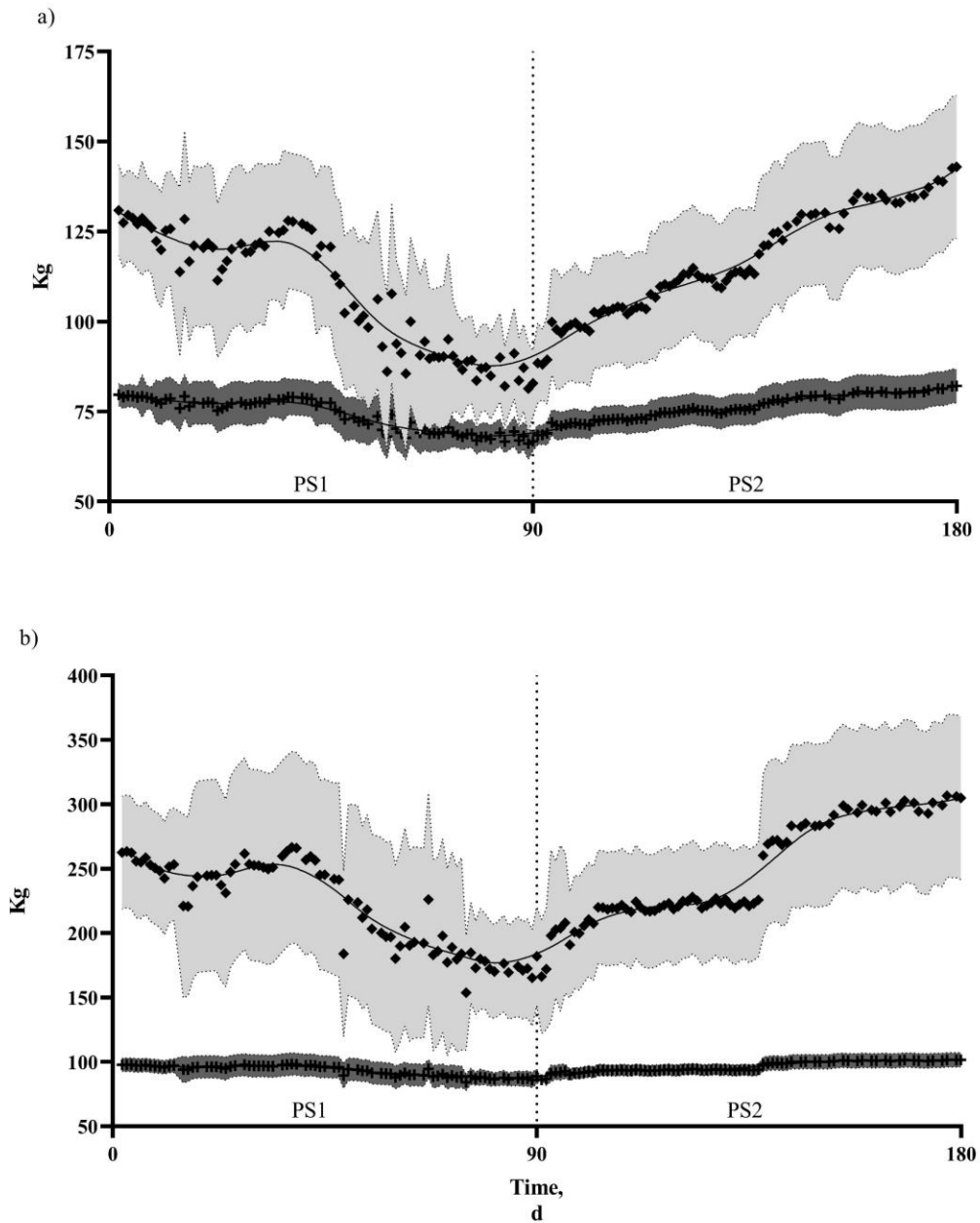


Figure 4-1: Empty body fat (kg, \blacklozenge , a) and empty body protein (kg, \blackplus , b) changes of young (n=6, a) and mature (n=6, b) breeding bulls undergoing periods of negative energy balance (PS1) and subsequent compensatory growth (PS2) over time. Light and dark shaded area indicates 95% confidence interval.

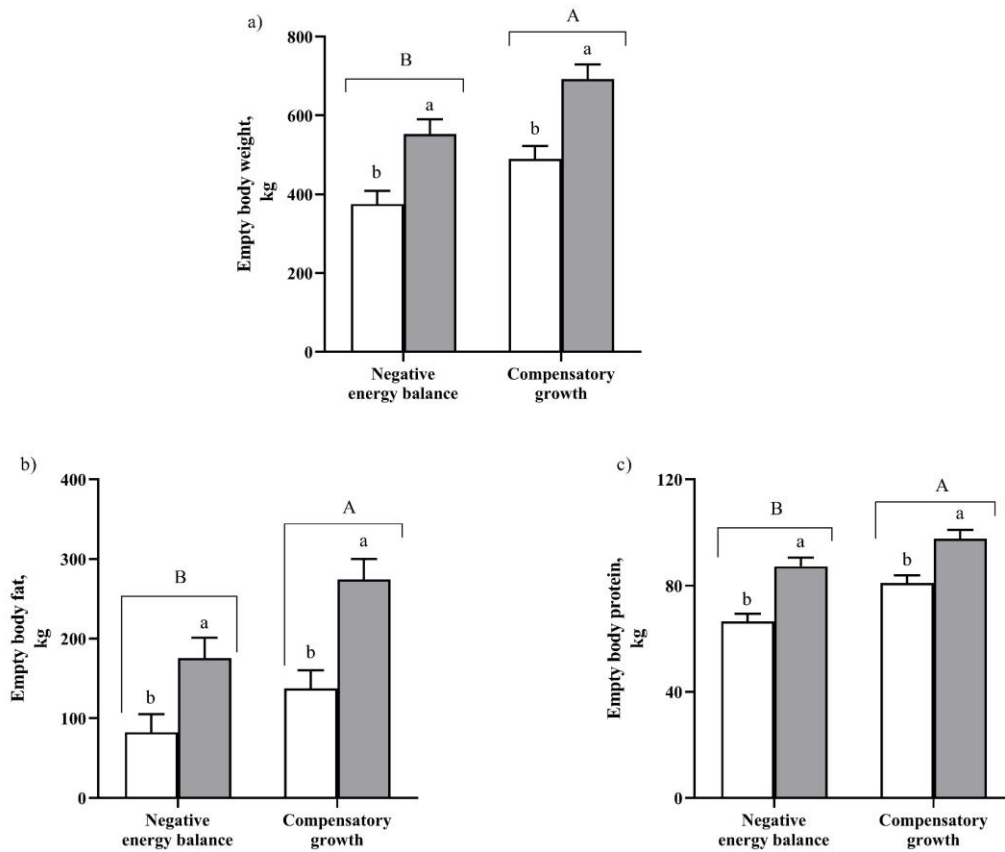


Figure 4-2: Empty body weight (kg, a), empty body fat (kg, b), and empty body protein (kg, c) of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Different uppercase letters indicate that periods are statistically different (Tukey's test; $P \leq 0.05$). Different lowercase letters within of periods indicate effect of age between young and mature bulls ($P \leq 0.05$).

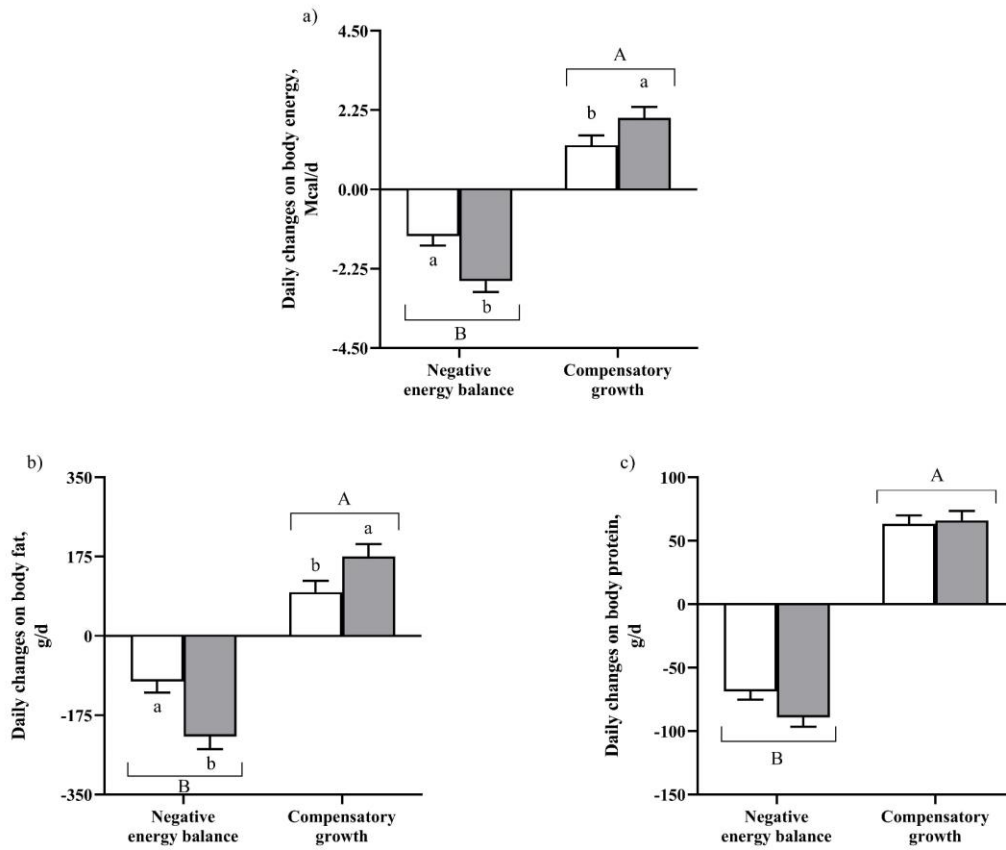


Figure 4-3: Daily changes on body energy (Mcal/d, a), body fat (g/d, b), body protein (g/d, c) (kg) of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Different uppercase letters indicate that periods are statistically different (Tukey's test; $P \leq 0.05$). Different lowercase letters within of periods indicate effect of age between young and mature bulls ($P \leq 0.05$).

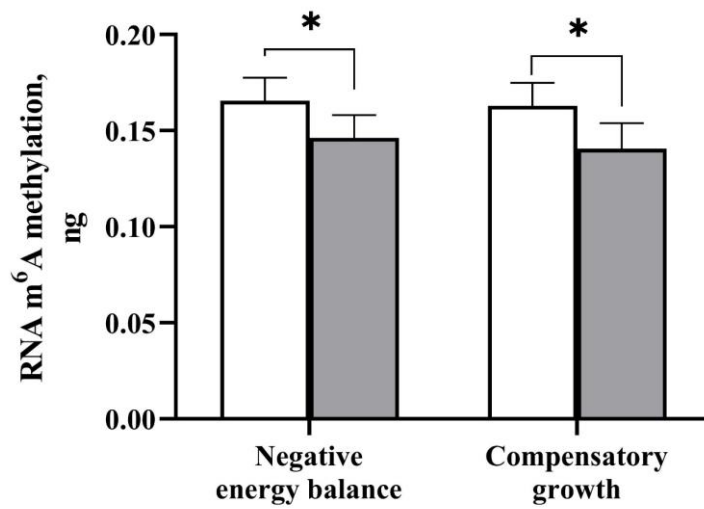


Figure 4-4: N6-methyladenosine (RNA m⁶A) methylation on hepatic tissue of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Means followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$).

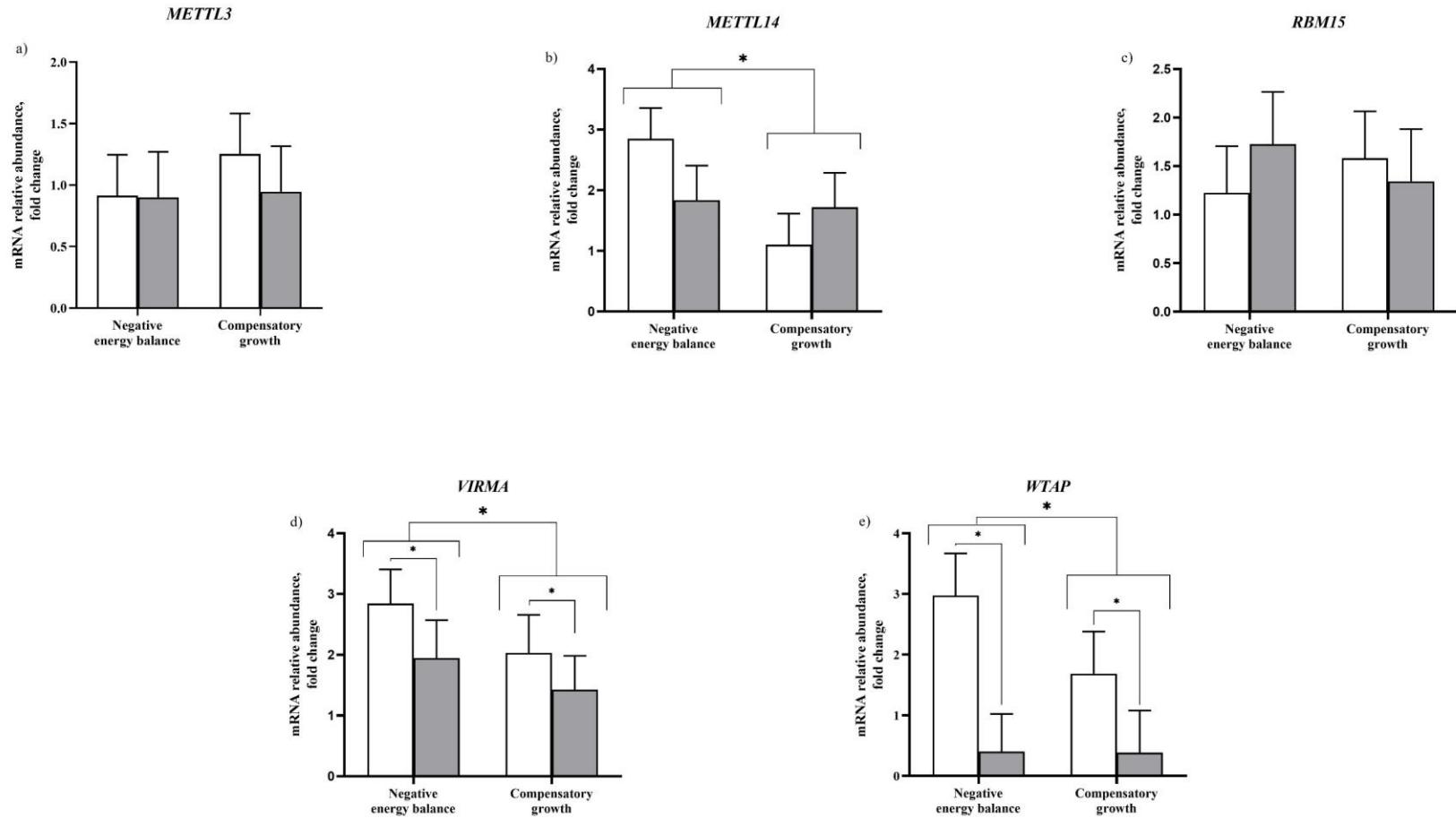


Figure 4- 5: Gene expression of the N6-methyladenosine methyltransferase complex on hepatic tissue of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Error bars show the standard error of the mean. Means within the sampling period followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$). *METTL3* = methyltransferase like 3 (a); *METTL14* = methyltransferase like 14 (b); *RBM15* = RNA binding motif protein 15 (c); *VIRMA* = vir like m⁶A methyltransferase associated (d); *WTAP* = WT1 associated protein (e)

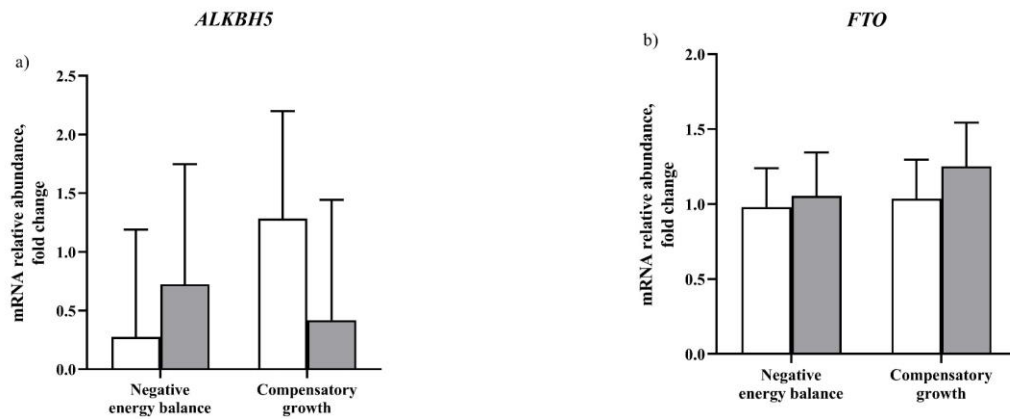


Figure 4-6: Gene expression of RNA demethylases on hepatic of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Error bars show the standard error of the mean. Means followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$). *ALKBH5* = alkB homolog 5 (a); *FTO* = alpha-ketoglutarate dependent dioxygenase (b)

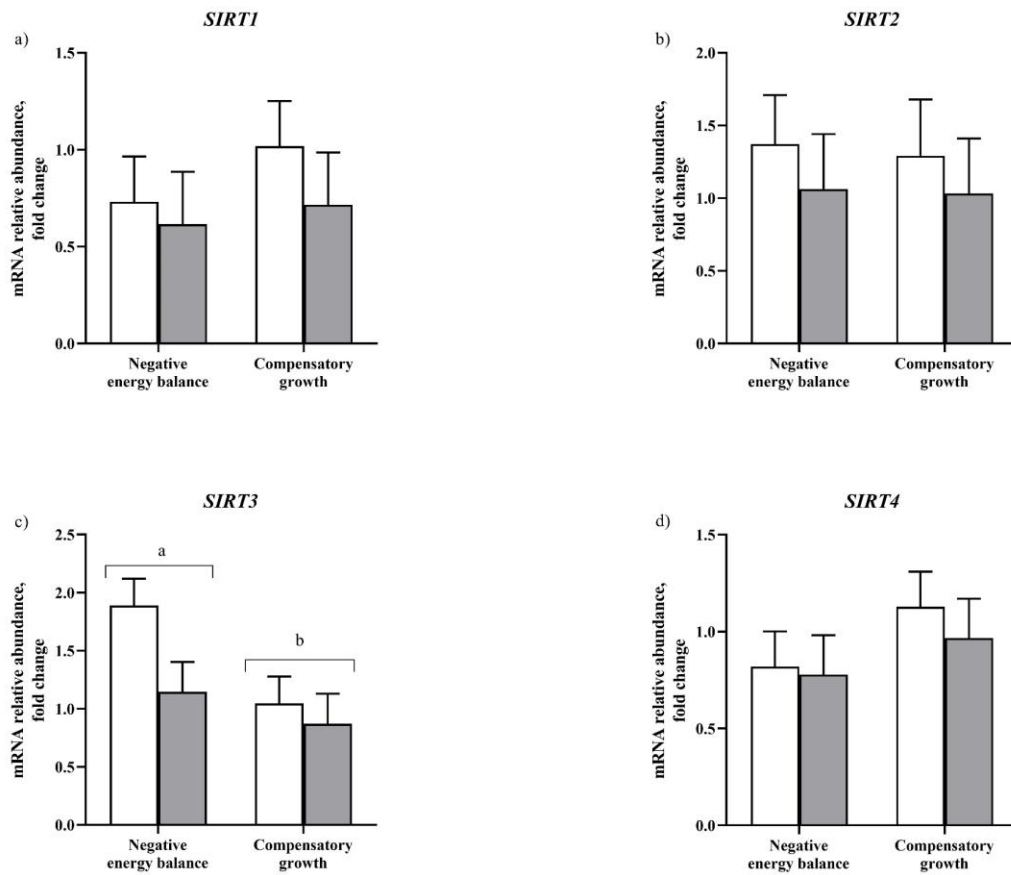


Figure 4- 7: Gene expression of sirtuins on hepatic tissue of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Error bars show the standard error of the mean. Means followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$). *SIRT1* = sirtuin 1 (a); *SIRT2* = sirtuin 2 (b); *SIRT3* = sirtuin 3 (c); *SIRT4* = sirtuin 4 (d)

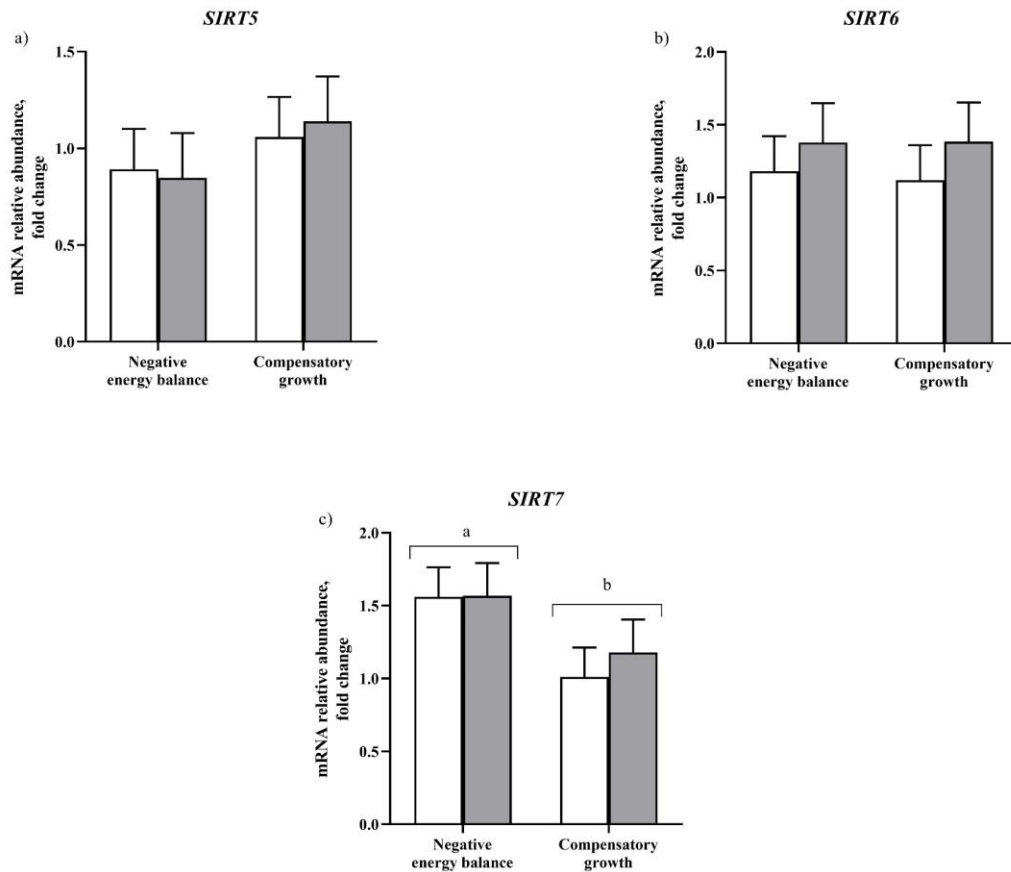


Figure 4- 8: Gene expression of sirtuins on hepatic tissue of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Error bars show the standard error of the mean. Means followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$). *SIRT5* = sirtuin 5 (a); *SIRT6* = sirtuin 6 (b); *SIRT7* = sirtuins 7 (c)

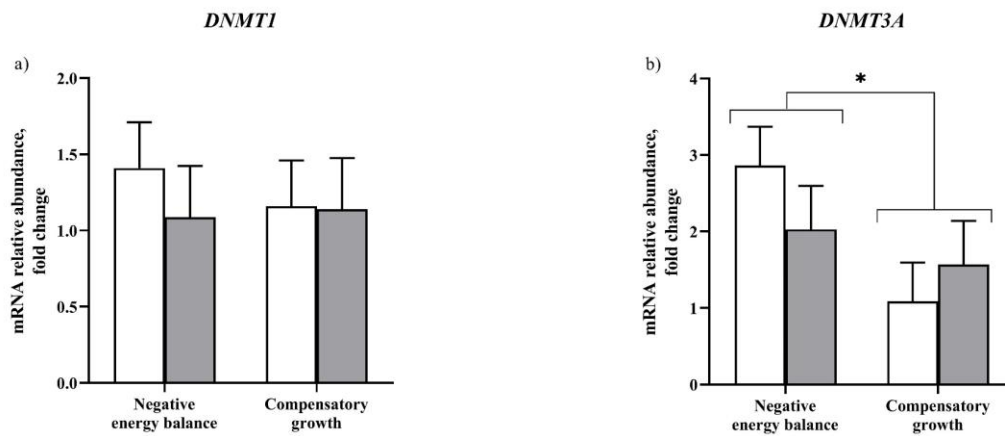


Figure 4- 9: Gene expression of DNA methyltransferase on hepatic tissue of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Error bars show the standard error of the mean. Means followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$). *DNMT1* = DNA methyltransferase 1 (a); *DNMT3A* = DNA methyltransferase 3 alpha (b)

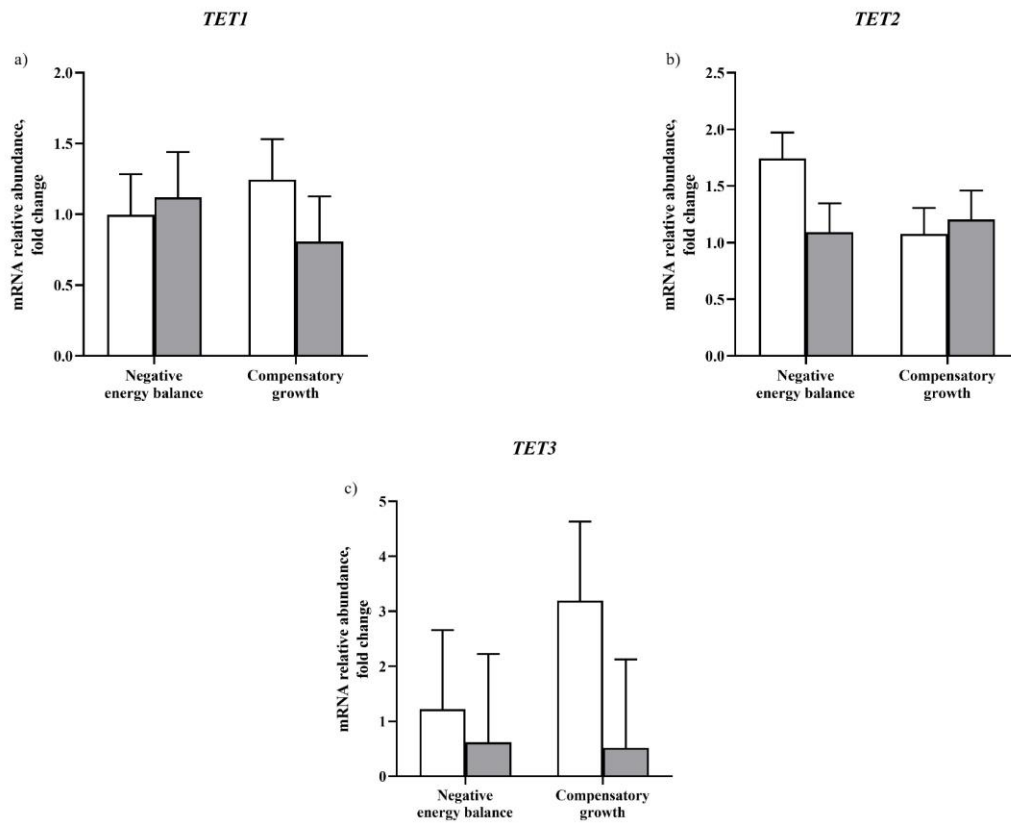


Figure 4- 10: Gene expression of DNA demethylases on hepatic tissue of young (n=6, unshaded, □) and mature (n=6, shaded, ■) breeding bulls undergoing periods of negative energy balance and subsequent compensatory growth. Error bars show the standard error of the mean. Means followed by different letters are statistically different (Tukey's test; $P \leq 0.05$). Means followed by asterisk (*) indicate statistical tendency ($0.05 < P \leq 0.10$). *TET1* = methylcytosine dioxygenase 1 (a); *TET2* = methylcytosine dioxygenase 2 (b); *TET3* = methylcytosine dioxygenase 3 (c)