

A METABOLOMICS APPROACH FOR THE STUDY OF LONG-TERM
PROGESTERONE IN DOMESTIC SHEEP AND PHYSIOLOGICAL
PROCESSES IN DOMESTIC AND BIGHORN SHEEP

by

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DEDICATION

To my sister, Kallie, and my husband, Steven, for which I would not be where I am without you and this work would not have been possible.

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ABSTRACT

Metabolomics allows for a snapshot of global metabolisms by studying metabolic intermediates and products of cellular metabolism. Experiments 1 and 2's objectives were to evaluate the effects of long-term P4 treatment, independent of the influence of the placenta and fetus, on changes in feed efficiency, BW, body composition, NEFA, metabolic hormones, and metabolites identified through nuclear magnetic resonance (NMR) metabolic profiling in mature Rambouillet ewes. Thirty, multiparous, 5- and 6-yr-old Rambouillet ewes were stratified by age and metabolic BW and assigned randomly to receive long-term P4 administration using controlled intravaginal releasing devices (CIDR) or no P4 (CIDRX; CIDR backbone only). Sera samples and body weights were collected every 14-d, along with CIDR/CIDRX replacement. Sera samples were assayed for metabolic hormones, NEFA, and metabolites. There were no differences in BW, RFI, STDMI, body composition, or temporal patterns of T3, T4, NEFA, or metabolites between CIDR- and CIDRX-treated ewes. Insulin concentrations were greater in CIDR-treated ewes than in CIDRX-treated ewes. Long-term P4 did not affect metabolism or body composition, independent from the presence of a fetus or placenta. Progesterone may increase tissue sensitivity to INS. In Experiment 3, the primary aim was to determine if NMR metabolic profiling has the potential to serve as a management tool for evaluating herds of bighorn (*Ovis canadensis*) sheep. Bighorn sheep herds were sampled between December of 2014 to March of 2015 in Montana and Wyoming. The sampling included 240 bighorn sheep ewes from 13 herds from geographically distinct locations at different times of the year. Metabolites identified by NMR in bighorn sheep serum were analyzed by pathway enrichment analyses, PLS-DAs, and biomarker analyses to determine if bighorn sheep herds can be distinguished by pregnancy status, geographic location, or time of year. NMR metabolic profiling could not distinguish between pregnant and non-pregnant bighorn sheep. Metabolic profiling did differentiate bighorn sheep herds and identified a subset of potential biomarkers that discriminated distinct geographic locations and time of year. Thus, NMR metabolic profiling has the potential to develop a suite of metabolites that wildlife managers can use to assess bighorn sheep nutrition and overall health.

CHAPTER ONE

INTRODUCTION

Metabolic aspects are an integral part of physiology and help to control homeostasis. Understanding the complex relationships of metabolism would allow us to solve problems that are associated with stresses related to nutrition, disease, reproduction, social interactions and changes in environment so we could increase the efficiency of reproduction, decrease the incidence of disease, and development management of domestic and wild ungulates. This thesis comprises of three experiments that examine the physiology of Rambouillet ewes and bighorn sheep. Experiment 1 examines long-term progesterone that mimics concentrations during pregnancy, without the influence of the placenta or fetus, effects on body composition, feed efficiency, non-esterified fatty acid concentration, and metabolic hormone concentrations. This study was based on the observation by Swartz et al. (2014) who reported that the total kg of TDN consumed per ewe per kg of lamb born was 24% greater in Rambouillet ewes from lines selected for high reproductive rate (HL) than in ewes selected for low reproductive rate (LL). Interestingly, nutrient intake and TDN did not differ between lines of ewes. Additionally, the only endocrinological difference between ewes of these lines was that systemic concentrations of progesterone were greater in HL ewes than in LL ewes between 60- and 120-d of gestation. The physiological mechanism for these observations was not clear; however, one could hypothesize that HL ewes were more efficient in partitioning nutrients into fetal growth and development. These results may be interpreted to mean

that the apparent increase in efficiency of nutrient utilization in HL ewes during gestation was the result of increased concentrations of progesterone between d-60 and d-120 of gestation. Again, it is not clear whether progesterone only may increase efficiency or the partitioning of nutrients, or whether signals from the placenta and/or fetus need to be present to observe this change in efficiency. Therefore, in the present study, the effect of progesterone was examined to determine if progesterone increases feed efficiency or the partitioning of nutrients, without the influence of placental or fetal signals. It is well documented that maternal system shifts protein, lipid, and carbohydrate metabolism to accommodate the stresses of pregnancy. Using traditional assays limits the number of metabolites that can be investigated in terms of metabolic products associated with the effect of progesterone.

After the Experiment 1 was completed, this lab had the opportunity to study metabolic profiling using nuclear magnetic resonance spectroscopy (NMR). The reason for this is that NMR metabolic profiling can dramatically expand the number of metabolites involved in protein, lipid, and carbohydrate metabolism; thus, opening new avenues for the study of long-term effects of progesterone on metabolism. Using NMR metabolic profiling, presented an opportunity to study relationships that could not have been possible using traditional assay methods for metabolites. Therefore, the aim in Experiment 2 was to examine the effects of progesterone on changes in protein, lipid, or carbohydrate metabolism using NMR metabolic profiling.

In Experiment 3, the objective was to develop a potential suite of metabolites using NMR technology that may be important in assessing nutritional health, disease

health and body condition in free-ranging ungulates, in this case, bighorn sheep. Bighorn sheep populations were decimated with the colonization of North America. Restoration efforts over the past hundred years has increased bighorn sheep populations 4-fold; however, certain herds of bighorn sheep have had a difficult time recovering to historical population size. Since there is paucity of literature regarding the physiology of bighorn sheep, wildlife managers have a difficult time accessing herd health, nutritional status, and disease states to determine the cause(s) that contribute to differences in bighorn sheep herds have not experienced an increase in population size compared to other bighorn sheep herds. If NMR metabolic profiling can identify a potential suite of metabolites that can distinguish bighorn sheep based on pregnancy status, geographic location, or nutritional status, it will bring researchers one step closer to understanding bighorn sheep physiology and how physiology drives population dynamics. Furthermore, it will provide wildlife managers with a potential tool to effectively develop management strategies to evaluate bighorn sheep herds.

In Chapter 2, contains a review of literature that encompasses overviews of pregnancy and the partitioning of nutrients to the fetus; metabolism and reproduction; the theory of nuclear magnetic resonance and metabolic profiling; and, bighorn sheep history and physiology.

CHAPTER TWO

LITERATURE REVIEW

Pregnancy and Nutrient Partitioning to the Fetus

Successful reproduction is essential for the survival of a species. Therefore, the fundamentals of a successful pregnancy have been intensely studied and are well understood. A healthy pregnancy involves the correct combination of hormones and a hospitable environment that can support the fetus. Reproduction in livestock is greatly impacted by the interaction between nutrition and metabolism. Nutrition affects reproduction by directly providing energy to develop and sustain the embryo or fetus (Robinson et al., 2006). Nutrition also impacts the regulation of hormones that control reproduction and influences the development of the neonate (Boland et al., 2001). Furthermore, the nutritional status of ewes has been shown to interact with systemic concentrations of progesterone to alter the maintenance of pregnancy (Parr et al., 1987).

Endocrinology of Pregnancy in the Ewe

A successful pregnancy includes maternal recognition of pregnancy, involving hormones and signals which regulate the function of the uterus to create a hospitable environment to ensure the survival of an embryo (Spencer et al., 2004). Progesterone, also known as the hormone of pregnancy, is crucial for short and long term maintenance of pregnancy. Progesterone is a steroid hormone that is produced by theca interna cells of tertiary ovarian follicles and by luteal cells from a functioning corpus luteum during the

estrous cycle, and during most, if not all, of the stages of pregnancy (for review, Senger, 2012). In the ewe, after 55 days of gestation, the placenta begins to synthesize and secrete progesterone at high enough concentrations to maintain pregnancy without a functioning corpus luteum (Kindahl, 2007). Progesterone prevents the resumption of the estrous cycle, creates a hospitable environment in the uterus for embryonic and fetal development, and prevents uterine contraction to enhance the establishment of pregnancy and embryonic and fetal development (Lye, 1996). Furthermore, Arck et al. (2007) reviewed the immunosuppressant qualities of progesterone that prevent the maternal immune system from recognizing the embryo as foreign and destroying it. During pregnancy, progesterone blocks early T-cell development, as well helps to create a pregnancy-protective environment through the involvement of a protein called P-induced blocking factor.

The physiological mechanism for the maternal recognition of pregnancy is a combination of signals that are released by the growing blastocyst, in the ewe, between days 12-15 of pregnancy. This must occur before the blastocyst attaches to the endometrium to prevent luteolysis of the corpus luteum, which in turn maintains concentrations of progesterone essential to establishing pregnancy. One of the signals that is of primary importance is a cytokine known as interferon-tau which acts locally on the endometrium to inhibit the synthesis and secretion of prostaglandin $F_{2\alpha}$ (Nasar and Rahman, 2006). This is an important physiological event to the establishment and maintenance of progesterone for the duration of gestation. The consequence of a failure

to maintenance of progesterone consequences anytime during pregnancy results in embryonic death or fetal abortion.

There are many factors that are involved with the events of pregnancy that are associated with progesterone and complex hormonal interactions that are important in shifting the maternal metabolism that accommodates changes in lipid, protein, and carbohydrate metabolism to support the fetus and placenta. Some of these will be discussed in the following sections.

Pregnancy Specific Protein B

Pregnancy Specific Protein B (PSPB) is a glycoprotein synthesized by the ruminant placenta which plays a role in the establishment of pregnancy during early embryonic and fetal development. The role of PSPB in establishing pregnancy is thought to be mediated by the immunosuppression of the maternal immune system (Weems et al., 2003). In fact, changes in PSPB concentrations can be used to detect pregnancy at an accuracy of 99% after day 30 of pregnancy in ewes (Redden and Passavant, 2013). Furthermore, PSPB concentrations have been used in wild ungulates, like elk, to accurately detect pregnancy (Drew et al., 2001 and Noyes et al., 1997). Assay of PSPB has provided wildlife managers with a effective tool to determine pregnancy rates in herds of wild ungulates, rather than the use of ultra-sound technology. The primary reason for this is that the uses of ultra-sound technology requires extensive training and expertise and is relatively expensive.

Progesterone Receptors

This thesis involves the physiological effects of progesterone. To understand those effects, it is important to understand the mechanism of action of progesterone. It is well established that progesterone acts through a receptor sites of target tissues such as: the reproductive tract and mammary tissue of females (For review, Senger, 2012). Generally, there are two classes of progesterone receptors: nuclear receptors and membrane bound receptors.

These classes of progesterone receptors have been found primarily in tissues directly associated with reproduction and lactation. Currently, there are no reports in the literature that have identified either class of progesterone receptors in liver or adipose tissue; these tissues are essential for the partitioning of nutrients in mammals. However, there is one study in humans by Copas et al. (2001), who identified a nuclear progesterone receptor in the levator ani muscle. This muscle is located on the pelvic floor and is critical in maintaining bowel functions of the urethra and rectum, as well as, accommodating the expansion of the pelvic canal during parturition. However, the function of this receptor was not clear from the explanation given in the report. Even though progesterone receptors were identified in this muscle, no other skeletal muscle to our knowledge has been reported to contain progesterone receptors related to the partitioning of nutrients.

The nuclear progesterone receptor has two isoforms, A and B, and the two mRNA transcripts come from one gene. PR-A and PR-B are identical in the C-terminal, but PR-A is truncated 164 amino acid short at the N-terminal than PR-B. PR-B is the stronger

transcriptional factor, while PR-A can suppress PR-B (Li, et al., 2003). PR-A has two activation factors, while PR-B has three activation factors. Activation factor 1 is located at the amino-terminal domain and activation factor 2 is located at the ligand binding domain. Activation factor 1 and 2 can function independently or together, depending on the cell target. Activation factor 3, only located in PR-B, works together with activation factors 1 and 2 (Hill, et al., 2012). These receptors are found primarily in the smooth muscle of the reproductive tract of females (Hodges et al., 1999). Progesterone receptors in smooth muscle have a close relationship with estrogen. The interaction between progesterone receptors and estrogen is complex and not well understood. Further research needs to be conducted to determine the function of smooth muscle progesterone receptors in the presence of estrogen and not in the presence of estrogen (Hodges et al., 1999).

The second class of a progesterone receptor are membrane localized progestin receptors (mPR). The mPR gene has three isoforms: mPR α , mPR β , mPR γ . All three isoforms when bound to a progestin have fast G-protein coupled activation. All three mPR have been identified as having functions in the endometrium and in oocyte maturation. The mPR α isoform has been identified in the mid-piece and flagellum of human sperm. Interestingly, the mPR β isoform can be found in high concentrations throughout pregnancy until 2 days before parturition, while the mPR γ isoform remains at high concentrations throughout the entire pregnancy. It is still unclear the exact functions of the mPR in these tissues; however, they seem to have a regulatory function related to short durational effects in reproductive processes (Dressing et al., 2010). To our

knowledge, there are no reports in the literature of these receptors related to the partitioning of nutrients or metabolism during pregnancy.

Partitioning of Nutrients to the Fetus

Nutrients utilized by the fetus are under strict endocrinologic signals created by the fetus and placenta. Glucose, lactate, amino acids and beta-hydroxybutyrate are the main substrates that are used for energy by the fetus. Glucose is the main source of energy, providing 50-70% of the substrate utilized by the fetus. Lactate comprises around 20-25% of the substrate that utilized by the fetus (Bauman and Currie, 1980). Lactate is hypothesized to accumulate because of the use of glucose through glycolytic pathways in the fetus. The rest of the substrates that are utilized in the fetus can be attributed to amino acids and beta-hydroxybutyrate. There is a high production of urea from the fetus which may be due to fetal metabolism of amino acids for energy. Furthermore, a study by Harding et al. (1991) showed that in addition to using glucose, the fetal brain also uses 3-hydroxybutyrate as an energy source. This was an important study, because before this discovery it was assumed that the fetal brain only used glucose for energy consumption. However, especially in glucose starved states, the fetal brain may use other substrates, such as 3-hydroxybutyrate, for energy to keep the fetus alive.

Homeostasis and Homeorhesis

Homeostasis and homeorhesis are different kinds of regulatory mechanisms that balance and synchronize regulation of the partitioning of nutrients and metabolism. Homeostasis regulation that maintains the basal or normal physiological functions of the

body. One basic example of homeostasis and metabolites would be glucose concentrations and energy balance. Glucose is needed in high concentrations to provide energy for the organism, concentrations that are too high (hyperglycemia) can cause metabolic disorders such as diabetes (Bauman and Currie, 1980). Low concentrations (hypoglycemia) places an animal in a negative energy balance. Thus, the concept of homeostasis, i.e. to maintain a 'balance' for nominal function. Homeorhesis is the second type of regulation and is defined as the changing in the metabolism of specific tissues to support changes in physiological states. An example of homeorhesis is lactation. Mammary tissues must alter their metabolism to support lactation for the support of a newborn (Bauman et al., 1980). These two physiological conceptions appear to operate in concert to maintain metabolism and the partitioning of nutrients in metazoan animals.

Metabolism and Reproduction

Endocrinology and Control of Metabolism

Metabolism are complex biochemical reactions that occur in cells which maintain life and ensure proper growth. Metabolism may be broken down into two distinct physiological processes: catabolism and anabolism. Catabolism is the form of metabolism that involves a set of metabolic pathways that breakdown relatively complex molecules, known as nutrients, to simpler forms and the release of energy from these nutrients. Anabolism is the form of metabolism that constructs new molecules through a different set of metabolic pathways that utilize the products of catabolic pathways for the energy necessary to maintain the functions of an organism. Metabolism is highly

regulated and controlled through complex interactions among the products of these two processes. These products are metabolites and regulatory hormones known as metabolic hormones. The following sections are a brief overview of some of the metabolic hormones, however, not all the metabolic hormones were considered in this thesis.

Insulin

Insulin is a metabolic hormone that regulates glucose moving into and out of tissues and is crucial in maintaining glucose concentrations within a “homeostatic” range. Changing concentrations of glucose in the systemic concentrations of mammals are intimately correlated with changes in insulin concentrations (Hadley and Levine, 2006). Insulin is a protein hormone synthesized and secreted by beta cells of the pancreas. As concentrations of glucose increase in the blood, insulin is released by these cells. Insulin functions by binding to a tyrosine kinase receptor, which becomes activated through phosphorylation to cause the uptake of glucose, which in turn is utilized for energy homeostasis primarily by adipose, skeletal muscle, and liver (Hadley and Levine, 2006). Also, insulin stimulates the storage of glucose in the liver as glycogen. If glycogen levels become too high, insulin promotes the synthesis of fatty acids by the liver and synthesis and storage of these as fats by adipose tissue (Cadorniga-Valino et al., 1997). Insulin is important in metabolism because glucose is the major source of energy utilization by all cells. Interestingly, the brain is known as an “insulin-insensitive” organ, because even though insulin receptors have been identified in the brain, the function of these receptors is unknown. Researchers have recently hypothesized that insulin receptors in the central nervous system, i.e. the brain, may play a diverse role in regulating neural circuits

associated with life span, learning and memory (Chiu and Cline, 2010). Alterations in insulin in circulation is also very 'resistant' to the changing of physiological states such as pregnancy. Pregnancy is known to increase insulin resistance, which decreases the utilization of glucose (Pettersen et al., 1993). As a result, the decrease in the uptake of glucose in the maternal system, increases glucose utilization of glucose, increases the amount of energy that can be used for fetal and placental metabolism.

Thyroid Hormones

There are two main thyroid hormones synthesized and secreted by the thyroid gland: triiodothyronine (T3) and thyroxine (T4). They are synthesized by the amino acid tyrosine. The synthesis and secretion of T3 and T4 are primarily regulated by thyroid stimulating hormone (TSH). Thyroid stimulation hormone is secreted and synthesized by the anterior pituitary gland. Triiodothyronine and T4 are the main regulators of the basal metabolic rate (Hadley and Levine, 2006). An increase in thyroid hormones stimulates fat catabolism, which increases the amount of fatty acids that are in the blood. These fatty acids are then converted to glucose by the liver and used for energy metabolism. Thyroid hormones also have a major effect on carbohydrate metabolism; stimulating gluconeogenesis by the liver and glucose concentrations in the blood. Along with increasing glucose concentrations, thyroid hormones also increase the ability for cells to uptake glucose for energy production (Bowen, 2010).

Leptin

Leptin is a protein hormone that is synthesized and secreted from white adipocytes as those cells increase the storage of lipids. When leptin concentrations increase in the systemic circulation, it is indicative of an increase in body fat, which is a good indicator of a positive energy balance. Leptin acts through specific receptors of specific nuclei in the hypothalamus that are thought to be involved with metabolic regulation. If an animal is energy deficient, in a negative energy balance, leptin concentrations decrease. In turn, low levels of leptin will increase appetite and decrease energy expenditure. On the other hand, high concentrations of leptin tend to decrease appetite and increase energy expenditure. Again, these changes in energy balance and the role of leptin appear to be mediated through the complex interaction between the hypothalamus and the hindbrain (Park et al., 2015).

Cortisol

Cortisol, otherwise known as the “stress hormone”, is a steroid hormone that is synthesized and released from the adrenal cortex in response to stressful stimuli. The function of cortisol to regulate the changes that occur in the body in response to stress, many of which are changes in metabolism. Cortisol is known as a glucocorticoid because one of its effect on its target tissues is to stimulate the release of glucose stores, fatty acids and/or amino acids that can be used for energy. This aspect of cortisol is thought to be related to a conserved, evolutionary response, i.e., a quick release of energy could aid in prey escaping a predator. On the other hand, long-term or sustained releases of cortisol may be detrimental and catabolic in muscle. The type of action reduces muscle mass and

lean body mass by breaking down muscle to compensate for the increase in energy expenditure in response to a long-term stress. In fact, long-term elevations in cortisol are known to increase feed intake, body fat percentage and insulin resistance (Christiansen et al., 2007).

Changes in Maternal Metabolism During Pregnancy

Pregnancy may be defined as a period during the lifetime of a mammalian female in which she makes the appropriate physiological and anatomical changes necessary for her to accommodate the physiological and anatomical demands of the developing placenta, the conceptus, embryo, and fetus, and prepare for the demands of parturition and lactation. These physiological changes include cardiovascular, hematologic, renal, respiratory, and metabolic changes. In other words, pregnancy is a stress, but a stress that does not exceed the limits of homeostasis: rather, the female accommodates this stress through homeorhetic processes. That is her body must change its physiological and homeostatic mechanisms in pregnancy to ensure the fetus the continued survival of the embryo and fetus. Increases in blood sugar, breathing, and cardiac output are all required. Metabolically, this means continuously altering carbohydrate, protein, and fat metabolism (Bauman and Currie, 1980). During the first stages of pregnancy, an increase in energy intake supports the increase demands of glucose. However, towards the end of pregnancy as estrogen concentrations increase, energy intake decreases (Grummer, 1995). The maternal metabolism compensates for this decrease that occurs because of an increase in efficiency of glucose mobilization and utilization in the liver (Bauman and Bell, 1997).

Even though the mother is in a negative energy balance, this increase in carbohydrate utilization allows for the final stages of growth for the fetus.

As previously stated, pregnancy induces insulin resistance which decreases the amount of glucose utilized by maternal tissues. In humans, decreasing the uptake of glucose results in a chain of events that decreases the amount of carbon that enters the tricarboxylic acid cycle, ultimately leading to a decreased production of nitrogen receptors and therefore a decreased production of the amino acids alanine and glutamine. These events result in a decrease in amino acid nitrogen and lower urea synthesis (Kalhan, 2000). It is unknown why these adaptations occur, however, it is hypothesized that it is a maternal response to conserve nitrogen for protein synthesis (Kalhan, 2000). An increase in the utilization of dietary protein and conservation of nitrogen occurs in late pregnancy, because there is no evidence that the maternal tissues store nitrogen for the fetus in early pregnancy (Herrera and Ortega, 2008). In fact, nitrogen utilization is so important that in late pregnancy amino acid concentrations are higher in the fetal serum than in the maternal serum because of the active transport of amino acids through transporters and energy (Herrera and Ortega, 2008).

Pregnancy also affects maternal lipid metabolism. During early pregnancy, the maternal metabolism stores large amounts of fatty acids to be used during late pregnancy for increased energy demands (Vernon et al., 1981). The switch from lipid accumulation to lipid mobilization corresponds to a decrease in the rate of fatty acid and acylglycerol-glycerol synthesis, as well as a decreased activity of the enzyme lipoprotein lipase (Vernon et al., 1981). As the adipocytes decrease in volume, the decreased activity of

lipoprotein lipase, fatty acids, and acylglycerol-glycerol parallels with a decrease in lipid synthesis (Vernon et al., 1981). Furthermore, in late pregnancy the number of insulin receptors in adipocytes decreases, which may play a role in promoting the switch from lipid accumulation to lipid mobilization (Vernon et al., 1981). A decrease in insulin receptors may be related to an increase in progesterone concentrations that will be discussed in the following section.

Progesterone and Metabolism

An interesting relationship between progesterone and insulin exists as there is evidence that progesterone increased insulin resistance in rats (Kumagai et al., 1993). An insulin resistant cell requires higher concentrations of insulin to uptake the same amount of glucose as a normal cell. Another study on the relationship between progesterone and insulin in rats, reported that progesterone increased plasma levels of insulin to improve glucose tolerance, with no effect on feed intake or fat deposition (Wade and Gray, 1978). This study also reported that any changes associated with feed intake or body composition will occur even in the absence of insulin.

Recently, Swartz et al. (2014) reported that progesterone concentrations were greater in Rambouillet ewes selected for high reproductive rates than in ewes selected for low reproductive rate during pregnancy. In their study, nutrient intake and total digestible nutrients did not differ between the two lines. However, the total kg of total digestible nutrients consumed per ewe per kg of lamb born was 24% greater in low reproductive rate line ewes than in high reproductive rate ewes. One could hypothesize that high reproductive rate ewes were more efficient in partitioning nutrients into fetal growth and

development. The physiological mechanism involved in this observation is not clear, but maybe related to greater systemic concentrations of progesterone between day 60 and day 120 of gestation in high reproductive rate ewes than in low reproductive rate ewes. Also, the nutritional status of ewes has been shown to interact with systemic progesterone concentrations and influence the maintenance of pregnancy (Parr et al., 1987).

The purpose of this study was to determine if progesterone can mimic the same pattern of protein, fat, and carbohydrate alterations associated with metabolic regulations of pregnancy without the influence of placental and fetal interactions. First we have to ask the question, is there any evidence that progesterone metabolic effects in mammals. Interestingly, many studies have been conducted in human females to determine if progesterone effects changes in body composition. The perception of human females is that progesterone makes them gain weight and body fat; however, there are few studies that corroborate this assertion. A review on progestin-only contraceptives and its effect on weight gain and body composition was conducted by Lopez et al., in 2016. This review examined 22 different studies that have been conducted in human females that use progestin-only contraceptives. This review stated that 15 of the studies reported no changes in body weight or in body composition between females taking a progestin-only contraceptive and females not taking a progestin-only contraceptive. Furthermore, five of the studies reported that there were slight changes in body weight and body composition; however, the studies methods were questionable. Finally, two of the studies reported significant changes in body composition and body weight between females taking a progestin-only contraceptive and females not taking progestin. Again, these two studies

have questionable methods and the evidence was low-quality. The conclusions of the review by Lopez et al. (2016) was that oral progestin contraceptives did not cause a change in body weight or body composition associated with fat deposition.

The effect on progesterone and metabolism in ruminants is equivocal. A study by Busby et al. (2002) reported that feeding melengesterol acetate (MGA), a synthetic progestin, to beef heifers required at least 57-d to affect an increase in ADG, marbling score, and tenderness relative to these characteristics in heifers not fed MGA. Additional studies conducted in heifer feedlot cattle reported that feeding heifers MGA increased average daily gain and gain to feed ratios when compared to heifers not feed MGA (Bloss, et al., 1966 and Kreikemeier and Mader, 2004). In these same studies, MGA did not differ in carcass characteristics between heifers fed MGA or not. The mechanism for these observations is not known. Again, there is a lack of literature as to how progesterone affects metabolism in the absence of a fetus or placenta.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) was discovered in 1947 by researchers at Stanford and Harvard universities. NMR initially was used in identifying structures of small molecules; however, in recent years it has been crucial in the study of metabolomics. NMR has benefits over traditional metabolite assays. The benefits include:

- 1) The number of metabolites identified per sample. While traditional metabolite assays can only identify one metabolite per assay, NMR metabolic profiling has the potential to identify hundreds of metabolites. Increasing the number of

metabolites identified gives researchers a better understanding of the global metabolism of an individual.

- 2) The cost per sample. Given the amount of data generated by one sample, NMR is much more cost effective than traditional metabolite assays.
- 3) The time commitment. NMR metabolic profiling needs expertise training to analyze samples. The time commitment to learn how to analyze the spectra produced by NMR metabolic profiling may be high, taking about 25-30 minutes to analyze one spectra. After gaining experience analyzing samples, one spectra can be analyzed between 10 and 15 minutes. Traditional metabolite assays can take anywhere between 1 hour and 6 hours for 35 samples.

Theory of Nuclear Magnetic Resonance

Protons in the nucleus are constantly spinning, much like electrons spin in the outer orbit of the atom. The spin associated with the proton can be thought of as a rotation, however, this is just a metaphorical term as protons are not round but more “phantom particles” (Moskowitz, 2014). Researchers only have theories as to why protons have this intrinsic property known as a spin.

When exposed to a magnetic field, the nucleus acts as a magnet and the intrinsic spin of the protons align with either a low- or high-energy state. The low-energy state is when the spin of the protons aligns with the external magnetic field, while the high-energy state is when the spin of the protons is against the external field. Without the presence of a magnetic field, protons spin in random orientations. For a signal to be measured, an

electromagnetic radiation, generally, radio frequency radiation, at a given frequency is applied to the sample, which in turn flips the spin of the protons. The relaxation time is when the protons flip back to the previous state that before the next electromagnetic pulse. NMR measures the resonance frequency associated with the protons flipping their spin states and then flipping back (Carr and Purcell, 1954).

The entire theory of NMR is based on a concept called nuclear shielding. The external magnetic field that the atom is exposed to, “locks” the electrons into a position in the orbit of the nucleus. This electron interaction with the applied field creates a small magnetic field within the atom (Ando and Webb, 1983). This internal magnetic field adds to the external magnetic field, resulting in each proton spin flip requiring a different frequency to cause an energy change. This different frequency is what is measured by the NMR machine and creates the peaks in the associated spectra (Ando and Webb, 1983). Each peak produced in the spectrum is proportional to the number of nuclei present in the associated chemical environment. The number of nuclei present is used for chemical identification of the chemical composition of the metabolites that are present in the sample. From the chemical shifts associated the peaks, we can determine the concentration of a metabolite in a sample.

NMR Metabolic Profiling and Pregnancy

NMR studies in pregnancy have been conducted in human females to determine if biomarkers exist to identify healthy pregnancies. Preeclampsia is a syndrome in the second half of pregnancy that is associated with high blood pressure and proteinuria (Austdal et al., 2014). Even though fetal and maternal death is high in women with preeclampsia, not

much is known about the disorder. A study by Diaz et al. (2013) reported 21 metabolites that significantly change throughout a healthy pregnancy. These metabolites include choline, creatinine, 4-deoxyerthronic acid, 4-deoxythreonic acid, furolyglycine, guanidoacetate, 3-hydroxybutyrate, and lactate. A normal pregnancy has isoleucine and threonine involved in oxidation/ketone body synthesis, urea cycle regulation, and a relationship with to furolyglycine and creatinine (Diaz et al., 2013). These identified metabolites indicate the changes of metabolism associated with pregnancy in healthy human females that have normal caloric intakes. Women with preeclampsia have increase concentrations of choline and decreased concentrations of glycine, p-cresol sulfate, and hippurate in urine (Austdal et al., 2014). These changes in metabolites indicates the potential of biomarkers to indicate diseases associated with pregnancy. There are no studies that have used NMR metabolic profiling for the study of metabolic changes associated with pregnancy in ruminant species.

Bighorn Sheep History and Physiology

In this thesis, we examined the population dynamics of bighorn sheep (*Ovis canadensis*) by studying their physiology through the assay of systemic concentration patterns of metabolites. To understand the population dynamics, it is important to understand the history and any previous studies associated with the physiology of this species.

Bighorn sheep populations were decimated after colonization of western North America. Historic populations were thought to be in the millions, but even after major

restoration efforts are only currently around 80,000 (Buechner, 1960). Most herds of bighorn sheep are in isolated and small populations (Butler et al., 2013). The limited size of bighorn populations has been attributed to respiratory pathogens. However, the limited knowledge of bighorn sheep physiology limits researchers and wildlife managers understanding of how respiratory pathogens affect bighorn sheep herds. Using NMR metabolic profiling would give researchers the opportunity to study the global metabolism of bighorn sheep and create a potential panel of metabolites that may assess herd health in relation to nutritional states, environmental conditions, and disease states.

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CHAPTER THREE

EFFECT OF LONG-TERM PROGESTERONE ON FEED EFFICIENCY, BODY COMPOSITION, NON-ESTERIFIED FATTY ACIDS, AND METABOLIC HORMONES IN MATURE RAMBOUILLET EWES

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Contributions: Was the main person for data collection, data analysis, interpretation of article, and drafting of the paper.

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Contributions: Was critical in experiment design, data collection, data analysis, data interpretation, and the critical revision of this paper

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ABSTRACT

The objectives of this study were to evaluate the effects of long-term progesterone (P4) treatment on changes in feed efficiency, BW, body composition, NEFA and metabolic hormones in mature Rambouillet ewes. Thirty, multiparous, 5- and 6-yr-old Rambouillet ewes were stratified by age and metabolic BW and assigned randomly to receive long-term P4 administration using controlled intravaginal releasing devices (CIDR) or no P4 (CIDRX; CIDR backbone only). Initially, ewes were synchronized for estrus using a 7-d CIDR and PGF_{2α} protocol. All ewes exhibited estrus within 72-h after PGF_{2α}. Twelve-d after estrus (d = 0), each ewe received either a CIDR (n = 15) or a CIDRX (n = 15). Every 2-wk thereafter, the CIDR or CIDRX was removed from each ewe and replaced with a new CIDR or CIDRX for 126-d. Jugular venous blood samples were collected from each ewe at the time of CIDR or CIDRX replacement. Serum samples were assayed for P4, NEFA, insulin (INS), triiodothyronine (T3) and thyroxine (T4). Individual feed intake was recorded using the GrowSafe units beginning at d-0 following a 3-wk adaptation period. Ewes were fed a mixed grass hay diet ad libitum that met the nutrient requirements for maintenance. BW for each ewe was collected every 2-wk when CIDR or CIDRX were replaced. Back fat (BF) and rib-eye area (REA) were measured for each ewe every 28-d using ultrasonography. BW, RFI, BF and REA did not differ ($P > 0.10$) between CIDR- and CIDRX-treated ewes. Calculated estimates of body composition did not differ ($P > 0.10$) between CIDR- and CIDRX-treated ewes. NEFA, T3 and T4 concentrations did not differ ($P > 0.10$) between CIDR- and CIDRX-treated ewes. However, NEFA concentrations did differ ($P < 0.05$) between d-56 and d-126.

Furthermore, concentrations of T3 and T4 did differ ($P < 0.05$) between d-84 and d-126. Insulin concentrations did differ ($P < 0.05$) between CIDR- and CIDRX-treated ewes. In conclusion, long-term P4 treatment did not appear to alter feed efficiency and portioning of nutrients. However, maintaining P4 may alter the homeostatic relationship between INS and carbohydrate metabolism in ewes.

Key words: carcass traits, ewe, metabolism, progesterone, residual feed intake, hormones, metabolites

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INTRODUCTION

Nutrition and metabolism are known to affect reproduction in livestock. It is established that nutrition affects reproduction by not only providing energy directly to the embryo or fetus for development and growth, but also through regulation of hormones that control reproduction and impact development of the neonate (Robinson et al., 2006; Boland et al., 2001). Furthermore, the nutritional status of ewes has been shown to interact with systemic progesterone (P4) concentrations and influence the maintenance of pregnancy (Parr et al., 1987).

In 2014, Swartz et al. reported that the total kg of TDN consumed per ewe per kg of lamb born was 24% greater in Rambouillet ewes from lines selected high reproductive rate (HL) than in ewes selected for low reproductive rate (LL). In their study, nutrient

intake and TDN did not differ between lines of ewes. Additionally, the only endocrinological difference between ewes of these lines was that systemic concentrations of P4 were greater in HL ewes than in LL ewes between 60- and 120-d of gestation. The increase in P4 was higher in the HL ewes because the HL ewes had a higher number of twins than the LL ewes. However, the increase in P4 does not explain why there is a 24% increase in efficiency between the two lines. Even though the physiological mechanism in this observation is not clear, one could hypothesize that HL ewes were more efficient in partitioning nutrients into fetal growth and development. These results may be interpreted to mean that the apparent increase in efficiency of nutrient utilization in HL ewes during gestation was the result of increased concentrations of P4 between d-60 and d-120 of gestation.

Based on the results of Swartz et al. (2014) we hypothesized that long-term, systemic P4 concentrations may be related to increased feed efficiency and to changes in partitioning of nutrients. Thus, the objectives of this study were to evaluate the effects of long-term P4 treatment, independent of the influence of the placenta and fetus, on changes in feed efficiency, BW, and body composition, NEFA, and metabolic hormones in mature Rambouillet ewes. The hypotheses tested in this experiment were that feed efficiency, BW, back fat (BF), rib-eye area (REA), body composition, NEFA and metabolic hormones do not differ between Rambouillet ewes treated with a long-term P4 regimen, maintained with controlled intravaginal releasing devices (CIDR), or ewes not treated with the long-term P4 (CIDRX) regimen for 126-d.

MATERIALS AND METHODS

Animals and Housing

This experiment was conducted at the Montana State University Bozeman Area Research and Teaching Facility (BARTF). Animal care, handling, and protocols used in this experiment were approved by the Montana State University Agricultural Animal Care and Use Committee.

Thirty, multiparous, 5- and 6-yr-old commercial Rambouillet ewes from the Montana State University, Red Bluff Research Ranch flock in Norris, Montana were used for this study. Additionally, two 2-yr-old crossbred Suffolk x Rambouillet and one 4-yr-old Rambouillet, sexually experienced, epididymectomized rams were used for detection of estrus.

At the beginning of the study, each ewe received an electronic individual ID ear tag that was used to record feed intake in the GrowSafe units (GrowSafe Systems Ltd., Airdrie, AB, Canada). Ewes were housed in four open-shed pens (33 m x 11 m) each with an individual GrowSafe unit. Each pen included 8 or 7 ewes. Each pen had its own individual watering system. During sample collection, the ewes were walked to a facility next to the pens, which contained an alleyway and an enclosed scale. This enclosed scale is where the blood samples and weights were collected.

Treatments

Before the beginning of the feeding trial adaptation period, individual BW were collected on two consecutive days and averaged. The average of the BW for each ewe

was used to calculate individual metabolic BW ($MBW = BW^{0.75}$). At the same time, estimates of BF and REA were obtained by ultrasonography over the 12th rib of each ewe.

Ewes were stratified by age and MBW, then assigned randomly to one of two treatments. Treatments were:

- 1) long-term P4 maintenance using P4-containing CIDR (CIDR; n = 15)
- 2) no long-term P4 maintenance using a non-P4-containing CIDR backbone (CIDRX; n = 15).

To make the CIDR backbone, the outer, P4-containing silastic membrane was removed by slicing down the long axis of the CIDR with a scalpel blade and peeling the membrane from the plastic T-shaped backbone. All CIDR backbones were soaked in 80% ethanol (vol/vol H₂O). They were dried and coated with three layers of Flex Seal Liquid Rubber (Swift Response, Weston, FL, USA) to minimize the abrasive properties of the backbone on the vaginal wall of ewes. An in-depth procedure on how to make the non-P4-containing CIDR backbone is included in Appendix A.

In this experiment, it was necessary to normalize the length of the long-term P4 treatment to estrus or the estrous cycle of each ewe. This was accomplished using the 7-d CIDR and PGF₂ protocol. Each ewe received a CIDR for 7-d. On d-7, CIDR were removed and each ewe was injected (i.m.) with 12.5 mg of PGF₂ (dinoprost tromethamine; ProstaMate®, Vedico, Inc., St. Joseph, MO, USA). Ewes were then exposed to epididymectomized rams that had painted briskets to mark the rumps of any ewe that exhibited estrus. All ewes showed estrus within 72-h after PGF₂. Twelve-d after

estrus each CIDR-treated and CIDRX-treated ewe received a CIDR or CIDRX, respectively. This event was the beginning of the feeding trial and d-0 of the experiment. Maintenance of long-term P4 concentrations in each ewe was accomplished by replacing a CIDR every 14-d with a new CIDR. The backbones of the CIDRX-treated ewes were replaced every 14-d with fresh CIDR backbones. Figure 1 shows the schematic representation of the progesterone concentrations in the CIDR- and CIDRX- treated ewes in relation to the estrous cycle, along with the replacement of the CIDR and CIDRX for each of the treatments. The timeline for CIDR and CIDRX replacement is in Figure 2.

Blood Sampling Procedures

Blood samples were obtained by venepuncture of the jugular vein from each ewe every 2-wk along with the CIDR or CIDRX was replacement (Figure 2). Blood samples were cooled, allowed to clot and centrifuged into serum within 24-h after collection. Serum samples were stored in aliquots at -20°C and -80°C until assayed for metabolites and hormones.

BW and Ultrasonography for BF and REA

Body weights of each ewe were collected on two consecutive days beginning on d-0 and every 14-d associated with the replacement of either a CIDR or CIDRX (Figure 2). The averages of the two consecutive BW were considered the BW for that day. Estimates of BF and REA were obtained by ultrasonography every 28-d beginning at d-0 (Figure 2).

Feeding Intake and Nutrition

A 126-d trial was conducted to estimate the feed efficiency of CIDR- and CIDRX-treated ewes using the GrowSafe feed intake system. Ewes were given ad libitum access to mixed grass hay, water, and mineralized salt blocks. The chemical composition is given in Table 1. The chemical composition of the mixed grass hay on an as fed basis met the NRC (NRC, 2006) nutrient requirements for maintenance of a 60-kg adult ewe. Ewes were allowed a 3-wk adaptation period where the feed bars were removed from the GrowSafe units so that multiple ewes could eat at the same time. At the beginning of the experiment feed bars were replaced to ensure accurate measurements of individual ewe feed intake.

Residual Feed Intake Calculations

Daily intakes were computed for each of the ewes from the feed intakes derived from the GrowSafe Data software. Days that had scale noise greater than 12% and with assigned feed disappearance less than 92% were not used for feed intakes. Average daily gain (kg/d) of individual ewes were modeled by linear regression of bi-weekly BW using the PROC GLM procedure of SAS (SAS Inst., Inc., Cary, NC, USA). The regression coefficients were used to compute the ADG, initial and final BW, and mid-test MBW as described by Lancaster et al. (2009). Expected feed intake (EFI) was modeled using PROC GLM by linear regression of DMI against the modeled mid-test MBW and ADG (Koch et al., 1963). The model used to estimate EFI was:

$$Y_i = \beta_0 + \beta_1 ADG_i + \beta_2 \text{mid-test MBW}_i + \epsilon_i$$

where Y_i is the DMI of the ewe, β_0 is the regression intercept, β_1 is the partial regression coefficient of DMI on modeled ADG, β_2 is the partial regression coefficient of DMI modeled on mid-test MBW, and ε_i is the residual error in the DMI of the ewes. Residual feed intake (RFI) was calculated for each ewe as the difference between DMI and EFI.

Calculated Estimates of Body Composition

Estimates of muscle mass (kg) and intra-muscular fat (kg) were calculated from BF, REA and BW based on regression equations reported by Silva et al. (2006) for mature ewes. Estimates of empty body weight (kg), and proportions of empty body weight dry matter (%), empty body weight fat (%), empty body weight protein (%), carcass weight (kg), carcass weight dry matter (%), carcass weight fat (%), and carcass weight protein (%) were calculated from BW based on regression equations reported by Sanson et al. (1993) for mature ewes.

Progesterone Hormone Assay

Progesterone concentrations were assayed using radioimmunoassay (RIA) kits (MP Biomedical, Costa Mesa, CA) validated for sheep serum. Intra- and inter-assay CVs for a pooled sample that contained 0.31 ng/mL of P4 were 12% and 2.1%, respectively. The sensitivity of this assay was 0.62 ng/mL.

Thyroid Hormones Assays

Thyroxine (T3) and triiodothyronine (T4) concentrations were assayed using radioimmunoassay (RIA) kits (MP Biomedical, Costa Mesa, CA) validated for sheep serum. For the T3 and T4 assays, all the samples were assayed on a single run and the

intra-assay CVs for a pooled sample that contained 0.18 ng/mL of T3 and 44 ng/mL of T4 were 9.6% and 3.6%, respectively. The sensitivity of the T3 and T4 assays were 0.013 ng/mL and 0.411 ng/mL, respectively.

Insulin Hormone Assay

Insulin (INS), concentrations were assayed using an RIA kit (EMD Millipore, Darmstadt, Germany) validated for sheep serum. All the samples were assayed in a single run and the intra-assay CVs for a pooled sample that contained 5.6 ng/mL of INS was 4.7%. The sensitivity of this assay was 0.10 ng/mL.

Non-Esterified Fatty Acid Assay

Concentrations of NEFA was quantified with a commercially available enzymatic-colorimetric assay (HR Series NEFA – HR [2]., Wako Diagnostics, Richmond, VA) validated for sheep serum. Intra- and inter-assay CVs for a pooled sample that contained 0.21 mEq/L of NEFA were 5.1% and 3.7%, respectively. The sensitivity of this assay was 0.017 mEq/L.

Statistical Analysis

Data for BW, RFI, BF, and REA at 70-d were analyzed by ANOVA for completely randomized design using PROC ANOVA of SAS. The model included treatment (CIDR and CIDRX). Means from each analysis were separated using Bonferroni's adjustment.

Data for muscle mass (kg), intra-muscular fat (kg), empty body weight (kg), empty body weight dry matter (%), empty body weight fat (%), empty body weight

protein (%), carcass weight (kg), carcass weight dry matter (%), carcass weight fat (%), and carcass weight protein (%) were analyzed by ANOVA using separate PROC MIXED models for repeated measures of SAS. The covariance structure was compound symmetry. The model included treatment (CIDR and CIDRX), day (ultrasound day), and the treatment by day interaction. Ewe within treatment was the subject and d of ultrasound was the repeated measure. Means were separated using Bonferroni's multiple comparison adjustment.

Data for P4, T3, T4, INS and NEFA concentrations, and the T3:T4 ratio were analyzed by ANOVA using separate PROC MIXED models for repeated measures of SAS (SAS, Cary, NC). Again, the covariance for this model was compound symmetry. The model included treatment (CIDR and CIDRX), day (ultrasound day), and the treatment by day interaction. Ewe within treatment was the subject and day of ultrasound was the repeated measure. Means were separated using Bonferroni's multiple comparison adjustment.

RESULTS

Progesterone concentrations on d-0 differed ($P < 0.05$) between those ewes that received a CIDR and those that received a CIDRX (Figure 3). There was a treatment by day interaction ($P < 0.05$) for P4 concentrations over the 126-d experimental period (Figure 3). Progesterone concentrations in CIDR-treated ewes decreased by d-14 and remained constant until d-84 then increased and remained high from d-96 to d-126.

Whereas, P4 concentrations remain the same in CIDRX-treated ewes until d-98 and remain low (< 1.5 ng/mL) until d-126 (Figure 3).

Body weight, STDMI, RFI, BF and REA did not differ between CIDR- and CIDRX-treated ewes (Table 2). Likewise, calculated estimates of muscle mass, intra-muscular fat, empty body weight, carcass weight; percentages of empty body weight as dry matter, fat, and protein; and, percentages of carcass weight as dry matter, fat, and protein did not differ between CIDR- and CIDRX-treated ewes (Table 3).

Non-esterified fatty acids, T3 and T4 concentrations did not differ between CIDR- and CIDRX-treated ewes (Table 4). However, NEFA concentrations differed ($P < 0.05$) between d-56 and d-126. Concentrations of T3 and T4 differed between d-84 and d-126 (Table 4). Concentrations of INS were greater ($P < 0.05$) in CIDRX-treated ewes than in CIDR-treated ewes and between d-0 and d-126 (Table 5).

DISCUSSION

To our knowledge this is the first study using CIDRs for maintenance of long-term P4 concentrations in the systemic circulation. We confirmed that long-term P4 can be sustained using sequential replacement of CIDRs for 126-d. The difference between CIDRX- and CIDR- treated ewes on day-0 (d-12 of the estrous cycle) may be due to individual ewe differences at the time of ovulation relative to estrus. Progesterone concentrations at d-14 in CIDRX- ewes is consistent with a normal estrous cycle length and luteal function in the next cycle. Whereas, P4 concentrations in CIDR- treated ewes reflect early regression of the corpus luteum (Ottobre et al., 1980) and inhibition of estrus

and ovulation. For CIDRX- treated ewes, d-42 represents the periovulatory period, which is characterized by low concentrations of P4. Thereafter, P4 concentrations in CIDRX- treated ewes continue to decrease from d-56 to d-126 because of the change in photoperiod associated with the onset of the anestrus season. This is reflected in the progressive increase in the proportion of anestrus ewes from 25% at d 56, 57% at d 84, and 95% at d 126.

On the other hand, P4 concentrations were maintained at ≥ 2 ng/ml between d-14 to d-84 because of P4 release from sequential replacement of CIDRs (Figure 3). In the study by Sarda et al. (1973), P4 concentrations in pregnant ewes did not markedly increase until between d-80 and d-90 of pregnancy. Furthermore, Swartz et al. (2014) reported that P4 concentrations did not differ between HL and LL ewes until after d-60. One of our hypotheses for this study was that P4 concentrations needed to be maintained for longer than 70-d to cause a change in metabolism in sheep. To mimic pregnancy, two CIDRs were inserted at d-84 to increase P4 concentrations mimicking the change associated with pregnancy. Indeed, at d-98, P4 concentrations markedly increased indicating that two CIDRs do in fact increase P4 concentrations similar to those observed in pregnant ewes.

More importantly, the focus of this study was to evaluate the effects of long-term P4 treatment on changes in feed efficiency, BW, body composition, metabolic hormones and NEFA concentrations in mature Rambouillet ewes, independent of placental and fetal functions. Mimicking pregnancy related changes in P4 concentrations in ewes using sequential replacement and number of CIDRs did not influence feed efficiency, BW,

estimates of muscle mass, intra-muscular fat, empty body weight, carcass weight; percentages of empty body weight as dry matter, fat, and protein; and, percentages of carcass weight as dry matter, fat, and protein. Moreover, long-term P4 treatment did not differ in T3, T4 or NEFA concentrations between CIDR- and CIDRX- treated ewes.

The studies conducted in heifer feedlot cattle reported that feeding heifers MGA increased average daily gain and gain to feed ratios when compared to heifers not fed MGA (Bloss, et al., 1966 and Kreikemeier and Mader, 2004). One reason that MGA may increase feed efficiency in these studies and not in the present study is because these heifers were growing and receiving a high caloric intake diet. Without outside variables such as high caloric diet or growth present, P4 does not seem to increase feed efficiency.

However, long-term P4 had lower concentrations of INS throughout the study. There is evidence that P4 increased INS resistance in rats (Kumagai et al., 1993). Another study on P4 influence on INS in rats, reported that P4 increases plasma levels of INS to improve glucose tolerance, with no effect on feed intake or fat deposition (Wade and Gray, 1978). This study also reported that any changes associated with feed intake or body composition will occur even in the absence of INS. Yet, our results indicate that in sheep, the higher concentrations of P4 have higher concentrations of INS and the lower concentrations of P4 have lower concentrations of INS. It appears that in sheep, P4 increases tissue responsiveness to INS, because glucose concentrations do not differ. In other words, the higher concentrations of P4 are associated with lower concentrations of INS for the same amount of glucose. One difference between the previous studies and the current study was the first studies were conducted in monogastrics and this study is

conducted in ruminants. Whether ruminants and monogastrics differ in P4's influence on INS is unknown. Further research needs to be conducted to determine if the results from this study concerning INS is repeatable or not.

In conclusion, it appears that maintaining systemic P4 concentrations that mimic those observed during pregnancy for 126-d in the ewe does not affect changes in BW, feed efficiency, body composition, or temporal concentrations of NEFA, T3 or T4. However, it appears that long-term P4 treatment may alter mechanisms associated with carbohydrate metabolism. Our results indicate that systemic P4 concentrations are not directly related to increased feed efficiency and changes in partitioning of nutrients over a 126-d. Any changes observed in the Swartz et. al., 2014 study was due to signals either from the placenta, fetus, or a combination of progesterone with fetal and placental signaling. However, maintaining P4 may alter the homeostatic relationship between INS and carbohydrate metabolism.

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Table 1. Chemical composition of mixed grass hay diet¹

Item	Mixed Grass Hay diet
Nutrient analyses	
Moisture, %	13.3
Dry Matter, %	86.7
Crude Protein, %	7.8
Fiber (acid det.), %	37.8
TDN, %	59.4
Net energy (maint.), mCal/lbs	0.6
Net energy (gain), mCal/lbs	0.3
Sulfur, %	0.2
Phosphorous, %	0.2
Potassium, %	2.2
Magnesium, %	0.2
Calcium, %	0.5
Sodium, %	n.d.
Iron, ppm	79.1
Manganese, ppm	90.2
Copper, ppm	7.7
Zinc, ppm	18.0

¹ Ewes had free access to the mixed grass hay diet.

Table 2. Least square means of final body weight (BW), final standard dry matter intake (STDMI), residual feed intake (RFI), final back fat depth (BF), and final rib-eye area (REA) in Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for 126-d

Item	Treatment		SEM ^a	<i>P</i> -value
	CIDR	CIDRX		
n	15	15		
BW, kg	57.8	58.7	8.40	0.70
STDMI, kg	1.32	1.40	0.04	0.49
RFI, kg/d	-0.03	0.02	0.20	0.50
BF, mm	1.9	2.0	0.20	0.46
REA, mm ²	26.4	26.8	0.50	0.60
^a Pooled SEM				

Table 3. Muscle mass (M), intra-muscular fat (IMF), empty body weight (EMW), empty body weight dry matter (EBWDM), empty body weight fat (EBWF), empty body weight protein (EBWP), carcass weight (CW), carcass weight dry matter (CWD), carcass weight fat (CWF), and carcass weight protein (CWP) of Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for 126-d

Item	Treatment		SEM ^a	P-value
	CIDR	CIDRX		
n	15	15		
M, kg	13.3	13.7	2.2	0.60
IMF, kg	1.9	2.0	0.1	0.57
EMW, kg	48.9	49.1	2.9	0.92
EMWDM, %	45.4	45.5	0.4	0.92
EBWF, %	18.2	18.4	1.4	0.92
EBWP, %	17.82	17.79	0.05	0.92
CW, kg	26.9	27.1	1.0	0.92
CWD, %	50.2	50.3	0.3	0.92
CWF, %	18.7	18.8	1.2	0.92
CWP, %	19.48	19.45	0.06	0.92
^a Pooled SEM				

Table 4. Least square means of non-esterified fatty acids (NEFA), thyroxine (T3) and triiodothyronine (T4) concentrations of Rambouillet ewes for both treatments (received a P4-containing controlled intravaginal releasing device or received a non-progesterone containing CIDR backbone) for 126-d

Item	Day				
	0	28	56	84	126
NEFA, mEq/L ¹	0.35 ^{a, b}	0.37 ^{a, b}	0.27 ^a	0.37 ^{a, b}	0.43 ^b
T3, ng/mL	0.99 ^a	0.96 ^a	0.90 ^a	0.79 ^b	0.98 ^a
T4, ng/mL	38.9 ^a	38.5 ^a	35.2 ^a	30.7 ^b	35.7 ^a
T3:T4	0.025	0.025	0.026	0.026	0.028

^{a, b} Means within rows with different superscripts letters differ; $P < 0.05$.

^{1,2,3,4} Pooled SEM = 0.033 mEq/L; 0.05 ng/mL; 24.9 ng/m; 0.00004, respectively.

Table 5. Least square means of insulin (INS) concentrations of Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for 126-d

Item	Treatment		Mean ¹
	CIDR	CIDRX	
n	15	15	
0	1.3	2.3	1.8 ^a
28	1.4	1.9	1.7 ^a
56	1.3	2.0	1.7 ^a
84	1.2	1.9	1.6 ^a
126	2.0	2.7	2.3 ^b
Mean ²	1.4 ^a	2.2 ^b	

^{a,b} Means within a column or row with different letters differ; $P < 0.05$.

¹ Pooled SEM = 0.05 ng/mL.

² Pooled SEM = 0.03 ng/mL.

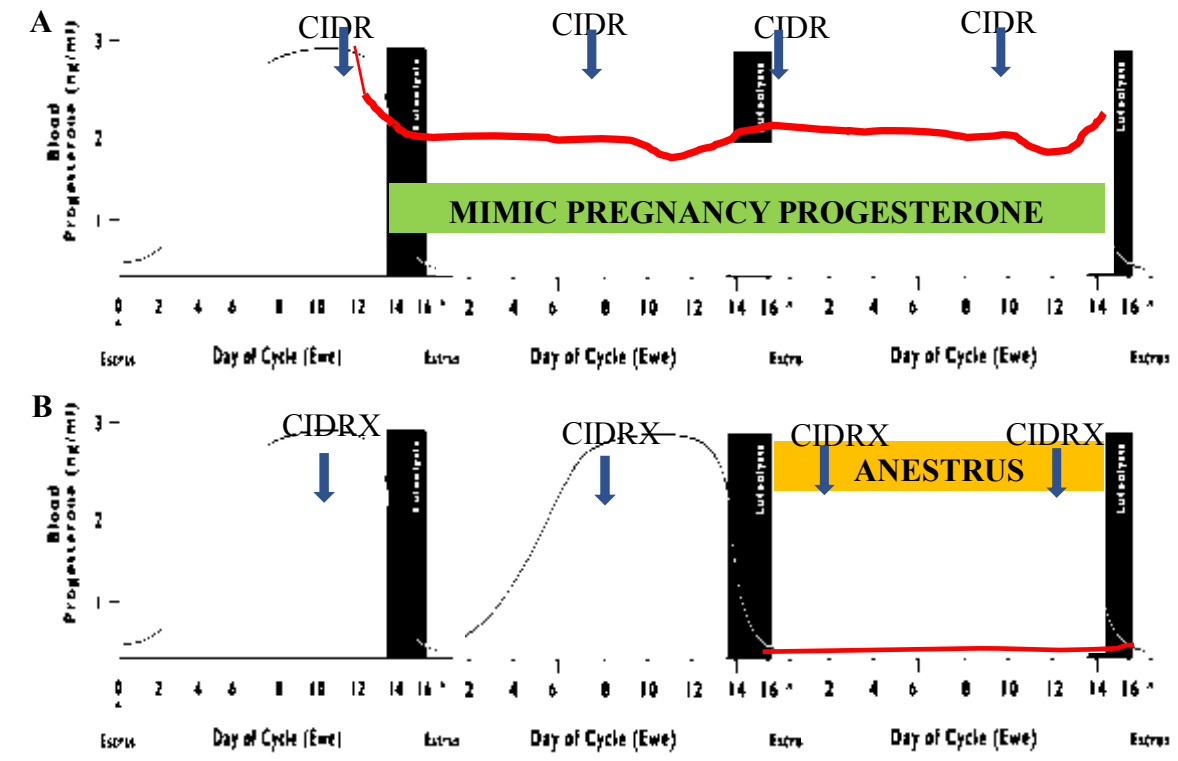


Figure 1. Schematic representation (adapted from Senger, 2012) of progesterone (P4) concentrations at 14-d intervals in relation to the estrous cycle in (A) Rambouillet ewes given a P4-containing, controlled internal drug release device (CIDR; n = 15) to mimic P4 concentrations of pregnancy and in (B) a non-P4-containing CIDR (CIDRX; n = 15). Both CIDR- and CIDRX- treatments began on d-12 (d 0 insertion of devices) of the estrous cycle relative to the estrus of the ewes.

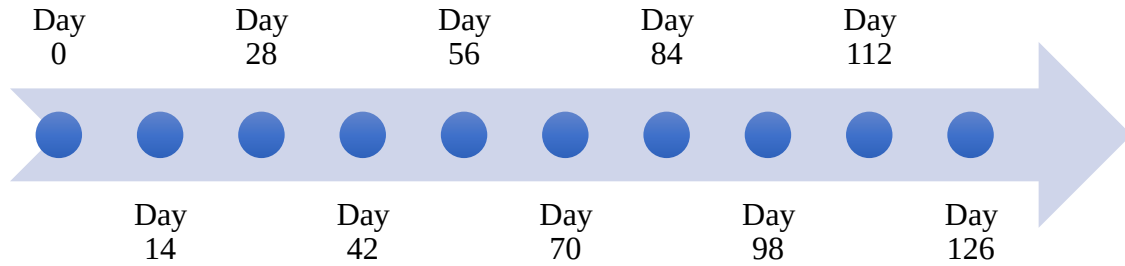


Figure 2. Timeline for sampling protocols during the 126-d experiment. Every 14-d CIDR or CIDRX were changed, blood samples obtained, and body weights recorded. Every 28-d rib-eye area and back fat thickness were ultra-sounded.

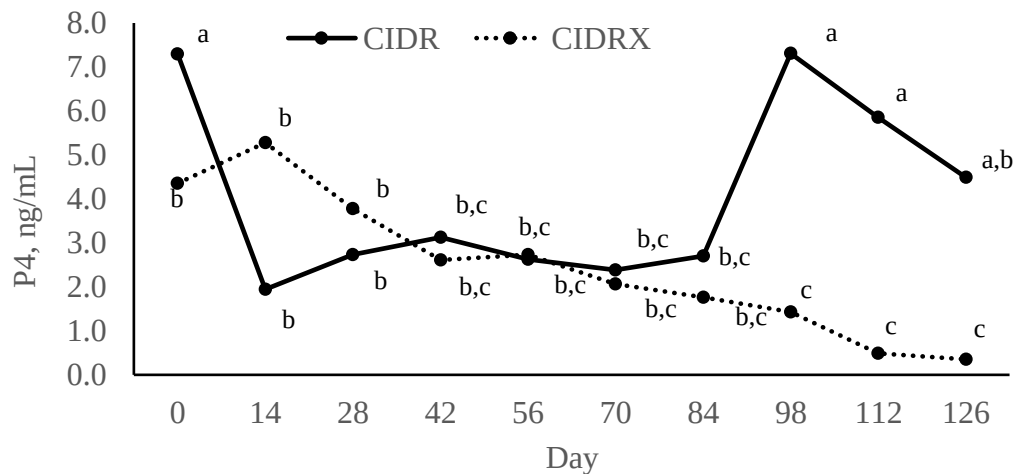


Figure 3. Progesterone (P4) concentrations at 14-d intervals in Rambouillet ewes given a P4-containing, controlled internal drug release device (CIDR; $n = 15$) or a non-P4-containing CIDR (CIDRX; $n = 15$) beginning on d-12 (d 0 insertion of devices) of the estrous cycle relative to estrus. Interaction of treatment $\times$ d; $P < 0.05$. Different letters among points indicate differences at $P < 0.05$. Pooled SEM = 5.1 ng/mL.

CHAPTER FOUR

USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR) METABOLIC PROFILING TO STUDY THE EFFECT OF LONG-TERM PROGESTERONE ON METABOLIC PROFILES IN RAMBOUILLET EWES

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Contributions: Operates the NMR spectrophotometer critical for samples to be assayed for metabolic profiling.

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Contributions: Was critical in experiment design, data collection, data analysis, data interpretation, and the critical revision of this paper

Manuscript Information Page

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Abstract

Introduction

Progesterone has been examined in multiple studies on its effects on body composition and feed efficiency in ruminant animals. However, it is unknown if the effects of long-term progesterone only can change protein, lipid, or carbohydrate metabolism associated with shifts in maternal metabolism.

Objectives

The objective of this study was to determine if NMR metabolic profiling can distinguish between Rambouillet ewes treated with long-term progesterone that mimics concentrations of pregnancy and Rambouillet ewes not treated with long-term progesterone.

Methods

Metabolites were identified in Rambouillet ewe serum through NMR metabolic profiling. The metabolites were analyzed through PLS-DA and time-series analysis to determine if NMR metabolic profiling can distinguish between Rambouillet ewes treated with long-term progesterone that mimics concentrations of pregnancy and Rambouillet ewes not treated with long-term progesterone.

Results

There was no difference in BW, RFI, REA, BF, or body composition between Rambouillet ewes treated with long-term progesterone and Rambouillet ewes not treated

with long-term progesterone. There was no metabolic shift associated with the CIDR- or CIDRX- treated ewes in the PLS-DA or time-series analyses.

Conclusion

This is the first study to our knowledge that illustrates long-term homeostasis. Any metabolic shift associated with pregnancy must originate from signals from the placenta or fetus and not from progesterone.

Keywords

NMR, Metabolomics, Rambouillet ewe, Homeostasis

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1 Introduction

Metabolomics is the study of metabolic intermediates and products of cellular metabolism. Metabolomics allows for a snapshot of the global metabolism of an individual, and explains the functional nutritional and health states that an animal is currently in. The global metabolism of an individual may be measured and assessed using the analytical technology of NMR. Metabolomics first was applied in human medicine to study disease states of cancer, diabetes, and autoimmune disorders; as well as the application of pharmaceuticals. Currently, metabolomics has expanded to domestic animals to study infectious diseases and metabolic disorders. Metabolomics is also being applied in domestic livestock research in feed efficiency trials. Metabolites that are identified by NMR are associated with known metabolic pathways that can be directly linked to an animal's physiological states (Sun et al., 2016).

Nuclear magnetic resonance metabolic profiling has many advantages and a few disadvantages. One advantage is minimal amount of time needed for sample preparation, which is only about nine hrs. total for 96 samples. Another advantage is the number of metabolites that can be identified using NMR spectroscopy. While traditional metabolite assays only produce concentrations of one metabolite, NMR has the potential to identify 40-200 metabolites depending what extraction procedure is used. Since NMR can identify multiple metabolites from one sample, it is very cost-effective when compared with traditional metabolites. The last advantage of NMR is that it is highly reproducible and sample recovery is high. One disadvantage of NMR is the training and expertise associated with analyzing the samples. The initial training on the machine may be time

consuming. However, once training is complete, the baselining and profiling of the samples takes about 20 minutes per sample.

Progesterone (P4) is a hormone that is essential for the maintenance of pregnancy. Progesterone has been examined in numerous studies on its effects on body composition in feedlot cattle. One study reported that feeding heifers melengesterol acetate (MGA) increased average daily gain and gain to feed ratios when compared to heifers not feed MGA (Bloss, et al., 1966). In this same study, MGA did not differ in carcass characteristics between heifers fed MGA or not. A second study found the same results that MGA increased feed efficiency, but did not improve carcass characteristics (Kreikemeier and Mader, 2004). Furthermore, in ewes, the nutritional status has been shown to interact with systemic progesterone concentrations and influence the maintenance of pregnancy (Parr et al., 1987).

In 2014, Swartz et al. reported that the total kg of TDN consumed per ewe per kg of lamb born was 24% greater in Rambouillet ewes from lines selected high reproductive rate (HL) than in ewes selected for low reproductive rate (LL). In their study, nutrient intake and TDN did not differ between lines of ewes. Additionally, the only endocrinological difference between ewes of these lines was that systemic concentrations of P4 were greater in HL ewes than in LL ewes between 60- and 120-d of gestation. The increase in P4 was higher in the HL ewes because the HL ewes had a higher number of twins than the LL ewes. However, the increase in P4 does not explain why there is a 24% increase in efficiency between the two lines. Even though the physiological mechanism

in this observation is not clear, one could hypothesize that HL ewes were more efficient in partitioning nutrients into fetal growth and development.

Based on the results of Swartz et al. (2014) we hypothesized that long-term, systemic P4 concentrations may be related to a shift of metabolism that could increase feed efficiency and change partitioning of nutrients. Therefore, the objectives of this study were to evaluate the effects of long-term P4 treatment, independent of the influence of the placenta and fetus, on changes in metabolites using NMR spectroscopy metabolic profiling in mature Rambouillet ewes. The hypothesis tested in this experiment was that there is no metabolic difference between Rambouillet ewes that received long-term P4 maintained with a controlled intravaginal releasing devices (CIDR), or Rambouillet ewes not treated with the long-term P4 (CIDRX) regimen for 126-d.

2 Materials and Methods

2.1 Animals and Housing

This experiment was conducted at the Montana State University Bozeman Area Research and Teaching Facility (BARTF). Animal care, handling, and protocols used in this experiment were approved by the Montana State University Agricultural Animal Care and Use Committee.

Thirty, multiparous, 5- and 6-yr-old commercial Rambouillet ewes from the Montana State University, Red Bluff Research Ranch flock in Norris, Montana were used for this study. Additionally, two 2-yr-old crossbred Suffolk x Rambouillet and one 4-yr-

old Rambouillet, sexually experienced, epididymectomized rams were used for detection of estrus.

At the beginning of the study, each ewe received an electronic individual ID ear tag that was used to record feed intake in the GrowSafe units (GrowSafe Systems Ltd., Airdrie, AB, Canada). Ewes were housed in four open-shed pens (33 m x 11 m) each with an individual GrowSafe unit. Each pen included 8 or 7 ewes. Each pen had its own individual watering system. During sample collection, the ewes were walked to a facility next to the pens, which contained an alleyway and an enclosed scale. This enclosed scale is where the blood samples and weights were collected.

2.2 Treatments

Before the beginning of the feeding trial adaptation period, individual BW were collected on two consecutive days and the BW were averaged. The average of the BW for each ewe was used to calculate individual metabolic BW ($MBW = BW^{0.75}$). At the same time, estimates of BF and REA were obtained by ultrasonography over the 12th rib of each ewe.

Ewes were stratified by age and MBW, then assigned randomly to one of two treatments. Treatments were:

- 1) long-term P4 maintenance using P4-containing CIDR (CIDR; n = 15)
- 2) no long-term P4 maintenance using a non-P4-containing CIDR backbone (CIDRX; n = 15).

To make the CIDR backbone, the outer, P4-containing silastic membrane was removed by slicing down the long axis of the CIDR with a scalpel blade and peeling the

membrane from the plastic T-shaped backbone. All CIDR backbones were soaked in 80% ethanol (vol/vol H₂O). They were dried and coated with three layers of Flex Seal Liquid Rubber (Swift Response, Weston, FL, USA) to minimize the abrasive properties of the backbone on the vaginal wall of ewes. An in-depth procedure on how to make the non-P4-containing CIDR backbone is included in Appendix A.

In this experiment, it was necessary to normalize the length of the long-term P4 treatment to estrus or the estrous cycle of each ewe. This was accomplished using the 7-d CIDR and PGF₂ protocol. Each ewe received a CIDR for 7-d. On d-7, CIDR were removed and each ewe was injected (i.m.) with 12.5 mg of PGF₂ (dinoprost tromethamine; ProstaMate®, Vedico, Inc., St. Joseph, MO, USA). Ewes were then exposed to epididymectomized rams that had painted briskets to mark the rumps of any ewe that exhibited estrus. All ewes showed estrus within 72-h after PGF₂. Twelve-d after estrus each CIDR-treated and CIDRX-treated ewe received a CIDR or CIDRX, respectively. This event was the beginning of the feeding trial and d-0 of the experiment. Maintenance of long-term P4 concentrations in each ewe was accomplished by replacing a CIDR every 14-d with a new CIDR. The backbones of the CIDRX-treated ewes were replaced every 14-d with fresh CIDR backbones. Figure 1 shows the schematic representation of the progesterone concentrations in the CIDR- and CIDRX- treated ewes in relation to the estrous cycle, along with the replacement of the CIDR and CIDRX for each of the treatments. The timeline for CIDR and CIDRX replacement is in Figure 2.

2.3 Blood Sampling Procedures

Blood samples were obtained by venepuncture of the jugular vein from each ewe every 2-wk along with the CIDR or CIDRX replacement (Figure 2). Blood samples were cooled, allowed to clot and centrifuged into serum within 24-h after collection. Serum samples were stored in aliquots at -20°C and -80°C until assayed for metabolites and hormones.

2.4 BW and Ultrasonography for BF and REA

Body weights of each ewe were collected on two consecutive days beginning on d-0 and every 14-d associated with the replacement of either a CIDR or CIDRX (Figure 2). The averages of the two consecutive BW were considered the BW for that day. Estimates of BF and REA were obtained by ultrasonography every 28-d beginning at d-0 (Figure 2).

2.5 Feeding Intake and Nutrition

A 126-d trial was conducted to estimate the feed efficiency of CIDR- and CIDRX-treated ewes using the GrowSafe feed intake system. Ewes were given ad libitum access to mixed grass hay, water, and mineralized salt blocks. The chemical composition is given in Table 1. The chemical composition of the mixed grass hay on an as fed basis met the NRC (NRC, 2006) nutrient requirements for maintenance of a 60-kg adult ewe. Ewes were allowed a 3-wk adaptation period where the feed bars were removed from the GrowSafe units so that multiple ewes could eat at the same time. At the beginning of the experiment feed bars were replaced to ensure accurate measurements of individual ewe feed intake.

2.6 Residual Feed Intake Calculations

Daily intakes were computed for each of the ewes from the feed intakes derived from the GrowSafe Data software. Days that had scale noise greater than 12% and with assigned feed disappearance less than 92% were not used for feed intakes. Average daily gain (kg/d) of individual ewes were modeled by linear regression of bi-weekly BW using the PROC GLM procedure of SAS (SAS Inst., Inc., Cary, NC, USA). The regression coefficients were used to compute the ADG, initial and final BW, and mid-test MBW as described by Lancaster et al. (2009). Expected feed intake (EFI) was modeled using PROC GLM by linear regression of DMI against the modeled mid-test MBW and ADG (Koch et al., 1963). The model used to estimate EFI was:

$$Y_i = \beta_0 + \beta_1 ADG_i + \beta_2 \text{mid-test MBW}_i + \varepsilon_i$$

where Y_i is the DMI of the ewe, β_0 is the regression intercept, β_1 is the partial regression coefficient of DMI on modeled ADG, β_2 is the partial regression coefficient of DMI modeled on mid-test MBW, and ε_i is the residual error in the DMI of the ewes. Residual feed intake (RFI) was calculated for each ewe as the difference between DMI and EFI.

2.7 Calculated Estimates of Body Composition

Estimates of muscle mass (kg) and intra-muscular fat (kg) were calculated from BF, REA and BW based on regression equations reported by Silva et al. (2006) for mature ewes. Estimates of empty body weight (kg), and proportions of empty body weight dry matter (%), empty body weight fat (%), empty body weight protein (%), carcass weight (kg), carcass weight dry matter (%), carcass weight fat (%), and carcass weight protein (%)

were calculated from BW based on regression equations reported by Sanson et al. (1993) for mature ewes.

2.8 Progesterone Hormone Assay

Progesterone concentrations were assayed using radioimmunoassay (RIA) kits (MP Biomedical, Costa Mesa, CA) validated for sheep serum. Intra- and inter-assay CVs for a pooled sample that contained 0.31 ng/mL of P4 were 12% and 2.1%, respectively. The sensitivity of this assay was 0.62 ng/mL.

2.9 Insulin Hormone Assay

Insulin (INS), concentrations were assayed using an RIA kit (EMD Millipore, Darmstadt, Germany) validated for sheep serum. All the samples were assayed in a single run and the intra-assay CVs for a pooled sample that contained 5.6 ng/mL of INS was 4.7%. The sensitivity of this assay was 0.10 ng/mL. Insulin concentrations were converted to mM and included with the metabolites identified using NMR spectroscopy. Insulin was included in these analyses because in a previous study, INS was the only metabolic hormone that differed between CIDR- and CIDRX- treated ewes.

2.10 Statistical Analysis for Insulin

Data for INS concentrations were analyzed by ANOVA using separate PROC MIXED models for repeated measures of SAS (SAS, Cary, NC). The covariance of the model is compound symmetry. The model included treatment (CIDR and CIDRX), day (ultrasound day), and the treatment by day interaction. Ewe within treatment was the

subject and day of ultrasound was the repeated measure. Means were separated using Bonferroni's multiple comparison adjustment.

2.11 Nuclear Magnetic Resonance Sample Procedure

Serum samples were prepared for nuclear magnetic resonance spectroscopy using the following protocol. Five-hundred μL of serum and 1,500 μL of acetone were added to 2 mL plastic, flat-cap, conical vials. Each vial was inverted 10 times and frozen for at -20°C for 1 hour. After 1 hour, vials were centrifuged at 4°C , at 13,000 $\times g$ for 30 minutes. The supernatant of each sample was then transferred to fresh 2 mL flat-cap vials and dried using a centrifuge-vacuum overnight. The next day, 500 μL of NMR buffer (phosphate buffer plus D_2O , TSP and imidazole) was mixed with the dried samples and transferred to 5 mm NMR tubes (Bruker©, 2017). The specific protocol used for extracting small metabolites from sera for NMR spectroscopic analysis is included in Appendix B.

2.12 NMR Spectroscopy

All NMR spectra were analyzed at 25°C on a Bruker 600 MHz (^1H Larmor frequency) AVANCE III solution spectrometer. The spectrometer is equipped with an automatic sample loading system (SampleJet™), a 5-mm helium-cooled ^1H -optimized TCI NMR probe (Cryoprobe™), and Topspin software (Bruker version 3.5). 1D ^1H NMR experiments were completed using the “zgpg30” Bruker pulse sequence and recorded with 256 scans and a ^1H spectral window of 9615.38 Hz. Free induction decays (FIDs) were sampled at 32 K data points and a dwell time interval of 52 μs totaling to an

acquisition time of around 1.7 seconds and a 1 second relaxation recovery delay between acquisitions.

Spectra were collected from the NMR operating system, Topspin 3.5 (Bruker©, 2017). The raw spectra were imported into the Topspin program, Fourier transformed, phased, and the standard was set to 0 ppm before being imported into the Chenomx© NMR Suite program software (Chenomix NMR Suite 8.1). Once the spectra were imported into the Chenomx software, they were baseline corrected. In the baseline correction, the water resonance region was deleted between 4.3 and 5.6 ppm. For metabolite identification, the Chenomix small molecule library for 600-MHz (^1H Larmor frequency) magnetic field strength NMR spectrometers were used and NMR spectral profiles were fitted for each sample. Our laboratory has established a NMR compound database containing 58 small molecule metabolites; all identified in ruminant serum or plasma. An internal DSS standard was used for the quantification of the 58 small molecule metabolites. Furthermore, one buffer control and one quality control serum from a ruminant were included with every sample-run to adjust for run to run variation. A more detailed description of the NMR spectroscopy procedure is included in Appendix B.

2.13 Chemometrics

Concentrations of compounds were statistically analyzed using MetaboAnalyst 3.0 (Xia and Wishart, 2016). The analyses conducted was a pathway enrichment analysis and a partial least squares discriminant analysis (PLS-DA). The PLS-DA model was used to visualize the data set and to accurately measure the covariance among the response. The R^2 and Q^2 variables were used to ensure the PLS-DA model was accurate and

appropriate. The R^2 variable is equal to the sum of squares captured by the model and the Q^2 variable is the cross-validated value of the R^2 . The PLS-DA model was used to identify any significantly different metabolites between Rambouillet ewes treated with long-term P4 and Rambouillet ewes not treated with long-term P4. From the PLS-DA, the variables importance for the projection (VIP) score for each metabolite that were greater than 1.0 were considered significantly different. The VIP scores are the metabolites that contribute to any differences identified through the PLS-DA if the model is appropriate. Comparisons were made to determine if NMR metabolic profiling can distinguish Rambouillet ewes that received long-term P4 (CIDR) for 126-d and Rambouillet ewes that did not receive long-term P4 (CIDRX) for 126-d. A time-series analysis was also conducted to determine if the interactive PCA plot can distinguish any differences associated with the treatment of long-term P4, time, or the interaction between treatment and time. In the time series analysis, the model is validated using permutation test statistics. A permutation test statistic is the ability of the model to distinguish between the treatment of long-term P4, time, and the interaction between treatment and time better than chance. A permutation test with a $p > 0.05$ cannot distinguish the treatment of long-term P4, time, or the interaction between treatment and time better than chance. The last step in determining model quality was to look at the leverage plots. The leverage points are observations with extreme values or predictor capability. The leverage points identify outliers in the model and any metabolites that can be used for predictor capabilities for the model.

3 Results

3.1 Identification of metabolites in the spectra of Rambouillet serum

The 58 small molecule metabolites that were identified in ruminant serum are in Appendix C. Every metabolite identified was cross referenced with the Human Metabolome Database to ensure the correct identification of metabolites.

3.2 Distinguishing Rambouillet ewes treated with long-term progesterone and Rambouillet ewes not treated with long-term progesterone

Progesterone concentrations on d-0 differed ($P < 0.05$) between those ewes that received a CIDR and those that received a CIDRX (Figure 3). There was a treatment by day interaction ($P < 0.05$) for P4 concentrations over the 126-d experimental period (Figure 3). Progesterone concentrations in CIDR-treated ewes decreased by d-14 and remained constant until d-84 then increased and remained high from d-96 to d-126. Whereas, P4 concentrations remain the same in CIDRX-treated ewes until d-98 and remain low (< 1.5 ng/mL) until d-126 (Figure 3).

Body weight, STDMI, RFI, BF and REA did not differ between CIDR- and CIDRX-treated ewes (Table 2). Likewise, calculated estimates of muscle mass, intramuscular fat, empty body weight, carcass weight; percentages of empty body weight as dry matter, fat, and protein; and, percentages of carcass weight as dry matter, fat, and protein did not differ between CIDR- and CIDRX-treated ewes (Table 3). Concentrations of INS were greater ($P < 0.05$) in CIDRX-treated ewes than in CIDR-treated ewes and between d-0 and d-126 (Table 4).

The PLS-DA illustrates that there is no clear metabolic shift between Rambouillet ewes treated with long-term progesterone and Rambouillet ewes not treated with long-term progesterone for the full 126-d (Figure 4). Furthermore, the R^2 value is 0.21 and the Q^2 value is -0.09. These values indicate that this model is highly inappropriate to use and the model's ability to identify treatments is no better than chance.

Table 5 is the R^2 value and the Q^2 value of the PLS-DA models for d-0, d-28, d-56, d-84, and d-126 of the experiment between Rambouillet ewes treated with long-term progesterone and Rambouillet ewes not treated with long-term progesterone. These values indicate that none of the PLS-DA models are effective in distinguishing between CIDR- and CIDRX- treated ewes and no metabolic shifts can be detected between the two treatments.

The full model of the time series analysis indicates that there is no metabolic difference between the CIDR- and CIDRX- treated ewes and that there is no metabolic difference between d-0 and d-126 (Figure 5). The permutation test statistics (Figure 6) are relatively small for the treatment and time aspect of the model. However, the permutation test statistics are almost one in the interaction between treatment and time. This indicates that the model cannot distinguish between CIDR- and CIDRX- treated ewes in a time series analysis. However, the leverage plots in Figure 7 indicate that there are 3 outliers identified in the treatment aspect of the model, 4 outliers identified in the time aspect of the model, and 2 outliers identified in the interaction between treatment and time. The next step was to determine whether reducing the model to containing no outliers would be able to distinguish CIDR- and CIDRX- treated ewes.

The reduced model of the time series analysis indicates that there is no metabolic difference between the CIDR- and CIDRX- treated ewes and that there is no metabolic difference between d-0 and d-126 when containing no outliers (Figure 8). The permutation test statistics (Figure 9) for the treatment, time and interaction between the two for the model is > 0.1 . This indicates that the model cannot distinguish between CIDR- and CIDRX- treated ewes in a time series analysis. Furthermore, the leverage plots in Figure 10 indicate that with all the outliers removed, there is one significantly important metabolite in the treatment, time, and the interaction between the two. However, because permutation test statistics are > 0.1 , the model cannot use these metabolites to distinguish between time or the treatment.

4 Discussion

Long-term P4 mimicking concentrations of pregnancy did not cause a change in the relationships of small metabolites identified through NMR metabolic profiling. Any metabolic changes that are associated with pregnancy are not due to progesterone alone. The combination of fetal or placental signals along with progesterone may cause metabolic shifts that are associated with pregnancy. In Figure 4, the PLS-DA cannot distinguish between CIDR- and CIDRX- treated ewes. This analysis was for all times and all treatments. For the full 126-d period, the analysis cannot distinguish between the two treatments and there does not appear to be a drift associated with time either. The full and reduced time-series analysis (Figure 5 and 8) does not distinguish a treatment, time or an interaction between treatment and time. This confirms that there was not a metabolic shift

between CIDR- and CIDRX- treated ewes or a metabolic shift associated with time. Furthermore, no changes in small metabolites corresponds to no differences in body composition, STDMI, RFI, REA and BF.

Many studies have been conducted in human females to determine if progesterone cause any changes in body composition. Human females say that progesterone makes them gain weight and body fat, however there are few studies that report this is true. A review on progestin-only contraceptives and its effect on weight gain and body composition was conducted by Lopez et al., in 2016. This review examined 22 different studies that have been conducted in human females on progestin-only contraceptives. This review stated that 15 of the studies reported no changes in body weight or in body composition between females taking a progestin-only contraceptive and females not taking a progestin-only contraceptive. Furthermore, 5 of the studies reported that there were slight changes in body weight and body composition, however, the studies methods were questionable. Finally, 2 of the studies reported significant changes in body composition and body weight between females taking a progestin-only contraceptive and females not taking progestin. Again, these 2 studies have questionable methods and the evidence was low-quality. This review that progestin-only contraceptives does not increase body weights or change body composition corresponds to the data that Rambouillet ewes that received long-term P4 and the Rambouillet ewes that did not receive long-term P4.

The studies conducted in heifer feedlot cattle reported that feeding heifers MGA increased average daily gain and gain to feed ratios when compared to heifers not feed

MGA (Bloss, et al., 1966 and Kreikemeier and Mader, 2004). One reason that MGA may increase feed efficiency in these studies and not in the present study is because these heifers were growing and receiving a high caloric intake diet. Without outside variables such as high caloric diet or growing, P4 does not seem to change feed efficiency. In this same study, MGA did not differ in carcass characteristics between heifers fed MGA or not.

This is the first study to our knowledge that demonstrated long-term homeostasis. Homeostasis is the property within the body to maintain a condition of balances or equilibrium within the internal environment, even when exposed to outside conditions. There are studies that show long-term homeostasis, but only for one or two key metabolites. For example, long-term homeostasis of glutamate, glucose, or phosphorous. In this study, homeostasis was maintained for a full 126-d period with no changes in metabolites, body composition or body weights.

In conclusion, the effects of long-term P4 treatment mimicking those concentrations of pregnancy, independent of the influence of the placenta and fetus, does not change metabolite profiles identified using NMR metabolic profiling in mature Rambouillet ewes. In this study, we identified long-term homeostasis for a full 126-d with no changes in metabolic profiles associated with treatment or time, no changes in body composition, no changes in body weights.

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Table 1. Chemical composition of mixed grass hay diet¹

Item	Mixed Grass Hay diet
Nutrient analyses	
Moisture, %	13.3
Dry Matter, %	86.7
Crude Protein, %	7.8
Fiber (acid det.), %	37.8
TDN, %	59.4
Net energy (maint.), mCal/lbs	0.6
Net energy (gain), mCal/lbs	0.3
Sulfur, %	0.2
Phosphorous, %	0.2
Potassium, %	2.2
Magnesium, %	0.2
Calcium, %	0.5
Sodium, %	n.d.
Iron, ppm	79.1
Manganese, ppm	90.2
Copper, ppm	7.7
Zinc, ppm	18.0
¹ Ewes had free access to the mixed grass hay diet.	

Table 2. Least square means of final body weight (BW), final standard dry matter intake (STDMI), residual feed intake (RFI), final back fat depth (BF), and final rib-eye area (REA) in Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for 126-d

Item	Treatment		SEM ^a	<i>P</i> -value
	CIDR	CIDRX		
n	15	15		
BW, kg	57.8	58.7	8.40	0.70
STDMI, kg	1.32	1.40	0.04	0.49
RFI, kg/d	-0.03	0.02	0.20	0.50
BF, mm	1.9	2.0	0.20	0.46
REA, mm ²	26.4	26.8	0.50	0.60
^a Pooled SEM				

Table 3. Least square means of muscle mass (M), intra-muscular fat (IMF), empty body weight (EMW), empty body weight dry matter (EBWDM), empty body weight fat (EBWF), empty body weight protein (EBWP), carcass weight (CW), carcass weight dry matter (CWDM), carcass weight fat (CWF), and carcass weight protein (CWP) of Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for 126-d

Item	Treatment		SEM ^a	P-value
	CIDR	CIDRX		
n	15	15		
M, kg	13.3	13.7	2.2	0.60
IMF, kg	1.9	2.0	0.1	0.57
EMW, kg	48.9	49.1	2.9	0.92
EMWDM, %	45.4	45.5	0.4	0.92
EBWF, %	18.2	18.4	1.4	0.92
EBWP, %	17.82	17.79	0.05	0.92
CW, kg	26.9	27.1	1.0	0.92
CWDM, %	50.2	50.3	0.3	0.92
CWF, %	18.7	18.8	1.2	0.92
CWP, %	19.48	19.45	0.06	0.92
^a Pooled SEM				

Table 4. Least square means of insulin (INS) concentrations of Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for 126-d

Item	Treatment		Mean ¹
	CIDR	CIDRX	
n	15	15	
0	1.3	2.3	1.8 ^a
28	1.4	1.9	1.7 ^a
56	1.3	2.0	1.7 ^a
84	1.2	1.9	1.6 ^a
126	2.0	2.7	2.3 ^b
Mean ²	1.4 ^a	2.2 ^b	

^{a,b} Means within a column or row with different letters differ; $P < 0.05$.

¹ Pooled SEM = 0.05 ng/mL.

² Pooled SEM = 0.03 ng/mL.

Table 5. Results from the parameters for assessing whether the model quality is appropriate in discriminating Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for different days for the 126-d experiment. The larger the R^2 and Q^2 values are, the better quality and more appropriate the model is for discrimination

Day of Experiment	R^2	Q^2
Day 0	0.40	-0.16
Day 28	0.52	-0.39
Day 56	0.39	-0.48
Day 84	0.45	-0.50
Day 126	0.52	-0.23

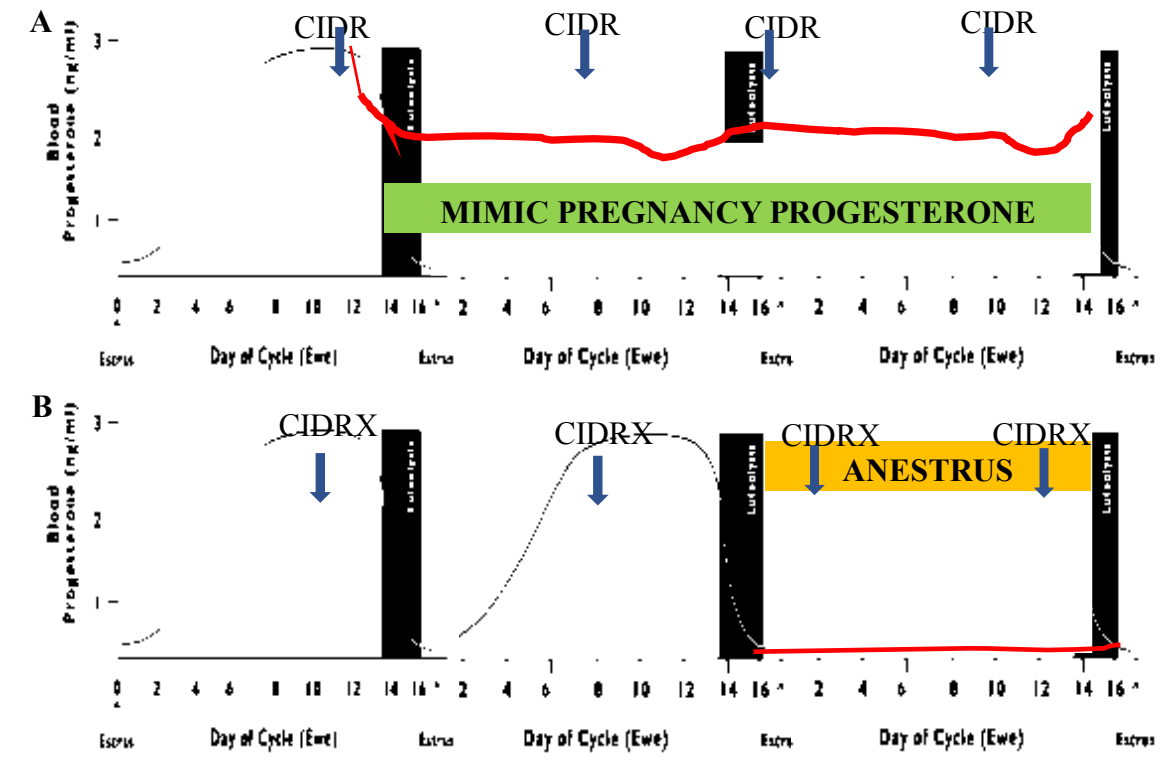


Figure 1. Schematic representation (adapted from Senger, 2012) of progesterone (P4) concentrations at 14-d intervals in relation to the estrous cycle in (A) Rambouillet ewes given a P4-containing, controlled internal drug release device (CIDR; n = 15) to mimic P4 concentrations of pregnancy and in (B) a non-P4-containing CIDR (CIDRX; n = 15). Both CIDR- and CIDRX- treatments began on d-12 (d 0 insertion of devices) of the estrous cycle relative to the estrus of the ewes.

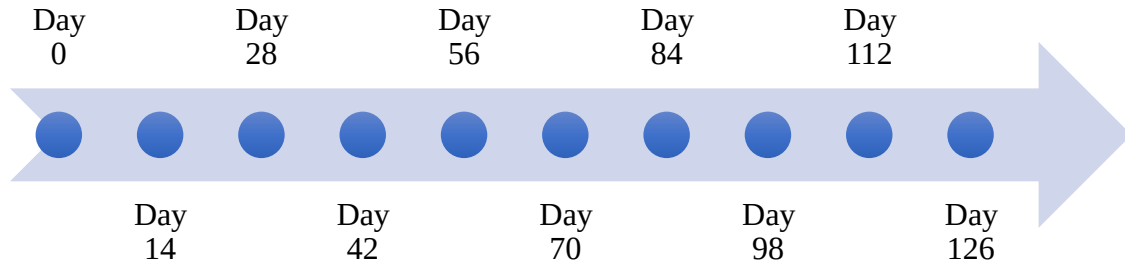


Figure 2. Timeline for sampling protocols during the 126-d experiment. Every 14-d CIDR or CIDRX were changed, blood samples obtained, and body weights recorded. Every 28-d rib-eye area and back fat thickness were ultra-sounded.

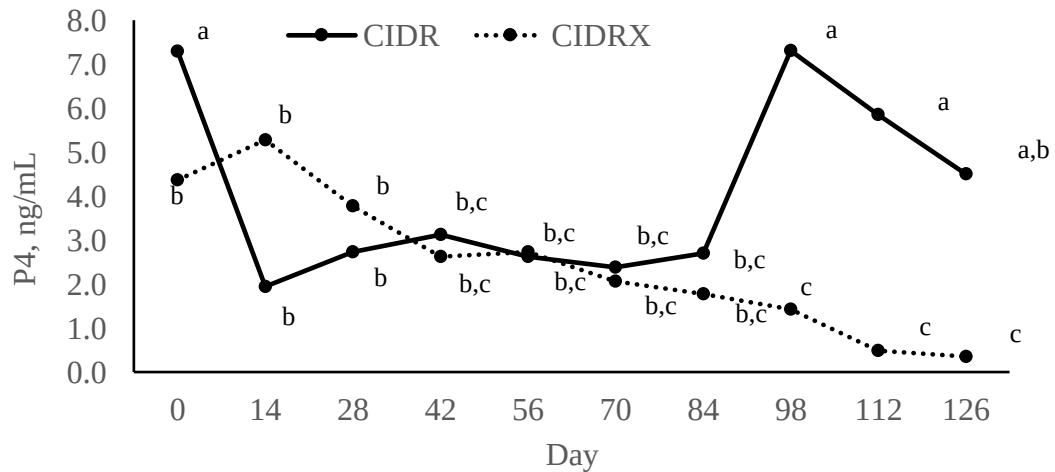


Figure 3. Progesterone (P4) concentrations at 14-d intervals in Rambouillet ewes given a P4-containing, controlled internal drug release device (CIDR; $n = 15$) or a non-P4-containing CIDR (CIDRX; $n = 15$) beginning on d-12 (d 0 insertion of devices) of the estrous cycle relative to estrus. Interaction of treatment $\times$ d; $P < 0.05$. Different letters among points indicate differences at $P < 0.05$. Pooled SEM = 5.1 ng/mL.

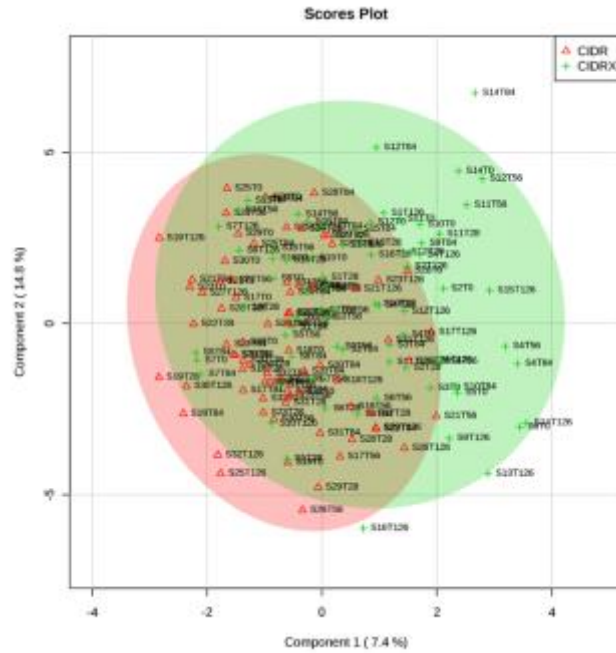


Figure 4. Partial least squares discriminant analysis (PLS-DA) score map between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for a 126-d period. CIDR- treated ewes are represented in red and CIDRX- treated ewes are represented in green.

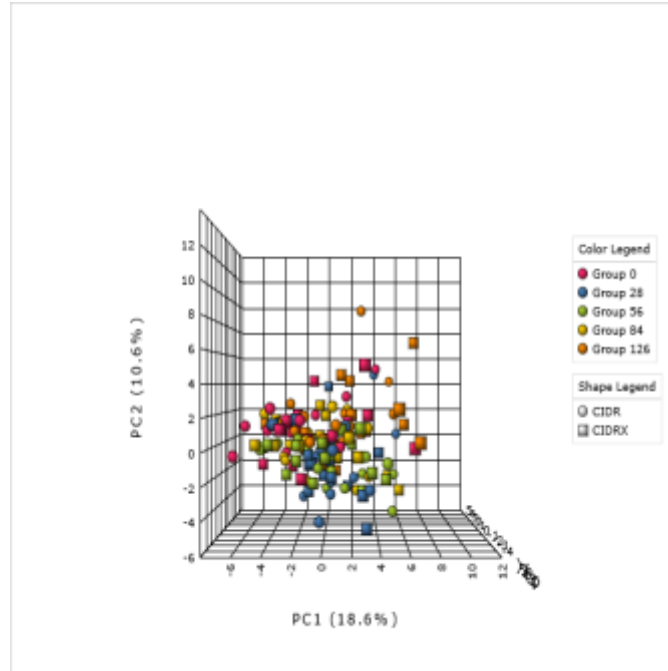


Figure 5. Full model of a time-series analysis 3-dimensional principal component visualization score map between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for the 126-d period. CIDR-treated ewes are represented by an oval and CIDRX-treated ewes are represented by a square. Day-0 is represented by red, day-28 is represented by blue, day-56 is represented by green, day-84 is represented by yellow, and day-126 is represented in orange.

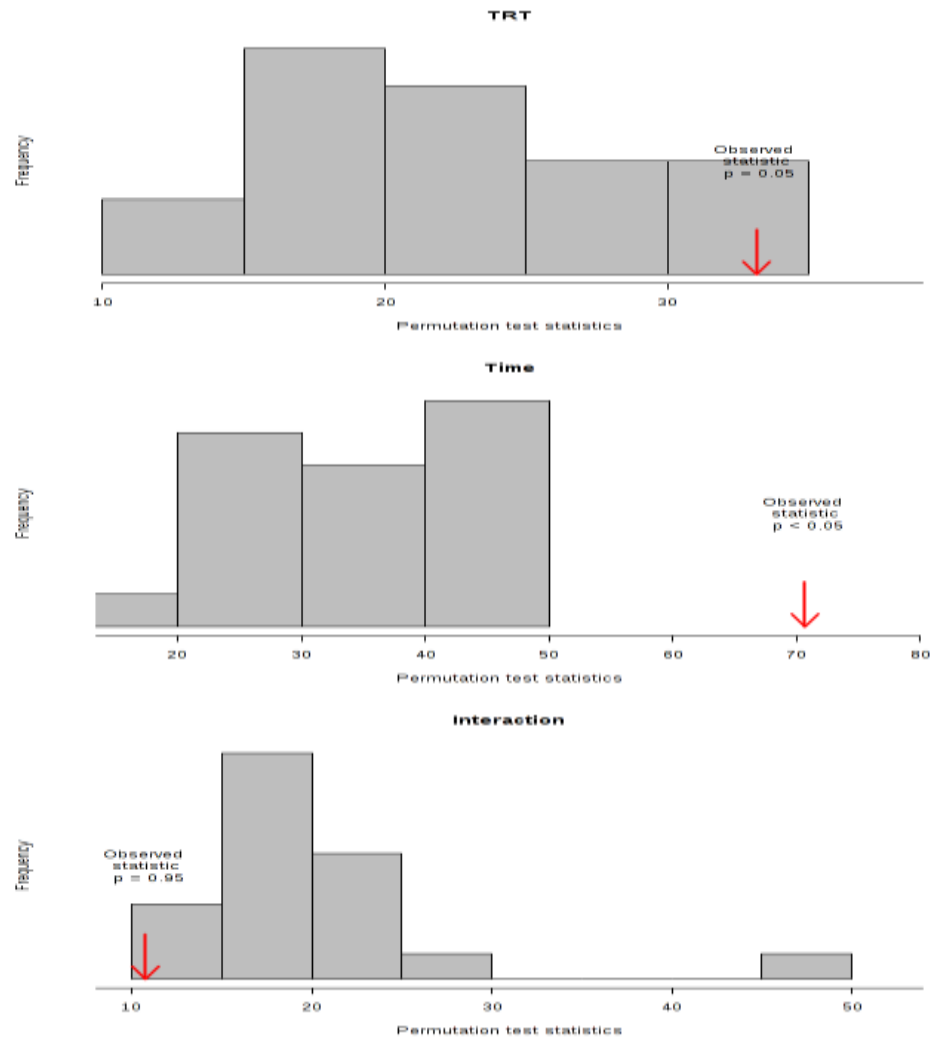


Figure 6. Full model permutation test statistics between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for the 126-d period. The model included treatment, time and the interaction between those two variables.

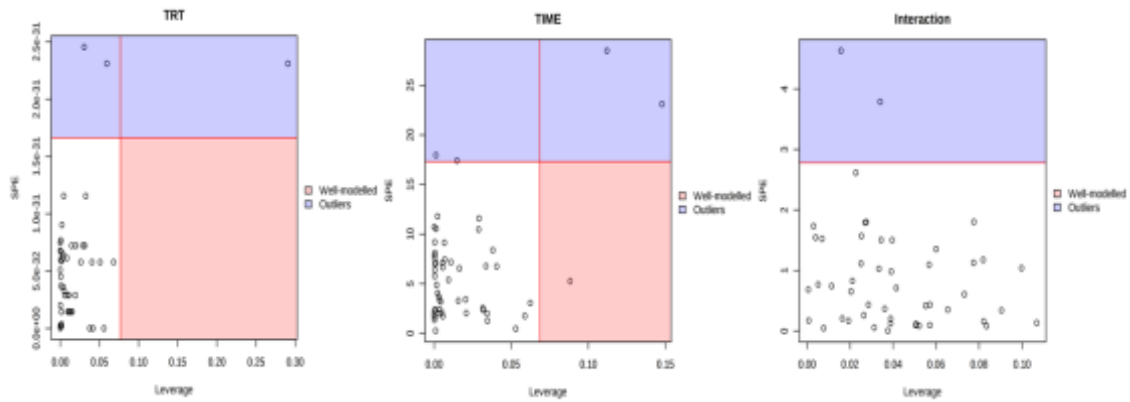


Figure 7. The full model leverage plots from the time series analysis between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for the 126-d period. CIDR-treated ewes are represented by an oval and CIDRX-treated ewes are represented by a square. The model includes treatment, time and the interaction between the two variables.

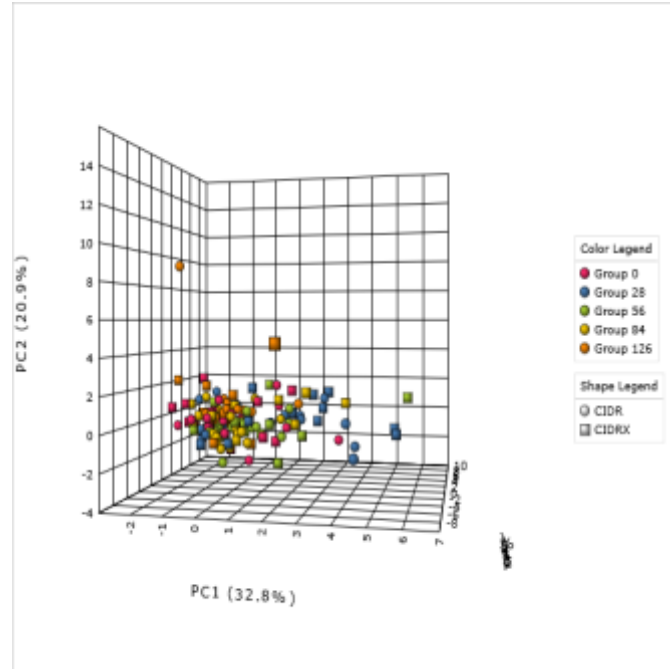


Figure 8. Reduced model of a time-series analysis 3-dimensinal principal component visualization score map between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for the 126-d period. CIDR-treated ewes are represented by an oval and CIDRX-treated ewes are represented by a square. Day-0 is represented by red, day-28 is represented by blue, day-56 is represented by green, day-84 is represented by yellow, and day-126 is represented in orange.

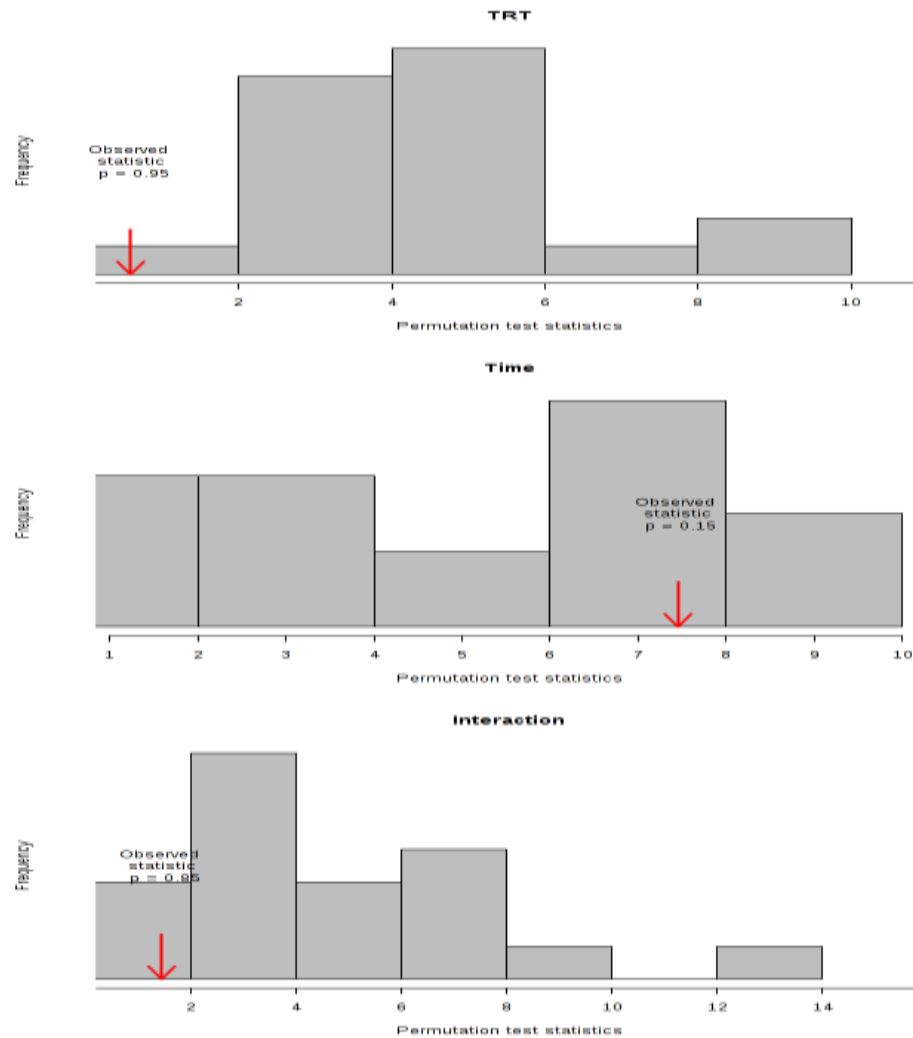


Figure 9. Reduced model permutation test statistics between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for the 126-d period. The model included treatment, time and the interaction between those two variables.

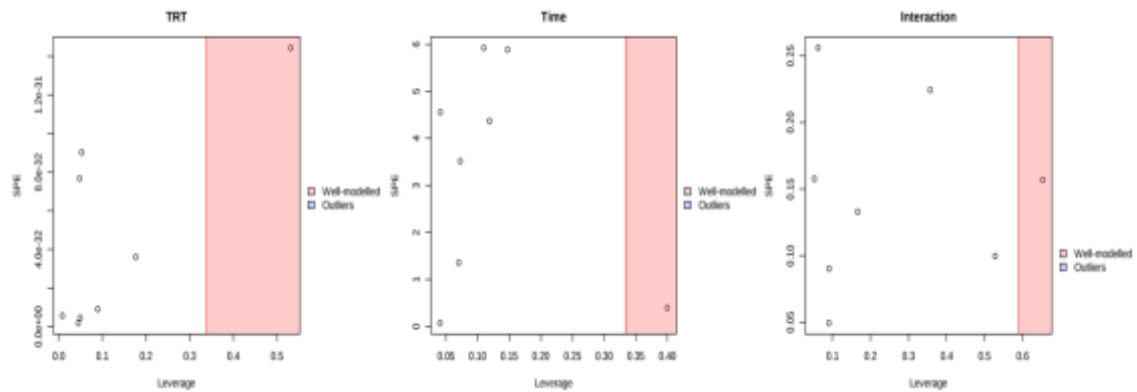


Figure 10. The reduced model leverage plots from the time series analysis between Rambouillet ewes that received a P4-containing controlled intravaginal releasing device (CIDR) or a CIDR backbone (no P4; CIDRX) for the 126-d period. CIDR-treated ewes are represented by an oval and CIDRX-treated ewes are represented by a square. The model includes treatment, time and the interaction between the two variables.

CHAPTER FIVE

POTENTIAL IDENTIFICATION OF METABOLIC BIOMARKERS USING
NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR) METABOLIC
PROFILING FOR NUTRITION STATUS, SEASON, AND
LOCATION OF BIGHORN SHEEP (*OVIS CANADENSIS*)
IN MONTANA AND WYOMING

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Contributions: Was critical in experiment design, data collection, data analysis, data interpretation, and the critical revision of this paper

Manuscript Information Page

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Abstract

Introduction

Bighorn sheep populations are currently well below historic abundance despite nearly a century of restoration efforts by natural resource agencies. However, wildlife managers have access to limited tools and techniques to assess bighorn sheep physiology status to determine what may increase populations. Nuclear magnetic resonance (NMR) spectroscopy metabolic profiling has the potential to create a panel of metabolites that wildlife managers may use to assess physiological states.

Objectives

The objective of this study was to determine if NMR metabolic profiling has the potential to serve as a management tool for evaluating the physiological status of herds of bighorn (*Ovis canadensis*) sheep.

Methods

Bighorn sheep herds were sampled between December of 2014 to March of 2015 in Montana and Wyoming. The sampling included 240 bighorn sheep ewes from 13 herds from geographically distinct locations at different times of the year. Metabolites identified in bighorn sheep serum were analyzed in a pathway enrichment analysis, through PLS-DA, and in a biomarker analysis to determine if bighorn sheep herds can be distinguished by pregnancy status, geographic location, and time of year.

Results

NMR metabolic profiling could not distinguish between pregnant and non-pregnant bighorn sheep. Metabolic profiling could distinguish between bighorn sheep herds and a subset of potential biomarkers can be identified based on distinct geographic locations and time of year.

Conclusion

NMR metabolic profiling has the potential to develop a suite of metabolites that wildlife managers can use to assess bighorn sheep health, including disease and physiological status.

Keywords

NMR, Bighorn sheep, Metabolomics

1 Introduction

Over a hundred years of restoration efforts in bighorn sheep (*Ovis canadensis*) herds have increased populations; however, the number is still 10-folds less than historic population estimates (Buechner et al., 1960; Toweill and Geist et al., 1999). Currently, it is thought that respiratory disease may play a significant role in limiting population increases and therefore, the recovery of bighorn sheep. However, there is little known about the physiology of bighorn sheep to validate what is limiting these populations. There are also limited tools and techniques for wildlife biologists and physiologists to evaluate the nutrition, health, and physiological status of bighorn sheep populations. The tools and techniques that wildlife managers use also can be expensive and inaccurate. An economical, analytical tool called nuclear magnetic resonance (NMR) spectroscopy metabolic profiling has the potential to identify potential biomarkers that can determine whether nutritional and/or environmental factors may be contributing to the differences in population sizes and recovery rates.

Metabolomics is the study of metabolic intermediates and products of cellular metabolism. Metabolomics allows for a snapshot of the global metabolism of an individual, and explains the functional nutritional and health states that an animal is currently in. The global metabolism of an individual may be measured and assessed using the analytical technology of NMR. Metabolomics first was applied in human medicine to study disease states of cancer, diabetes, and autoimmune disorders; as well as the application of pharmaceuticals. Currently, metabolomics has expanded to domestic animals to study infectious diseases and metabolic disorders. Metabolomics is also

being applied in domestic livestock research in feed efficiency trials. Metabolites that are identified by NMR are associated with known metabolic pathways that can be directly linked to an animal's physiological states (Sun et al., 2016).

The purpose of this study was to develop a suite of metabolites that can be used to assess nutritional health, disease health and body condition of bighorn sheep in a similar way that is successfully done in the livestock industry and human medicine. We hypothesized that because metabolomics has been successful in human medicine and in the livestock industry, that metabolomics maybe used in bighorn sheep management to identify potential biomarkers that can be used to assess herd health. The specific objectives of this study were to determine if:

- 1) NMR metabolic profiling can distinguish between non-pregnant and pregnant ewes and if potential biomarkers can be identified. This analysis would determine whether any differences associated with geographical location or time of year is not associated with pregnancy.
- 2) NMR metabolic profiling can distinguish the Fergus herd collected in December and the Absaroka herd collected in March and if potential biomarkers can be identified. This objective was to determine whether two herds from geographically distinct locations that were sampled at different times of the year could be distinguished from one another. If there is a metabolic shift between the two herds, then NMR metabolic profiling has the potential to be used as a management tool.
- 3) NMR metabolic profiling can distinguish between all herds collected in December and all herds collected in March and if potential biomarkers can be

identified. This objective was to determine if differences in metabolism could be identified in different times of the year. The winter diet for all herds collected will be at sub-maintenance because of a decrease in energy and protein due to plant senescence. The bighorn sheep sampled in December should be catabolizing fat reserves. The bighorn sheep sampled in March will most likely have exhausted most if not all the fat reserves and be using lean body mass to reach maintenance requirements. Therefore, there should be a metabolic shift between bighorn sheep that have been at sub-maintenance for a short period of time and bighorn sheep that have survived at sub-maintenance for an extended period of time.

4) NMR metabolic profiling can distinguish bighorn herds from one another within a season to determine whether using these analyses can separate herds from one another within the same time period and if potential biomarkers can be identified. This objective was to determine if metabolic differences may be identified on geographic location, because of the forage availability of different winter ranges. Forage availability may differ based on the type of plant communities located on the range, competition among other herbivores, and the severity of snowpack which buries forage.

The specific hypotheses in this study was that NMR metabolic profiling cannot distinguish between:

- 1) pregnant and non-pregnant bighorn ewes
- 2) bighorn sheep herds that have access to good quality forage and those that do not collected from in Fergus and the bighorn sheep collected in Absaroka

- 3) bighorn sheep sampled in December and bighorn sheep sampled in March
- 4) bighorn sheep within a season compared to a control population.

2 Materials and Methods

2.1 Animals and Sampling

Bighorn sheep herds were sampled between December of 2014 to March of 2015 in Montana and Wyoming. The sampling included 240 bighorn sheep ewes from 13 herds (Table 1). The herd location and the number of bighorn sheep from each location is in Table 2.

Blood samples were obtained by venipuncture of the jugular vein from each bighorn sheep ewe. Blood samples were cooled, allowed to clot and centrifuged to harvest serum within 24-h of collection. Serum samples were stored in aliquots at -20°C until assayed for pregnancy specific protein B (PSPB), progesterone (P4), non-esterified fatty acids (NEFA), and nuclear magnetic resonance spectroscopy metabolic profiling.

2.2 Determining pregnancy in Bighorn sheep

Sera was assayed for PSPB (BioPRYN™; Biotracking, Moscow, ID, USA). The PSPB enzyme-linked immunosorbent assay (ELISA) is used to indicate pregnancy and is 98% accurate at predicting pregnancy at 30 days after conception (Drew et al., 2001). Pregnancy specific protein B is a glycoprotein synthesized by the ruminant placenta. Sera samples were assayed with two male samples to ensure the ELISA was standardized. The optical density (OD) from the ELISA was used to determine pregnancy status for each bighorn ewe. Progesterone (P4) concentrations were assayed using enzyme-linked immunoassay

(ELISA) kits (ENZO Life Sciences©, Farmingdale, NY, USA) validated for sheep serum. Intra- and inter-assay CVs for a pooled sample that contained 1.4 ng/mL of P4 were 5.1% and 8.9%, respectively. Intra- and inter-assay CVs for a pooled sample that contained 0.2 ng/mL of P4 were 4.9% and 12.0%, respectively. The sensitivity of this assay was 1.3 pg/mL. The pregnancy status and corresponding P4 concentrations and PSPB values is in Table 3. PSPB values with an OD > 0.210 and P4 concentrations > 1.5 ng/mL were determined as pregnant. PSPB values with an OD > 0.210 and P4 concentrations < 1.5 ng/mL were considered to have embryonic death and not pregnant. Samples with PSPB OD values between 0.210 and 0.135, P4 concentrations > 1.5 ng/mL or P4 concentrations < 1.5 ng/mL were considered in the early stages of pregnancy or to have embryonic death. Samples with PSPB values with an OD < 0.135 and P4 concentrations > 1.5 ng/mL were considered estrous cycling or in the early stages of pregnancy. Samples with PSPB values with an OD < 0.135 and P4 concentrations < 1.5 ng/mL were considered not pregnant and not cycling.

2.3 Non-esterified fatty acid assay

Concentrations of non-esterified fatty acids (NEFA) was quantified with a commercially available enzymatic-colorimetric assay (HR Series NEFA – HR [2], Wako Diagnostics, Richmond, VA) validated for sheep serum. Intra- and inter-assay CVs for the first pooled sample that contained 0.20 mM of NEFA were 6.7% and 6.5%, respectively. Intra- and inter-assay CVs for the second pooled sample that contained 0.34 mM of NEFA were 4.5% and 5.9%, respectively. The sensitivity of this assay was 0.021 mM. NEFA concentrations

were included in the statistical analysis along with the small metabolites identified through NMR metabolic profiling.

2.3 NMR Sample Preparation

Serum samples were prepared for nuclear magnetic resonance spectroscopy using the following protocol. Five-hundred μL of serum and 1,500 μL of acetone were added to 2 mL plastic, flat-cap, conical vials. Each vial was inverted 10 times and frozen for at -20°C for 1 hour. After 1 hour, vials were centrifuged at 4°C , at 13,000 $\times g$ for 30 minutes. The supernatant of each sample was then transferred to fresh 2 mL flat-cap vials and dried using a centrifuge-vacuum overnight. The next day, 500 μL of NMR buffer (phosphate buffer plus D_2O , TSP and imidazole) was mixed with the dried samples and transferred to 5 mm NMR tubes (Bruker©, 2017). The specific protocol used for extracting small metabolites from sera for NMR spectroscopic analysis is included in Appendix B.

2.4 NMR Spectroscopy

All NMR spectra were analyzed at 25°C on a Bruker 600 MHz (^1H Larmor frequency) AVANCE III solution spectrometer. The spectrometer is equipped with an automatic sample loading system (SampleJet™), a 5-mm helium-cooled ^1H -optimized TCI NMR probe (Cryoprobe™), and Topspin software (Bruker version 3.5). 1D ^1H NMR experiments were completed using the “zgsgp” Bruker pulse sequence and recorded with 256 scans and a ^1H spectral window of 9615.38 Hz. Free induction decays (FIDs) were sampled at 32 K data points and a dwell time interval of 52 μs totaling to an

acquisition time of around 1.7 seconds and a 1 second relaxation recovery delay between acquisitions.

Spectra were collected from the NMR operating system, Topspin 3.5 (Bruker©, 2017). The raw spectra were imported into the Topspin program, Fourier transformed, phased, and the standard was set to 0 ppm before being imported into the Chenomx© NMR Suite program software (Chenomix NMR Suite 8.1). Once the spectra were imported into the Chenomx software, they were baseline corrected. In the baseline correction, the water resonance region was deleted between 4.3 and 5.6 ppm. For metabolite identification, the Chenomix small molecule library for 600-MHz (^1H Larmor frequency) magnetic field strength NMR spectrometers was used and NMR spectral profiles were fitted for each sample. Our laboratory has established a NMR compound database containing 58 small molecule metabolites; all identified in ruminant serum or plasma (Appendix C). An internal DSS standard was used for the quantification of the 58 small molecule metabolites. Furthermore, one buffer control and one quality control serum from a ruminant were included with every sample-run to adjust for run to run variation. A more detailed description of the NMR spectroscopy procedure is included in Appendix B.

2.5 Chemometrics

Concentrations of the small metabolites were statistically analyzed using MetaboAnalyst 3.0 (Xia and Wishart, 2016). The analyses conducted was a pathway enrichment analysis, a partial least squares discriminant analysis (PLS-DA), and a biomarker analysis. Analysis procedures of these small metabolites is similar to those described by Sun et al.

(2016) to identify potential biomarkers between herds describing different nutritional and health states as well as identify significantly important pathways.

2.5.1 Pathway enrichment analysis

The pathway analysis from Metaboanalyst 3.0 uses an algorithm for an enrichment analysis from the *Bos Taurus* (cow) to identify pathways that are significantly impacted by the metabolites identified. The enrichment analysis identified 7 pathways that are impacted by the 54 metabolites identified by NMR metabolic profiling (Table 4). These 7 pathways were the same significant pathways in all the analyses. For pathways to be considered significantly important, the pathway impact values needed to be greater than 0.1. Furthermore, all p-values were corrected using the Holm-Bonferroni correction. The Holm-Bonferroni correction is important to counteract the issue of error rates associated with multiple comparisons. After the correction, any p-value less than 0.05 along with an impact value < 0.1 is considered significant.

2.5.2 Partial least squares discriminant analysis

The PLS-DA model was used to visualize the data set and to accurately measure the covariance among the response. The R^2 and Q^2 variables were used to ensure the PLS-DA model was accurate and appropriate. The R^2 variable is equal to the sum of squares captured by the model and the Q^2 variable is the cross-validated value of the R^2 . The PLS-DA model was used to identify significantly different metabolites between the different comparisons made among bighorn sheep sampled from various herds. From the PLS-DA, the variables importance for the projection (VIP) score for each metabolite that

were > 1.0 were considered significantly different among the herds being compared. The VIP scores are the metabolites that contribute to any differences identified through the PLS-DA. Comparisons were made to determine if NMR metabolic profiling can distinguish:

- 1) between non-pregnant and pregnant bighorn sheep ewes.
- 2) herds that are geographically distinct with access to different nutritional resources. The Fergus herd collected in December ($n = 29$) was compared to the Absaroka herd collected in March ($n = 18$).
- 3) between all herds collected in December and all herds collected in March.
- 4) different bighorn herds within a season to determine whether using these analyses can separate herds from one another within the same time month.

2.5.3 Biomarker analysis

The metabolites with VIP scores > 1.0 were used in a potential biomarker analysis. These metabolites were used because they are contributing to any differences identified in the PLS-DA. Biomarker analysis is used to identify metabolite biomarkers that are associated with disease states and nutritional state. The biomarker analysis used in these analyses was a classical multivariate ROC curve in MetaboAnalyst 3.0. Metabolites with frequency scores > 0.1 were considered potential biomarkers. Any metabolites identified by the biomarker analysis are the least number of metabolites that can distinguish bighorn sheep herds from one another. To be considered a potential biomarker, the metabolite identified in the biomarker analysis also needed to be found in the pathways identified in

the pathway enrichment. This last part of the analysis not only reduces the number of metabolites that can be used as biomarkers but also ensures that the metabolites identified are found in significantly important pathways.

3 Results

3.1 Identification of metabolites from the spectra of bighorn sheep sera

The 58 small molecule metabolites that were identified in ruminant serum are in Appendix C. Every metabolite identified was cross referenced with the Human Metabolome Database to ensure the correct identification of metabolites.

3.2 Distinguishing differences in metabolic profiles between non-pregnant and pregnant bighorn sheep ewes

Pregnancy Specific Protein B was used to determine pregnancy rates of bighorn sheep herds. One-hundred and seventy-nine bighorn sheep were pregnant because of a positive PSPB test and P4 concentrations > 1.5 ng/mL. Fifty-nine bighorn sheep were considered non-pregnant because of a negative PSPB test and P4 concentrations < 1.5 ng/mL.

Bighorn sheep serum samples were analyzed to determine if there was a metabolic difference between non-pregnant and pregnant bighorn sheep. The PLS-DA could not discriminate between non-pregnant bighorn sheep and pregnant bighorn sheep (Figure 1) with the suite of metabolites that we have identified in the metabolic library developed for bighorn sheep sera. Even though there was minor separation in metabolic shifts between pregnant and non-pregnant bighorn sheep, the PLS-DA consisted of a majority overlap. The parameters for assessing the model quality in discriminating non-pregnant

and pregnant bighorn sheep indicate the model is highly ineffective, with the R^2 value being 0.26 and the Q^2 value being 0.12. No further analysis for the identification of potential biomarkers was conducted because of the ineffectiveness of the model to distinguish pregnant and non-pregnant bighorn sheep.

3.3 Distinguishing differences in metabolic profiles between bighorn sheep collected in Fergus and bighorn sheep collected in Absaroka

The PLS-DA could completely discriminate between the bighorn sheep sampled from the Fergus population in December and the bighorn sheep sampled from the Absaroka population in March. Figure 2A validates that there is a metabolic shift between the two herds and that they are completely separated from each other metabolically. The parameters for assessing the model quality in discriminating the Fergus and Absaroka herd indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.86. The VIP scores identified 18 metabolites that differed between the two herds (Figure 2B) and contributed to the metabolic shifts shown in the PLS-DA (Figure 2A).

Figure 3 displays the 14 potential biomarkers, from the 18 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis. These 14 potential biomarkers are the least number of metabolites that can separate the Fergus and Absaroka herds.

The metabolites that are potential biomarkers between the Fergus and Absaroka herds and are identified to be found in significantly important pathways (Table 4) are creatinine found in arginine/proline metabolism, choline found in glycine, serine, and threonine metabolism, trimethylamine n-oxide found in methane metabolism, urea found

in arginine/proline metabolism, threonine found in methane metabolism and glycine, serine and threonine metabolism.

3.4 Distinguishing differences in metabolic profiles between all bighorn herds collected in December and all bighorn herds collected in March

While looking at Figure 4A, the PLS-DA illustrates that even though populations of bighorn sheep sampled in December and populations of bighorn sheep sampled in March are not completely metabolically separated from each other, that they are mostly separated with minor overlap. The parameters for assessing the model quality in discriminating all herds from December and all herds from March indicate the model is effective, with the R^2 value being 0.81 and the Q^2 value being 0.67. The VIP scores identified 18 metabolites that were changed between the herds collected in December and the herds collected in March (Figure 4B) and contributed to the metabolic shifts shown in the PLS-DA (Figure 4A).

The 15 potential biomarkers, from the 18 metabolites identified as significantly different from the VIP scores, found from the biomarker analysis are shown in Figure 5. These 15 potential biomarkers are the least number of metabolites that can separate herds sampled in December and herds sampled in March. The metabolites that are identified as potential biomarkers and are found in pathways identified as significantly important (Table 4) are 2-oxoisocaproate found in valine, leucine, and isoleucine metabolism, choline found in glycine, serine, and threonine metabolism, tyrosine found in methane metabolism, creatinine found in arginine/proline metabolism and trimethylamine n-oxide found in methane metabolism.

3.5 Distinguishing differences in metabolic profiles between bighorn sheep herds within a season compared to a control population of Rambouillet ewes

The final analyses were to compare bighorn herds to one another within a season to determine whether NMR spectroscopy metabolic profiling can separate herds from one another within the same month. One problem was to find a control population to compare each herd to where the environmental conditions and the nutritional status were known. To overcome this, we used a control population of Rambouillet ewes that were fed a maintenance diet and maintained weight from December through April of 2014-2015. Each herd was compared to the control population in December, January, and March.

3.5.1 Distinguishing differences in metabolic profiles between bighorn sheep herds in December compared to a control population of Rambouillet ewes

There were five herds sampled in December: Castle Reef, Fergus, NE Yellowstone, Paradise and Stillwater. Starting with the Castle Reef herd, the PLS-DA shows that the Castle Reef herd and the control Rambouillet population are completely separated, which illustrates the metabolic shift between the Castle Reef bighorn sheep and the control population (Figure 6A). The parameters for assessing the model quality in discriminating the Castle Reef herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.96 (Table 5). This means this model is appropriate to use to discriminate the Castle Reef population of bighorn sheep and the Rambouillet control population. In Figure 6B, the VIP scores identified 22 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 6A). Figure 6C displays the 11 potential

biomarkers, from the 22 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA (Figure 7A) shows that the Fergus herd and the Rambouillet population are completely separated which, again, indicates a metabolic shift between the bighorn sheep and the Rambouillet ewes' population. The parameters for assessing the model quality in discriminating the Fergus herd from the control Rambouillet ewes indicate the model is highly effective and appropriate, with the R^2 value being 0.94 and the Q^2 value being 0.94 (Table 5). In Figure 7B, the VIP scores identified 21 metabolites that were changed between the two Fergus population and the control Rambouillet population and contributed to the metabolic shifts shown in the PLS-DA (Figure 7A). Figure 6C displays the 6 potential biomarkers, from the 21 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The next herd analyzed was the NE Yellowstone herd, the PLS-DA illustrates that the NE Yellowstone herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the NE Yellowstone bighorn sheep and the control population (Figure 8A). The parameters for assessing whether the model quality is appropriate in discriminating the NE Yellowstone herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.98 and the Q^2 value being 0.96 (Table 5). In Figure 8B, the VIP scores identified 20 metabolites that were changed between the NE Yellowstone population and the control Rambouillet population and contributed to the metabolic shifts shown in the PLS-DA (Figure 8A).

Figure 8C displays the 15 potential biomarkers, from the 20 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA shows that the Paradise herd and the control Rambouillet population are completely separated, illuminating the metabolic shift between the Paradise bighorn sheep and the control population (Figure 9A). The parameters for assessing the model quality in discriminating the Paradise herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.96 (Table 5). With the previous observations, the model is appropriate to use to discriminate the Paradise population and the control Rambouillet population. In Figure 9B, the VIP scores identified 25 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 9A). Figure 9C displays the 9 potential biomarkers, from the 25 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The final herd analyzed in December was the Stillwater herd. The PLS-DA shows that the Stillwater herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Stillwater bighorn sheep and the control population (Figure 10A). The parameters for assessing whether the model quality is appropriate in discriminating the Stillwater herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.93 (Table 5). In Figure 10B, the VIP scores identified 18 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in

the PLS-DA (Figure 10A). Figure 10C displays the 8 potential biomarkers, from the 18 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The final step in analyzing the herds from December compared to the Rambouillet control sheep was to determine if there was a subset of metabolites that are potential biomarkers in each of the five herds. Potential biomarkers identified in all 5 herds were sarcosine, methionine, and trimethylamine n-oxide. Each potential biomarker identified also needs to be found in metabolic pathways identified as significantly important (Table 4) to be considered a potential biomarker. The potential biomarkers in December that were also found in pathways identified as significantly important (Table 4) were trimethylamine n-oxide found in methane metabolism and sarcosine found in glycine, serine, threonine, arginine and proline metabolism.

3.5.2 Distinguishing differences in metabolic profiles between bighorn sheep herds in January compared to a control population of Rambouillet ewes

There were six herds sampled in January: Castle Reef, Jackson, Lost Creek, Stillwater, Taylor Hilgard and Ferris-Simone (sampled in February, but included in the January analysis). Starting with the Castle Reef herd, the PLS-DA shows that the Castle Reef herd and the control Rambouillet population are completely separated, showing a metabolic shift between the Castle Reef herd and the control population (Figure 11A). The parameters for assessing the model quality in discriminating the Castle Reef herd from the control Rambouillet ewes indicate the model is highly effective and appropriate, with the R^2 value being 0.97 and the Q^2 value being 0.95 (Table 5). In Figure 11B, the VIP

scores identified 23 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 11A). Figure 11C displays the 7 potential biomarkers, from the 23 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA (Figure 12A) shows that the Jackson herd and the Rambouillet control population are completely separated which, again, indicates a metabolic shift between the two populations. The parameters for assessing the model quality in discriminating the Jackson herd from the control Rambouillet ewes indicate the model is highly effective and appropriate, with the R^2 value being 0.94 and the Q^2 value being 0.92 (Table 5). In Figure 12B, the VIP scores identified 25 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 12A). Figure 12C displays the 15 potential biomarkers, from the 25 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The next herd analyzed was the Lost Creek herd, the PLS-DA illustrates that the Lost Creek herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Lost Creek bighorn sheep and the control population (Figure 13A). The parameters for assessing whether the model quality is appropriate in discriminating the Lost Creek herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.95 and the Q^2 value being 0.94 (Table 5). In Figure 13B, the VIP scores identified 24 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in

the PLS-DA (Figure 13A). Figure 13C displays the 9 potential biomarkers, from the 24 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA shows that the Stillwater herd and the control Rambouillet population are completely separated, showing the metabolic shift between the Paradise bighorn sheep and the control population (Figure 14A). The parameters for assessing the model quality in discriminating the Stillwater herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.95 (Table 5). With the previous observations, the model is appropriate to use to discriminate the two populations. In Figure 14B, the VIP scores identified 17 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 14A). Figure 14C displays the 7 potential biomarkers, from the 17 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The next herd analyzed in January was the Taylor Hilgard herd. The PLS-DA shows that the Stillwater herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Taylor Hilgard bighorn sheep and the control population (Figure 15A). The parameters for assessing whether the model quality is appropriate in discriminating the Taylor Hilgard herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.98 and the Q^2 value being 0.98 (Table 5). In Figure 15B, the VIP scores identified 25 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in

the PLS-DA (Figure 15A). Figure 15C displays the 13 potential biomarkers, from the 25 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The final herd analyzed was the Ferris-Seminoe herd. This bighorn sheep herd was sampled in February, however, since this herd was the only herd sampled in February it was included in the January analysis. The PLS-DA shows that the Ferris-Seminoe herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Ferris-Seminoe bighorn sheep and the control population (Figure 16A). The parameters for assessing whether the model quality is appropriate in discriminating the Ferris-Seminoe herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.96 (Table 5). In Figure 16B, the VIP scores identified 18 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 16A). Figure 16C displays the 5 potential biomarkers, from the 18 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Again, the final step in analyzing the herds from January compared to the Rambouillet control sheep was to determine if there was a subset of metabolites that are potential biomarkers in each of the six herds. Potential biomarkers that were identified in all six herds creatinine, dimethyl sulfone, asparagine and carnitine. It is important that the potential biomarkers identified have an impact on the metabolic pathways identified as significantly important (Table 4). Therefore, the potential biomarkers that would be used

are biomarkers that are also found in these identified pathways. The potential biomarkers in January that were also found in pathways identified as significantly important (Table 4) were creatinine found in arginine/proline metabolism and asparagine found in alanine, aspartate and glutamate metabolism.

3.5.3 Distinguishing differences in metabolic profiles between bighorn sheep herds in March compared to a control population of Rambouillet ewes

There were eight herds sampled in March: Absaroka, Castle Reef, Cody, Devil's Canyon, Dubois, Jackson, Lost Creek, and Stillwater. Starting with the Absaroka herd, the PLS-DA shows that the Absaroka herd and the control Rambouillet population are completely separated, showing a metabolic shift between the Absaroka herd and the control population (Figure 17A). The parameters for assessing the model quality in discriminating the Absaroka herd from the control Rambouillet ewes indicate the model is highly effective and appropriate, with the R^2 value being 0.97 and the Q^2 value being 0.96 (Table 5). In Figure 17B, the VIP scores identified 13 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 17A). Figure 17C displays the 9 potential biomarkers, from the 13 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA shows that the Castle Reef herd and the control Rambouillet population are completely separated, showing the metabolic shift between the Paradise bighorn sheep and the control population (Figure 18A). The parameters for assessing the model quality in discriminating the Castle Reef herd from the control Rambouillet ewes

indicate the model is highly effective and appropriate, with the R^2 value being 0.95 and the Q^2 value being 0.90 (Table 5). In Figure 18B, the VIP scores identified 15 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 18A). Figure 18C displays the 15 potential biomarkers, from the 15 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA shows that the Cody herd and the control Rambouillet population are completely separated, showing the metabolic shift between the Cody bighorn sheep and the control population (Figure 19A). The parameters for assessing the model quality in discriminating the Cody herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.94 and the Q^2 value being 0.88 (Table 5). With the previous observations, the model is appropriate to use to discriminate the two populations. In Figure 19B, the VIP scores identified 15 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 19A). Figure 19C displays the 15 potential biomarkers, from the 15 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The next herd analyzed in March was the Devil's Canyon herd. The PLS-DA shows that the Devil's Canyon herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Devil's Canyon bighorn sheep and the control population (Figure 20A). The parameters for assessing whether the model quality is appropriate in discriminating the Devil's Canyon herd from the control

Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.97 and the Q^2 value being 0.96 (Table 5). In Figure 20B, the VIP scores identified 14 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 20A). Figure 20C displays the 7 potential biomarkers, from the 14 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Next, the PLS-DA shows that the Dubois herd and the control Rambouillet population are completely separated, showing the metabolic shift between the Dubois bighorn sheep and the control population (Figure 21A). The parameters for assessing the model quality in discriminating the Dubois herd from the control Rambouillet ewes indicate the model is highly effective, with the R^2 value being 0.95 and the Q^2 value being 0.93 (Table 5). With the previous observations, the model is appropriate to use to discriminate the two populations. In Figure 21B, the VIP scores identified 13 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 21A). Figure 21C displays the 13 potential biomarkers, from the 9 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The next herd analyzed in March was the Jackson herd. The PLS-DA shows that the Jackson herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Jackson bighorn sheep and the control population (Figure 22A). The parameters for assessing whether the model quality is appropriate in discriminating the Jackson herd from the control Rambouillet ewes

indicate the model is highly effective, with the R^2 value being 0.96 and the Q^2 value being 0.96 (Table 5). In Figure 22B, the VIP scores identified 15 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 22A). Figure 22C displays the 5 potential biomarkers, from the 15 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The next herd analyzed in March was the Lost Creek herd. The PLS-DA shows that the Lost Creek herd and the control Rambouillet population are completely separated, indicating a metabolic shift between the Lost Creek bighorn sheep and the control population (Figure 23A). The parameters for assessing whether the model quality is appropriate in discriminating the Lost Creek herd from the control Rambouillet ewes indicate the model is effective, with the R^2 value being 0.86 and the Q^2 value being 0.72 (Table 5). In Figure 23B, the VIP scores identified 10 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 23A). Figure 23C displays the 10 potential biomarkers, from the 10 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

The final herd analyzed in March was the Stillwater herd. The PLS-DA indicates that the Stillwater herd and the control Rambouillet population are completely separated, showing a metabolic shift between the Stillwater bighorn sheep and the control population (Figure 24A). The parameters for assessing whether the model quality is appropriate in discriminating the Stillwater herd from the control Rambouillet ewes

indicate the model is highly effective, with the R^2 value being 0.96 and the Q^2 value being 0.93 (Table 5). In Figure 24B, the VIP scores identified 9 metabolites that were changed between the two populations and contributed to the metabolic shifts shown in the PLS-DA (Figure 24A). Figure 24C displays the 6 potential biomarkers, from the 9 metabolites identified as significantly different from the VIP scores, identified by the biomarker analysis.

Finally, the last step in analyzing the herds from March compared to the Rambouillet control sheep was to determine if there was a subset of metabolites that are potential biomarkers in each of the eight herds. Potential biomarkers that were identified in all eight herds were creatinine, dimethyl sulfone, and carnitine. It is important that the potential biomarkers identified have an impact on the metabolic pathways identified as significantly important (Table 4). Therefore, the potential biomarkers that would be used are biomarkers that are also found in these identified pathways. The only potential biomarker also found in a significantly important pathway (Table 4) is creatinine, which is found in arginine/proline metabolism.

4 Discussion

In the multiple analyses performed in this study, many key biomarkers were successfully identified through statistical validation and biomarker analysis. This suggests that certain key metabolites can serve as potential biomarkers in distinguishing bighorn sheep herds.

4.1 Distinguishing differences in metabolic profiles between non-pregnant and pregnant bighorn sheep ewes

The first objective of this study was to determine if non-pregnant and pregnant bighorn sheep could be distinguished using NMR metabolic profiling. The PLS-DA (Figure 1) model quality was highly ineffective and not appropriate to use, therefore, we were not able to distinguish differences between non-pregnant and pregnant bighorn sheep. The inability to distinguish non-pregnant and pregnant bighorn sheep using NMR small metabolic profiles indicates that any differences in the other analyses in this study is not related to pregnancy.

4.2 Distinguishing differences in metabolic profiles between bighorn sheep collected in Fergus and bighorn sheep collected in Absaroka

The second objective of this study was to determine if NMR metabolic profiling can distinguish the Fergus herd collected in December and the Absaroka herd collected in March. The Fergus herd is located at a low elevation on the plains, while the Absaroka herd is located at a high elevation and sampled in March. The purpose of studying these two herds were that these herds are geographically distinct from one another with access to different nutritional resources and should be metabolically shifted from one another based on region and time of year. If a metabolic shift exists between the two herds, then NMR metabolic profiling had the potential to be used in distinguishing differences among herds. The complete metabolic shift, illustrated in Figure 2A, between the two herds makes physiological sense as the Fergus herd in December should be in better condition than the Absaroka herd in March, which has been on a sub-maintenance diet for three months. Shown in Figure 2B, 18 metabolites were identified as having variable

of importance (VIP) scores greater than 1.0. Having VIP scores greater than 1.0 indicates that metabolite having contributed significantly to the changes in the PLS-DA.

Fourteen potential biomarkers (Figure 3) were identified from the biomarker analysis from the 18 metabolites identified by the VIP scores. These potential biomarkers are the least number of compounds that can be used to separate these herds and explain the known metabolic shifts between them. To narrow the list of potential biomarkers, only metabolites also identified in significantly important pathways (Table 4) were considered potential biomarkers. These metabolites were creatinine found in arginine/proline metabolism, choline found in glycine, serine, and threonine metabolism, trimethylamine n-oxide found in methane metabolism, urea found in arginine/proline metabolism, threonine found in methane metabolism and glycine, serine and threonine metabolism.

Creatinine is a waste product of creatine muscle catabolism and higher concentrations of this metabolite in serum is indicative of greater muscle breakdown (Hosten, 1990). Creatinine may be a potential biomarker between the Absaroka herd and the Fergus herd because of creatinine's relationship with muscle breakdown. The Absaroka herd is in greater metabolic distress than the Fergus herd and should be using more muscle for energy. Creatinine is identified in the arginine/proline metabolism pathway.

Choline is essential metabolite that is important in the synthesis of the neurotransmitter acetylcholine, is critical in the process of exporting triacylglycerol from the liver, and is found in most membrane structures (Artegoitia et al., 2014). Choline

deficiencies have been described as having decreased numbers of acetylcholine which is related to depression. However, there has been no research on whether increased concentrations of choline are indicative of membrane breakdown due to an energy deficiency. The significantly important pathway that choline can be identified in is glycine, serine, and threonine metabolism.

The importance of trimethylamine n-oxide in mammals is starting to gain more attention in recent years. Studies have shown that trimethylamine n-oxide stabilizes proteins against urea-induced denaturation. Concentrations of trimethylamine n-oxide depend on many factors such as diet, gut microbial flora, liver enzymes and kidney function (Velasquez et al., 2016). Trimethylamine n-oxide may be a potential biomarker because of its function in protecting proteins against urea. The significantly important pathway the trimethylamine n-oxide can be identified in is methane metabolism.

Urea is a metabolite considered a waste product of protein metabolism. Urea in ruminants also functions in a “protein regeneration cycle.” This includes the recycling of urea in the gastrointestinal tract as a source of nitrogen for the microbial community (Marini et al., 2006). Urea could be a potential biomarker because higher concentrations could be indicative of increased protein metabolism for energy when in a low energy state. The significantly important pathway that urea is identified in is arginine/proline metabolism

Threonine is an essential amino acid that has many functions in the body. Threonine aids in making antibodies, is required for collagen formation, prevents fatty deposition in the liver, and is critical for central nervous system functions. The significant

metabolic pathways that threonine is identified in are methane metabolism and glycine, serine and threonine metabolism.

The potential biomarkers that were identified in this analysis determined it is possible to discriminate herds from different geographic areas, at different times of the year using NMR metabolic profiling. These potential biomarkers, along with the significantly important pathways these small metabolites are identified in, have the possibility to identify herds in different nutritional states and geographic locations.

4.3 Distinguishing differences in metabolic profiles between all bighorn herds collected in December and all bighorn herds collected in March

The third objective of this study was to determine if all bighorn sheep sampled in December could be distinguished from all bighorn sheep sampled in March. The purpose of conducting these analyses was to determine if NMR metabolic profiling can be used to distinguish differences in bighorn sheep between seasons. The PLS-DA (Figure 4A) shows that there is a clear metabolic shift between seasons, which may be attributed to the different nutritional and physiological states that the bighorn sheep are in in December while compared to March. Again, this follows the observations that the bighorn sheep collected in December are in better physiological condition than the bighorn sheep collected in March. Shown in Figure 4B, 18 metabolites were identified as having VIP scores greater than 1.0. Having VIP scores greater than 1.0 indicates that metabolite having contributed significantly to the changes identified in the PLS-DA.

The biomarker analysis identified 15 potential biomarkers (Figure 5) from the 18 metabolites identified by the VIP scores. These potential biomarkers are the least number

of small metabolites that can be used to separate these herds and explain the known metabolic shifts the herds sampled in December and the herds sampled in March. Again, to narrow the list of potential biomarkers, only small metabolites also identified in significantly important pathways (Table 4) were considered potential biomarkers. These potential biomarkers and are found in pathways identified as significantly important (Table 4) are 2-oxoisocaproate found in valine, leucine, and isoleucine metabolism, choline found in glycine, serine, and threonine metabolism, tyrosine found in methane metabolism, creatinine found in arginine/proline metabolism and trimethylamine n-oxide found in methane metabolism.

Creatinine, trimethylamine n-oxide, and choline were previously described because they were also identified as potential biomarkers in distinguishing the Fergus herd and the Absaroka herd. The significantly important pathways that these small metabolites are identified in are the same as previously described. The fact that these three metabolites were identified as potential biomarkers in distinguishing the Fergus herd and the Absaroka herd, as well as all the bighorn sheep sampled in December and all the bighorn sheep sampled in March indicates the potential of NMR metabolic profiling in identifying potential biomarkers in bighorn sheep in different physiology and nutritional states.

Two more small metabolites were identified as potential biomarkers in distinguishing the bighorn sheep sampled in December and the bighorn sheep sampled in March. The first small metabolite that was different than the previous analysis was 2-oxoisocaproate. 2-oxoisocaproate is a metabolite which is an intermediate in the

metabolism of leucine. Higher concentrations of 2-oxoisocaproate may be indicative of making more leucine. Leucine regulates protein metabolism and research indicates leucine may inhibit protein degradation. December herds had higher concentrations of 2-oxoisocaproate, and therefore higher leucine metabolism, than the herds sampled in March. In December, leucine may have been protecting muscle and protein degradation so the bighorn sheep would be utilizing carbohydrate and lipid metabolism for energy. In March, when lipid and carbohydrate stores were depleted, protein metabolism was needed for energy. The second small metabolite that was different than the previous analysis was tyrosine. Tyrosine is a non-essential amino acid that is critical in the formation of neurotransmitters. The significant pathway that tyrosine is identified in is methane metabolism.

The third objective of this study was to determine if all bighorn sheep sampled in December could be distinguished from all bighorn sheep sampled in March. The potential biomarkers that were identified in this analysis determined it is possible to discriminate herds from different geographic areas and at different times of the year using NMR metabolic profiling. These potential biomarkers, along with the significantly important pathways that these small metabolites are identified in, have the possibility to identify herds in different nutritional states and geographic locations. These small metabolites have the potential to be used by wildlife managers in identifying herds that have less nutritional resources.

4.4 Distinguishing differences in metabolic profiles between bighorn sheep herds within a season compared to a control population of Rambouillet ewes

The final analysis of this study was to determine if using NMR metabolic profiling could distinguish bighorn sheep herds from one another within the same season. The purpose of conducting these analyses was to determine if NMR metabolic profiling can be used to distinguish differences in bighorn sheep from different geographic locations within the same time period or season. The first month that was analyzed was the December herds collected in 2014. The control population used for this analysis were Rambouillet ewes that were collected at the same time of year for all time periods. The Rambouillet ewes were on a maintenance diet and had no environmental stressors for the whole collection period. The PLS-DA for the Castle Reef herd (Figure 6A), the Fergus herd (Figure 7A), the NE Yellowstone herd (Figure 8A), the Paradise herd (Figure 9A), and the Stillwater herd (Figure 10A) all illustrate that there is a clear metabolic shift between these herds and the control Rambouillet ewes. The clear metabolic separations follow the known physiological states that populations are in, all the five bighorn herds were in a sub-maintenance nutritional state, while the control Rambouillet ewes were in a maintenance nutritional state. The VIP scores [the Castle Reef herd (Figure 6B), the Fergus herd (Figure 7B), the NE Yellowstone herd (Figure 8B), the Paradise herd (Figure 9B), and the Stillwater herd (Figure 10B)] greater than 1.0 indicates that small metabolite having contributed significantly to the changes identified in the PLS-DA.

Potential biomarkers that are obtained from the VIP scores [the Castle Reef herd (Figure 6C), the Fergus herd (Figure 7C), the NE Yellowstone herd (Figure 8C), the Paradise herd (Figure 9C), and the Stillwater herd (Figure 10C)] are the least number of

small metabolites that can be used to separate the bighorn herds from the control population of Rambouillet ewes. Only small metabolites that are potential biomarkers in all five herds are considered potential biomarkers in distinguishing bighorn sheep within a season. Potential biomarkers identified in all 5 herds were sarcosine, methionine, and trimethylamine n-oxide. Each potential biomarker identified also needs to be found in metabolic pathways identified as significantly important (Table 4) to be considered a potential biomarker. To narrow the list of potential biomarkers, only small metabolites also identified in significantly important pathways (Table 4) were considered potential biomarkers. The potential biomarkers in December that were also found in pathways identified as significantly important pathways (Table 4) were trimethylamine n-oxide found in methane metabolism and sarcosine found in glycine, serine, threonine, arginine and proline metabolism. Again, trimethylamine n-oxide was already identified as a biomarker and its functions are previously listed. Sarcosine is a metabolite identified as a byproduct of glycine metabolism (Cernei et al., 2013). Glycine metabolism is critical in the synthesis of glucose, through serine and pyruvate. Higher concentrations of sarcosine indicate the animal is using glucose for energy and is mostly utilizing carbohydrate metabolism for energy. Sarcosine is identified in the significantly important pathway glycine, serine, threonine, arginine and proline metabolism.

The second month that was analyzed was the January herds collected in 2015. The Rambouillet ewes used in this analysis were also collected in January. The PLS-DA for the Castle Reef herd (Figure 11A), the Jackson herd (Figure 12A), the Lost Creek herd (Figure 13A), the Stillwater herd (Figure 14A), the Taylor Hilgard herd (Figure 15A),

and the Ferris-Seminole herd (Figure 16A) all illustrate that there is a clear metabolic shift between these herds and the control Rambouillet ewes. The clear metabolic separations follow the known physiological states that these populations are in, all the five bighorn herds were in a sub-maintenance nutritional state, while the control Rambouillet ewes were in a maintenance nutritional state. The VIP scores [Castle Reef herd (Figure 11B), the Jackson herd (Figure 12B), the Lost Creek herd (Figure 13B), the Stillwater herd (Figure 14B), the Taylor Hilgard herd (Figure 15 B), and the Ferris-Seminole herd (Figure 16B)] greater than 1.0 indicates that small metabolite having contributed significantly to the changes identified in the PLS-DA.

Potential biomarkers [Castle Reef herd (Figure 11C), the Jackson herd (Figure 12C), the Lost Creek herd (Figure 13C), the Stillwater herd (Figure 14C), the Taylor Hilgard herd (Figure 15 C), and the Ferris-Seminole herd (Figure 16C)] that are obtained from the VIP scores are the least number of small metabolites that can be used to separate the bighorn herds from the control population of Rambouillet ewes. Only small metabolites that are potential biomarkers in all six herds are considered potential biomarkers in distinguishing bighorn sheep within a season. Potential biomarkers identified in all six herds were creatinine, dimethyl sulfone, asparagine and carnitine. Each potential biomarker identified also needs to be found in metabolic pathways identified as significantly important (Table 4) to be considered a potential biomarker. To narrow the list of potential biomarkers, only small metabolites also identified in significantly important pathways (Table 4) were considered potential biomarkers. The potential biomarkers in January that were also found in pathways identified as

significantly important (Table 4) were creatinine found in arginine/proline metabolism and asparagine found in alanine, aspartate and glutamate metabolism. Creatinine has been identified as a potential biomarker in previous analyses and functions have been described. Higher concentrations of creatinine in the bighorn sheep indicate that these herds are beginning to utilize protein metabolism for energy. Asparagine is a non-essential amino acid that is important in removing ammonia, aids in DNA synthesis, and increases insulin resistance when present in high concentrations (Kraus, et al., 2004). Asparagine at lower concentrations indicate the animal may be using glucose for energy and utilizing carbohydrate metabolism. Asparagine is in lower concentrations in the bighorn sheep herds collected in January, indicating these herds are still using carbohydrate metabolism. Asparagine is identified in the significantly important pathway alanine, aspartate and glutamate metabolism. Creatinine and asparagine indicate that in January, the bighorn sheep herds are still utilizing carbohydrate metabolism, however, are beginning to shift to protein metabolism for energy.

The final month that was analyzed was the March herds collected in 2015. The Rambouillet ewes used in this analysis were also collected in March. The PLS-DA for the Absaroka herd (Figure 17A), the Castle Reef herd (Figure 18A), the Cody herd (Figure 19A), the Devil's Canyon herd (Figure 20A), the Dubois herd (Figure 21A), the Jackson herd (Figure 22A), the Lost Creek herd (Figure 23A), and the Stillwater herd (Figure 24A) all illustrate that there is a clear metabolic shift between these herds and the control Rambouillet ewes. The clear metabolic separations follow the known physiological states that these populations are in, all the eight bighorn herds were in a

sub-maintenance nutritional state, while the control Rambouillet ewes were in a maintenance nutritional state. The VIP scores [Absaroka herd (Figure 17B), the Castle Reef herd (Figure 18B), the Cody herd (Figure 19B), the Devil's Canyon herd (Figure 20B), the Dubois herd (Figure 21B), the Jackson herd (Figure 22B), the Lost Creek herd (Figure 23B), and the Stillwater herd (Figure 24B)] greater than 1.0 indicates that small metabolite having contributed significantly to the changes identified in the PLS-DA.

Potential biomarkers [Absaroka herd (Figure 17C), the Castle Reef herd (Figure 18C), the Cody herd (Figure 19C), the Devil's Canyon herd (Figure 20C), the Dubois herd (Figure 21C), the Jackson herd (Figure 22C), the Lost Creek herd (Figure 23C), and the Stillwater herd (Figure 24C)] that are obtained from the VIP scores are the least number of small metabolites that can be used to separate the bighorn herds from the control population of Rambouillet ewes. Only small metabolites that are potential biomarkers in all eight herds are considered potential biomarkers in distinguishing bighorn sheep within a season. Potential biomarkers identified in all eight herds were creatinine, dimethyl sulfone, and carnitine. Each potential biomarker identified also needs to be found in metabolic pathways identified as significantly important (Table 4) to be considered a potential biomarker. To narrow the list of potential biomarkers, only small metabolites also identified in significantly important pathways (Table 4) were considered potential biomarkers. The only potential biomarker also found in a significantly important pathway (Table 4) is creatinine, which is found in arginine/proline metabolism. Creatinine has been identified as a potential biomarker in previous analyses and functions have been described. Creatinine concentrations were higher in the bighorn sheep herds,

indicating that they were mostly utilizing muscle for energy and have shifted their metabolisms to protein degradation for energy.

An exciting find in these analyses was demonstrating a shift in metabolism from December to March. In December, the bighorn sheep herds were mostly using carbohydrate metabolism for energy. In January, the bighorn sheep herds were using a combination of carbohydrate and protein metabolism. In March, the bighorn sheep herds were mostly using protein metabolism. The change from carbohydrate metabolism to protein metabolism corresponds with what is physiologically hypothesized to be occurring in the bighorn sheep at the parallel time of year.

5 Conclusion

Through the potential biomarkers that were identified in this study, it is possible to discriminate herds from each other based on geographic location and nutritional states. Potential biomarkers were identified between herds in different seasons and different geographic locations. These results will give us insights to the physiology of the members of those herds and could provide information to bighorn sheep managers to better manage their herd. These biomarkers represent a potential panel of metabolites that may be used for assessing nutritional status, environmental stress, and herd health through the identification of significantly important metabolic pathways related to energy and protein balance.

For future research, herds from the 2015-2017 season will be processed, profiled and analyzed through NMR metabolic profiling. This will provide researchers a

longitudinal view of herd health for various populations and provide insight on how metabolic shifts vary through time. Furthermore, bighorn sheep samples from a South Dakota Captive Wildlife Facility will be processed, profiled and analyzed using NMR metabolic profiling. These samples are longitudinal serum samples collected from adult bighorn sheep that experienced a cross-strain of *Mycoplasma ovipneumoniae* (MOVI) transmission event. Serial samples include samples from: 1) when the bighorn sheep were transferred to the captive facility; 2) after acclimation to captivity; 3) during the transmission event and infection period; and, either at the end of the study (survivors) or soon after death (non-survivors). After the transmission event, some of the individual bighorn sheep became severely ill and died, while others only showed mild signs of the disease and then recovered. The combination of metabolomics and temporal samples from bighorn sheep before exposure to a transmission event of MOVI will allow for a greater understanding of the mechanisms of respiratory disease and potentially develop biomarkers that would allow wildlife managers to make decisions regarding the management of bighorn sheep populations regarding disease.

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Table 1. General attributes of the 13 bighorn sheep populations investigated in this study.

	Hunt-Area	Connectivity Level	Migratory	Ecological Region	Elevation Range (m)
Absaroka	-	-	-	Cen Rocky Mtn.	
Castle Reef	MT-422	Meta Pop.	Yes	Cen. Rocky Mtn.	1400-2900
Fergus	MT-482	Meta Pop.	No	N. Rolling Plains	700-900
Hilgard	MT-302	Isolated	Yes	Cen. Rocky Mtn.	2100-3400
Lost Creek	MT-213	Limited	Yes	Cen. Rocky Mtn.	1700-3200
NE Yellowstone	MT-9995	Meta Pop.	Yes	Cen. Rocky Mtn.	1500-3400
Paradise	MT-124	Isolated	No	N. Rocky Mtn.	800-2000
Stillwater	MT-500a	Limited	Yes	Cen. Rocky Mtn.	1500-3900
Cody	-	-	-	Cen. Rocky Mtn	-
Devil's Canyon	-	-	-	Cen. Rocky Mtn	-
Dubois	WY-22	Meta Pop.	Yes	Cen. Rocky Mtn.	2200-3800
Ferris-Seminoe	-	-	-	Cen. Rocky Mtn	-
Jackson	WY-7	Meta Pop.	Yes	Cen. Rocky Mtn.	2100-3600

Table 2. Geographic locations of bighorn sheep herds and number of bighorn sheep samples collected in December of 2014, January of 2015, February of 2015, and March of 2015

Bighorn sheep herds	December 2014	January 2015	February 2015	March 2015
Castle Reef	5	8	0	3
Fergus	30	0	0	0
Lost Creek	0	6	0	6
Absaroka	0	0	0	18
Cody	0	0	0	10
Devil's Canyon	0	0	0	25
Dubois	0	0	0	20
Ferris-Seminoe	0	0	8	0
Jackson	0	12	0	12
NE Yellowstone	4	0	0	0
Paradise	30	0	0	0
Stillwater	5	5	0	6
Taylor Hilgard	0	27	0	0

Table 3. Criteria for determining pregnancy rates based on optical density (OD) values of systemic pregnancy specific protein B (PSPB) and concentrations of progesterone (P4; ng/mL).

	P4 > 1.5 ng/mL	P4 < 1.5 ng/mL
PSPB OD > 0.21	Pregnant	Embryonic death
PSPB OD = 0.15 to 0.135	Early stages of pregnancy ^a or embryonic death	Early stages of pregnancy or embryonic death ^a
PSPB OD < 0.135	Estrous cyclicity or in the early stages of pregnancy ^a	Non-pregnant and no estrous cyclicity
^a < 30 days of pregnancy		

Table 4. Results from the serum metabolomic pathway analysis from all metabolites identified in the serum of bighorn sheep. Metabolic pathways with impact values greater > 0.1 were considered important, along with a p -value of > 0.05 . The p -values were corrected with the Holm-Bonferroni correction to reduce the error of the multiple comparisons. The metabolic pathways that are significantly affected by the compounds identified are indicated on the right.

Pathway Impact	Impact value	p – value	Holm-Bonferroni Correction	Significant Pathway
Aminoacyl- tRNA biosynthesis	0.14	0.0001	0.001	Significant
Glycine, serine, threonine metabolism	0.60	0.0001	0.001	Significant
Valine, isoleucine, leucine biosynthesis	0.99	0.0001	0.001	Significant
Alanine, aspartate, glutamate metabolism	0.28	0.0001	0.001	Significant
Arginine & proline metabolism	0.17	0.001	0.006	Significant
Methane metabolism	0.40	0.004	0.020	Significant
Phenylalanine, tyrosine, tryptophan biosynthesis	1.00	0.008	0.032	Significant
Cysteine & methionine metabolism	0.27	0.020	0.060	Non-significant
Phenylalanine metabolism	0.41	0.040	0.080	Non-significant
Pyruvate metabolism	0.24	0.050	0.080	Non-significant

Table 5. Results from the parameters for assessing whether the model quality is appropriate in discriminating bighorn sheep herds from the control Rambouillet ewes. The larger the R^2 and Q^2 values, the better quality and more appropriate the model is for discrimination.

Herd	Month Sampled	R^2	Q^2
Castle Reef	December	0.97	0.96
Fergus	December	0.94	0.94
NE Yellowstone	December	0.98	0.96
Paradise	December	0.97	0.96
Stillwater	December	0.97	0.93
Castle Reef	January	0.97	0.95
Jackson	January	0.94	0.92
Lost Creek	January	0.95	0.94
Stillwater	January	0.97	0.95
Taylor Hilgard	January	0.98	0.98
Ferris-Seminole	February	0.97	0.96
Absaroka	March	0.97	0.96
Castle Reef	March	0.95	0.90
Cody	March	0.94	0.88
Devil's Canyon	March	0.97	0.96
Dubois	March	0.96	0.93
Jackson	March	0.96	0.96
Lost Creek	March	0.86	0.72
Stillwater	March	0.96	0.93

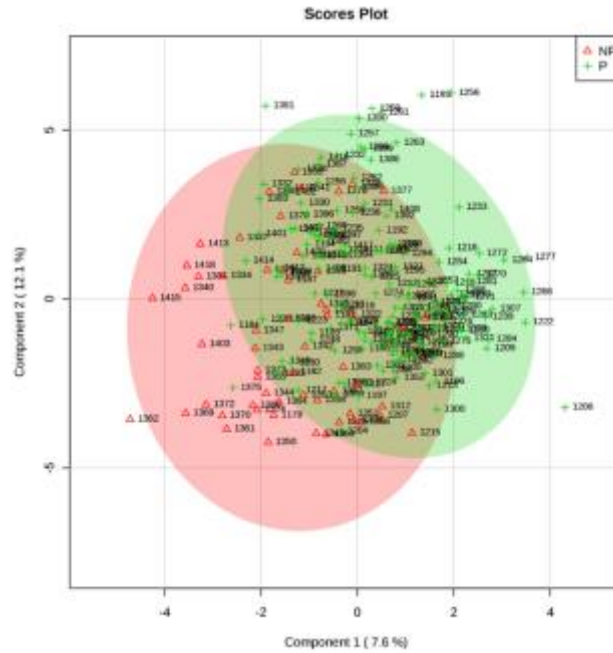


Figure 1. Partial least squares discriminant analysis (PLS-DA) score map between non-pregnant bighorn sheep ($n = 59$) and pregnant bighorn sheep ($n = 179$). Pregnant is represented in green and non-pregnant is represented in red.

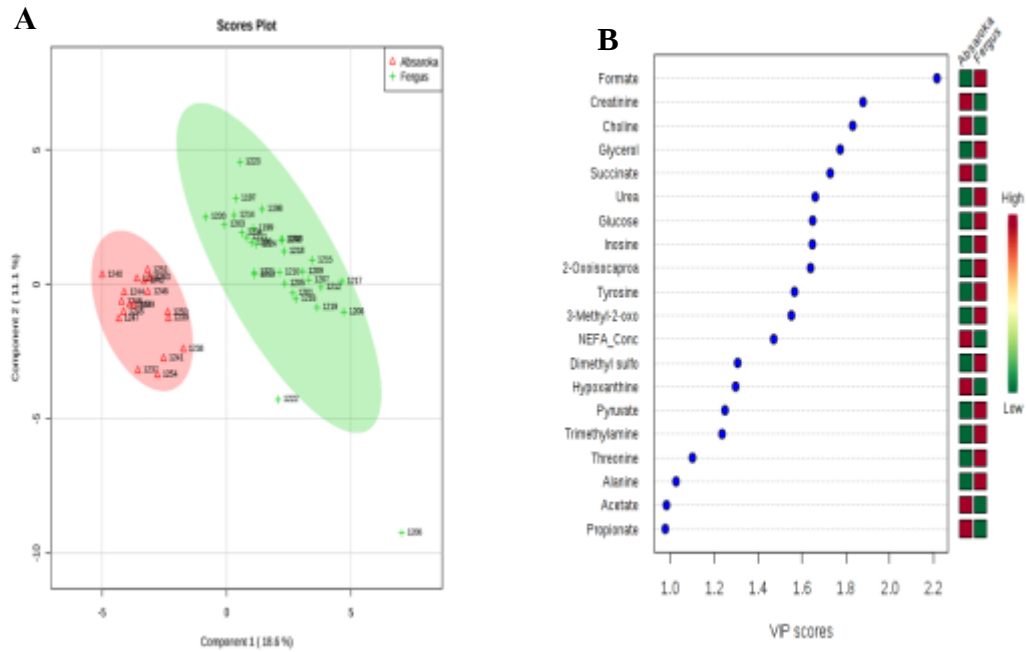


Figure 2. Partial least squares discriminant analysis (PLS-DA) score map (A) and variable of importance (VIP) scores (B) between Fergus (n = 29, collected in December) and Absaroka (n = 18, collected in March) bighorn sheep herds. In A, Fergus is represented in green and Absaroka is represented in red. In B, metabolites with a VIP score > 1.0 are considered important.

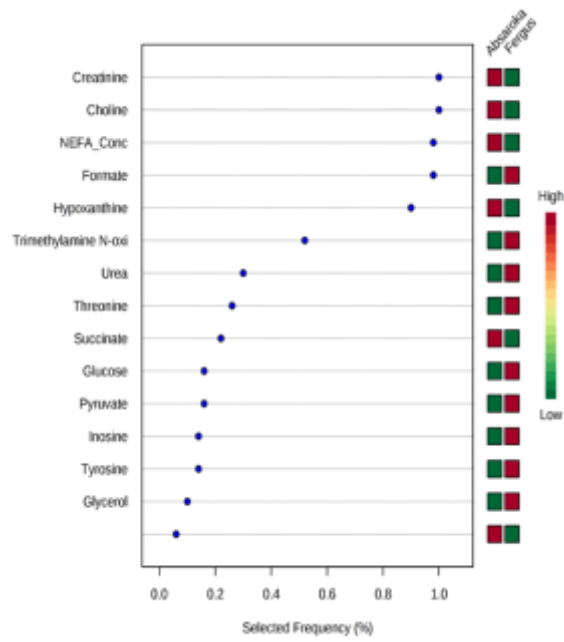


Figure 3. Biomarker analysis between Fergus (n = 29, collected in December) and Absaroka (n = 18, collected in March) bighorn sheep herds. Metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying herds.

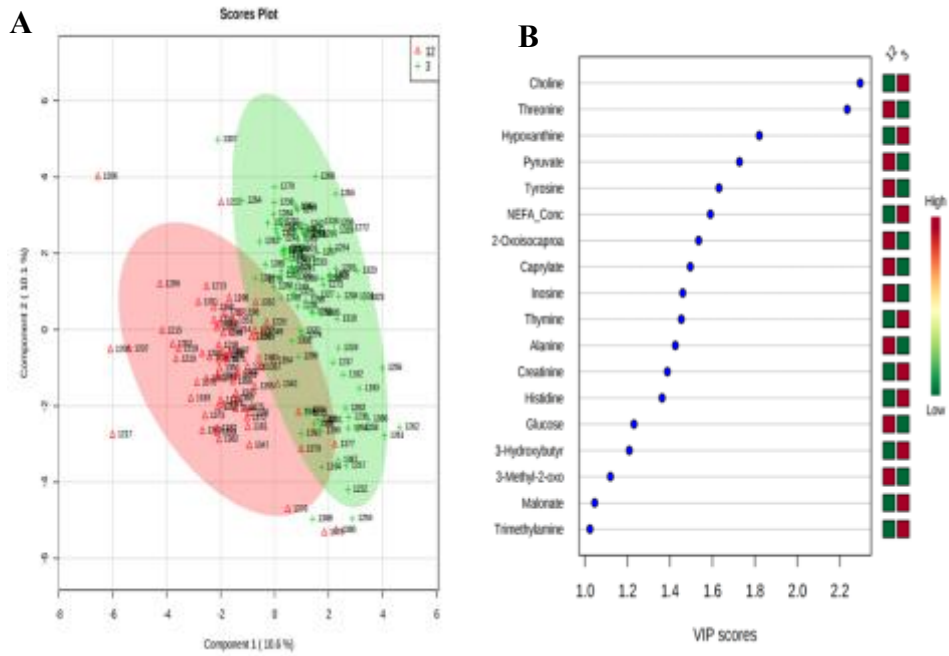


Figure 4. Partial least squares discriminant analysis (PLS-DA) score map (A) and variable of importance (VIP) scores (B) between herds sampled in December ($n = 73$) and herds sampled in March ($n = 99$). In A, December is represented in red and March is represented in green. In B, metabolites with a VIP score > 1.0 are considered important.

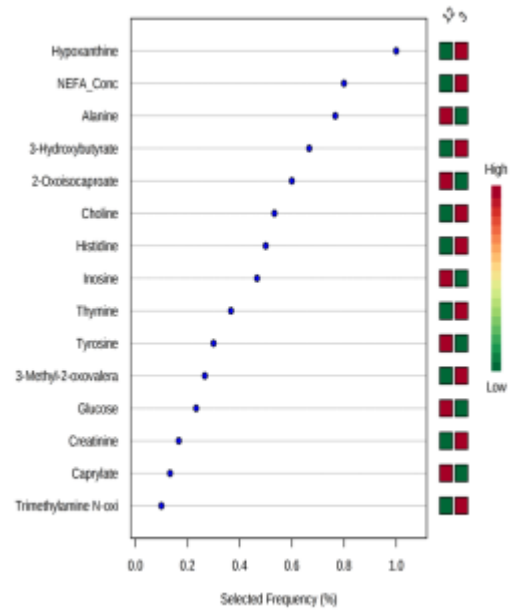


Figure 5. Biomarker analysis between herds collected in December ($n = 73$) and herds collected in March ($n = 99$). Metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying herds.

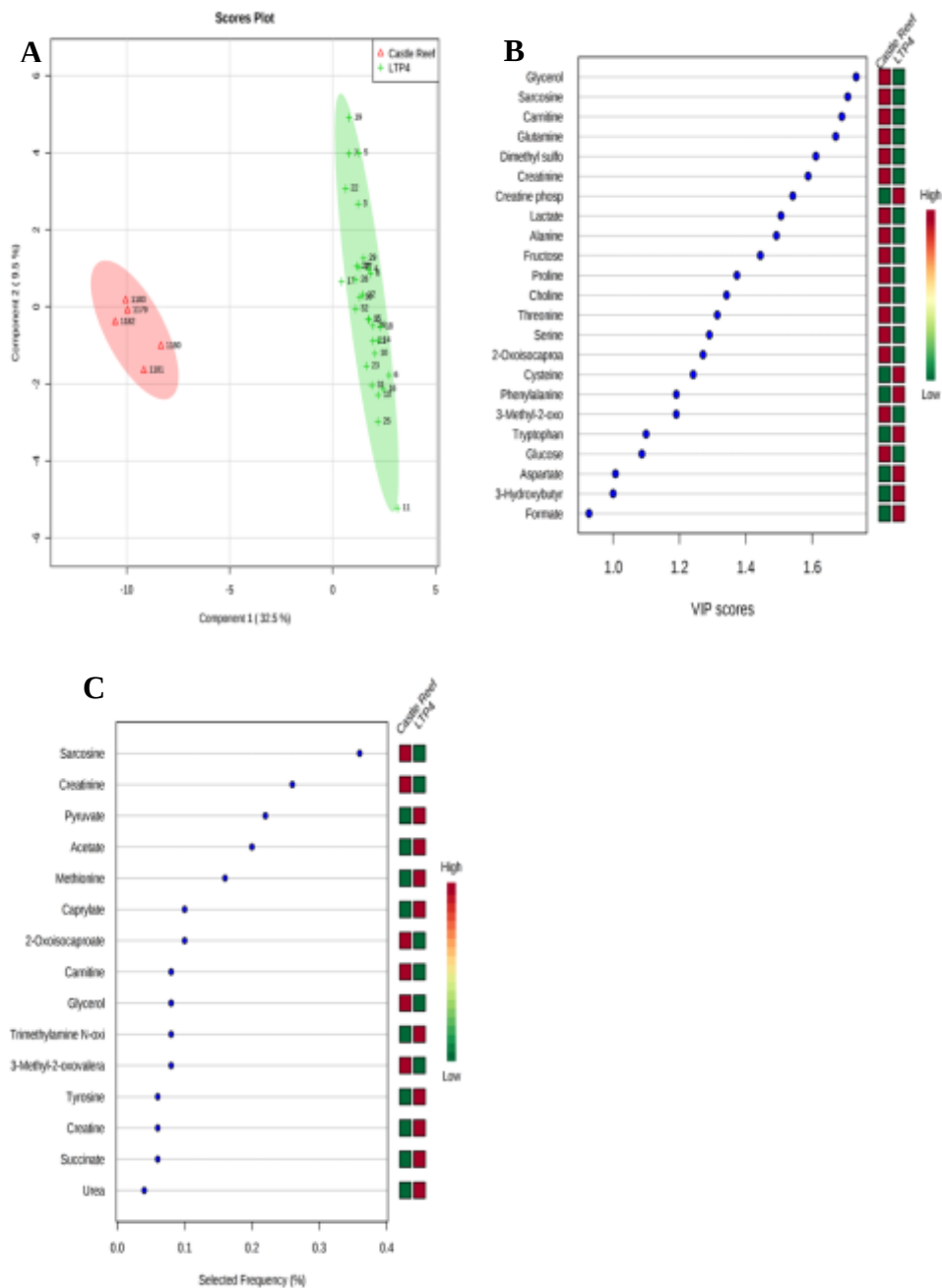


Figure 6. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Castle Reef ($n = 5$, sampled in December) and control Rambouillet ewes ($n = 29$, sampled in December). In A, Castle Reef is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Castle Reef herd from the control Rambouillet ewes.

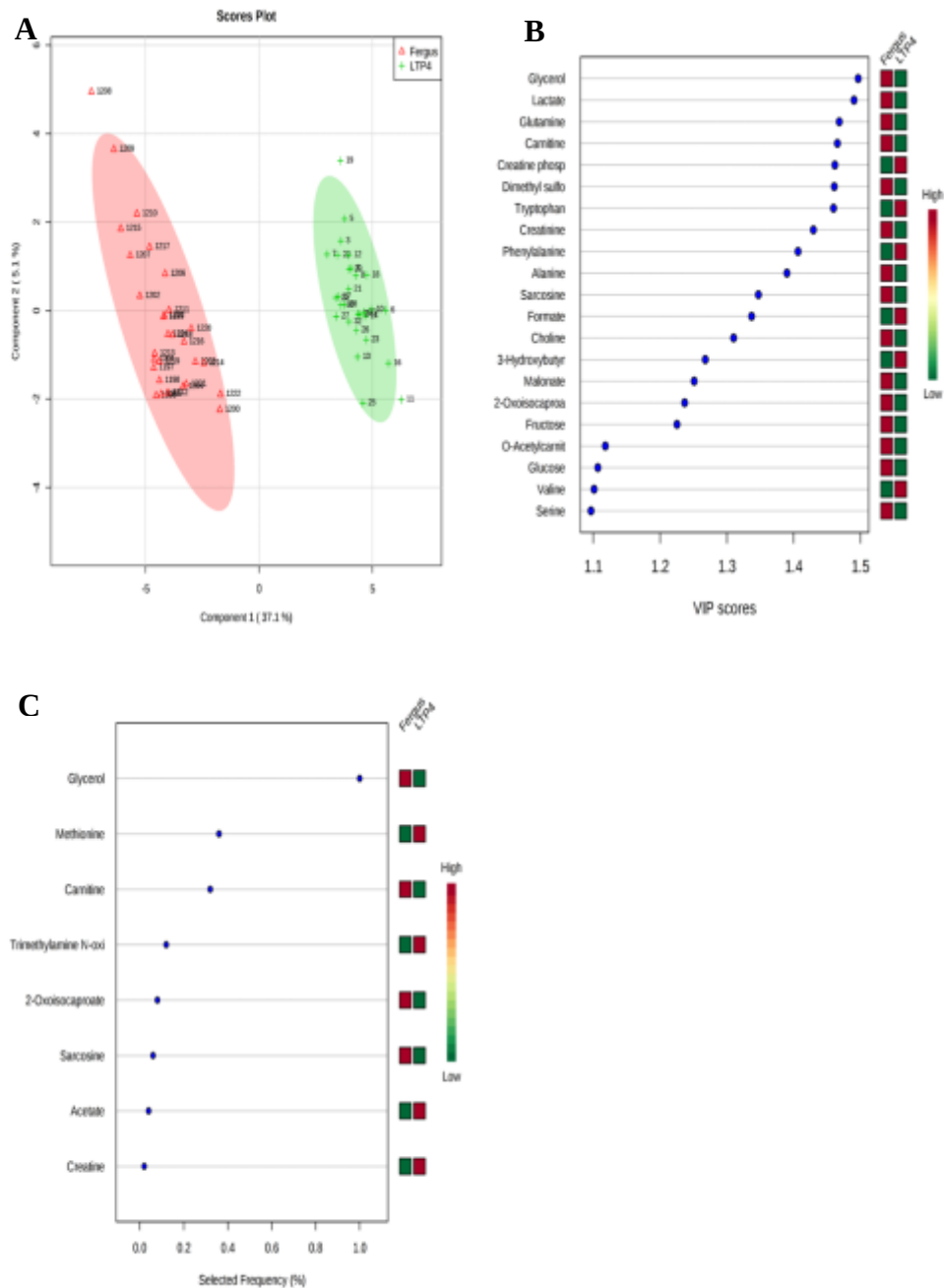


Figure 7. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Fergus (n = 29, sampled in December) and control Rambouillet ewes (n = 29, sampled in December). In A, Fergus is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score >1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Fergus herd from the control Rambouillet ewes.

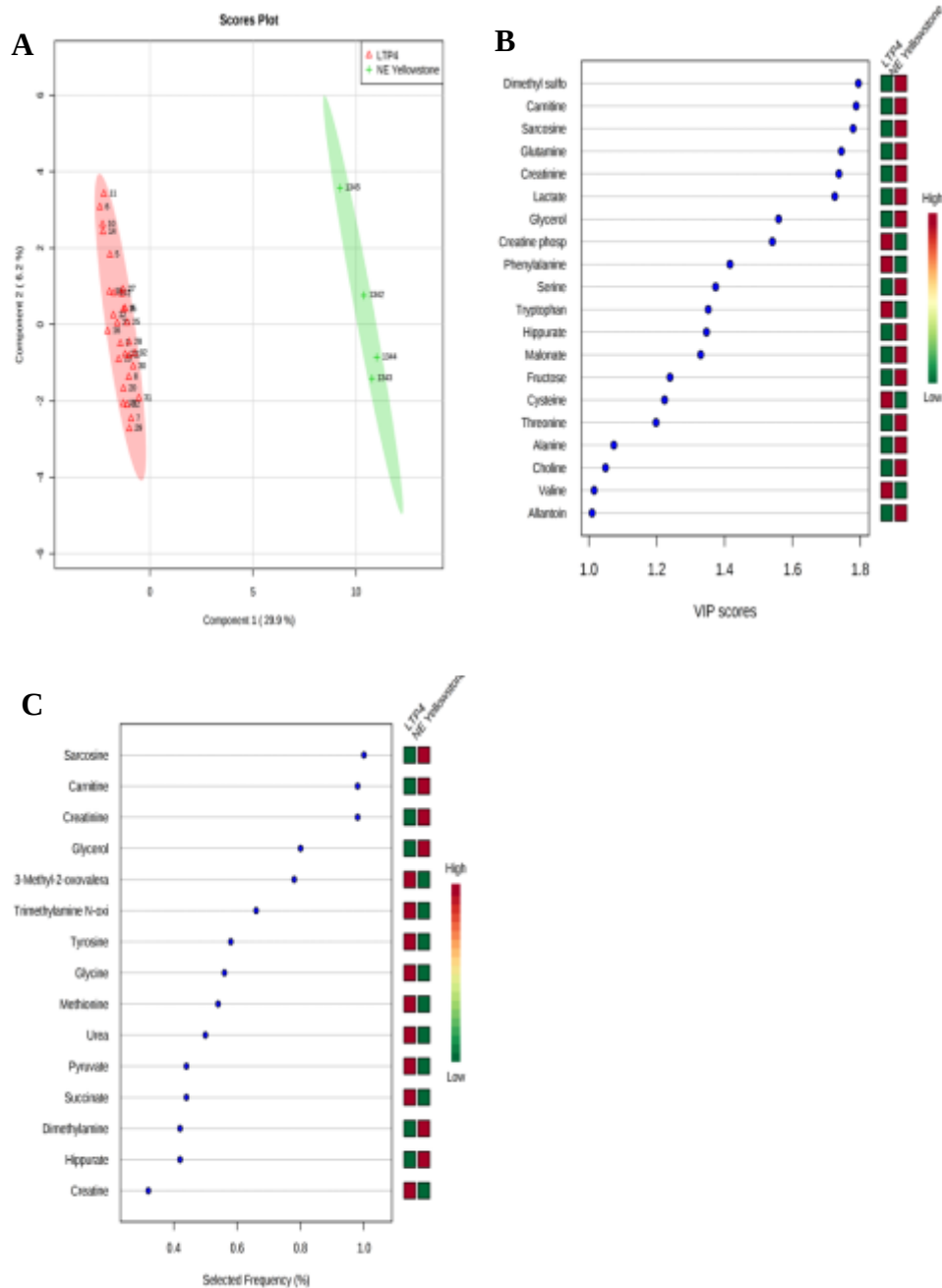


Figure 8. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from NE Yellowstone (n = 4, sampled in December) and control Rambouillet ewes (n = 29, sampled in December). In A, NE Yellowstone is represented in green and the Rambouillet ewes is represented in red. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the NE Yellowstone herd from the control Rambouillet ewes

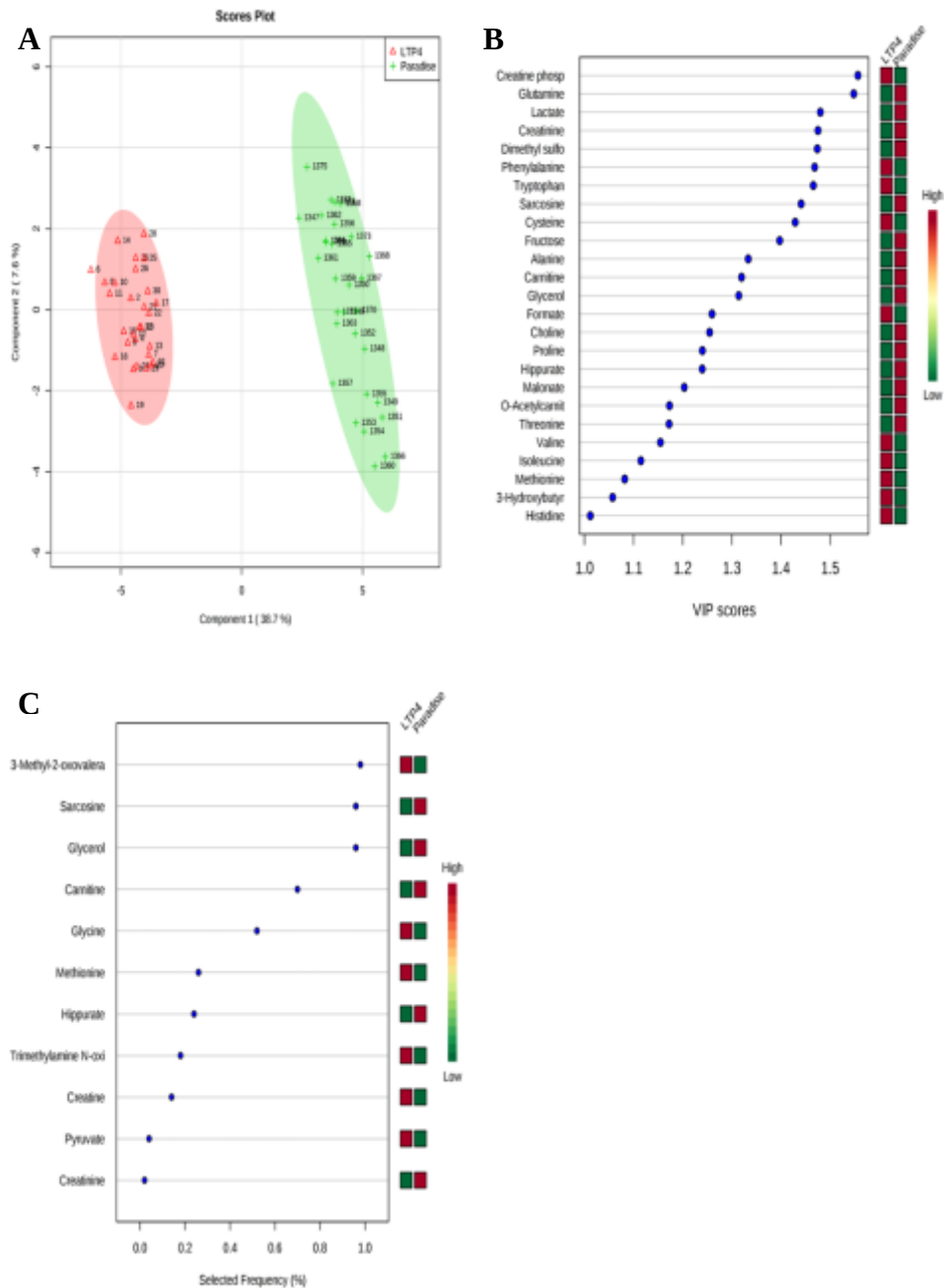


Figure 9. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Paradise ($n = 30$, sampled in December) and control Rambouillet ewes ($n = 29$, sampled in December). In A, Paradise is represented in green and the Rambouillet ewes is represented in red. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Paradise herd from the control Rambouillet ewes.

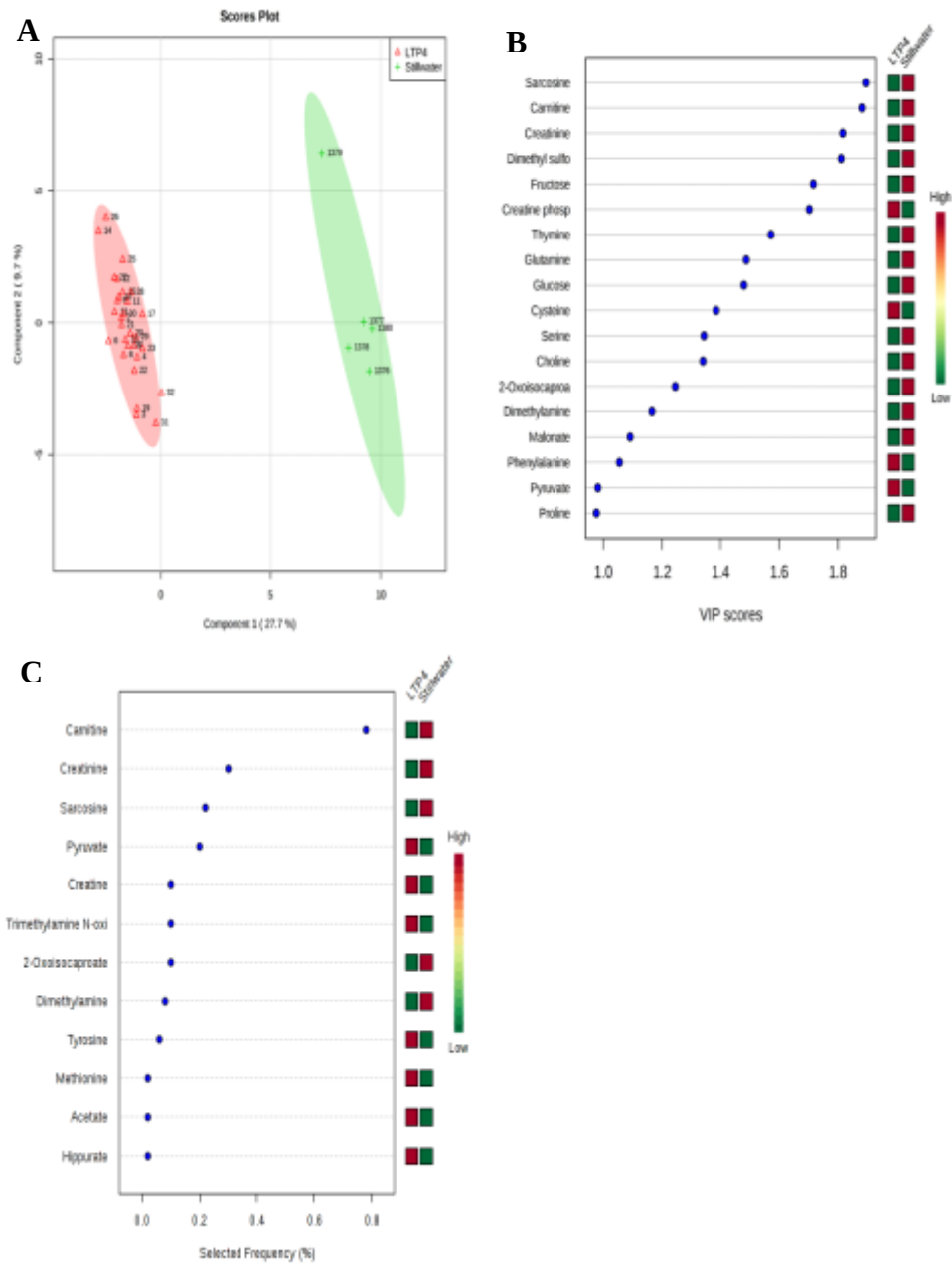


Figure 10. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Stillwater (n = 5, sampled in December) and control Rambouillet ewes (n = 29, sampled in December). In A, Stillwater is represented in green and the Rambouillet ewes is represented in red. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Stillwater herd from the control Rambouillet ewes.

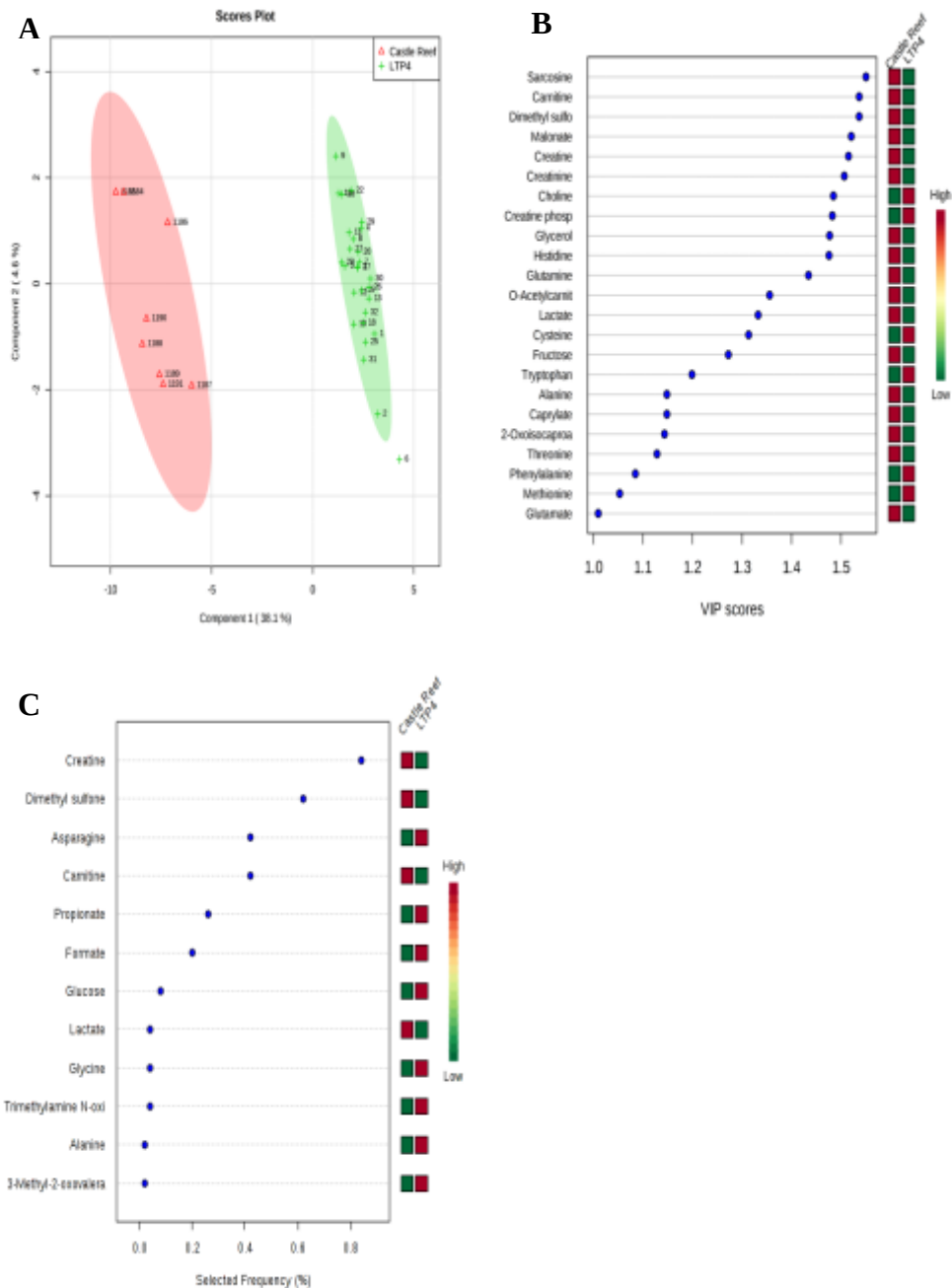


Figure 11. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Castle Reef ($n = 8$, sampled in January) and control Rambouillet ewes ($n = 28$, sampled in January). In A, Castle Reef is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Castle Reef herd from the control Rambouillet ewes.

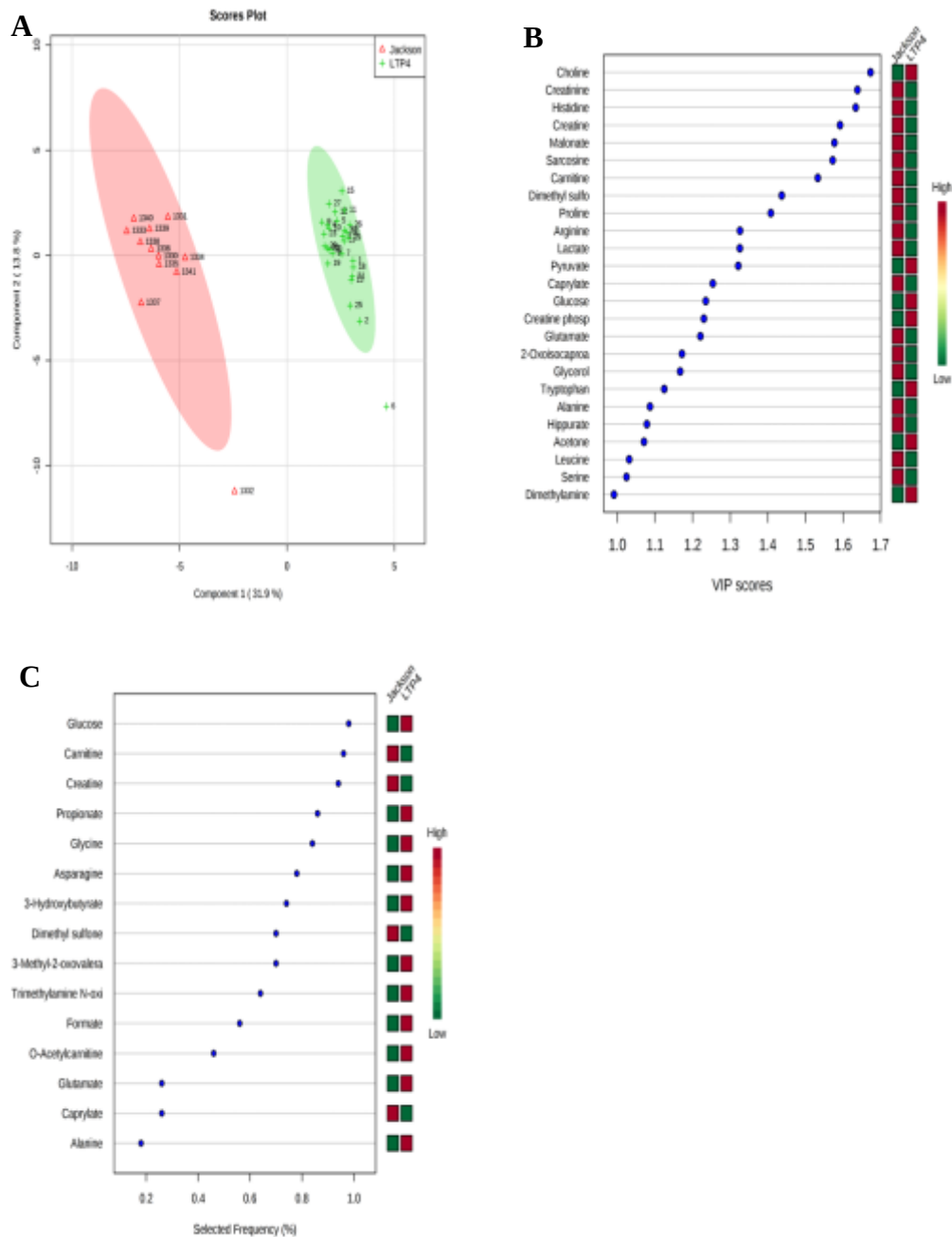


Figure 12. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Jackson ($n = 12$, sampled in January) and control Rambouillet ewes ($n = 28$, sampled in January). In A, Jackson is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Jackson herd from the control Rambouillet ewes.

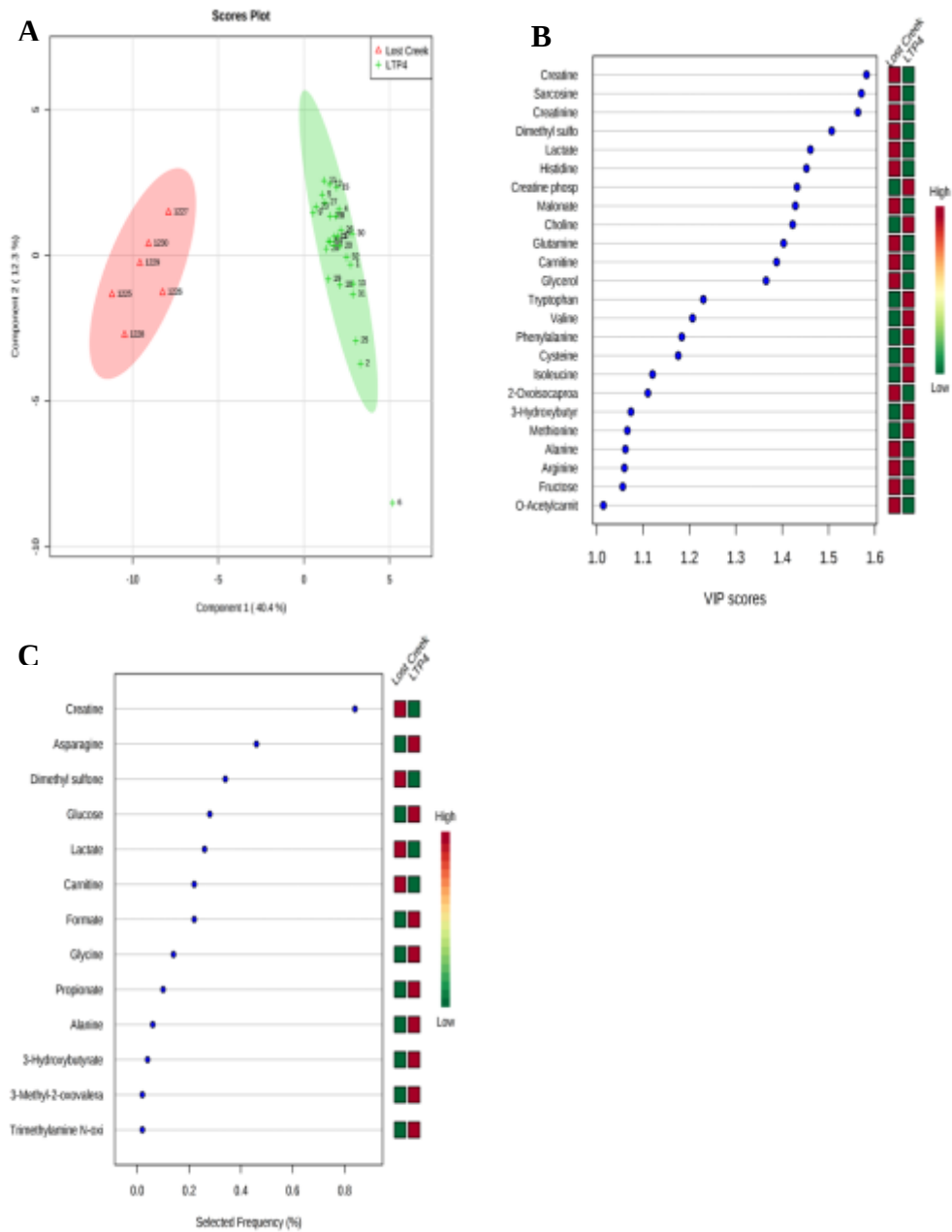


Figure 13. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Lost Creek ($n = 6$, sampled in January) and control Rambouillet ewes ($n = 28$, sampled in January). In A, Lost Creek is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Lost Creek herd from the control Rambouillet ewes.

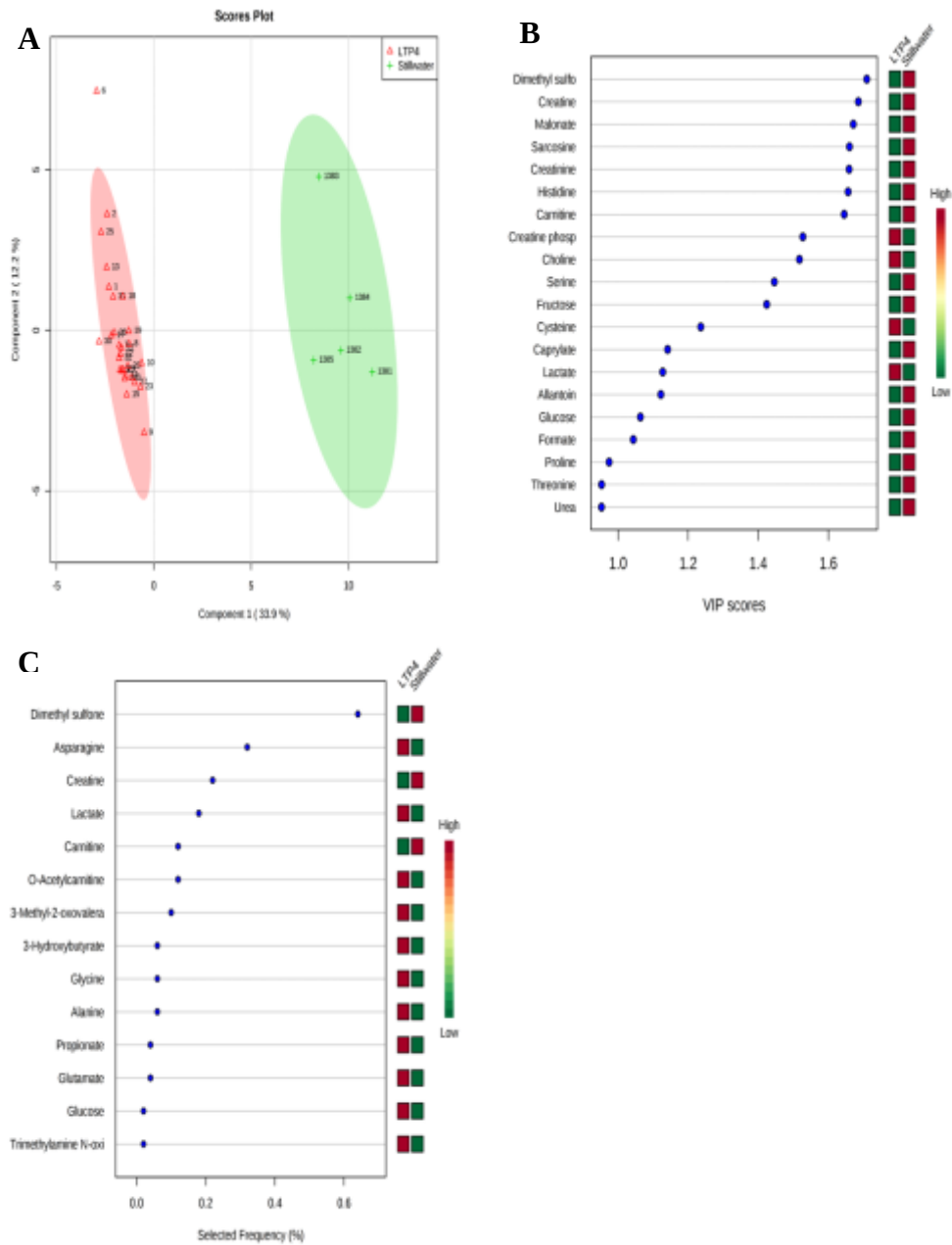


Figure 14. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Stillwater (n = 5, sampled in January) and control Rambouillet ewes (n = 28, sampled in January). In A, Stillwater is represented in green and the Rambouillet ewes is represented in red. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Stillwater herd from the control Rambouillet ewes.

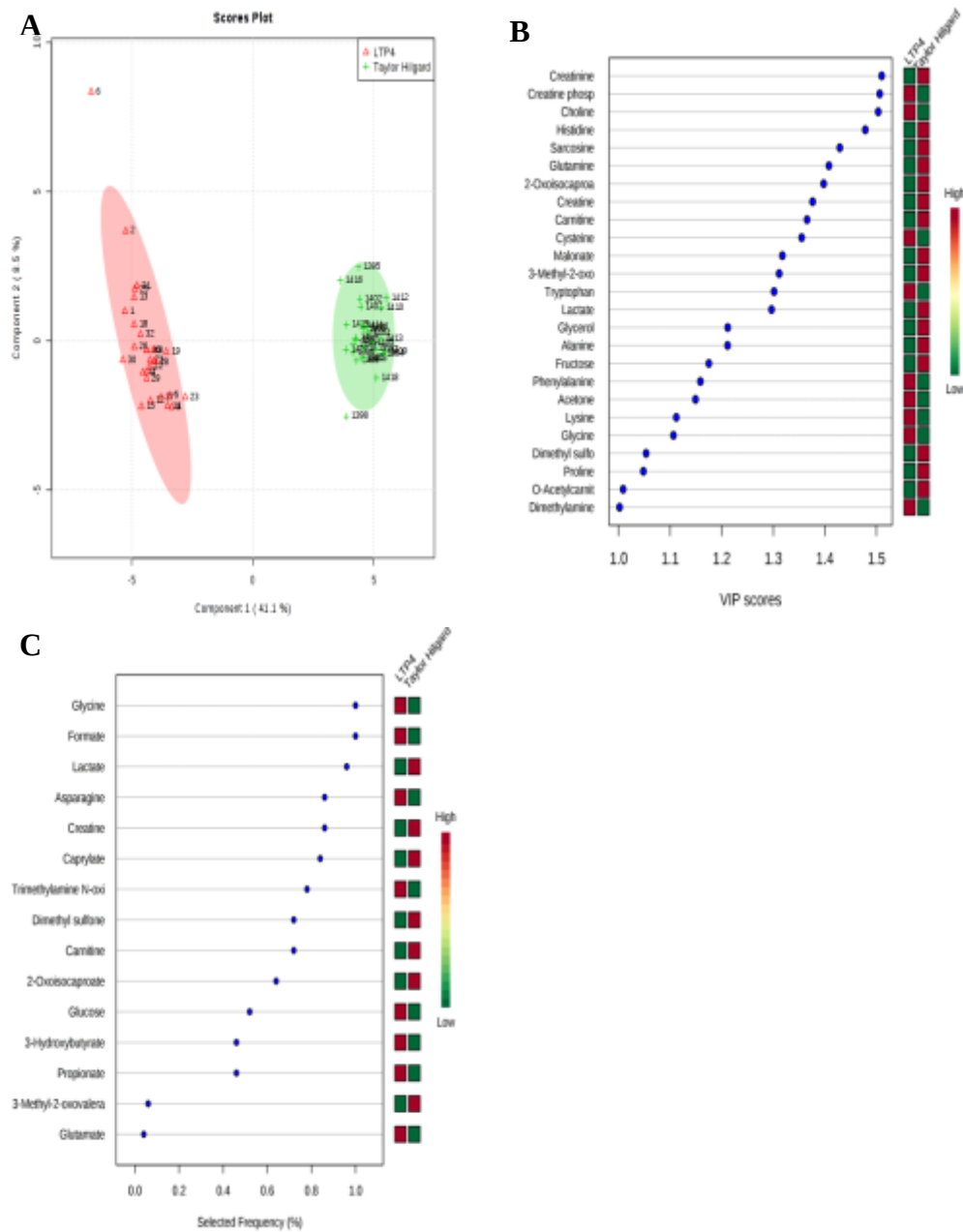


Figure 15. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Taylor Hilgard ($n = 27$, sampled in January) and control Rambouillet ewes ($n = 28$, sampled in January). In A, Taylor Hilgard is represented in green and the Rambouillet ewes is represented in red. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Taylor Hilgard herd from the control Rambouillet ewes.

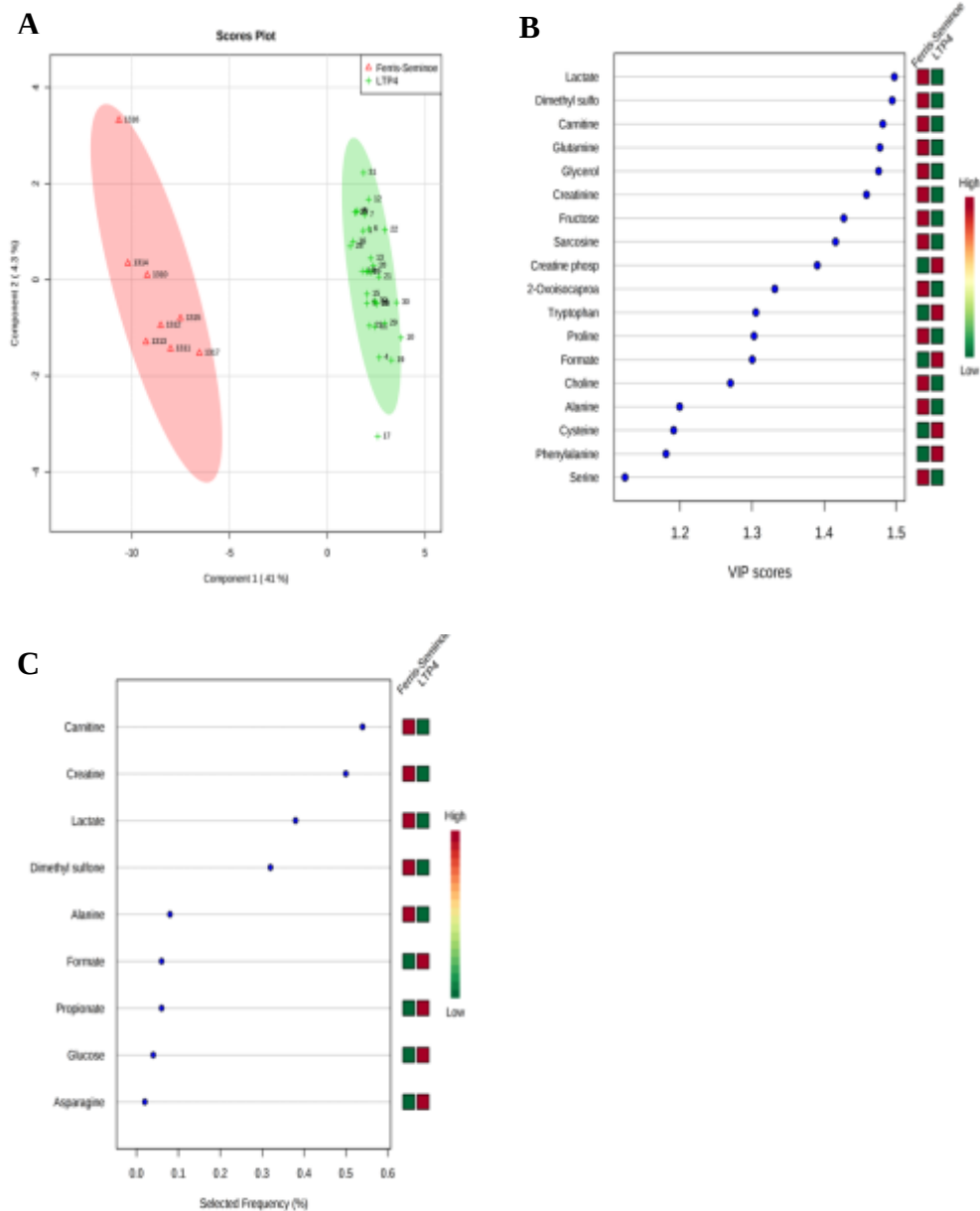


Figure 16. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Ferris Seminoe ($n = 8$, sampled in February; in January analysis) and control Rambouillet ewes ($n = 31$, sampled in February). In A, Ferris Seminoe is represented in red and the control ewes are represented in green. In B, metabolites with a VIP score > 1.0 are considered important. In C, metabolites with selected frequencies > 0.1 are considered potential biomarkers for identifying the Ferris Seminoe herd from the control Rambouillet ewes.

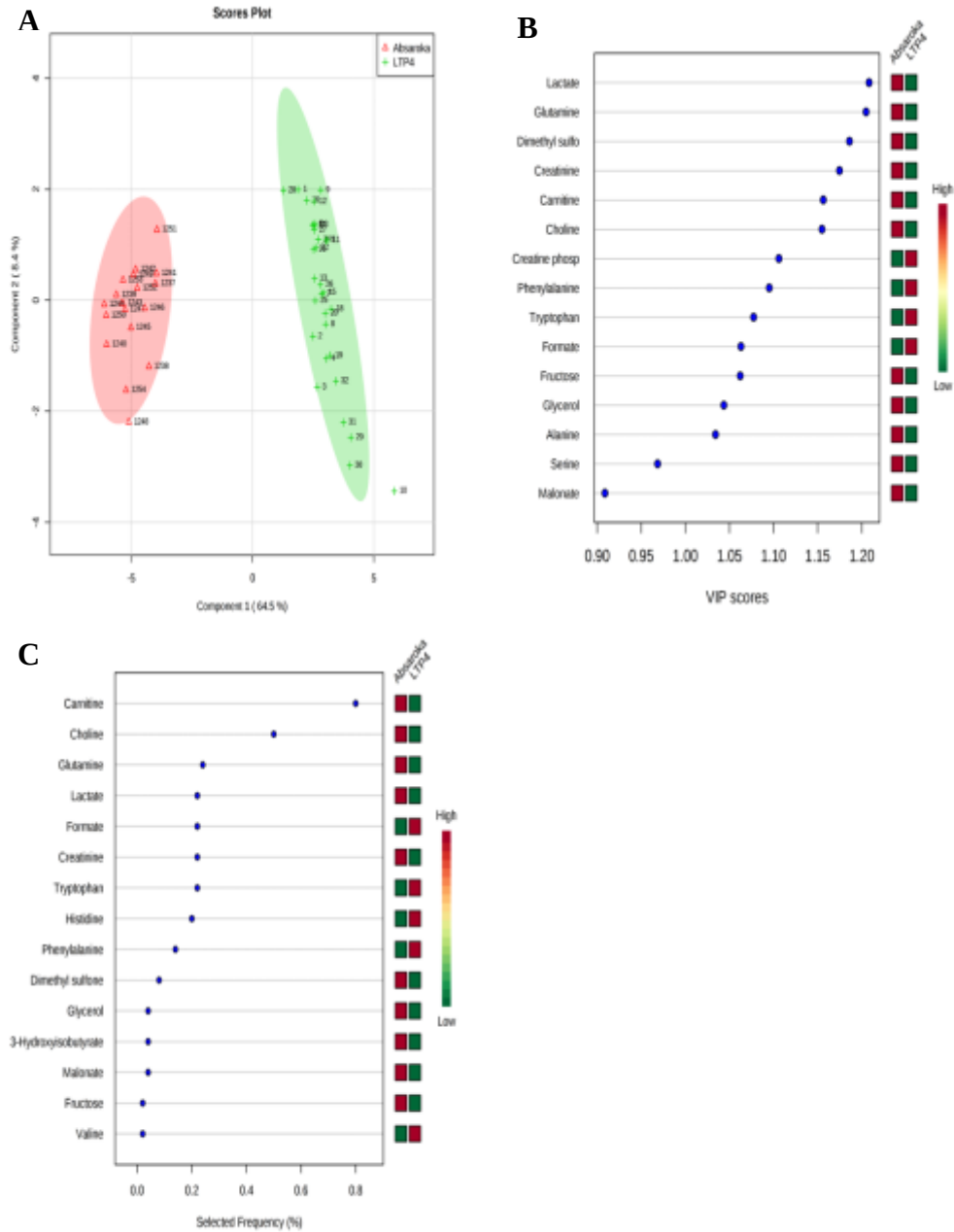


Figure 17. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Absaroka ($n = 18$, sampled in March) and control Rambouillet ewes ($n = 31$, sampled in March). In A, Absaroka is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Absaroka herd from the control Rambouillet ewes.

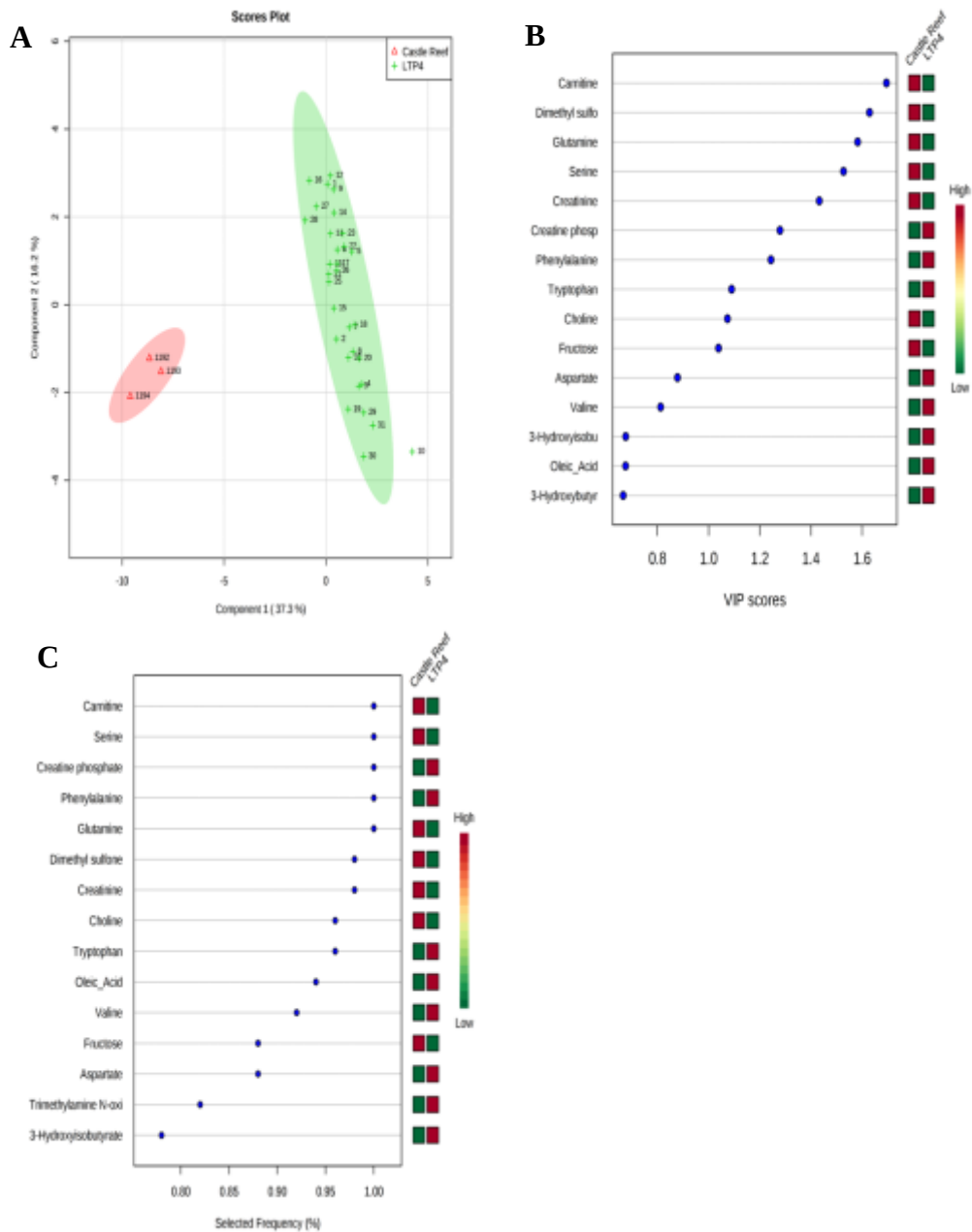


Figure 18. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Castle Reef (n = 3, sampled in March) and control Rambouillet ewes (n = 31, sampled in March). In A, Castle Reef is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Castle Reef herd from the control Rambouillet ewes.

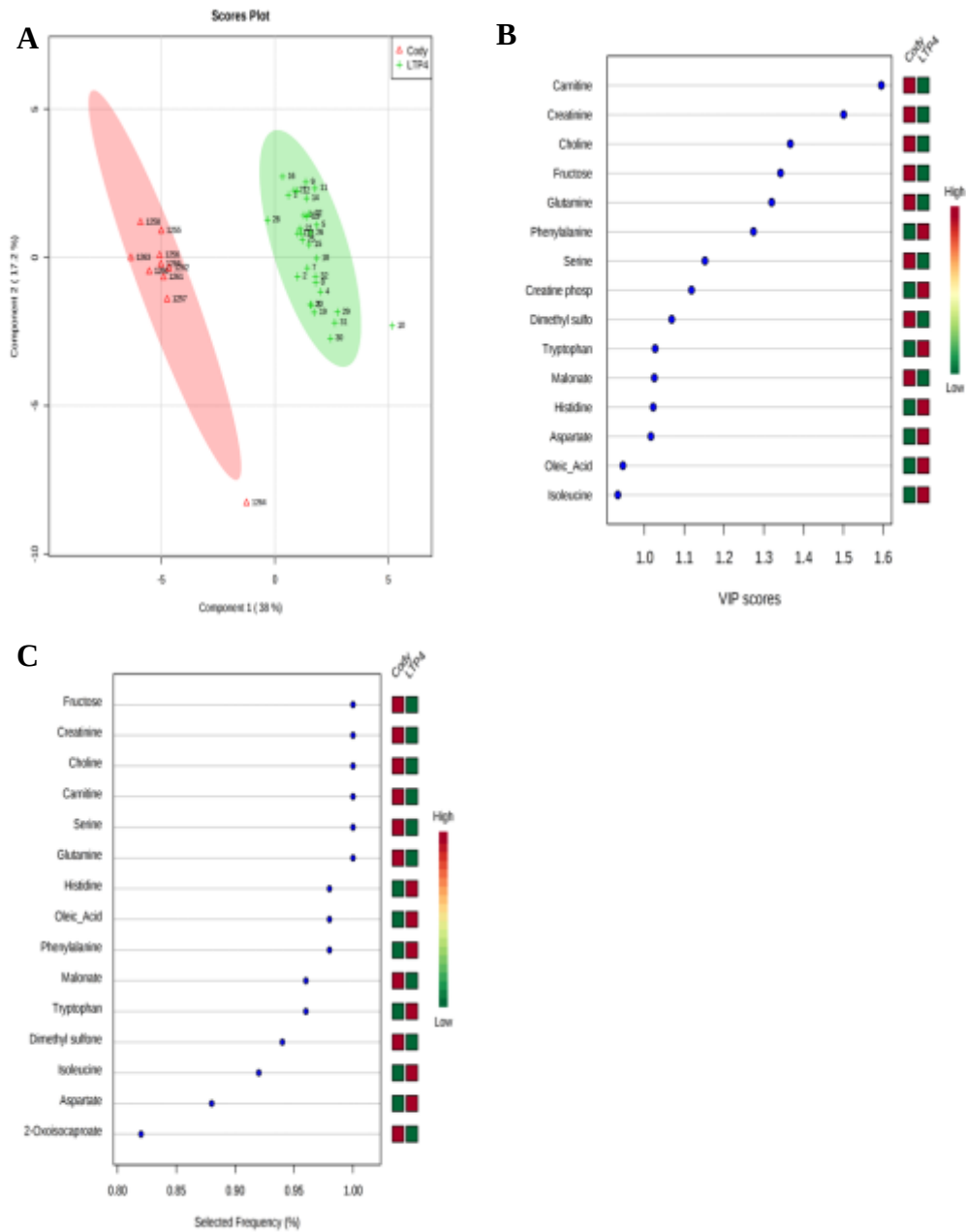


Figure 19. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Cody (n = 10, sampled in March) and control Rambouillet ewes (n = 31, sampled in March). In A, Cody is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Cody herd from the control Rambouillet ewes.

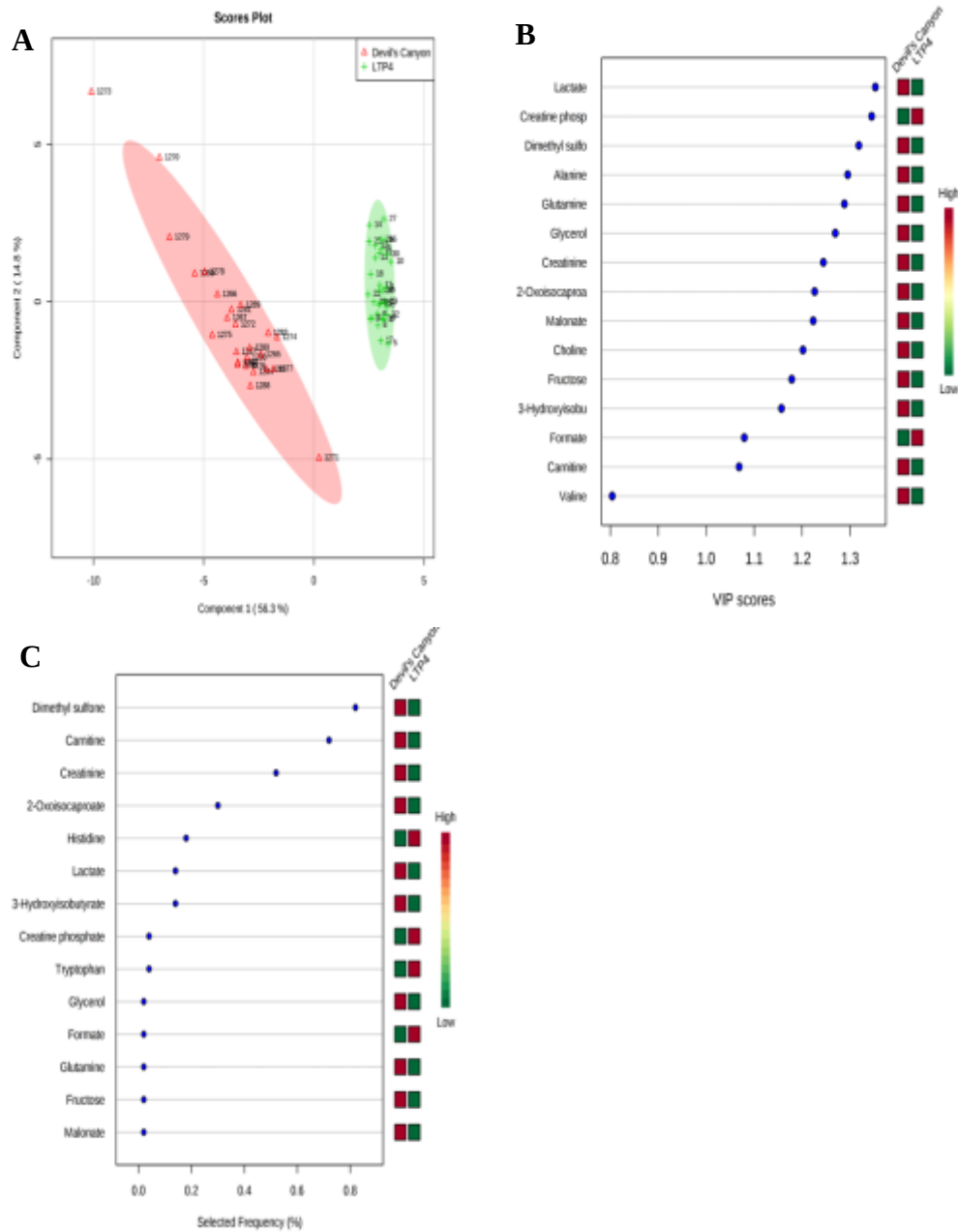


Figure 20. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Devil's Canyon ($n = 25$, sampled in March) and control Rambouillet ewes ($n = 31$, sampled in March). In A, Devil's Canyon is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Devil's Canyon herd from the control Rambouillet ewes.

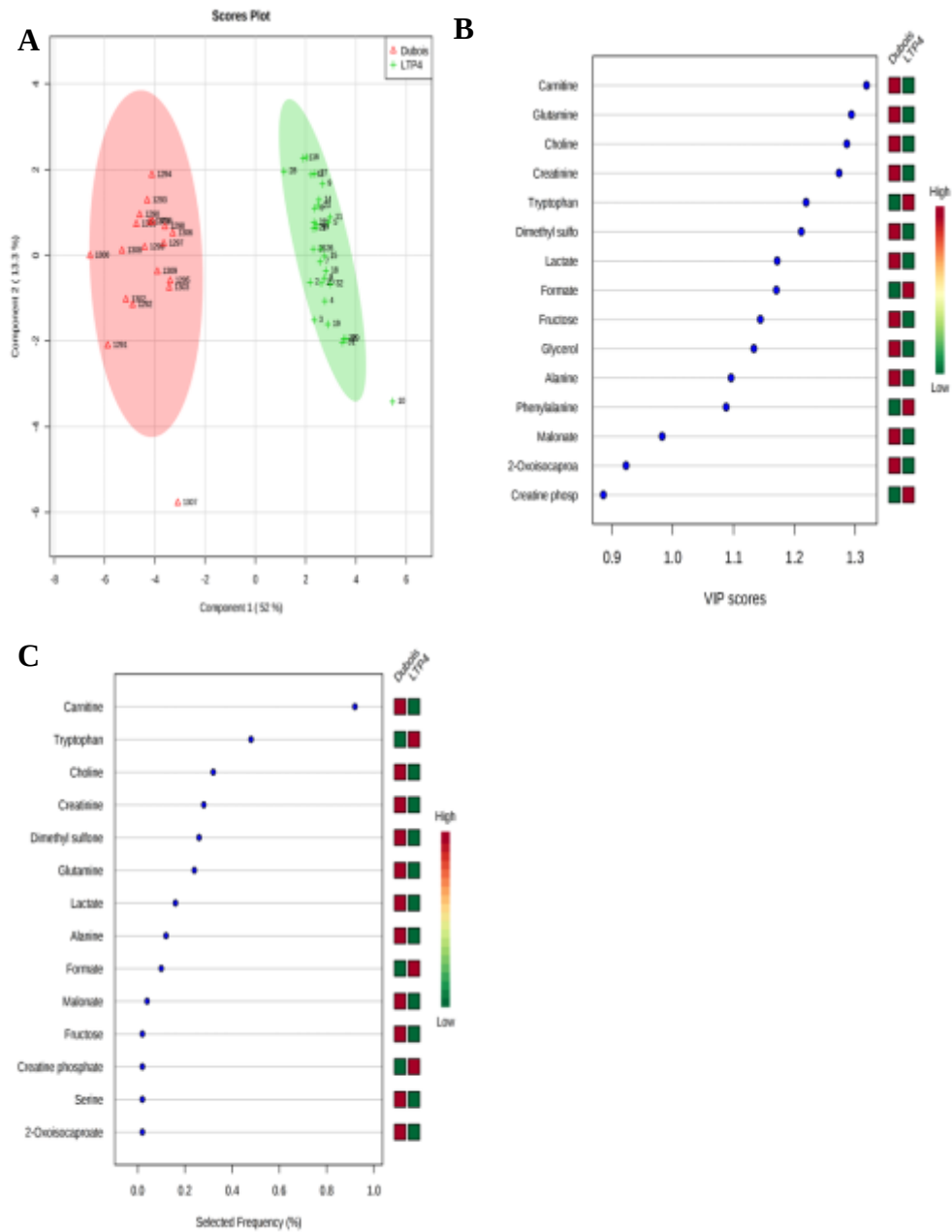


Figure 21. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Dubois ($n = 19$, sampled in March) and control Rambouillet ewes ($n = 31$, sampled in March). In A, Dubois is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Dubois herd from the control Rambouillet ewes.

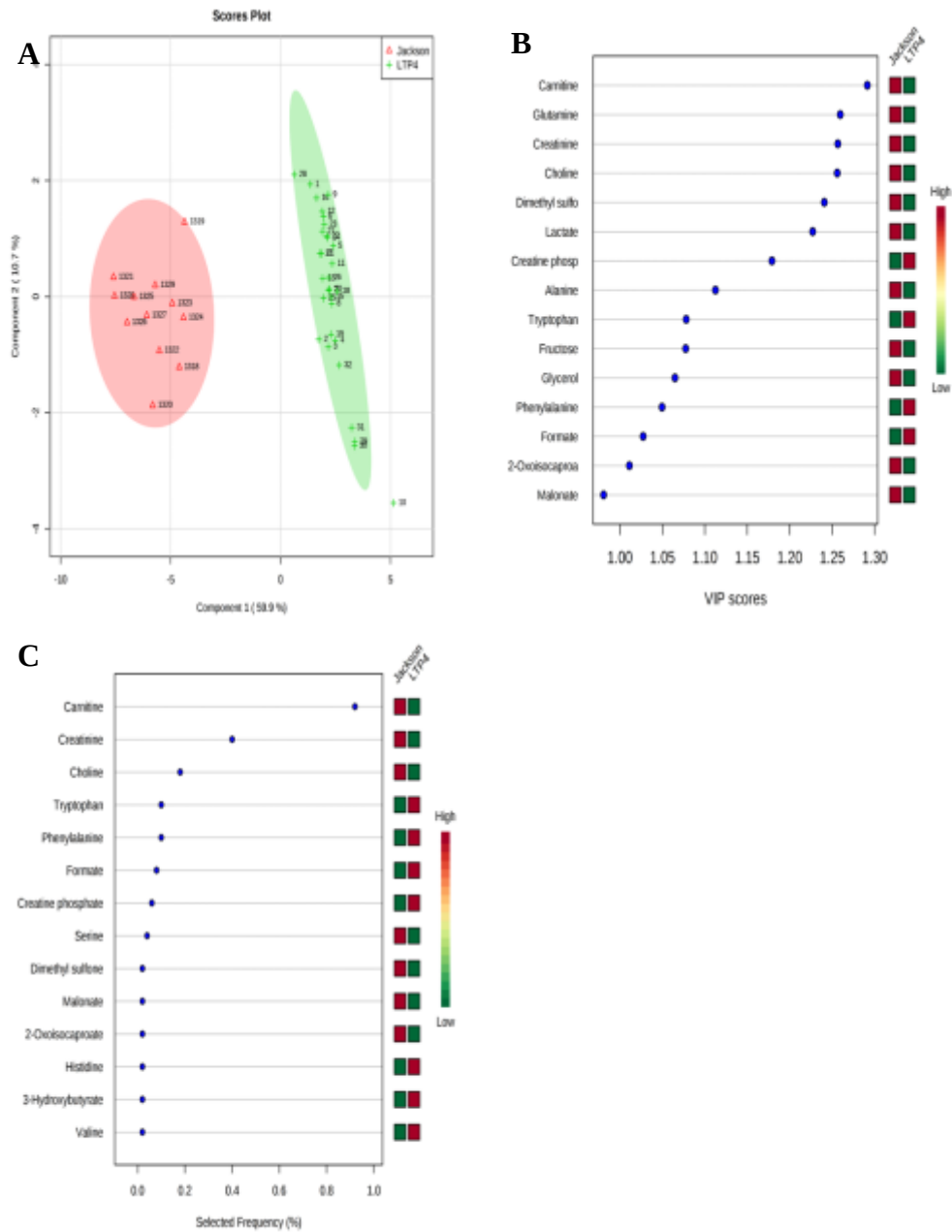


Figure 22. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Jackson ($n = 12$, sampled in March) and control Rambouillet ewes ($n = 31$, sampled in March). In A, Jackson is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Jackson herd from the control Rambouillet ewes.

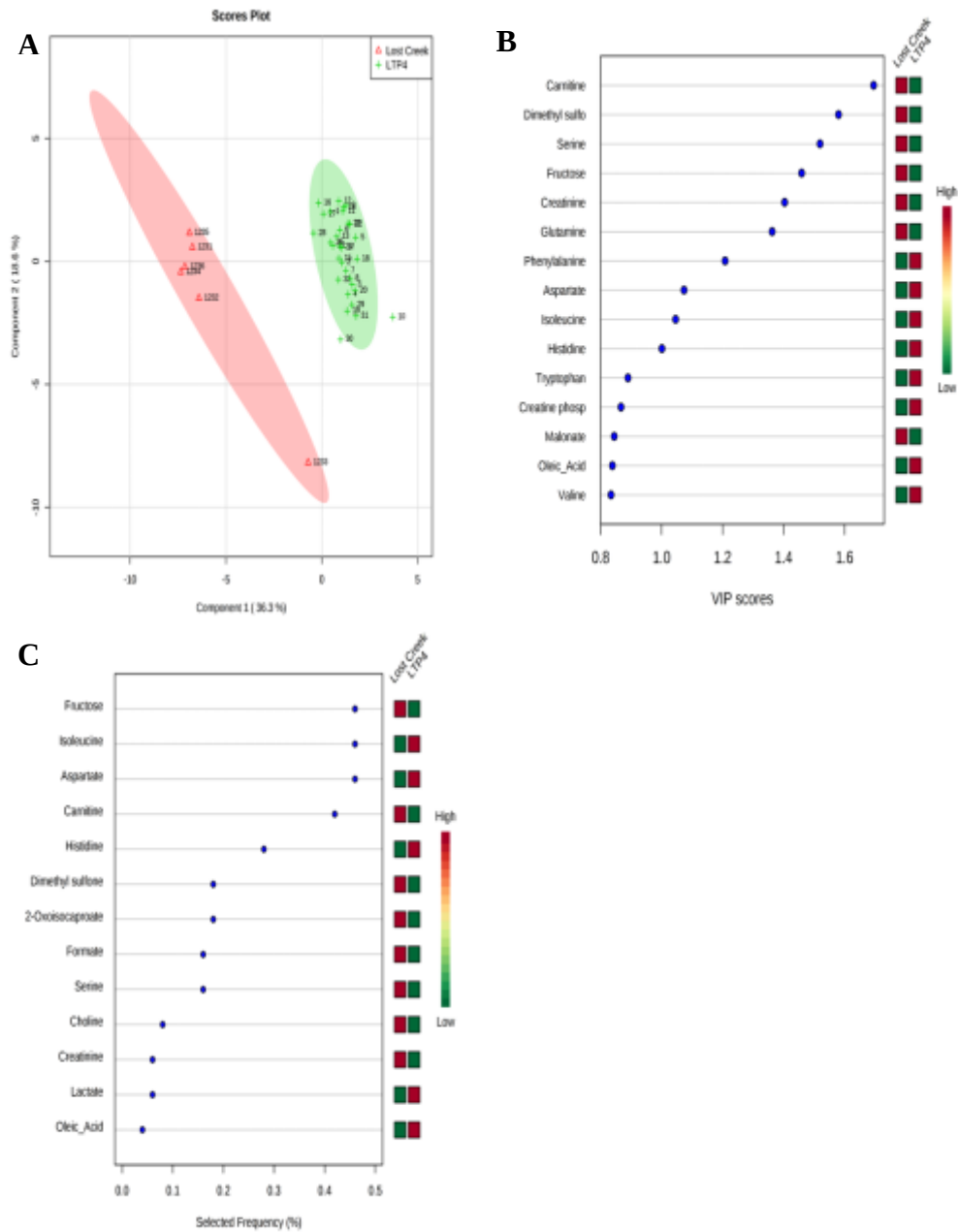


Figure 23. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Lost Creek ($n = 6$, sampled in March) and control Rambouillet ewes ($n = 31$, sampled in March). In A, Lost Creek is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Lost Creek herd from the control Rambouillet ewes.

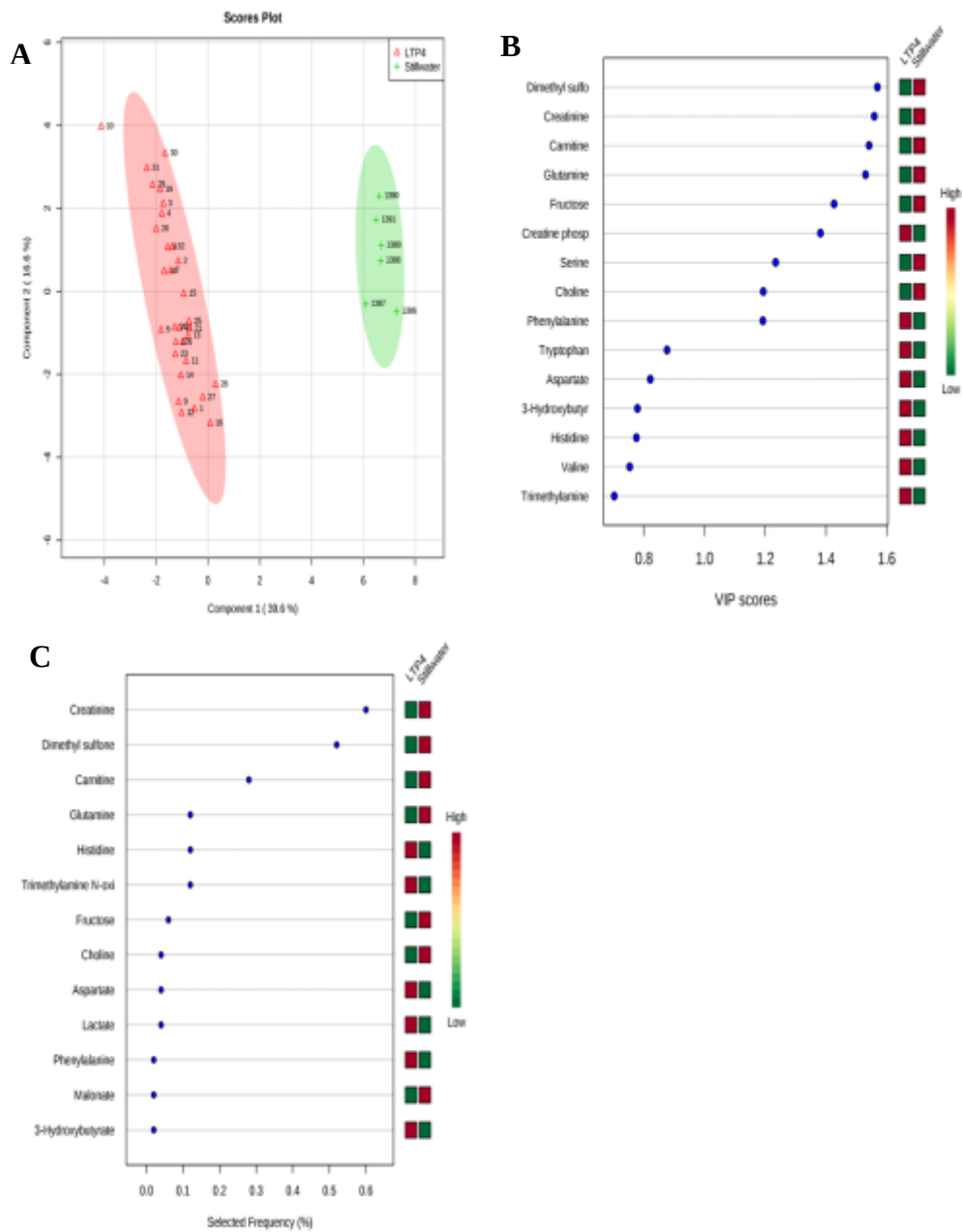


Figure 24. Partial least squares discriminant analysis (PLS-DA) score map (A), variable of importance (VIP) scores (B) and the biomarker analysis (C) between the bighorn sheep herd from Stillwater ($n = 6$, sampled in March) and control Rambouillet ewes ($n = 31$, sampled in March). In A, Stillwater is represented in red and the Rambouillet ewes is represented in green. In B, metabolites with a VIP score greater than 1.0 are considered important. In C, metabolites with selected frequencies greater than 0.1 are considered potential biomarkers for identifying the Stillwater herd from the control Rambouillet ewes.

CHAPTER SIX

CONCLUSIONS

Metabolomics allows for a snapshot of global metabolisms through the study of metabolic intermediates and products of cellular metabolism. In Experiments 1 and 2, the objectives were to evaluate the effects of long-term progesterone (P4) treatment, independent of the influence of the placenta and fetus, on changes in feed efficiency, final body weight (BW), body composition, non-esterified fatty acids (NEFA), metabolic hormones, and metabolites identified through nuclear magnetic resonance (NMR) metabolic profiling in mature Rambouillet ewes. There were no differences in BW, RFI, body composition, or temporal patterns of thyroid hormones, NEFA, or metabolites between Rambouillet ewes that received long-term P4 for 126-d and Rambouillet ewes that did not receive long-term P4. Long-term P4 did not affect metabolism or body composition independent from the presence of a fetus or placenta. Any changes associated with maternal metabolism during pregnancy may require fetal and/or placental signals along with P4 to be initiated. However, without these signals, P4 cannot initiate the change in maternal metabolism associated with efficiency. Interesting, insulin (INS) concentrations were greater in CIDR- treated ewes than in CIDRX- treated ewes. Progesterone may increase tissue sensitivity to INS, which is opposite of many literature. Further studies need to be conducted to determine if the relationship discovered between P4 and INS in this study is repeatable.

In Experiment 3, the primary aim was to determine if NMR metabolic profiling has the potential to serve as a management tool for evaluating herds of bighorn (*Ovis canadensis*) sheep. NMR metabolic profiling could not distinguish between pregnant and non-pregnant bighorn sheep indicating that any further differences discovered is not related to pregnancy. Metabolic profiling did differentiate bighorn sheep herds and identified a subset of potential biomarkers that discriminated distinct geographic locations and time of year. Thus, NMR metabolic profiling has the potential to develop a suite of metabolites that wildlife managers can use to assess bighorn sheep nutrition and overall health. These biomarkers, derived by NMR metabolic profiling, have the potential to provide wildlife managers with a tool to effectively develop management strategies to evaluate bighorn sheep herds.

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APPENDICES

APPENDIX A

PREPARATION OF THE NON-PROGESTERONE-CONTAINING CIDR
BACKBONE FOR THE CIDRX-TREATED EWES IN THE
LONG-TERM PROGESTERONE STUDY

Eazi-Breed™ CIDR® sheep inserts were used in this study. The controlled intravaginal drug releasing devices (CIDR; Figure 1) consist of a T-shaped nylon backbone. A silicone rubber skin is molded over the nylon backbone. The silicone outside contains 0.3 g of progesterone that is released into the bloodstream after insertion.

To make the CIDR backbone, the outer, progesterone-containing silastic membrane was removed by slicing down the long axis of the CIDR with a scalpel blade and peeling the membrane from the plastic T-shaped backbone. Removing the outer progesterone containing membrane leaves the T-shaped backbone (Figure 2).

All the CIDR backbones were soaked in 80% ethanol (vol/vol DI) overnight in a circular motion. After soaking in ethanol, the CIDR backbones were then soaked in phosphate buffer overnight. To ensure the CIDRs contained no progesterone, the phosphate buffer that they were soaked in were tested for progesterone using a radioimmunoassay (RIA) kits (MP Biomedical, Costa Mesa, CA). The RIA assay confirmed that all CIDR backbones were leached of all progesterone.

The CIDR backbones after the above procedure had abrasive properties that could cause irritation on the vaginal wall of the ewes. Therefore, they were coated with three layers of Flex Seal Liquid Rubber (Swift Response, Weston, FL, USA). They were then dried over a 48-hr period to ensure that the backbones were dry. After treatment, the backbones no longer had abrasive properties and could be inserted in the vaginal walls of the ewes without damage.



Figure 1. A controlled intravaginal drug releasing device (CIDR) that contains 0.3 g of progesterone per insert. The CIDR consists of a T-shaped nylon insert molded with a silicone rubber skin containing progesterone. The silicone outside is was contains the 0.3 g of progesterone. CIDRs were used for the CIDR- treated ewes in the long-term progesterone studies. 7



Figure 2. A controlled intravaginal drug releasing device (CIDR) backbone that contains no progesterone. The silicone outside of the CIDR was removed from the original CIDR to leave a T-shaped nylon backbone. This backbone was used for the CIDRX- treated ewes in the long-term progesterone studies.

APPENDIX B

NUCLEAR MAGNETIC RESONANCE PROTOCOL

Making NMR Buffer

Making 0.5 M Phosphate Buffer: The goal of making 0.5 M phosphate buffer was to make 100-mL of a dibasic solution and 100-mL of a monobasic solution. The monobasic solution needs to be titrated to the dibasic solution to a pH of 7.05. The monobasic solution was made by adding 6.0 g of monobasic sodium phosphate (NaH_2PO_4) into 100-mL of ultrapure water (UPW). A 100-mL volumetric flask was used and filled with about 25-mL UPW. Using a funnel, 6.0 g of NaH_2PO_4 was added to the 25-mL of UPW (very slowly so that the NaH_2PO_4 did not clump at the bottom of the flask). The weight boat used to measure the NaH_2PO_4 was rinsed out using UPW into the funnel to assure that the full amount of NaH_2PO_4 was added to the solution. Then UPW was added to the 100-mL mark and swirled into solution. The dibasic solution was made by adding 7.98 g of dibasic sodium phosphate (Na_2HPO_4) into 100-mL of UPW. The same procedure as above was used to get the Na_2HPO_4 into solution. Before titration, the pH meter needed to be calibrated. The entire Na_2HPO_4 solution was added to a 300-mL beaker with the pH calibrator. The NaH_2PO_4 solution was slowly titrated into the beaker until the pH was at 7.05. At around pH 7.02, smaller and smaller amounts of the NaH_2PO_4 solution was added because the solution was reaching the end of the buffering zone. If not careful, the pH would jump extremely fast and it could go past 7.05.

Making the 0.5 M Phosphate Buffer Stock Solution for 60-mL: The first step was to add 6-mL of deuterium oxide (D_2O) to 54-mL of 0.5 M of 0.5 M phosphate buffer.

Making the DSS Stock Solution for 50-mL: Using a 50-mL volumetric flask, 5-mL of D₂O was added. Using a weigh boat, 71.5 mg of 3-(Trimethylsilyl)-1-Propanesulfonic acid (NaDSS; FW 218.33) was weighed and added to the 50-mL volumetric flask. The weigh boat was rinsed off into the 50-mL flask using UPW to ensure all the NaDSS was added to the flask. Using a second weigh boat, 117.5 mg of sodium azide (NaN₃; FW 65.02) was added to the flask. Again, the weigh boat was rinsed off into the 50-mL flask using UPW to ensure all the NaN₃ was added to the flask. The rest of the 50-mL flask was filled with UPW for a total of 45-mL of UPW.

Making the Imidazole Solution: Using a 15-mL test tube, 0.4 mL of D₂O was added. Using a weigh boat, 26.1 mg of imidazole (C₃N₂H₄) was added to the test tube. The weigh boat was rinsed off into the 15-mL tube using 1 mL of UPW. Next, a second weigh boat was used to measure 10.8 mg of NaN₃ and was added to the flask. The weigh boat was rinsed off into the 15-mL tube using 1 mL of UPW. Another 1.6 mL of UPW was added to the solution for a total of 3.6 mL of UPW.

Making 1L of Working NMR Buffer DSS: The working NMR buffer contains 38.170 mL of the stock DSS solution, 2.090 mL of the stock imidazole solution, 50.000 mL of the 0.5 M Phosphate Buffer Stock Solution, 90.970 mL of D₂O, and 818.770 mL of UPW.

Acetone Extraction Procedure of Serum for NMR

NMR Sample Preparation: Each sample should contain at least 750 μ l of serum or plasma and can be frozen at either -20°C and -80°C. Each sample should not have been through any freeze/thaw cycles. The limitations of the speed vacuum, means that only 48 samples could be extracted a day. The NMR facility can hold only 96 samples at a time. Furthermore, every sample run needed to include 2 quality controls (the same controls for every run) and 1 blank tube which only contains the DSS buffer. The quality controls were made by freezing a large quantity of serum in aliquots that is pulled from the same animal, in the same day. This ensures that the quality control will only go through the one freeze/thaw cycle, the same as the samples. The night before the extractions, the appropriate number of samples were thawed in the fridge. This means only 48 samples thawed at a time, including the control sample.

NMR Sample Extraction Procedure: The next day after the samples were thawed in the fridge, the samples were warmed to room temperature. Using 2 mL micro-centrifuge tubes that have a flat cap (Catalog Number 02-681-258), 500 μ l of serum sample is added. HPLC Grade Acetone was brought to room temperature. Acetone was added to the serum in a 3-fold change or 1.5 mL of acetone. No special tips are needed to add the acetone, only blue 1,000 μ l tips. After the acetone was added, the tubes were inverted 10 times. The samples were then put in the 4°C freezer for one hour. After one hour, the samples were spun on a small centrifuge at 12,000 rpm or 13, 000 xg for 30 min at 4°C. The liquid at the top of the micro-centrifuge was pipetted off into new clean

micro-centrifuge tubes. The samples were then spun over night with no heat with the caps open so the acetone would dry off. After the samples are taken off the speed vacuum the next day, this was the point in the procedure where the samples can be frozen in the 4°C freezer, until needed. If the samples do not need to be frozen, then 500 µl of DSS buffer was added to the dried micro-centrifuge tubes. After adding the DSS buffer, the tubes were immediately vortexed and allowed to sit at room temperature for 15 minutes. After 15 minutes, the samples were spun on a small centrifuge at 12,000 rpm or 13,000 xg for another 15 minutes at 4°C. The samples were then pipetted into the appropriate NMR tubes. The NMR tubes were then transferred to the NMR facility for processing.

NMR Spectra Profiling

NMR Machine: All NMR spectra were analyzed at 25°C on a Bruker 600 MHz (¹H Larmor frequency) AVANCE III solution spectrometer. The spectrometer is equipped with an automatic sample loading system (SampleJet™), a 5-mm helium-cooled ¹H-optimized TCI NMR probe (Cryoprobe™), and Topspin software (Bruker version 3.5). 1 D ¹H NMR experiments were completed using the “zgesgp” Bruker pulse sequence and recorded with 256 scans and a ¹H spectral window of 9615.38 Hz. Free induction decays (FIDs) were sampled at 32 K data points and a dwell time interval of 52 µseconds totaling to an acquisition time of around 1.7 seconds and a 1 second relaxation recovery delay between acquisitions.

NMR Spectra Phasing: Spectra were collected from the NMR operating system, Topspin 3.5 (Bruker©, 2017). The raw spectra were imported into the Topspin program.

The first step was to Fourier transform the data using the ft function. A Fourier transformation mathematically takes a waveform and breaks them down into sinusoidal components. The DSS standard was set to 0 ppm before being phased. Phasing consisted of bringing the whole spectra up to baseline 0. The final step in finishing the spectra was to add the sample number to spectra and save them. The saved file could then be opened in the Chenomx© NMR Suite program software (Chenomix NMR Suite 8.1).

Processing the Spectra: Once the spectra were imported into the Chenomx software, they were baseline corrected. In the baseline correction, the water resonance region was deleted between 4.3 and 5.6 ppm. Baseline correction is fine tuning the spectra to remove any distortions in the spectra. The next step was to calibrate the pH at 7.05 ppm. The pH indicator in the buffer is imidazole. The pH indicator is fit to the highest peak at the imidazole range. All the samples were between the pH 7.03 ppm and 7.05 ppm. The last step of processing was to calibrate the DSS peak. The DSS peak is found at 0 ppm and is set at the concentration of 25 mM. The DSS peak is calibrated by fitting the peak as closely as possible. The rest of the metabolites concentrations is based on the DSS concentration being at the correct concentration, so this step is critical.

Identification and Quantification of Metabolites: For metabolite identification, the Chenomix small molecule library for 600-MHz (^1H Larmor frequency) magnetic field strength NMR spectrometers were used and NMR spectral profiles were fitted for each sample. Each metabolite that was identified in the spectra of ruminant serum was validated through the Human Metabolome Database. Our laboratory has established a

NMR compound database containing 58 small molecule metabolites. An internal DSS standard was used for the quantification of the 58 small molecule metabolites.

Metabolomics 3.0: Whether doing a statistical analysis (PLS-DA), a time-series analysis, or a biomarker analysis, the first step is to upload the data file into a csv file. The data file was uploaded as concentrations and as the samples in rows. All the labels for the metabolites were included in the first row, while the sample numbers were in the first column. Either the treatment or herd was in the second column. The second step in uploading the data file was to use the missing value estimation function. In step one the button was checked to delete any feature with greater than 50% of the values missing. In step two, the estimated value for the missing values was replaced with the column mean. The third step would be to normalize the data. The data was normalized by the median, the data was not transformed, and the data scaling was auto-scaled. All the options were used to see which method of the above optimized normalization.

PLS-DA: This analysis can be found in the statistical analysis package of MetaboAnalyst. The 2-D plot from the PLS-DA visualized the metabolic shifts associated with the treatment population or herds. The cross validation showed the model quality for distinguishing treatment population or herds. Finally, the important features identified the variable of importance (VIP) scores for the model. The VIP scores are the metabolites that contribute the most to any differences seen in the 2-D plot. However, the VIP scores are only important if the model quality from the cross validation is appropriate.

Biomarker Analysis: The variable of importance scores identified in the PLS-DA were the only metabolites placed into the biomarker analysis. This was because we were trying to use the least number of metabolites to distinguish differences. In the biomarker analysis, the multivariate exploratory ROC analysis overview tool was used. The classification model used was linear SVM and the ranking method was the SVM built-in. The number of latent variables used in this analysis was 2. ROC view was used to visualize the model quality, with the best models being near one. The probability view showed the best way the model could assign treatments to the correct treatment or herd. If the ROC values are appropriate and the probability view can correctly assign the treatments population and herds, then the significant features were used. The significant features are the potential biomarkers that could be used to identified either the treatment population or the herds from one another. These potential biomarkers are the least number of compounds that could be used to distinguish the herds or treatment population.

Time Series Analysis: To run the time series analysis correctly, the excel spreadsheet needs to be correctly set up. In the first column, the sample number and its associated time. For example, if a sample number was 2 and this sample was taken on day 28, then the label would be S2T28. The second column would be the treatment, in this experiment CIDR or CIDRX. The third column would be the time, for example 0, 28, 56, 84, and 126. The rest of the spreadsheet consists of the metabolites label in the first row and their associated concentrations. To evaluate the time series, the interactive PCA visualization 3-D plot is used. The interactive PCA plot breaks down any time or treatment differences, along with the associated components. The ASCA module is used

to access model quality. The model validation illustrates the permutation test statistics. The permutation test statistics are the ability of the model to correctly identify treatments, times, and the interaction between treatment and time. In the permutation test, a $p > 0.05$ cannot correctly identify treatments, times, and the interaction between treatment and time better than chance. The significant features are the leverage plots for treatment, time, and the interaction between treatment and time. The leverage points are observations with extreme values or predictor capability. The leverage points identify outliers in the model and any metabolites that can be used for predictor capabilities for the model.

Pathway Analysis in MetaboAnalyst 3.0: In the pathway analysis, the metabolites identified through NMR metabolic profiling were used. Only the names were uploaded into the one-column compound list. After the metabolites were uploaded, the metabolites needed to be standardized. Some metabolites had slightly different names than what was in the query and needed to be individually added from the Human Metabolome Database. After the unknown metabolites were identified through the Human Metabolome Database, the analysis was continued. The pathway library used was the *Bos Taurus* (cow), because this library was for ruminants. The over representation analysis used was the hypergeometric test and the pathway topology analysis used was the relative-betweenness centrality. The analysis identified pathways that were significantly impacted by the metabolites that were identified in the sera of bighorn sheep and Rambouillet ewes. Metabolic pathways with impact values greater > 0.1 were considered important, along with a p -value > 0.05 . The p -values were corrected with the Holm-Bonferroni

correction to reduce the error of the multiple comparisons. The metabolic pathways that were significantly affected by the compounds identified are indicated on the right.

APPENDIX C

IDENTIFICATION AND CHARACTERIZATION METABOLITES IDENTIFIED BY
NUCLEAR MAGNETIC RESONANCE

Table 1: Chemical shifts (ppm) and Multiplicities for the Pool of 58 Metabolites identified in Ruminant Serum by Nuclear Magnetic Resonance and the chemical shifts (ppm) and multiplicities for the 58 authenticated compounds.

metabolite	ruminant serum	authentic compounds
2-aminobutyrate	0.97 (t), 1.888 (m), 3.715 (dd)	0.982 (t), 1.906 (m), 3.718 (dd)
2-hydroxyisobutyrate	1.342 (s)	1.34 (s)
2-oxoisocaproate	0.925 (d), 2.082 (m), 2.599 (d)	0.932 (d), 2.085 (m), 2.507 (d)
3-hydroxybutyrate	1.188 (d), 2.295 (m), 2.396 (m), 4.145 (m)	1.204 (d), 2.314 (m), 2.414 (m), 4.160 (m)
3-hydroxyisobutyrate	1.058 (d), 2.475 (m), 3.531 (m), 3.695 (m)	1.114 (d), 2.649 (m), 3.683 (m)
3-hydroxyisovalerate	1.255 (s), 2.355 (s)	1.28 (s), 2.35 (s)
3-methyl-2-oxovalerate	0.88 (t), 1.085 (d), 1.445 (m), 1.695 (m), 2.922 (m)	0.90 (t), 1.1(d), 1.46 (m), 1.7 (m), 2.93 (m)
acetate	1.195 (s)	1.91 (s)
acetone	2.22 (s)	2.22 (s)
alanine	1.465 (d), 3.765 (q)	1.46 (d), 3.76 (q)
allantoin	5.38 (s), 6.00 (s)	5.38 (s)
arginine	1.64 (m), 1.719 (m), 1.90 (m), 3.237 (t), 3.76 (t)	1.68 (m), 1.90 (m), 3.23 (t), 3.76 (t)
asparagine	2.845 (m), 2.94 (m), 3.99 (dd)	2.84 (m), 2.94 (m), 4.0 (dd)
aspartate	2.67 (dd), 2.80 (dd), 3.89 (dd)	2.66 (dd), 2.80 (dd), 3.89 (dd)
betaine	3.25 (s), 3.89 (s)	3.25 (s), 3.89 (s)
caprylate	0.86 (m), 1.265 (d), 1.54 (m), 2.16 (t)	0.85 (m), 1.27 (d), 1.53 (m), 2.16 (t)
choline	3.19 (s), 3.507 (dd), 4.057 (ddd)	3.189 (s), 3.507 (dd), 4.056 (ddd)
creatine	3.024 (s), 4.045 (s)	3.02 (s), 3.92 (s)
creatine phosphate	3.03 (s), 4.045 (s)	3.03 (s), 4.05 (s)
creatinine	3.025 (s), 3.92 (s)	3.03 (s), 3.93 (s)
cysteine	3.02 (m), 3.09 (m), 3.965 (dd)	3.06 (m), 3.97 (dd)
dimethylamine	2.7 (s)	2.5 (s)
dimethyl sulfone	3.14 (s)	3.138 (s)

ethanol	1.17 (t), 3.65 (q)	1.17 (t), 3.65 (q)
formate	8.44 (s)	8.44 (s)
fructose	3.545 (m), 3.57 (m), 3.69 (m), 3.80 (m), 3.89 (dd), 4.0 (m), 4.1 (m)	3.577 (m), 3.695 (m), 3.903 (dd), 4.005 (m), 4.029 (m), 4.118 (m)
glucose	3.23 (dd), 3.40 (m), 3.46 (m), 3.525 (dd), 3.715 (m), 3.76 (m), 3.84 (dd), 5.225 (d)	3.233 (dd), 3.398 (m), 3.458 (m), 3.524 (dd), 3.728 (m), 3.824 (m), 3.889 (dd), 4.634 (d), 5.223 (d)
glutamate	2.03 (m), 2.12 (m), 2.32 (m), 2.37 (m), 3.75 (dd)	
glutamine	2.12 (m), 2.44 (m), 3.765 (t)	2.125 (m), 2.446 (m), 3.766 (t)
glycerol	3.55 (m), 3.645 (m), 3.78 (tt)	3.551 (m), 3.644 (m), 3.775 (tt)
glycine	3.545 (s)	3.54 (s)
hippurate	3.955 (d), 7.54 (none), 7.65 (tt), 7.82 (dd)	3.96 (d), 7.54 (none), 7.62 (tt), 7.82 (dd)
histidine	3.123 (dd), 3.23 (dd), 3.96 (dd), 7.07 (d), 7.8 (s)	3.16 (dd), 3.23 (dd), 3.98 (dd), 7.09 (d), 7.90 (d)
hypoxanthine	8.17 (s), 8.19 (s)	8.17 (s), 8.20 (s)
inosine	3.83 (dd), 3.97 (dd), 4.27 (dd), 4.43 (dd), 6.08 (d), 8.215 (s), 8.32 (s)	3.832 (dd), 3.902 (dd), 4.26 (dd), 4.42 (dd), 4.798 (s), 6.055 (d), 8.187 (s), 8.305 (s)
isoleucine	0.925 (t), 0.995 (d), 1.25 (m), 1.46 (m), 1.97 (m), 3.66 (d)	0.926 (t), 0.996 (d), 1.248 (m), 1.457 (m), 1.968 (m), 3.66 (d)
lactate	1.315 (d), 4.10 (q)	1.32 (d), 4.10 (q)
leucine	0.95 (t), 1.7 (m), 3.725 (m)	0.948 (t), 1.7 (m), 3.722 (m)
lysine	1.43 (m), 1.71 (m), 1.88 (m), 3.02 (t), 3.75 (t)	1.46 (m), 1.71 (m), 1.89 (m), 3.02 (t), 3.74 (t)
malonate	3.119 (s)	3.11 (s)
methionine	2.12 (m), 2.63 (t), 3.85 (dd)	2.157 (m), 2.631 (t), 3.851 (dd)

o-acetylcarnitine	2.13 (s), 2.5 (dd), 2.631 (dd), 3.18 (s), 3.59 (d), 3.84 (dd), 5.69 (q)	2.13 (s), 2.48 (dd), 2.61 (dd), 3.18 (s), 3.61 (d), 3.82 (dd), 5.57 (q)
phenylalanine	3.17 (m), 3.99 (dd), 7.325 (d), 7.36 (m), 7.415 (m)	3.19 (m), 3.98 (dd), 7.32 (d), 7.36 (m), 7.42 (m)
proline	1.98 (m), 2.59 (m), 2.34 (m), 3.34 (dt), 3.414 (dt), 4.12 (dt)	1.99 (m), 2.06 (m), 2.34 (m), 3.33 (dt), 3.41 (dt), 4.12 (dt)
propionate	1.043 (t), 2.168 (q)	1.04 (t), 2.17 (q)
propylene glycol	1.13 (d), 3.439 (dd), 3.544 (dd), 3.878 (m)	1.130 (d), 3.434 (dd), 3.537 (dd), 3.87 (m)
pyroglutamate	2.02 (m), 2.4 (m), 2.49 (m), 4.17 (dd)	2.02 (m), 2.39 (m), 2.50 (m), 4.17 (dd)
pyruvate	2.30 (s)	2.46 (s)
sarcosine	2.73 (s), 3.62 (s)	2.73 (s), 3.61 (s)
serine	3.84 (dd), 3.975 (m)	3.832 (dd), 3.958 (m)
succinate	2.39 (s)	2.393 (s)
threonine	1.32 (d), 3.58 (d), 4.245 (m)	1.316 (d), 3.575 (d), 4.244 (m)
thymine	1.86 (s), 7.375 (s)	1.86 (s), 7.37 (s)
trimethylamine n-oxide	3.25 (s)	3.25 (t)
tryptophan	3.295 (dd), 3.47 (dd), 4.05 (dd), 7.19 (m), 7.275 (m), 7.31 (s), 7.533 (d), 7.725 (d)	3.292 (dd), 3.472 (dd), 4.05 (dd), 7.194 (m), 7.274 (m), 7.31 (s), 7.53 (d), 7.723 (d)
tyrosine	3.042 (dd), 3.19 (dd), 3.393 (dd), 6.88 (m), 7.18 (m)	3.024 (dd), 3.17 (dd), 3.921 (dd), 6.877 (m), 7.17 (m)
urea	5.79 (s)	5.78 (s)
valine	0.975 (d), 1.03 (d), 2.259 (m), 3.595 (d)	0.976 (d), 1.029 (d), 2.261 (m), 3.601 (d)
s, singlet; d, doublet; dd, doublet of doublets; t, triplet; dt, doublet of triplets; q, quartet; m, multiplet		