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Effects of Providing Novel Feedstuffs to Livestock on Production and Skeletal Muscle Growth

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EFFECTS OF PROVIDING NOVEL FEEDSTUFFS TO LIVESTOCK ON PRODUCTION AND SKELETAL MUSCLE GROWTH

by

Laura A. Motsinger

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ABSTRACT

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by

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Feed accounts for the majority of input costs in livestock operations and, therefore, a deeper understanding of how feed impacts growth and production of livestock is necessary. Different feed sources can be utilized to improve efficiency of livestock animals. Inclusion of novel feed products in the diet may improve growth and production of livestock animals, however, research needs to be completed on novel feeds in order for producers to be able to make an informed decision on whether to feed them or not. As such, we investigated the effects of including two novel alfalfa products: ProLEAF MAX™, an alfalfa leaf pellet; and/or ProFiber Plus™, alfalfa stems, in the rations of finishing beef steers, developing dairy heifers, and lactating dairy cows. We hypothesized that inclusion of ProLEAF MAX™ would result in improved growth and performance of finishing beef steers, improved growth and development of developing dairy heifers, and improved milk yield and milk components of lactating dairy cows. Inclusion of ProFiber Plus™ in the diet of beef steers and dairy heifers decreases cost of gain and total feed cost, respectively, without affecting overall growth in steers, while

decreasing growth in dairy heifers. In lactating dairy cows, inclusion of ProLEAF MAX™ and ProFiber Plus™ in the diet resulted in improved milk yield and milk components. Additionally, to further explore the effects of including novel products in the diet on growth, we examined the effects of supplementing murine myoblasts with polyamines and polyamine precursors. We hypothesized that provision of adequate concentrations of polyamines or their precursors would result in increased proliferation and protein synthesis of murine myoblasts. Our results demonstrated that polyamines and their precursors increase proliferation rates as well as alter mRNA expression of genes involved in polyamine biosynthesis, cell proliferation, and protein synthesis in murine myoblasts. This work provides insight into how novel feedstuffs and other dietary supplements affect growth and performance of livestock using both *in vivo* and *in vitro* models.

(231 pages)

PUBLIC ABSTRACT

Effects of Providing Novel Feedstuffs to Livestock

on Production and Skeletal Muscle Growth

Laura A. Motsinger

As the population increases and available land for food production decreases, it is necessary for livestock producers to continually work towards increasing livestock production efficiency. In livestock operations, feed accounts for the majority of input costs associated with raising livestock. As such, it is necessary to improve growth and production of livestock animals, while also optimizing feed utilization. Different feedstuffs can be included in the diet of livestock animals to maximize growth and production. However, the effects of some of these novel feedstuffs on growth and production of livestock animals has not been elucidated. As such, we investigated the effects of including two novel alfalfa products, ProLEAF MAX™ (a pellet composed of alfalfa leaves) and ProFiber Plus™ (alfalfa stems), in the diets of beef steers, dairy heifers, and lactating dairy cows. We hypothesized that inclusion of ProLEAF MAX™ and ProFiber Plus™ in the diet would result in improved growth and performance of beef steers, growth and development of dairy heifers, and milk yield and milk components of lactating dairy cows. We found that inclusion of ProFiber Plus™ in the diet of beef steers and dairy heifers decreases feed costs without affecting overall growth in steers, but decreases growth in dairy heifers and inclusion of the two novel alfalfa products in the diet of lactating dairy cows results in improved milk yield and milk components. Additionally, we examined the effects of supplementing murine myoblasts with

polyamines and polyamine precursors to further investigate novel products that may be able to be utilized in the diets of livestock animals to increase growth. We hypothesized that supplementation of polyamines and their precursors would result in improved growth of skeletal muscle cells (myoblasts). Treatment of myoblasts with polyamines and their precursors improves proliferation rates and alters mRNA expression of genes involved in polyamine biosynthesis, cell proliferation, and protein synthesis. Collectively, our observations suggest that various novel feedstuffs, whether it be alfalfa processed differently or amino acid derivatives (polyamines), have the potential to improve various growth and/or production measures. However, additional research is required to fully understand the potential of including these products in the diet.

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CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

As the population continues to grow at an exponential rate, it is necessary to maximize food production with the available land. The global population is expected to reach upwards of 9 billion by 2050 [1] and as the population continues to increase, available land that can be used for food production is decreasing [2]. From 2019 to 2021, farmland in the United States decreased by 800,000 acres [3], therefore, livestock producers must continually look for ways to increase efficiency of livestock animals to keep up with growing consumer demands. To maximize efficiency of livestock animals, producers must pursue methods that maximize useable product of the animal at harvest, while minimizing input costs. Feed accounts for the majority of input costs in livestock operations [4] and, therefore, it is necessary to improve growth and production of livestock animals, while decreasing or optimizing feed utilization. A variety of different feedstuffs and exogenous compounds can be utilized in livestock diets to maximize production of livestock animals. Although much is known about using nutrition to maximize quantity and quality of consumable product from livestock, many unknowns about the mechanisms behind how different feeds and compounds affect production remains unknown. As such, the goal of this research is to gain an improved understanding of how novel feedstuffs and exogenous compounds affect production so that nutrition, as well as other exogenous compounds, can be used to increase efficiency of livestock animals.

Ruminant Digestion

Livestock species such as cattle, sheep, and goats are classified as ruminants due to the unique anatomy and physiology of their gastrointestinal tract [5]. Through the process of rumination, ruminant animals regurgitate and rechew their ingesta so that it can be broken down further [5]. Ruminants have a true stomach (abomasum) and a forestomach comprised of three compartments: the rumen, reticulum, and omasum [5]. Ingesta from the esophagus enters the rumen and reticulum, often referred to as the ruminoreticulum [5]. The rumen is comprised of many papillae that function to increase surface area and absorption of nutrients [6]. The rumen papillae begin to develop during weaning and continue to grow and change throughout the life of a ruminant [7]. Microbes such as bacteria, protozoa, and fungi are present in the ruminoreticulum and they function to ferment ingested feedstuffs [8]. The end-products of fermentation are then absorbed through the rumen wall and serve as a usable source of nutrients for the animal [5]. The main end-products of the microbial fermentation process are volatile fatty acids (VFA), which serve as the primary source of energy in a ruminant animal [5]. Acetate, propionate, and butyrate are the primary VFA that are produced during rumen fermentation [5]. Additionally, rumen microbes break down rumen degradable protein (RDP) during rumen fermentation to stimulate microbial growth, in turn, providing the ruminant animal with a source of microbial protein [9]. The omasum is the next chamber of the forestomach and the main function of this chamber is water absorption, along with continued fermentation and absorption of more VFA [10]. The omasum is comprised of omassal leaves, which function to increase surface area and maximize absorption [10]. Once ingesta leaves the omasum, it enters the abomasum, which has similar functions to

that of a true stomach found in a non-ruminant [5]. Lastly, ingesta proceeds through the small intestine and large intestine in a similar fashion to that of a non-ruminant animal [5].

Nutrient Requirements

Nutrient requirements of cattle depend largely on stage of production. Cattle at different stages of production (i.e. growing and finishing beef cattle, developing heifers, or lactating dairy cows) have different nutrient requirements that should be accounted for when formulating a total mixed ration (TMR). In growing and finishing beef cattle that weigh 250 to 500 kg, net energy of maintenance (NE_m) requirements range from 4.8 to 8.1 Mcal/day, increasing with body weight [11]. Metabolizable protein is required in concentrations of 239 to 402 g/day for maintenance of growing and finishing beef cattle, increasing with body weight [11]. Additional nutrients are needed during growth of growing and finishing cattle depending on average daily gain (ADG) [11]. For developing heifers with a body weight ranging from 150 to 400 kg and ADG ranging from 0.5 to 1.1 kg/day, total digestible nutrients are required at 55.4 to 69.2% of the diet dry matter (DM), increasing with weight [12]. Net energy for maintenance requirements for developing heifers range from 3.57 to 7.46 Mcal/day, increasing with weight [12]. Crude protein (CP) is required at concentrations of 12.8 to 13% of diet DM for developing heifers, decreasing as weight increases [12]. Lactating dairy cows have nutrient requirements that change depending on their current stage of lactation. Additionally, nutrient requirements increase as milk yield and milk components increase. In early lactation, net energy of lactation (NE_l) requirements range from 23 to 41.4 Mcals/kg DM, increasing with increases in milk yield and milk components [12]. Crude

protein is required in concentrations of 15.2 to 22.4% of the diet DM, depending on milk yield and milk components [12]. In midlactation, lactating cows have requirements of 32.2 to 52.8 Mcal/kg DM for NE_l which increase as production levels increase [12]. Crude protein requirements during midlactation range from 13.5 to 18.1% of DM, depending on level of production [12]. It is essential that nutrient requirements are being met when feeding ruminant animals so that maximum production potential can be reached.

Energy

Energy, the primary component of livestock feeds, is very important in ruminant nutrition and is often the first nutrient that is balanced for when developing a ration. In ruminants, energy has an effect on the amount of microbial protein that is able to be synthesized in the rumen [13]. A ruminant diet must also support the rumen microbial population and, as such, adequate energy is needed for efficient utilization of nitrogen in the rumen and energy use of the ruminant animal itself [13, 9]. Cattle primarily receive energy through consumption of carbohydrates, which are composed of carbon, hydrogen, and oxygen. Carbohydrates, as well as ammonia and amino acids, are required for growth of the rumen microbes, which play a major role in ruminant metabolism of nutrients [5]. Cattle receive the majority of their energy through consumption of two different types of carbohydrates, fibrous and non-fibrous carbohydrates.

Fibrous carbohydrates include feedstuffs such as forages, while non-fibrous carbohydrates include grains. Cattle primarily consume two types of carbohydrates, starch and cellulose, in order to meet their energy requirements [14]. Forages are primarily composed of the carbohydrate cellulose, and starch is the primary carbohydrate

that is present in grains. When forages are consumed by ruminant animals, the forages are regurgitated and chewed again during the process of rumination to allow for increased digestion of the forage through reduction of particle size [5]. Through the process of rumination, surface area of feed particles is increased, aiding in fermentation of the ingested feed [5]. Forage enters the ruminoreticulum for fermentation by the rumen microbes [5]. Unlike forages, grains do not require as much additional regurgitation, chewing, and rumination as fibrous carbohydrates [5]. Grains often consist of readily digestible carbohydrates, which are digested more rapidly than forages [5]. The rapid digestion of grains causes a greater concentration of VFA to be produced [5]. In the rumen, starch is fermented into VFA so that it can be used as an energy source for the animal [15]. Rumen microbes are able to ferment the ingested carbohydrates and convert them into VFA, methane, hydrogen sulfide, and carbon dioxide [5]. For ruminants, VFA comprise 50-85% of a ruminant animal's energy supply [14]. The three primary VFA produced as end-products of fermentation are acetate, propionate, and butyrate, which can be absorbed across the rumen epithelium [16]. Acetate is always the primary VFA that is produced, however, VFA proportions change depending on the diet. As the concentration of forage in the diet increases, acetate production increases [17] and when the concentration of grain/concentrate in the diet increases, propionate production increases [18]. Volatile fatty acids are then carried to the liver through the portal vein [5]. Once VFA reach the liver, acetate is primarily oxidized throughout the body to generate adenosine triphosphate (ATP) or used as a source of acetyl coenzyme A for lipid synthesis [19]. Propionate is utilized as a major substrate for gluconeogenesis, which is the process of converting a substrate into glucose, which can be used as an energy source

[20-22, 5]. Butyrate is absorbed by the rumen primarily as the ketone beta-hydroxybutyric acid and is utilized for maintenance of the rumen wall and oxidized for energy production throughout the body [19].

Ruminant diets require fiber for maintenance of a healthy rumen microbial population [23]. Fiber content in forage can be classified as acid detergent fiber (ADF) or neutral detergent fiber (NDF) [24]. Acid detergent fiber is fiber that is insoluble in an acid detergent and consists of the cellulose and lignin portions of the plant cell wall [24, 25]. Neutral detergent fiber is a measure of the total plant cell wall material that is insoluble in a neutral detergent and includes hemicellulose, cellulose, and lignin [24, 25]. Digestibility of the different portions of the plant cell wall varies such that hemicellulose and cellulose are slowly digested by the rumen microbes while lignin is largely undigestible [24]. As such, NDF is more digestible, while ADF is the least digestible portion of the fiber by the animal [25]. Physically effective fiber is dietary fiber that stimulates chewing, salivation, rumination, and rumen motility [23, 25]. The inclusion of physically effective fiber in a ruminant diet is important in maintaining a healthy rumen microbial population and rumen pH because chewing causes salivary buffers to flow into the rumen and neutralize fermentation acids [26, 23, 25].

Protein

There are two main types of protein that are consumed by ruminant animals, rumen degradable protein and rumen undegradable protein (RUP). Rumen degradable protein is the protein fraction that is degraded by rumen microorganisms [27]. Rumen degradable protein is necessary to feed the rumen microorganisms, which in turn, provide the ruminant animal with a source of microbial protein [9]. Microbial protein is the

highest quality protein that is available to a ruminant animal in most cases [9]. The requirement for RDP in ruminants relies, exclusively, on the amount of energy being consumed by the animal [28]. When energy intake increases, the animal's RDP requirement will also increase due to the rumen microorganisms needing additional fuel for their increased protein production [28]. When ruminants are deficient in RDP, growth of the rumen microbes and, in turn, the amount of protein that is produced by the microbes, is decreased [28]. In addition, RDP deficiencies can ultimately lead to decreased fiber digestion and dry matter intake [28].

The leftover protein that is not degraded by the rumen microorganisms is known as RUP [9]. Rumen undegradable protein bypasses the rumen and some of it can be absorbed in the small intestine of the animal [9]. Although RDP feeds the rumen microorganisms, providing the animal with high quality microbial protein, this is not enough to fulfill the protein requirement of animals with a high production requirement [29]. To make up for the difference between the protein requirement of the animal and the amount of protein provided by the rumen microorganisms, RUP is needed [30, 29]. For RUP to be useful to the animal, it is important that the RUP has adequate digestibility and a satisfactory amino acid profile [29].

In a ruminant diet, it is very important to balance for both RDP and RUP [28]. Rumen degradable protein is needed in order to provide a source of protein for the rumen microorganisms and, in turn, the microorganisms will provide a source of protein for the animal [28]. Rumen degradable protein is not able to satisfy the protein requirement of the ruminant animal alone, therefore, RUP is required in order to satisfy the remaining protein requirement [28]. When RDP is overfed, the RUP requirement remains

unchanged, therefore, the excess RDP that is being fed will be wasted, as it will be excreted in the urine or feces in the form of urea [31, 28]. If RUP is overfed it will be excreted in the urine as urea or feces as nitrogen unless there is a deficiency in RDP [31, 28]. Protein is often the most expensive nutrient in a ruminant diet and, as such, ensuring that rations are balanced for both RDP and RUP will minimize both the wastefulnes of excess protein excretion and the energy that is utilized to convert excesss protein to urea [28].

Protein that reaches the rumen is subjected to enzymatic activity of rumen bacteria and protozoa [12]. Approximately 40% of the rumen bacterial population are known to have proteolytic activity [32], which largely acts on the cell surface of rumen microbes [33]. Feed proteins are degraded by the rumen microbes into ammonia and branched chain fatty acids [31]. Non-protein nitrogen from feed particles also contributes to ammonia formation in the rumen [31]. The rumen microbes are able to utilize ammonia produced from the breakdown of protein in the rumen for their own growth, however, this process is dependent on the availability of energy in the rumen [31]. The proteolytic activity that takes place in the rumen affects the quality and quantity of RUP that passes into the small intestine [30]. The small intestine also receives protein from microbial protein synthesis [30]. Protein that is not digested in the small intestine is excreted in the feces or urine in the form of ammonia or urea, respectively [31].

Determining the amount of protein that is degraded in the rumen is an important indicator of both the amount of protein that the rumen microorganisms are receiving and the concentration of amino acids that a ruminant animal is ingesting [27, 31]. When determining the protein value of different feedstuffs, knowing the protein degradability is

essential [27]. The amount of protein that is degraded in the rumen is greatly dependent on the type of feedstuff that is being consumed [27]. Different processing methods and treatments of feedstuffs can alter the protein degradability as well.

If excess protein is fed to an animal, it is excreted as urea in the urine. In order for excess protein to be excreted as nitrogen in the urine, it must first be detoxified from ammonia into urea. The ammonia detoxification process requires energy, resulting in available energy being taken away from going towards the animal's energy requirements. Therefore, balancing ruminant rations for protein is an essential part of maintaining an efficient operation. Additionally, if a ruminant animal is in a negative energy balance, the presence of excess ammonia and its metabolites can cause negative energy balances to worsen [34].

To measure the protein status of ruminant animals, testing of blood urea nitrogen (BUN) [35], plasma urea nitrogen (PUN), or milk urea nitrogen (MUN) can be utilized. These measurements can also be used to measure the amount of nitrogen that is being excreted in the urine as waste [36]. Measurements of BUN, PUN, or MUN indicate the protein status of the animal by reflecting the amount of ammonia that is present in the rumen [35]. When nitrogen levels within the rumen are in excess compared to energy concentrations, the ammonia concentration within the rumen increases [35]. Additionally, when a ruminant animal consumes a diet that is deficient in protein, the ammonia concentrations within the rumen are low [35]. When the concentrations of ammonia within the rumen are low, more nitrogen is recycled back into the rumen as urea [35]. Regular testing of BUN, PUN, or MUN can be used by producers to ensure that their

ruminant animals are receiving adequate amounts of protein in their diet and that the energy and protein they are receiving is balanced.

Minerals

Minerals are essential components to ruminant diets; however, minerals are often times overlooked in production operations. Minerals can be organic, which are chemically bound to another compound, or inorganic, which are mined or chemically synthesized from a natural mineral source and are not bound to a carrier [37]. Minerals are classified as either macrominerals, which are required in greater concentrations, or microminerals (also known as trace minerals (TM)), which are required in smaller concentrations. Macrominerals include calcium, phosphorus, magnesium, potassium, sodium, chlorine, and sulfur [37]. Microminerals include iron, zinc, manganese, copper, iodine, cobalt, and selenium [37]. Many minerals have proven to be essential for optimal growth, productivity, and physiologic function [38]. A proper balance of minerals is critical because certain minerals can interact with one another, however, these interactions are not fully understood [37]. In ruminant diets, mineral supplements often times need to be provided because the primary components of ruminant diets usually do not satisfy mineral requirements [39, 40].

Beef Production

In a beef operation, it is estimated that feed accounts for over 70% of the total production costs [41, 42]. On average, the total cost of gain has increased over the past 30 years [43]. For these reasons, the beef industry is continually trying to improve animal efficiency without altering other important production traits, such as lean growth or meat

quality. In order to have an efficient and cost-effective nutrition program for beef cattle, an understanding of nutrient requirements for these animals under typical production parameters is necessary [44].

Nutrient requirements for beef cattle include those for protein, energy, minerals, vitamins, and water [44]. Deficiencies in these nutrients can have deleterious effects on growth and production. Diets deficient in protein will leave the rumen bacteria unable to efficiently digest roughages and negatively impact growth and development [44]. Energy deficiencies in ruminant diets can lead to reductions in microbial protein synthesis because rumen microbes utilize carbons from carbohydrates and ATP as an energy source for protein synthesis [28]. Mineral deficiencies can also have negative impacts on production because of their many functions throughout the body including skeletal development and maintenance, energy production and utilization, milk production, and basic body function [38, 45]. Trace minerals have become more widely studied over the recent years and their importance in ruminant diets has started to become further elucidated. Unfortunately, TM specifically are often times overlooked in beef production operations [46]. The current national TM recommendations [11] are designed to prevent TM deficiencies, however, they may not be set to optimize growth and performance of modern day cattle. Studies have shown that feedlot nutritionists often supplement TM at concentrations that are 125% to 300% of the national TM recommendations to optimize performance of their cattle [47]. Vitamins are essential in the diets of ruminant animals because they allow animals to efficiently utilize ingested nutrients [45]. While B vitamins and vitamin K can be synthesized by the rumen microbes [45], other vitamins must be provided in the diet. Vitamin deficiencies in a ruminant diet can negatively affect growth,

reproduction, bone development, immune function, maintenance of epithelial tissue, eyesight, and many other aspects of physiological function in a ruminant animal [45, 48]. Although much research has been completed to improve nutrition of beef cattle, further improvements in nutrition regimens are still needed to continue to increase production and efficiency of beef cattle.

Dairy Heifer Development

Sustainability of the dairy industry relies on a constant supply of properly developed heifers. Properly developed heifers are used to replace aging cows that have become less productive, and, therefore, replacement heifers are essential for maintaining a constant supply of milk. However, raising replacement heifers is a slow return on investment and is the second largest expense in a dairy operation, only behind feed costs [49, 50]. Through improved understanding of nutritional management practices and their impacts, efficiency, productivity, and profitability of developing heifers can be enhanced [51].

Of the total cost of raising dairy heifers, feed usually accounts for 50% of those total costs [52]. As such, the goal of dairy heifer development is to raise heifers that are efficient and profitable. Additionally, producers aim to breed their heifers at 55% of their mature body weight to achieve calving between 22 and 24 months of age [53, 54, 51, 55]. As such, optimal growth and development and timing of puberty and first calving is essential to achieving maximum future milk production [53].

To achieve optimal development of heifers, a ration that targets fast growth and high feed efficiency, but also minimizes the risk of over conditioning should be provided [51]. However, studies have demonstrated that accelerated growth of heifers during the

pre-pubertal stage can inhibit growth of the mammary gland, which can impact lifetime milk production [54]. Adequate concentrations of energy are required in developing dairy heifer diets to ensure optimal growth, development, and timing of onset of puberty. However, feeding excess energy to developing heifers could lead to excess adipose deposition in the mammary gland [51], which can lead to reduced milk production in the future [56]. In addition to adequate amounts of energy in the diets of developing dairy heifers, dietary metabolizable protein requirements should be met in order to achieve lean growth [51]. Protein requirements are higher in developing and growing animals than in mature animals and providing adequate amounts of protein in the diet is necessary to support the high demands of growth. Protein deficiencies in growing animals could lead to reductions in growth and development. Ultimately, through a better understanding of nutritional management practices for heifers, producers can achieve development of heifers that are efficient, profitable, and contribute to the maintenance of the milking herd for a constant supply of milk [51].

Lactating Dairy Cow Nutrition

Nutrition largely impacts milk production of dairy cows, which is very important to the global economy [12]. Successful feeding regimens include rations that provide the cow with adequate nutrients and optimize the synthesis of milk and milk components [12, 57]. Successful feeding regimens also allow the cow to maximize fermentation in the rumen, maximize growth of the rumen microbes, and minimize nutrients lost in excretion [12].

Optimal synthesis of milk and milk components requires adequate nutrients to be available in the diet of dairy cows [12]. Energy requirements can be met by feeding

adequate amounts of forages, concentrates, and some fat [8]. Protein is also required in sufficient concentrations for maintenance, growth, reproduction, and lactation in dairy cattle [12]. Protein is typically the most expensive commodity per unit in the ration, therefore, feeding excess protein should be avoided [8]. Additionally, excess dietary protein is excreted in the urine or feces and studies have shown that feeding excess protein in the diet can decrease production and reproduction in dairy cows, while also having negative effects on the environment through additional pollution of nitrogen in the form of ammonia [8, 58].

Excess amounts of protein in the diet of dairy cows can negatively affect reproduction [34]. In a study completed by Butler et al. (1996), researchers measured both PUN and MUN and found that PUN levels exceeding 19 mg/d in lactating dairy cows or heifers results in decreased conception and pregnancy rates. More recently, a study completed by Rhoads et al. (2006), determined that PUN levels of 25.2 mg/dL result in decreased embryo viability in lactating dairy cows. Ammonia and its metabolites can have toxic effects during gestation, causing amino acid deficiencies in gametes and early embryos [34]. Urea concentrations that are too high or too low indicate that changes need to be made in the ration to ensure that appropriate protein concentrations are present in the diet.

Balancing rations for dairy cows can prove to be very complex. Depending on the stage of lactation of the cow, nutrient requirements and nutritional goals can vary greatly. The different stages of lactation are illustrated in Figure 1.1. In early lactation, dairy cows, especially high producing dairy cows, are often times in a negative energy balance because nutrient requirements for maintenance and lactation exceed energy intake due to

the high demand for milk production [61, 8, 62]. Cows in a negative energy balance must rely on body reserves for an energy source until peak dry matter intake (DMI) is reached. In mid to late lactation, intake of dietary energy often exceeds the energy requirements for maintenance and lactation [62]. The goal of dairy cow production during mid to late lactation is to achieve improvement in body condition score to prepare for the next upcoming lactation [63], while avoiding over conditioning. Ensuring proper nutrition during the different stages of lactation is important because each lactation stage has different nutrient requirements. While much is known about nutritional requirements throughout the different stages of lactation, there are still many unknowns about nutritional practices in dairy operations that will best suit the dairy cow, while proving practical for the producer to implement with the space that they have available.

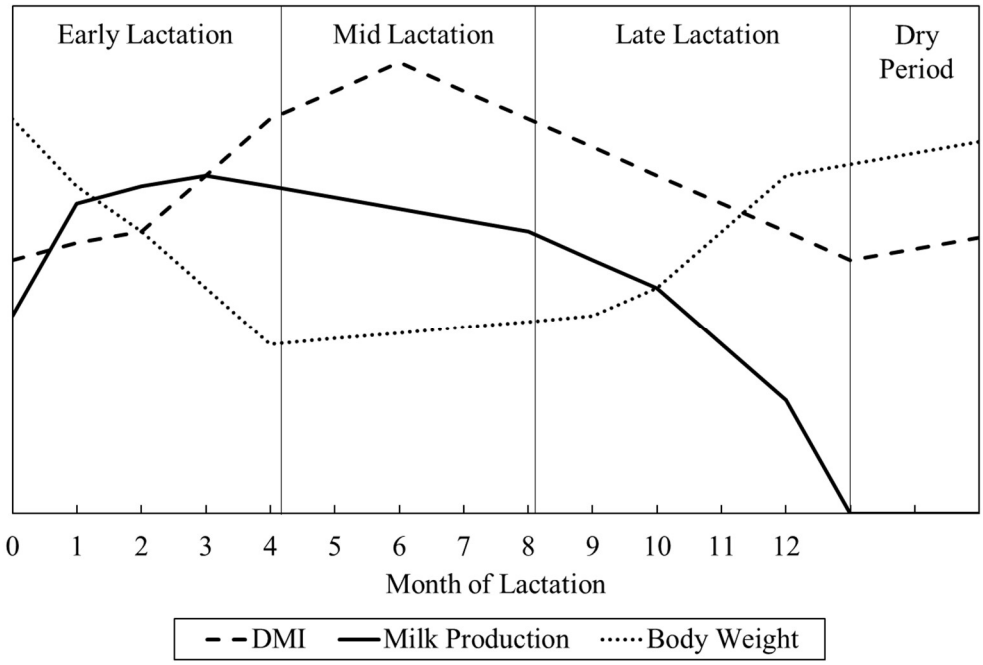

Figure 1.1. Stages of lactation in dairy cows. The stages of lactation are described in the text. Figure adapted from [64].

Alfalfa

Alfalfa has been cultivated for around 2,000 years [65]. Alfalfa is a perennial legume that grows 24 to 36 inches tall and grows best in sandy loam, silt loam, or clay loam soils [66] that can drain well and have a pH of 6.6-7.0 [67]. This legume can often be identified by its purple flowers and its trifoliate leaves with long narrow leaflets that are serrated at the tips. In the Intermountain West region of the United States, alfalfa is the most widely grown perennial forage [68] because it grows well in the arid region and the soil type that is found in this area [69].

The legume property of alfalfa allows the plant to fix nitrogen from the atmosphere into plant protein [70]. All organisms use ammonia (NH_3), a form of nitrogen, for making amino acids, proteins, nucleic acids, and other components [71]. Legumes have the ability to change the unusable form of nitrogen gas (N_2) into NH_3 that can be utilized by organisms [71]. Of note, no other plant type or animal that we know of can perform the process of nitrogen fixation like legumes. Biological nitrogen fixation is mediated by nitrogen-fixing bacteria called rhizobia that are in a symbiotic relationship with legumes [71]. These rhizobia reside in nodules on the roots of legumes and fix N_2 into NH_3, allowing for the legume to absorb the NH_3 [71]. As such, legumes do not require nitrogen fertilization like other plants. Alfalfa makes an ideal forage to include in the diets of ruminant animals because it has a similar energy content to other forages but has a higher protein content than non-legume forages due to the ability of legumes to fix atmospheric nitrogen.

Alfalfa is a legume that is grown throughout the United States and is a common ingredient in livestock rations. When feeding alfalfa to ruminant animals, producers must

be cautious because alfalfa has characteristics that can lead to bloat. Bloat occurs when gas production in the animal exceeds the animal's ability to expel excess gas via eructation [72]. Soluble proteins, saponins, absence of condensed tannins, rapid proliferation of rumen microbes, and increased fermentation and gas production in the rumen are causative agents of bloat from alfalfa [73, 74]. However, the alfalfa plant provides a vast amount of different nutrients for animals [65] and it is especially known for its high protein and fiber contents [65]. Not only does alfalfa provide a rich source of protein and fiber, but it is also a source of vitamins, minerals, and essential amino acids [65]. Alfalfa contains the vitamins C, K, D, E, provitamin A, B1, B2, B6, B12, folic acid/B9, biotin, and niacin [65]. Minerals found in alfalfa include calcium, phosphorus, iron, magnesium, potassium, zinc, copper, selenium, organic silicon, and manganese [65]. Alfalfa contains eight essential amino acids: alanine, lysine, arginine, histidine, cysteine, proline, methionine, and tyrosine [65]. As such, alfalfa is a nutrient rich plant that can be added to a ration in order to provide animals with energy, protein, vitamins, minerals, and essential amino acids [65].

Alfalfa has many benefits when compared to grass hay. Crude protein content in alfalfa ranges from 12-20%, depending on the time of cutting, which is much higher than that of an average grass hay, which has an average crude protein content of 8.4% [70]. Alfalfa also has a lower fiber content than that of grass, which average 31% crude fiber [70]. In ruminant animals, ruminal particulate passage rate depends on the fiber content of the feed [70] and, therefore, grass hay has a lower passage rate than alfalfa [70]. The passage rate of alfalfa is around 36 hours while the passage rate of grass hay can be up to 70 hours, almost double that of alfalfa [70]. Of note, digestibility of alfalfa is largely

dependent on the cutting and time of year that alfalfa is harvested [70]. The relatively lower fiber and high protein content of alfalfa when compared to other non-legume forages allows for higher feed consumption [70].

Nutrient composition of the alfalfa plant can be variable depending on the stage of growth, climate, soil, and region [75, 70]. Protein content of alfalfa varies from 16-20% in the early blooming stage to 12-15% later in the growing season [70]. However, alfalfa leaf protein declines slightly as the plant matures and the alfalfa stem protein declines to a much greater extent as the plant matures [76]. Fiber content can also vary and ranges from 20-28% depending on the stage of growth [70] with alfalfa leaf NDF concentration and digestibility decreasing slowly with maturity and alfalfa stem NDF and ADF increasing more rapidly with maturity [77]. The nutrient content variability that is present in forages, such as alfalfa, can make the process of formulating a TMR complex and can ultimately result in uncertainties in performance and production of livestock animals. As such, it is important that research is conducted to determine how novel harvesting and processing methods, such as alfalfa fractionation into leaf and stem portions, impacts performance of livestock animals when included in the diet.

Very few previous studies have investigated the effects of feeding fractionated alfalfa to cattle, however, previous research has examined the effects of feeding alfalfa leaf meal (ALM) to various livestock species at different stages of production [78, 79]. Alfalfa leaf meal has desirable nutritional composition with an energy content similar to small grain silage or a high-quality hay [79] and 22-28% crude protein [80, 79]. Additionally, ALM has been shown to have two to three times the crude protein as alfalfa stems [81-83] and decreased fiber content when compared to the whole alfalfa plant or

alfalfa stems [84-86]. In a study completed by Gossett and Riggs (1956), finishing feedlot steers exhibited increased weight gain when fed a diet that included ALM at concentrations of 7%, 14%, and 21% of the diet dry matter when compared to steers consuming a control diet that did not include ALM. Additionally, improved DMI in finishing feedlot steers was observed when ALM was included in the diet [79]. Past work has also examined the effects of feeding alfalfa stem haylage to dairy heifers and observed decreased weight gain, hip height, wither height, body condition score, and heart girth in heifers consuming a diet that included alfalfa stem haylage when compared to heifers consuming diets without alfalfa stem haylage [87]. While previous research has investigated the effects of feeding products such as ALM or alfalfa stem haylage on growth parameters of cattle, no studies have examined the effects of feeding alfalfa leaf pellets and alfalfa stems on growth and production of cattle. As such, additional research needs to be completed to determine the how fractionated alfalfa impacts beef and dairy cattle at various stages of production.

Skeletal Muscle Growth

In mammals, muscle fiber (myofiber) number is largely fixed at birth [88, 89]. Post-natal growth of myofibers primarily occurs through hypertrophy [89, 90], the increase in size of existing myofibers. Myofibers are not capable of division [92, 93] and, therefore, additional nuclei are needed to support hypertrophy of existing myofibers [91]. Muscle stem cells, called satellite cells, provide the additional nuclei that are required for muscle fiber hypertrophy [94]. In times of muscle growth, regeneration, or injury, satellite cells are activated and differentiate into myoblasts, which can fuse with existing muscle fibers to ultimately provide the nuclei required to support an increase in muscle

fiber size [95, 96]. Skeletal muscle is highly plastic, meaning it has the ability to change rapidly in response to different stimuli [97]. As such, muscle is continually remodeled through protein synthesis and protein degradation, which is collectively known as protein turnover [97]. Ultimately, for skeletal muscle growth to occur, skeletal muscle protein synthesis must exceed protein degradation [97].

Throughout the 19th century, the ability of muscle cells to regenerate was documented [98]. However, the mechanisms behind muscle regeneration was unknown [98]. In 1917, Lewis and Lewis noted that myofibers increase in size and nuclei, but no nuclear division was seen within the myofiber [99]. Satellite cells were first discovered in 1961 by two different researchers, Alexander Mauro and Bernard Katz, during two independent studies [98]. While studying the *tibialis anticus* muscle of a frog leg with an electron microscope, Alexander Mauro noticed that there were cells located on the periphery of the myofiber [100]. Due to the location on the peripheral region of the myofiber, Alexander Mauro named these cells, satellite cells [100]. Alexander Mauro noted that satellite cells are closely associated with the myofibers and he stated that more research needed to be completed in order to find out more about them [100]. More recently, satellite cells have been redefined as muscle stem cells, due to their ability to self-renew [98].

Satellite cells, located between the basement membrane and the sarcolemma of every muscle fiber [100], start out as quiescent satellite cells [101]. From the quiescent stage, satellite cells become activated during times of growth, muscle injury or increased training of the muscle [101]. After activation, satellite cells become myogenic precursor cells. These myogenic precursor cells can either go back to repopulate the existing

satellite cell population, or they can go on to become myoblasts [102]. The myoblasts can fuse with existing muscle fibers to support post-natal skeletal muscle growth [103-105].

Satellite cells, the stem cells of the muscle, can go through symmetric stem cell division, asymmetric stem cell division [94, 102], or progenitor proliferation [106]. When the gene Sprouty 1 (*Spry1*) is expressed, proliferating satellite cells will renew the quiescent satellite cell pool rather than go on to differentiate [107] (Figure 1.2). When satellite cells go through symmetric stem cell division, the new cells that are produced go on to expand the existing satellite cell population [106]. Asymmetric stem cell division results in some of the cells that are produced from the division going on to expand the existing satellite cell population and some of the cells progressing down the myogenic lineage to eventually fuse with existing muscle fibers [106]. Lastly, satellite cells can go through progenitor proliferation, resulting in stem cell commitment [108]. This type of division results in all of the satellite cells going on to fuse with existing myofibers. Ultimately, satellite cell differentiation is characterized by increased expression of the myogenic regulatory factors (Figure 1.2). Quiescent satellite cells express paired box transcription factor 3 (*Pax3*) and paired box transcription factor 7 (*Pax7*), proliferating myogenic cells express *Pax7* and myogenic factor 5 (*Myf5*), differentiating myogenic cells express *Pax7*, *Myf5*, myogenic differentiation factor (*MyoD*), and myogenin (*MyoG*), and fusing myogenic cells express *MyoG* and myogenic regulatory factor 4 (*MRF4*) [109, 99, 110, 111] (Figure 1.2). In addition to the myogenic regulatory factors, other genes are involved in myogenic progression. Proliferating myoblasts are marked by an increased expression of mitogen activated protein kinase (*MapK*) [112] and increased protein synthesis of fused muscle cells can be identified by increased abundance of

mammalian target of rapamycin (*mTOR*) [113] and eukaryotic translation initiation factor 2B subunit epsilon (*eIF-2Bε*) [114].

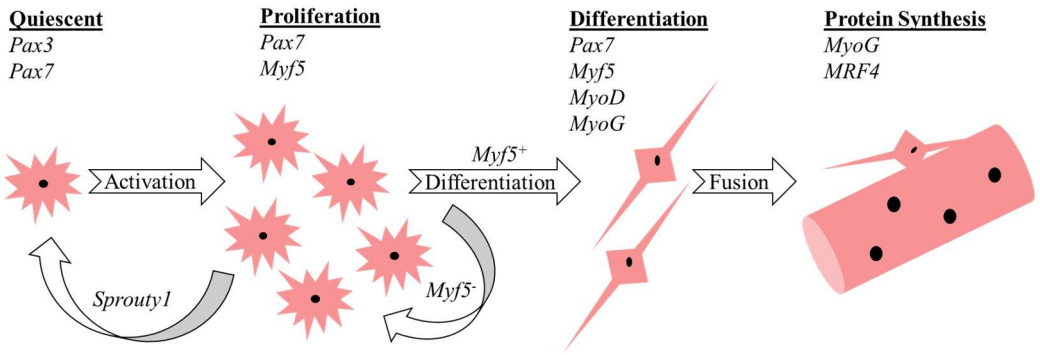

Figure 1.2. Satellite cell gene expression. The details of satellite cell gene expression throughout progression of the myogenic lineage are discussed in the text. Figure adapted from Bazgir et al. (2017).

Anabolic Hormones and Skeletal Muscle Growth

In the U.S., approximately 90% of cattle on feed receive at least one implant in their lifetime [116]. Anabolic implants serve as an important tool for increasing growth and efficiency within the beef industry. Implants that contain a combination of estrogens and androgens have been shown to increase cattle performance and feed efficiency by approximately 5-20%, depending on the type of implant used and the measurement being assessed [117-119]. Furthermore, combination implants increase satellite cell numbers in steers by 50% [118].

Androgens, such as testosterone, have many physiological effects when provided including increases in spermatogenesis, testicular function, hair growth, nitrogen retention, bone density, muscle mass and distribution, libido, and secondary male characteristics [120]. Androgens have been shown to increase skeletal muscle growth in healthy young men [121]. This increase in muscle growth is associated with an increase in satellite cell and myonuclei numbers [121]. However, the mechanisms behind the

ability of testosterone to increase satellite cell numbers are unknown [121]. Testosterone functions through both genomic and non-genomic actions [122-125]. Through genomic actions, the nuclear androgen receptor (AR) modulates target gene transcription by functioning as a ligand inducible transcription factor [126, 127]. Growth is affected by androgens binding to the AR inside of the cells [128], specifically through increases in protein synthesis and decreased protein degradation [129]. However, non-genomic mechanisms of androgens have also been shown to increase protein synthesis [130]. Through non-genomic actions, androgenic compounds have been found to activate the G protein-coupled receptors [131]. Subsequently, matrix metalloproteinases 2 and 9 are activated, causing heparin-binding epidermal growth factor-like growth factor to be released and activate the epidermal growth factor receptor [131, 130]. Epidermal growth factor-like growth factor affects insulin-like growth factor-1 receptor activity and expression, which results in improved cell growth through increases in protein synthesis [132, 131, 130]. Despite this, many uncertainties still remain surrounding the mechanism(s) through which androgens impact skeletal muscle growth.

Estrogens are a class of steroid hormones that are primarily known to be involved in the maintenance of sexual and reproductive function in women [133, 134]. However, estrogens have many other functions in both men and women [135]. Estrogens elicit their effects primarily through binding to the estrogen receptors [136], which function as transcription factors once bound to estrogen. In earlier studies, estrogens have been shown to mitigate inflammation following injury [137, 138, 133]. Additionally, estrogens have demonstrated antioxidant and membrane-stabilizing effects that help prevent oxidative stress and muscle damage [139, 140]. Estradiol-17β (E2), a form of estrogen,

has been shown to enhance satellite cell activation and proliferation [140]. In mice, reduction in E2 concentrations decreases force generating capacity of the muscles, suggesting that E2 plays a role in contraction of skeletal muscle [141]. Additionally, in culture, treatment with E2 demonstrated increased protein synthesis and decreases in protein degradation in fused bovine satellite cells (BSC) [142]. While androgenic and estrogenic steroids are known to be stimulators of growth and largely utilized in the beef industry to improve feed efficiency in beef cattle, the mechanisms through which these hormones improve production remains somewhat unknown [142].

One of the mechanisms through which implants function to improve growth is through modulation of circulating insulin-like growth factor-1 (IGF-1) [143]. When cattle receive E2, an increase in IGF-1 messenger RNA is observed in BSC isolated from these animals [144]. Another mechanism through which anabolic implants increase skeletal muscle growth is by increasing muscle fiber diameter, as well as increasing the number of myonuclei in existing muscle fibers [145, 146, 121]. Trenbolone acetate (TBA) is the most common androgenic compound used in anabolic implants because it has the androgenic activity of 3-5 times that of testosterone and anabolic activity that is 5-8 times greater than testosterone [147]. Additionally, TBA is non-estrogenic and has a higher affinity for the AR. Treating BSC with E2 or TBA results in increased cell proliferation and protein synthesis and decreased protein degradation, demonstrating that these are both potent modulators of satellite cell growth [148, 132, 142, 149, 131, 130] Additionally, anabolic hormones have been shown to stimulate cell proliferation in murine satellite cells [150] and C2C12 murine myoblasts [151] and differentiation in C2C12 myoblasts [151] and L6 rat myoblasts [152]. However, the exact mechanisms

through which androgens improve growth of skeletal muscle remains unknown [153, 121]. Previous research suggests that one way androgens may improve skeletal muscle growth is through modulation of the polyamine biosynthetic pathway [154-158]. In order to further elucidate the mechanisms behind skeletal muscle growth, it is important to determine how anabolic hormones impact proliferation and protein synthesis of myoblasts and whether the polyamine biosynthetic pathway is involved in this mechanism.

Polyamines and Skeletal Muscle Growth

Polyamines are naturally occurring amino acid derivatives that are essential for growth, cell proliferation, and cell differentiation [159-162]. In the polyamine biosynthetic pathway (Figure 1.3), the polyamine precursors methionine (Met), ornithine (Orn), and arginine are utilized as substrates for synthesis of putrescine (Put), spermidine (Spd), and spermine (Spe), which are the three naturally occurring polyamines that are found in mammalian cells [159, 163, 158]. Polyamines can be synthesized by cells [164, 165] and are found in high concentrations in many food sources including potatoes, tomatoes, most meats, and matured cheeses [166]. During times of muscle hypertrophy, the polyamine concentration increases, and during muscle atrophy, polyamine concentration decreases [167]. As such, polyamines could potentially be added in the diets of livestock animals to augment growth.

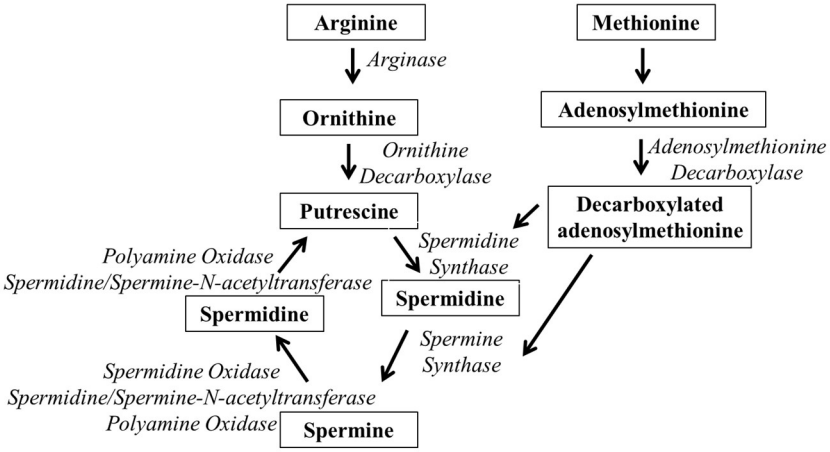

Figure 1.3. Polyamine biosynthesis and interconversion pathway. Polyamines (putrescine, spermidine, and spermine) and their precursors are shown in bold font and enzymes are shown in italic font. The details of the polyamine biosynthesis and interconversion pathway are described in the text. Figure adapted from Pegg and McCann (1982).

Previous studies have found that TBA, polyamine precursors, and polyamines are required in greater concentrations during periods of growth [168, 161, 158, 169]. Anabolic hormones, such as TBA and E2, are thought to modulate the biosynthesis of polyamines by increasing the expression of two enzymes that are involved in the polyamine biosynthetic pathway, ornithine decarboxylase (*ODC*) and S-adenosylmethionine decarboxylase (*AMD1*) [170-172]. Previous studies have also found that the *ODC* gene promotor contains an androgen response element and *AMD1* is likely a direct target gene of the AR [173]. Furthermore, when the AR is knocked out in mice, both *ODC* and *AMD1* expression are decreased [174]. Ornithine decarboxylase is involved in the production of Put from Orn and *AMD1* is involved in the production of decarboxylated S-adenosylmethionine from adenosylmethionine [158]. Previous studies have also looked at the effect of administration of an irreversible inactivator of *ODC*, an enzyme required for the synthesis of polyamines, on proliferation rates and intracellular polyamine concentrations of various cell types (i.e. 9L rat brain tumor cells, mouse

mammary carcinoma FM3A cells, and L6 myoblasts) and found that when *ODC* is inactivated, cell proliferation is decreased and intracellular concentrations of Put and Spd are decreased [175, 176, 6]. Previous studies have demonstrated that an interaction exists between anabolic hormones and the polyamine biosynthetic pathway [171, 172]. However, an improved understanding of how anabolic hormones, polyamine precursors, and polyamines impact myoblast proliferation and protein synthesis is necessary to further elucidate the mechanisms through which these compounds may function to increase skeletal muscle growth so that alternative growth promoting technologies for livestock species can be developed.

Summary

As the population continues to increase, and available farmland decreases, alternative feeds and growth-promoting compounds/technologies must be developed to increase feed efficiency of livestock animals to support the growing population. Improvements in technology have allowed for novel feed processing methods to be developed, such as fractionation of alfalfa. However, the effects of these feedstuffs on growth and production of livestock animals remains unknown. While other researchers have examined the effects of feeding ALM and [177, 78, 86, 80, 79] and alfalfa stem haylage [87] on growth and feed efficiency of cattle, no other studies have investigated the effects of feeding PLM and PFP to cattle. Polyamines are molecules that are known to be important for growth [159-162] and could also serve as a natural dietary supplement to improve growth and production of cattle. Previous research suggests that one of the mechanisms through which anabolic hormones increase growth is through interacting with the polyamine biosynthetic pathway [171, 172]. Although previous studies have

examined the effects of these compounds in bovine satellite cells [169, 178], no other studies have examined the effects of anabolic hormones, polyamines, and polyamine precursors on growth by assessing proliferation, protein synthesis, and changes in mRNA expression over time of genes involved in polyamine biosynthesis, protein synthesis, and growth in both C2C12 and Sol8 murine myoblast cells. As such, the goals of this research were to gain an improved understanding of how feeding novel fractionated alfalfa products to cattle affect growth, feed efficiency, and production and to determine how providing anabolic hormones, polyamines, and polyamine precursors to murine myoblasts affects skeletal muscle growth through assessment of proliferation, protein synthesis, and mRNA abundance.

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CHAPTER II

EFFECTS OF FEEDING A NOVEL ALFALFA LEAF PELLET PRODUCT (PROLEAF MAX) AND ALFLAFA STEMS (PROFIBER PLUS) ON PERFORMANCE IN THE FEEDLOT AND CARCASS QUALITY OF BEEF STEERS[1]

ABSTRACT

Alfalfa is often included in the diets of beef animals; however, the nutrient content of alfalfa is variable depending on the region in which it is grown, climate, soil, and many other factors. The leaf portion of alfalfa has a less variable nutrient composition than the stem portion of the plant. The variability that is present in the alfalfa plant can make the development of total mixed rations of consistent nutrient content difficult. As such, the purpose of this study was to determine how inclusion of fractionated alfalfa leaves and alfalfa stems impacts performance and carcass quality of finishing beef steers. Twenty-four steers were allocated to one of three treatments: a control group fed a typical finishing diet with alfalfa as the forage (CON; n=8), a typical diet that replaced alfalfa with fractionated alfalfa leaf pellets and alfalfa stems (ProLEAF MAX™ + ProFiber Plus™; PLM+PFP; n=8), or a typical diet that replaced alfalfa with alfalfa stems (PFP; n=8) for 63 days. Steers were fed individually twice daily, weighed every 14 days and ultrasound images were collected every 28 days. At the end of the feeding trial, steers were harvested at a commercial facility and carcass data was

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obtained. Analysis of dry matter intake demonstrated that steers receiving the PFP and CON diets consumed more feed ($P < 0.001$) than steers consuming the PLM+PFP diet. Steers receiving the PLM+PFP diet gained less ($P < 0.001$) weight than the steers receiving the other two dietary treatments. No differences ($P > 0.10$) in feed efficiency or carcass characteristics were observed. Steers receiving the PFP diet had improved ($P = 0.016$) cost of gain ($0.93 per kg) when compared to steers receiving PLM+PFP ($1.08 per kg) diet. Overall, our findings demonstrate that inclusion of PFP in place of alfalfa hay in a finishing diet has the potential to improve cost of gain, without negatively affecting growth, performance, or carcass characteristics of finishing feedlot steers.

INTRODUCTION

In a beef operation, feed accounts for the majority of total beef production costs (Hill, 2012). As such, the beef industry is continuously working to increase feed efficiency (FE) (Lines et al., 2018). Alfalfa is a common feedstuff included in the diets of many livestock species (Sen et al., 1998), including cattle. Alfalfa provides a source of protein, fiber, and other nutrients (Apostal et al., 2017). Alfalfa leaves have a high protein content and alfalfa stems are high in fiber (Palmonari et al., 2014). Alfalfa ranges from 12-20% crude protein, depending on the stage of maturity (Balliette and Torell, 2015) with alfalfa leaf protein only slightly declining with maturity and alfalfa stem protein declining to a much greater extent (Sheaffer et al., 2000). Crude fiber content is also variable depending on maturity (Church, 1977), and can range anywhere between 20-28% (Balliette and Torell, 2015). Alfalfa leaf neutral detergent fiber (NDF) concentration and digestibility decreases slowly with maturity and stem NDF and acid detergent fiber (ADF) increases more rapidly with increasing maturity (Fick and Onstad, 1988). The

nutrient content variability that is present in alfalfa can make the process of formulating a total mixed ration (TMR) of consistent nutrient content difficult and impact forage palatability and voluntary intake (Ademosum et al., 1968). As such, it is important to determine how novel harvesting and processing techniques, such as fractionation of alfalfa, may impact performance of livestock when included in the diet. The objective of this study was to examine the effects of including a novel alfalfa leaf pellet product [ProLEAF MAX (Scoular, Omaha, NE); PLM; (Pratt and Jackson, 2018)] and a novel alfalfa stem byproduct [ProFiber Plus; (Scoular, Omaha, NE); PFP; (Pratt and Jackson, 2018)] in the diet on feedlot performance and carcass quality of finishing beef steers when compared to steers fed a typical alfalfa hay based feedlot diet for the Intermountain West. We hypothesized that steers consuming diets that included alfalfa leaves would have improved growth and carcass characteristics when compared to steers consuming diets that included alfalfa hay.

MATERIALS AND METHODS

Steers

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Utah State University, approval number IACUC-2821, and steers were cared for in accordance with the Live Animal Use guidelines (FASS, 1999). Twenty-four Angus influenced steers that were approximately one year of age and similar in weight (420.6 kg ± 4.7 kg) were selected from the Utah State University beef herd. Twenty four steers was the maximum capacity of the University facilities that allowed for individual intake to be measured. Steers were housed in a covered barn in individual pens with free choice access to water. Steers were implanted at the start of the trial with Synovex Choice

(Zoetis, Parsippany, NJ). Synovex Choice implants contain 100 mg of trenbolone acetate and 14 mg of estradiol. Steers were initially stratified by weight so that there were no differences in starting weight and then randomly assigned to one of three treatment groups. Pre-trial, steers were subjected to a 14 d adjustment period. Over the course of the adjustment period, all steers were fed a typical alfalfa-based background diet that included the following ingredients (dry matter (DM) basis): alfalfa hay (26.9%), corn silage (38.5%), barley (16.2%), high moisture corn (15.4%), and a feedlot mineral supplement (3%). After the adjustment period, steers were fed their assigned experimental diets for an additional 63 d before harvest. During the 63 d feeding period, the experimental diets were fed in a series of two step-up diets (step-up diet and final diet) to allow for an increase in concentrate (grain) levels in the diets. The step-up diet was fed for 22 d and the final diet was fed for the final 41 d (Table 2.2).

The three treatment diets included corn silage, barley, high moisture corn, a feedlot mineral supplement, and either alfalfa hay (Table 2.1; control; CON; n=8), alfalfa leaf pellets and alfalfa stems (PLM+PFP; n=8) in place of alfalfa hay, or alfalfa stems (PFP; n=8) in place of alfalfa hay. The PLM and PFP products were included in their respective diets at concentrations required to replace the alfalfa hay in the CON diet, thus, allowing all treatment diets to have similar amounts of forage. In the PFP diet, the alfalfa hay was simply substituted for alfalfa stems and crude protein levels were matched by adding in urea. The PLM+PFP diet was designed to essentially create an ideal hay with the two products. The PLM was not included as the sole forage in its own treatment diet because the amount of physically effective fiber would not have been adequate to maintain rumen health. Forage nutrient compositions are shown in Table 2.1. The

nutrient compositions of the treatment diets can be seen in Table 2.2. Each of the three diets that were fed were formulated to be isocaloric and isonitrogenous using CowBytes (Government of Alberta, Canada). Of note, although all three treatment diets were balanced to be isocaloric and isonitrogenous, analyses of the diets provided to the steers showed that nutrient content of the diets slightly differed from formulated nutrient densities, likely due to inconsistencies when mixing the ration or sampling feeds (Table 2.2). Diets were mixed every two d and fed twice daily at 08:00 and 16:00 h. All feed ingredients for the diets, except urea and mineral supplement, were loaded into a commercial mixer, weighed, and mixed together for approximately 15 min. Because of the small amount required, both urea and the mineral supplement were pre-weighed and top dressed daily. Feed offered and feed refused was measured daily in order to determine individual daily dry matter intake (DMI) using the clean-bunk management system as described previously (Pritchard and Bruns, 2003). In brief, each individual bunk was cleaned out and feed refusals were weighed daily so that the amount of feed that was consumed in that 24 hour period could be recorded and any adjustments to the amount of feed provided to ensure animals were being fed *ad libitum* could be made. Bunks were managed to have approximately 0.9 kg of refusals per day to ensure that animals were receiving feed *ad libitum*. Every 14 d, steers were weighed at approximately 07:00 h. On d 0 and d 28, carcass ultrasound imaging was performed by a trained ultrasound technician to obtain 12^{th} rib fat thickness (FT) and ribeye area (REA) measurements using an EXAGO ultrasound (Universal Imaging, Bedford Hills, NY) to assess growth early on in the feeding trial. Feed efficiency, calculated as gain to feed (G:F), was determined from DMI and average daily gain (ADG). Although DMI was

calculated daily as described above, DMI will be presented as 14 d averages instead of daily averages in order to align with the weight gain data and calculated G:F for 14 d periods throughout the feeding period.

Harvest and preparation of fractionated alfalfa products

A self-propelled leaf combine (Pratt and Jackson, 2018) was used to fractionate the alfalfa plant into PLM, a pelleted alfalfa leaf product, and PFP, alfalfa stems. The leaf combine strips the alfalfa leaves from the standing alfalfa plant and the alfalfa leaf fraction was then transported by truck to a drying facility for curing and processing into pellets. The stem alfalfa fraction was cut, conditioned, and windrowed to be baled when dry.

Feed sample analysis

Samples of alfalfa hay, corn silage, barley, high moisture corn, PLM, and PFP were collected pre-trial and analyzed for nutrient compositions at a commercial lab (Cumberland Valley Analytical Services, Waynesboro, PA). Samples of the PLM and PFP were collected each time a new batch was delivered. A sample of the TMR was collected three times weekly immediately after feed was delivered to the bunks and urea and mineral supplement were top-dressed to the appropriate diets and a composite sample of each week was sent for analysis at a commercial lab. All samples were frozen at -20°C and sent for analysis at the completion of the trial.

Carcass data

All steers were harvested at a commercial harvest facility in Hyrum, UT once they reached approximately 550 kg and had approximately seven mm of ribeye fat thickness. This target weight and ribeye fat thickness were chosen to reflect the average weights of

cattle harvested in the state of Utah, as well as ensuring that the animals did not have too much fat while also working within the constraints of scheduling with the commercial facility (Troxel and Gadberry, 2015; USDA, 2018). All carcass data was obtained from the harvest facility including, hot carcass weight (HCW), marbling score (MS), ribeye area (REA), 12th rib fat thickness (FT), dressing percentage (DP), USDA yield grade (YG) and USDA quality grade (QG). Quality grade is the evaluation of the distribution of marbling within the lean (MS) and the degree of maturity of the animal, which are both factors that affect palatability of the meat (Hale et al., 2013). Yield grade is an estimate of the boneless, closely trimmed retail cuts from parts of the carcass that are considered to be of high value and is assigned based on HCW, REA, FT, and kidney, pelvic, and heart fat (Hale et al., 2013). Marbling to backfat ratio (M:BF) was calculated using previously described equations (Mohrhauser et al., 2015). Marbling to backfat ratio is a measure of the degree of marbling compared to the degree of backfat thickness and a smaller M:BF value represents more marbling that is present in the product, which is often favored by consumers of beef products.

Economic comparison

To make an economic comparison of the treatments, partial budgets were developed using the total feed costs (TFC), feedlot cost of gain (COG), feed cost per kilogram of hot carcass weight (FC/kg HCW), and the feed cost per marbling score (FC/MS). Total feed costs were calculated for each steer as the summed product of total feed (kg as-fed) and the weighted cost ($/kg) of each individual feed component where the weights were equal to the percentage of each feed component in the total diet. Five-year historical average prices (LMIC, 2020) were used for all feed components other than

the alfalfa leaf pellets, alfalfa stems, urea, and feedlot supplement for which actual prices were used. Total feed costs were then divided by total gain, hot carcass weight, and marbling score to calculate COG, FC/kg HCW, and FC/MS, respectively. Total feed cost is intuitively understood, greater relative TFC indicates additional expenses associated with feeding. Cost of gain estimated for this study considers marginal changes to the cost of feed only and represents the feed costs in dollars that could be anticipated by the feedlot to achieve one additional kg of weight gain. Feed cost/kg HCW represents the FC in dollars that are required to increase the hot carcass weight by one kg, while FC/MS represents the FC in dollars that are required to increase the MS by one MS.

Statistical analyses

A completely randomized design was used in this study. Steers were initially stratified by weight so that there were no differences in starting weight and then assigned to one of three blocks based on weight and randomly allocated to one of three treatment groups. All data were analyzed using the MIXED procedure of SAS® (version 9.4; SAS Institute Inc., Cary, NC). Treatment was the main effect and individual steer was included as a random variable in the model. The variables that were analyzed include: total weight gain, ADG, HCW, MS, REA, FT, DP, YG, QG, M:BF, TFC, COG, FC/kg HCW, and FC/MS. Repeated measures was used to analyze the following variables over time: weight, G:F, and DMI. A Tukey-Kramer adjustment was used in determining significant treatment differences by separation of the least square means. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered a tendency.

RESULTS

Feedlot performance

Analysis of body weight between the different treatment groups demonstrated that the steers increased in body weight over time ($P < 0.001$), and there was a tendency ($P = 0.10$) for treatment to have an effect on body weight such that steers that received the PFP diet tended to have increased body weight gain over the 63 d feeding period compared to the steers that received the PLM+PFP diet (Table 2.3). Average daily gain over the 63 d feeding period showed a tendency for an effect of treatment ($P = 0.058$) where the steers receiving the PFP diet had increased ($P = 0.047$) ADG compared to the PLM+PFP diet, but was not different ($P > 0.10$) from the CON (Fig. 2.1).

Average daily DMI between the different treatment groups demonstrated that intake increased over time ($P < 0.001$) and treatment had an effect ($P < 0.001$) on average daily DMI such that steers receiving the PFP and CON diets consumed more ($P < 0.001$) than steers receiving the PLM+PFP diet up until d 42 and the steers consuming the PFP diet consumed more ($P < 0.001$) than the steers consuming the other two diets from d 42 to d 63 of the feeding period (Fig. 2.2). Analysis of G:F showed that treatment had no effect ($P > 0.10$) on G:F throughout the 63 d trial (Fig. 2.3). While the steers that consumed the PFP diet gained the most weight and had the highest DMI, there was no difference in G:F between the treatment groups.

No differences ($P > 0.10$) were found between the different treatment groups in REA or FT measured by ultrasound on d 0 or d 28 of the feed trial. These data demonstrate that REA and FT are not affected when PFP or PLM+PFP are included in a diet for finishing feedlot steers.

Carcass characteristics

Analysis of carcass characteristics at harvest demonstrated that there were no differences ($P > 0.10$) in HCW, MS, REA, FT, YG, QG, DP, or M:BF (Table 2.4). It is important to note that although the animals consuming PFP had increased weight gain, they did not have an increased HCW and there were no differences in DP indicating that the extra weight gained did not yield more consumable product.

Economic analysis

Analysis of estimated economic metrics demonstrated that there were no differences ($P > 0.10$) in TFC, FC/kg HCW, or FC/MS between treatments (Table 2.5). However, COG over the 63 d feeding trial was affected ($P = 0.016$) by treatment such that the steers receiving the PFP diet had a lower COG ($P = 0.016$) than the PLM+PFP treatment, but were not different ($P > 0.10$) from the CON (Table 2.5). The estimated COG for PLM+PFP was $1.08/kg while the COG for PFP was $0.93/kg.

DISCUSSION

To the best of the authors' knowledge, this study is the first to investigate feeding pelletized alfalfa leaves or alfalfa stems to livestock species, however, many studies have examined the effects of feeding alfalfa leaf meal (ALM) or alfalfa leaf concentrate to livestock species. Similar to our alfalfa leaf pellet product, ALM provides a source of energy and protein, as it has similar energy content to that of a high quality hay or small grain silage (DiCostanzo et al., 1999) and has been reported to have 22 to 28% crude protein (Jorgensen et al., 1997; DiCostanzo et al., 1999), and two to three times the crude protein of alfalfa stems (Mowat et al., 1965; Mowat and Wilton, 1984; Albrecht et al., 1987). Additionally, ALM is more digestible and has a lower fiber content than whole alfalfa or alfalfa stems (Buxton and Brasche, 1991; Titgemeyer et al., 1992; Bourquin and

Fahey, 1994), making it a favorable supplement to low quality roughages (Gossett and Riggs, 1956). Alfalfa stems, on the other hand, can serve as an alternative to fiber sources such as straw (Su et al., 2017); however, alfalfa stems have more than twice the protein content of straw (Su et al., 2017), which is especially beneficial for growing animals. Gossett and Riggs (1956) performed a study similar to the present study in which they supplemented a diet for finishing beef steers that consisted of low-quality prairie hay, cottonseed meal, and ground milo grain with varying amounts (7 to 21% of the diet DM) of ALM in which all diets were, overall, isocaloric and isonitrogenous. In contrast to our study, Gossett and Riggs (1956) observed improved daily weight gains in steers consuming the diets supplemented with three different amounts of ALM (7%, 14%, and 21%, DM basis) when compared to steers consuming the control diet, which consisted of low-quality prairie hay, cottonseed meal, and ground milo grain. The contrasting results could be due, in part, to our study having supplemented alfalfa leaves (PLM) at approximately 14% DM (Table 2.2), while Gossett and Riggs (1956) observed the highest total weight gain and daily weight gains in steers consuming the treatment that consisted of 21% ALM (DM). Additionally, unlike the present study, Gossett and Riggs (1956) did not include alfalfa in their control diet, which could be another source of variation. In the present study, the animals that consumed the PFP diet gained more weight throughout the feeding trial when compared to steers that consumed the other two treatment diets. These results are likely due to the improved DMI observed in animals consuming the PFP treatment diet. While our study did not result in improved weight gain or ADG in animals that were supplemented with PLM, the findings of Gossett and Riggs (1956) and Klosterman et al. (1953) demonstrate that supplementation of ALM in

diets consisting of low quality forages has the potential to result in improved weight gain in cattle. However, more research needs to be done to determine the effects of including pelletized alfalfa leaves, such as PLM, in the ration of feedlot steers.

Additionally, few studies have been conducted on the inclusion of alfalfa stems in the diets of cattle, however, Su et al. (2017) investigated the effects of feeding alfalfa stem haylage on the performance of Holstein dairy heifers. Su et al. (2017) diluted a basal diet consisting of corn silage and alfalfa haylage with either alfalfa stem haylage or wheat straw and found that heifers consuming a diet diluted with alfalfa stem haylage had decreased weight gain and growth (as measured by heart girth, hip height, wither height, and body condition score) when compared to heifers consuming the other two treatment diets (corn silage and alfalfa haylage; corn silage, alfalfa haylage, and wheat straw), which contrasts the findings of the present study. The ADF and TDN of the alfalfa stems (PFP) used in the present study was higher than that of the alfalfa stemlage used by Su et. al. (2017), which could be a reason for the difference in results between the two studies. Additionally, this study analyzed finishing feedlot steers, whereas Su et al. (2017) analyzed growth of heifers.

After completion of the feeding trial, the nutrient composition of all treatment diets was analyzed. Although all treatment diets were initially balanced to be isocaloric and isonitrogenous, post-trial analyses showed small differences in nutrient content between the different treatment diets (Table 2.2). Forages tend to vary more in their nutrient composition than concentrates and the variation that was present between the different forage sources in our treatment diets (Table 2.1) was most likely the main cause of the nutrient composition variation between the formulated nutrient content and actual

nutrient content treatment diets. In addition, as with all large-scale feeding operations, there could have been variation in mixing and delivering the ration daily that contributed to differences in nutrient composition of the ration. Of note, for finishing feedlot diets, our treatment diets had a higher proportion of forage than is typical. However, treatment diets were balanced this way to ensure that enough of the PLM and PFP products were included in the diets to analyze their impact on feedlot performance. Variations that were present in nutrient composition between the different treatment diets included the PFP diet containing more ADF and NDF and less CP and TDN when compared to the other two treatment diets. The lower CP content that was present in the PFP diet could be due, in part, to the fact that the urea was top-dressed in this diet, therefore, samples of the PFP diet that were collected might not have been representative of the true CP content of this diet. These variations between the different treatment diets could have influenced our observed results.

In our study, the steers receiving the PLM+PFP diet had the lowest DMI throughout the trial; however, there is not a good explanation for this trend. The nutrient compositions of the different treatment diets were fairly similar, as such, the only explanation for the depressed DMI observed in steers receiving the PLM+PFP diet is that something about the PLM product in the diet caused decreased DMI. The inclusion of PLM in the PLM+PFP diet could have affected physically effective fiber content, which may be responsible for the decreased DMI observed in steers receiving the PLM+PFP diet. On days 56 and 63, steers consuming the PFP diet had numerically increased DMI compared to steers consuming the other two diets. The ADF of the PFP diet was higher than that of the other two diets (Table 2.2), indicating that perhaps the diet was not as

digestible and the steers needed to consume more feed to get a proper amount of nutrients. Alternatively, the large amount of fiber present in the PFP diet could have stimulated microbial fermentation and presence of rumen microbes, which could then result in decreased fermentation and increased DMI. Nonetheless, more research needs to be completed to determine how rumen characteristics change when PLM or PFP is included in the ration. In contrast to the present study, Su et al. (2017) did not observe improved DMI when alfalfa stem haylage was used to dilute a basal diet consisting of corn silage and alfalfa haylage when compared to wheat straw. Zehnder et al. (2010) observed similar results to the present study in that they did not observe improved DMI in beef heifers that were fed a diet that replaced soybean meal with ALM in a corn-based diet. However, DiCostanzo et al. (1999) observed improved DMI in finishing steers that were fed a diet that substituted ALM for hay and soybean meal, demonstrating that DMI has the potential to be improved when ALM is included in the diet. Although this study did not result in improved weight gain or DMI with supplementation of alfalfa leaves (PLM), it is important to note that inclusion of PFP, which is a cheaper alternative to alfalfa hay, even with the added costs of processing, in the diet of finishing steers results in similar performance when compared to the inclusion of alfalfa hay or PLM in the diet, and thus, may be an economically viable alternative forage for producers to use.

On day 63, G:F was higher for all three treatments when compared to the rest of the feeding period. The exact reasons for the increase in G:F towards the end of the feeding period as the steers approached their mature size are unknown. It is also important to point out that the steers had a sharp increase in weight gain the last week of the trial, without a change in DMI which is likely the reason why G:F increased.

However, there is no good explanation as to why the steers had such a sharp increase in weight gain during the last week of the feeding trial. Gossett and Riggs (1956) observed results that contrasted from our FE data and observed improved FE in steers that consumed the diets that were supplemented with ALM, which required 484 kg of feed to gain 45.4 kg of weight when compared to the steers consuming the diet that was not supplemented with ALM, which required 571.1 kg of feed for 45.4 kg of weight gain.

To our knowledge, the present study is the first to investigate the effects of feeding fractionated alfalfa on carcass characteristics of beef steers. However, there is one other study that examines the effects of feeding different forages to beef steers on carcass characteristics. Swanson et al. (2017) performed a study that investigated the effects of feeding a dry-rolled corn-based diet that included one of four different forage sources (alfalfa, corn silage, wheat straw, or corn stover) on carcass characteristics of beef steers. In agreement with our results, Swanson et al. (2017) observed no difference in HCW, MS, FT, or longissimus muscle area between the different treatments. However, Swanson et al. (2017) did observe a tendency for the steers consuming the diets including wheat straw or corn stover to have greater DP than alfalfa or corn silage treatments. In our study, although not statistically significant, MS was increased by approximately 13% and 15% in steers that consumed the PLM+ PFP diet when compared to the steers that consumed the CON or PFP diets, respectively. Additionally, the steers that consumed the PLM+PFP diet had numerically increased M:BF when compared to the steers in the other two treatment groups. These results indicate that the steers that consumed PLM+PFP were more efficient at depositing intramuscular fat than steers that received the other two treatments. However, it is important to note that these differences were not significant

and this trial needs to be replicated with a larger number of animals to determine whether fractionated alfalfa impacts fat deposition in the carcass when fed during the finishing period.

The economic analysis in the present study showed that the COG difference of $0.15/kg greater for the PLM+PFP diet when compared to the PFP diet. This COG difference has the potential to significantly alter return per head. The average total weight gain across all treatments was 118 kg. This would result in an average decrease in net return per head of $17.70 (118 kg x $0.15/kg) for PLM+PFP steers as compared to PFP. Additionally, while not statistically significant, PFP had a lower FC/kg HCW while PLM+PMP was shown to have a relatively lower FC/MS ratio. These results highlight trends within the data from this current study and demonstrate that the cost to produce hot carcass weight tends to be cheapest when feeding a PFP diet while the cost to produce better quality grade (i.e., increased marbling) tends to be cheapest when feeding the PLM+PFP diet.

In summary, our findings showed that replacing alfalfa hay with PFP in a finishing feedlot steer diet results in increased DMI and weight gain when compared to steers consuming the PLM+PFP diet. However, no differences were observed in G:F or carcass characteristics between the three treatment groups (CON, PLM+PFP, and PFP). Economic analysis demonstrated that steers receiving the PFP diet had improved cost of gain when compared to steers receiving PLM+PFP diet. Other studies have observed improved weight gain, DMI, and/or FE when animal diets are supplemented with ALM or alfalfa leaf protein concentrate, but we did not observe these differences in the present study. If we were able to have more than 8 animals per treatment in our study, we may

have observed similar trends to the previous studies. Additionally, our feeding period took place over 63 d and while we realize this is a limitation of the present study, it is an adequate amount of time to observe differences between the treatment groups with the parameters that we measured. As such, additional research needs to be completed to determine how including fractionated alfalfa in the diet impacts feedlot performance and carcass quality of beef steers.

CONCLUSION

As the population continues to grow exponentially and the amount of land available for food production decreases (Mayo, 2016), it is necessary to maximize efficiency of beef production. Feed accounts for the majority of costs associated with beef production (Archer et al., 1999; Hill, 2012; Lines et al., 2018) and, therefore, it is essential to develop nutrition regimens that will decrease cost of production without impacting efficiency of production. Overall, our findings demonstrate that inclusion of PFP in place of alfalfa hay in a finishing diet has the potential to improve COG and inclusion of alfalfa hay, PLM, or PFP in a finishing diet results in similar growth, performance, and end-product quality and quantity. Additionally, producers can purchase PFP for a lower price than alfalfa hay or PLM and improved COG when compared to animals receiving the PLM+PFP diet. However, more research needs to be completed on these products with a larger number of animals and also on how inclusion of different amounts of the products might impact production.

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Table 2.1. Nutrient composition of forage sources[1]

	Forage source			
Item	Alfalfa hay	PLM	PFP	Corn silage
DM, %	88.30	89.85	88.52	29.20
Analysis, DM basis				
Crude protein, %	14.40	24.05	12.07	9.90
ADF, %	41.60	26.40	50.05	25.40
aNDF, %	51.20	30.20	59.58	40.08
NFC, %	27.80	30.95	22.70	42.50
TDN, %	55.30	65.35	49.94	69.80
NE_m, Mcal/kg	0.24	0.30	0.22	0.34
NE_g, Mcal/kg	0.12	0.18	0.10	0.21
Ash, %	6.66	13.20	6.38	5.63
Calcium, %	1.24	2.17	0.70	0.24
Phosphorus, %	0.19	0.33	0.24	0.20
Magnesium, %	0.28	0.35	0.22	0.14
Potassium, %	1.85	3.25	2.26	1.40
Sodium, %	0.13	0.10	0.16	0.02
Iron, mg/kg	91.00	627.50	94.00	172.00
Manganese, mg/kg	23.00	57.00	17.50	75.00
Zinc, mg/kg	15.00	24.00	16.17	27.00
Copper, mg/kg	8.00	9.00	9.33	6.00

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE); ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, Non-fiber carbohydrates; TDN, total digestible nutrients; NE_m, net energy for maintenance; NE_g, net energy for gain; Mcal, megacalorie

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to finishing feedlot steers for 63 d.

Table 2.2. Composition and nutrient composition of treatment diets[1]

	Step-Up Diet			Final Diet		
Item	CON	PLM+PFP	PFP	CON	PLM+PFP	PFP
Composition of treatment diets						
Feed, % DM						
Alfalfa hay	16.5	-	-	14.0	-	-
PLM	-	16.3	-	-	13.8	-
PFP	-	6.0	16.6	-	5.8	14.0
Corn silage	24.6	21.2	24.6	13.2	10.3	13.2
Barley	27.7	26.8	27.6	35.8	34.8	35.8
High moisture corn	27.7	26.9	27.7	33.4	32.4	33.4
Feedlot supplement[2]	2.8	2.7	2.8	2.9	2.8	2.9
Urea	0.7	-	0.7	0.3	-	0.6
Nutrient composition of treatment diets						
DM, %	59.75	63.35	63.55	72.10	70.95	73.30
Analysis, DM basis						
Crude protein, %	13.85	14.1	12.40	13.20	13.35	12.40
ADF, %	19.05	18.80	27.55	18.00	19.90	29.15
aNDF, %	28.95	28.10	27.55	27.60	29.60	40.30
NFC, %	50.10	52.40	41.85	52.70	50.95	40.65
TDN, %	73.60	73.80	67.65	74.45	72.75	65.30
NE_m, Mcal/kg	0.36	0.36	0.32	0.37	0.35	0.31
NE_g, Mcal/kg	0.23	0.23	0.20	0.24	0.23	0.18
Ash, %	7.10	6.91	7.51	6.48	6.15	6.65

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE); ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, Non-fiber carbohydrates; TDN, total digestible nutrients; NE_m, net energy for maintenance; NE_g, net energy for gain; Mcal, megacalorie

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to finishing feedlot steers for 63 d (the step-up diet was fed for 22 d and the final diet was feed for the final 41 d).

[2]The guaranteed nutrient analysis for the feedlot supplement is as follows: 11.0% crude protein, 5.0% salt, 0.5% phosphorus, 8.0% calcium, 0.2% magnesium, 0.8% potassium, 0.5% sulfur, 2.0% sodium, 200.0 mg/kg copper, 400.0 mg/kg manganese, 650.0 mg/kg zinc, 2.0 mg/kg selenium, 22.0 mg/kg iodine, 9.0 mg/kg cobalt, 360.0 mg/kg Monensin.

Table 2.3. Effects of feeding fractionated alfalfa on weights of finishing feedlot steers

	Treatment[1]				
Day[2]	CON	PLM+PFP	PFP	SEM	P-value[3]
0	420.3	420.8	420.7	4.7	
14	438.6	435.6	439.3	4.9	
28	459.7	458.1	460.6	5.8	
42	489.9	482.9	497.8	7.1	
56	515.7	513.1	523.9	6.4	
63	539.2	531.0	545.7	6.5	
Treatment x day					0.97
Time					< 0.001
Treatment					0.10

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE)

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to finishing feedlot steers for 63 d. Values represent the least square mean ± SEM.

[2]Weights are displayed in kg.

[3]P-values for Treatment x day, Time, and Treatment when steer body weights were analyzed over time with repeated measures.

Table 2.4. Effects of feeding fractionated alfalfa on carcass characteristics of finishing feedlot steers

	Treatment[1]				
Carcass Characteristic	CON	PLM+PFP	PFP	SEM	P-value
Hot carcass weight, kg	302.7	299.5	309.4	5.02	0.38
Marbling score[2]	332.1	381.1	324.5	25.44	0.26
Cold camera ribeye area, (cm^2)[3]	69.68	70.89	70.89	1.93	0.88
12^{th} rib fat thickness, (mm)[3]	7.40	7.14	7.62	2.40	0.39
Dressing percent	56.00	56.40	56.60	0.57	0.75
Yield grade[3]	2.10	2.00	1.86	0.10	0.25
Quality grade[3]	2.38	2.25	2.13	0.22	0.73
Marbling to backfat ratio[4]	-0.19	0.82	-0.63	0.52	0.15

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE)

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to finishing feedlot steers for 63 d. Values represent the least square mean ± SEM.

[2]Marbling score is assessed visually by a USDA grader at the harvest facility.

[3]As measured by the camera at the commercial harvest facility.

[4]Marbling to backfat ratio identified in carcasses calculated using previously described equations (Mohrhauser et al., 2015). A lower number indicates more intramuscular fat deposition compared to 12^{th} rib fat deposition.

Table 2.5. Effects of feeding fractionated alfalfa on total feed costs, cost of gain, feed cost per kilogram of hot carcass weight, and feed cost per marbling score of finishing feedlot steers

	Treatment[1]				
Item	CON	PLM+PFP	PFP	SEM	P-value
TFC[2]	$120.43	$118.40	$116.11	3.52	0.69
COG[3]	$1.02[ab]	$1.08[a]	$0.93[b]	0.03	0.02
FC/kg HCW[4]	$0.40	$0.40	$0.38	0.01	0.29
FC/MS[5]	$0.37	$0.32	$0.36	0.01	0.09

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE); TFC, total feed costs, COG, cost of gain, FC/kg HCW, feed cost per kilogram of hot carcass weight; FC/MS, feed cost per marbling score

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to finishing feedlot steers for 63 d. Values represent the least square mean ± SEM. Different letters (a and b) are significantly different ($P < 0.10$) within each column.

[2]TFC ($) is the total cost associated with feeding each treatment for the 63 d feeding period.

[3]COG is equal to the TFC/total weight gain.

[4]FC/kg HCW is equal to the TFC/hot carcass weight.

[5]FC/MS is equal to TFC/marbling score.

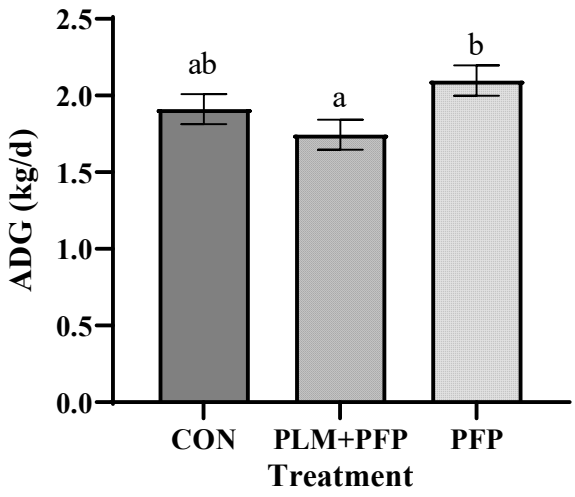

Figure 2.1. Average daily gains (ADG) of steers fed finishing diets consisting of corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (control; CON; n = 8), alfalfa leaf pellets [ProLEAF MAX (Scoular, Omaha, NE)] and alfalfa stems [ProFiber Plus (Scoular, Omaha, NE)] (PLM+PFP; n = 8), or alfalfa stems (PFP; n = 8) for 63 d. Values represent the least square mean ± SEM and bars with different letters indicate differences ($P \leq 0.05$) in ADG.

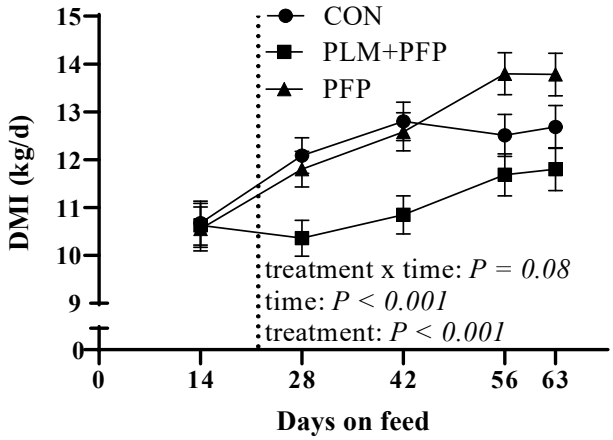

Figure 2.2. Average daily dry matter intake (DMI) of steers fed finishing diets consisting of corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (control; CON; n = 8), alfalfa leaf pellets [ProLEAF MAX (Scoular, Omaha, NE)] and alfalfa stems [ProFiber Plus (Scoular, Omaha, NE)] (PLM+PFP; n = 8), or alfalfa stems (PFP; n = 8) for 63 d. Values represent the least square mean ± SEM. Dotted vertical line denotes the end of feeding the step-up diet and the beginning of feeding the final diet.

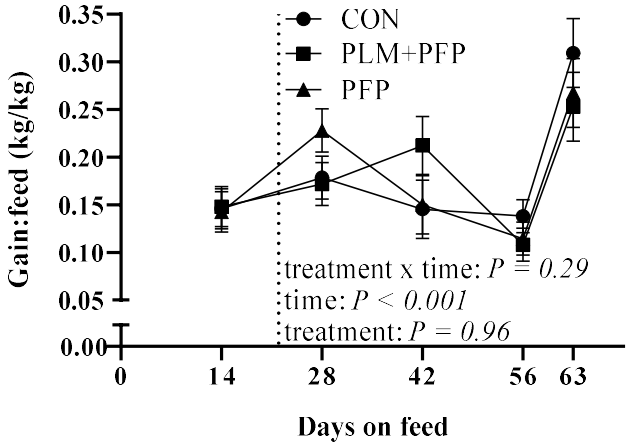

Figure 2.3. Average feed efficiency (gain to feed, G:F) of steers fed finishing diets consisting of corn silage, barley, high moisture corn, a feedlot supplement, and either alfalfa hay (control; CON; n = 8), alfalfa leaf pellets [ProLEAF MAX (Scoular, Omaha, NE)] and alfalfa stems [ProFiber Plus (Scoular, Omaha, NE)] (PLM+PFP; n = 8), or alfalfa stems (PFP; n = 8) for 63 d. Values represent the least square mean ± SEM. Dotted vertical line denotes the end of feeding the step-up diet and the beginning of feeding the final diet.

CHAPTER III

REPLACING ALFALFA HAY WITH A NOVEL ALFALFA LEAF PELLET PRODUCT (PROLEAF MAX) AND/OR ALFALFA STEMS (PROFIBER PLUS) IN THE DIET OF DEVELOPING DAIRY HEIFERS INFLUENCES GROWTH AND DEVELOPMENT WHILE HAVING NO IMPACT ON REPRODUCTIVE PERFORMANCE

ABSTRACT

Alfalfa is a commonly grown forage in the Intermountain West region of the US and is often included in the diet of dairy animals. Alfalfa provides a variety of different nutrients, but the nutrient content of alfalfa varies depending on factors such as the soil, region, cutting, and climate. However, alfalfa leaves tend to have less variation in their nutrient content than alfalfa stems. Fractionating alfalfa may be one way to improve the consistency of nutrients provided when included in the ration of dairy heifers. The purpose of this study was to determine how inclusion of fractionated alfalfa in the diet impacts growth and conception rates of developing dairy heifers. Heifers were allocated to one of three treatments: a control group fed a typical diet (CON; n=8), a typical diet that replaced alfalfa with fractionated alfalfa leaf pellets and alfalfa stems (ProLEAF MAX + ProFiber Plus; PLM+PFP; n=8), or a typical diet that replaced alfalfa with alfalfa stems (PFP; n=8) for 85 d. Heifers were fed individually twice daily and weight, hip height, and wither height were recorded every 14 d. Additionally, blood was collected every 28 d and conception rates were recorded at the end of the trial. Heifers receiving the PFP diet had lower weight gain ($P < 0.001$), hip height ($P = 0.045$), and wither height

(*P = 0.003*) compared to heifers receiving the CON or PLM+PFP diets when analyzed as repeated measures over time. However, total weight gain throughout the 85 d feeding period and ADG were not different (*P = 0.49)* between treatment groups. The interaction of treatment x time impacted dry matter intake (*P < 0.004*) and blood urea nitrogen (*P < 0.001*) when analyzed as repeated measures over time. Heifers receiving the PFP diet had decreased (*P < 0.001*) DMI when compared to heifers receiving the CON and PLM+PFP diets, however, feed efficiency was not different (*P = 0.82*) between the different treatment groups. Blood urea nitrogen decreased (*P < 0.001*) in heifers receiving the PFP diet as time went on. Conception rate did not differ (*P = 0.66)* between treatment groups. Total feed cost was lowest (*P < 0.001*) for the PFP diet and cost of gain tended (*P = 0.095*) to be increased for the PLM+PFP diet when compared to the CON diet. Overall, these data indicate that including alfalfa stems in a developing heifer diet may lower input costs and increase profitability but decrease growth.

INTRODUCTION

Dairy heifers are responsible for replacing older and less productive cows of the milking herd. Of the total cost of raising dairy heifers, feed usually accounts for 50% of those costs (Zwald, 2007). The overall goal of dairy heifer development is to raise heifers that are efficient and profitable. In order to achieve the development of efficient and profitable dairy heifers, the impacts of nutritional management on heifer development must be understood (Akins, 2016). Heifers should be fed a diet that targets high feed efficiency (FE), but also minimizes the risk of over conditioning (Akins, 2016), as excess adipose deposition in the mammary gland during development can lead to reduced milk production in the future (Sejrsen et al., 1982). Alfalfa is a nutrient dense feedstuff that is commonly included in the diets of beef and dairy cattle. However, the alfalfa plant varies in nutrient content depending on the stage of the soil, region, cutting and climate. Alfalfa ranges from 12-20% protein and 20-28% crude fiber (Balliette and Torell, 2015), depending on the stage of maturity of the plant. As the alfalfa plant matures, the alfalfa leaf protein content declines slightly, while the alfalfa stem protein content declines to a much greater extent (Sheaffer et al., 2000). The digestibility and neutral detergent fiber (NDF) concentration of alfalfa leaves decreases as the alfalfa plant matures, while the NDF and acid detergent fiber (ADF) concentrations of alfalfa stems increases as the plant matures (Fick and Onstad, 1988). The variability that is seen in the alfalfa plant can make it difficult to develop a total mixed ration (TMR) for animals. Therefore, it is important to investigate the effects of including fractionated alfalfa in the diets of livestock animals as providing a TMR that is consistent in nutrient density is desired. The objective of this study was to examine the effects of including a novel alfalfa leaf pellet product

[ProLEAF MAX (Scoular, Omaha, NE); PLM; (Pratt and Jackson, 2018)] and alfalfa stems [ProFiber Plus (Scoular, Omaha, NE); PFP; (Pratt and Jackson, 2018)] in the diet on growth, conception rates, and economic costs of developing dairy heifers when compared to heifers fed a typical developing heifer diet. We hypothesized that heifers consuming diets that included fractionated alfalfa products would have altered growth and development when compared to heifers that consumed diets that included alfalfa hay.

MATERIALS AND METHODS

Heifers

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Utah State University, approval number IACUC-2821, and heifers were cared for in accordance with the Live Animal Use guidelines (FASS, 1999). Twenty-four Holstein heifers that were approximately eight months of age and similar in weight (341 kg ± 9.7 kg) were selected from two different herds, the Utah State University Caine Dairy Farm herd (n=12) or the Ropelato Dairy Farm herd (n=12). Heifers were housed in a covered barn in individual pens with free choice access to water. Pre-trial, heifers were subjected to a 14 d adjustment period. Over the course of the adjustment period, all heifers were fed a typical background diet that included the following ingredients (dry matter (DM) basis): alfalfa hay (28%), oat hay (18.3%), barley straw (0.1%), corn silage (37.2%), steam flaked corn (14.6%), and a developing heifer mineral supplement (1.8%). The nutrient composition of all forages included in the background diet and treatment diets can be seen in Table 3.1. Initially, heifers were stratified by body weight and randomly assigned to one of the three different treatment groups. After the adjustment period, heifers were fed their assigned experimental diet for

85 d. The three different treatment diets included oat hay, barley straw, corn silage, steam flaked corn, a developing heifer supplement, and either alfalfa hay (control; CON; n=8), alfalfa leaf pellets and alfalfa stems (PLM+PFP; n=8) in place of alfalfa hay, or alfalfa stems (PFP; n=8) in place of alfalfa hay. Nutrient composition of the different treatments can be found in Table 3.2. ProLEAF MAX was included in a treatment diet with PFP because the goal was to create a hay out of the two products. Additionally, PLM was included in a treatment diet with PFP to ensure that there was an adequate amount of long-stem forage in the diet so rumen health was not compromised. All diets were balanced to include similar amounts of forage. Each of the three diets that were fed were formulated to be isocaloric and isonitrogenous using AMTS (Groton, NY). Although treatment diets in the present study were all balanced to be isocaloric and isonitrogenous, post-trial nutrient composition analysis of the diets revealed that there were slight differences between the nutrient composition of the diets that were initially balanced and the actual nutrient compositions of the treatment diets. Diets were mixed every 2 d and fed twice daily at 08:00 and 16:00 h. All feed ingredients for the diets were weighed, loaded into a commercial mixer, and then mixed for approximately 15 min to ensure a homogenous mixture of all ingredients.

Daily dry matter intake (DMI) was measured using the clean-bunk management system as previously described (Pritchard and Bruns, 2003). In brief, feed offered was weighed and the following day individual bunks were cleared out and feed refusals were weighed so that daily adjustments in feed offered could be made. To ensure that animals were receiving their feed *ad libitum*, feed bunks were managed to achieve 0.9 kg of refusals per day. The PFP diet required the addition of urea to ensure that all diets were

isonitrogenous, thus, urea was top-dressed to all bunks receiving the PFP diet and mixed in manually. Every 14 d, weight, hip height (HH), and wither height (WH) were recorded at approximately 0700 h. Every 28 d, blood serum was collected for blood urea nitrogen (BUN) analysis. Feed efficiency calculated as gain to feed (G:F) was determined by assessing DMI and average daily gain (ADG). Dry matter intake was calculated daily as described above, however, in this manuscript DMI will be presented as 14 d averages in order to align with the weight gain data.

Harvest and preparation of fractionated alfalfa products

A leaf combine (Pratt and Jackson, 2018), which is carried on a self-propelled vehicle, was used to fractionate the alfalfa plant into PLM, pelleted alfalfa leaves, and PFP, alfalfa stems. The leaf combine strips the alfalfa leaves from the standing alfalfa plant and the leaves were conveyed onto a trailer. The alfalfa leaf fraction was then transported by truck to a drying facility for curing and processing into pellets. The stem alfalfa fraction was cut, conditioned, and windrowed to be baled when dry.

Feed sample analysis

Samples of alfalfa hay, oat hay, barley straw, corn silage, steam flaked corn, PLM, and PFP were collected pre-trial and analyzed at a commercial lab (Cumberland Valley Analytical Services, Waynesboro, PA) for nutrient composition. Samples of the PLM and PFP were collected each time a new batch was delivered. A sample of the TMR was collected three times per week immediately following feed delivery to the bunks and urea top-dressing. All samples were frozen at -20°C and sent for nutrient composition analysis after completion of the trial. A composite sample of TMR for each diet each week was sent for analysis at a commercial lab.

Blood urea nitrogen

Blood was collected every 28 d into 10.0 mL, 16 x 100 mm BD Vacutainer Serum Blood Collection Tubes. After coagulation, blood was cooled at 4°C and serum was extracted the next day after a 15 min centrifugation at a speed of 1,500 rpm. Serum was stored at -20°C for future analyses. Blood urea nitrogen was determined using a commercially available BUN detection kit (Urea Nitrogen Colorimetric Detection Kit, Invitrogen™, Carlsbad, CA) and following the manufacturer specifications. Results of the BUN detection assays were analyzed on a BioTek® Synergy H1 plate reader (BioTek, Winooski, VT) using the program Gen5™ version 2.09. Intra-assay CV: 3.84%. Inter-assay CV: 1.69%.

Reproduction

Once heifers reached an approximate average of 55% of their mature body weight (340-363 kg), they were synchronized using a 5-Day CIDR Synchronization protocol and bred to sexed semen from the Holstein bull, DIAMONDBACK, using a single service of artificial insemination. A licensed veterinarian checked heifers for pregnancy using ultrasound imaging 30 d after artificial insemination.

Economic Analysis

To make an economic comparison of the treatments, the total feed cost (TFC) and cost of gain (COG) was calculated and compared for each treatment. Total feed costs were calculated for each heifer as the summed product of total feed (kg as-fed) and the weighted cost ($/kg) of each individual feed component where the weights were equal to the percentage of each feed component in the total diet. Five-year historical average prices (LMIC, 2020) were used for all feed components other than the alfalfa leaf pellets,

alfalfa stems, urea, and mineral supplement for which actual prices were used. As corn silage, oat hay, and barley straw prices are seldom collected and reported, the following assumptions were relied upon to estimate the prices of those feedstuffs within the diets: corn silage price ($/ton) = 9 x corn price ($/bu), oat hay price = 2/3 x grass hay price, barley straw price= 1/3 x grass hay price. Once the TFC for each treatment was calculated, comparisons were made with the intuitive understanding that greater relative TFC indicates additional expenses associated with feeding. Total feed costs were divided by total weight gain to calculate COG. Cost of gain estimated in the present study considers marginal changes to the cost of feed and represents the feed cost ($) that could be expected to achieve one additional kg of weight gain.

Statistical analyses

This study utilized a completely randomized design. Initially, heifers were stratified by weight so that no differences in starting weights were present and then randomly allocated to one of the three different treatment groups. All data was analyzed using the MIXED procedure of SAS® (version 9.4; SAS Institute Inc., Cary, NC). Treatment was the main effect. Individual heifer and farm origin were included as random effects in the model. The variables that were analyzed include: total weight gain, ADG, TFC, COG, and conception rates. Repeated measures was used to analyze the following variables over time: weight, HH, WH, DMI, G:F, and BUN. A Tukey-Kramer adjustment was used to determine treatment differences by separation of the least square means. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered to be a tendency for significance. Results are presented as the least squares mean ± standard error of the mean.

RESULTS

Heifer performance

Analysis of body weight as a repeated measure over time between different treatment groups showed that body weight increased ($P < 0.001$) over time for all heifers and treatment had an effect ($P < 0.001$) on body weight gain such that heifers that received the CON diet had increased ($P < 0.001$) body weight gain when compared to heifers receiving the PLM+PFP and PFP diets (Table 3.3). No effects of treatment x time ($P = 0.98$) were observed (Table 3.3). Treatment did not affect ADG ($P = 0.49$), the total amount of weight gained over the 85 d feeding period ($P = 0.49$), or G:F ($P = 0.82$) (Table 3.3). No effects of treatment x time were observed for HH ($P = 0.87$) or WH ($P = 0.80$) when analyzed as repeated measures (Table 3.4). Analysis of HH as a repeated measure over the 85 d feeding period showed that HH increased ($P < 0.001$) over time in all heifers and treatment had an effect ($P = 0.009$) on HH such that heifers that received the PFP diet had lower ($P = 0.009$) HH than heifers receiving the CON and PLM+PFP diets (Table 3.4). Analysis of WH throughout the 85 d feeding period demonstrated that WH increased ($P < 0.001$) over time in all heifers and treatment had an effect ($P = 0.003$) on WH such that heifers that received the PFP diet had lower ($P = 0.003$) WH than heifers receiving the CON diet and the PLM+PFP diet (Table 3.4).

Analysis of average daily DMI as a repeated measure over time showed that treatment x time impacted DMI ($P = 0.004$) (Fig. 3.1). Overall, heifers receiving the PFP diet had decreased DMI when compared to heifers receiving the CON ($P < 0.001$) and PLM+PFP ($P < 0.001$) diets throughout the 85 d feeding period (Fig. 3.1). As time goes on, repeated measures analysis shows that after d 28, DMI was increased ($P < 0.001$) in

heifers receiving the PFP diet (Fig. 3.1). Heifers receiving the CON diet had increased DMI when compared to heifers receiving the PLM+PFP ($P = 0.002$) and PFP ($P < 0.001$) diets on d 14 and d 42 through d 85 (Fig. 3.1).

Blood urea nitrogen

Analysis of BUN as a repeated measure over time of the heifers throughout the 85 d feeding period showed that the effect of treatment depended on time ($P < 0.001$) (Fig. 3.2). As time goes on, repeated measures analysis shows that BUN decreases ($P < 0.020$) in heifers receiving the PFP diet when compared to heifers receiving the CON diet (Fig. 3.2).

Conception rates

No differences ($P = 0.66$) in conception rate between the treatment groups were observed (data not shown). However, the heifers that received the PLM+PFP diet had the highest conception rates, numerically, out of the three different treatment groups (values represent the LSM ± SEM; CON: 42.4% ± 27.4, PLM+PFP: 62.5% ± 27.3, PFP: 59.9% ± 28.0).

Feed cost comparison

Least square means for TFC were calculated for each treatment equal to $123.48, $136.52, and $97.27 for heifers receiving the CON, PLM+PFP, and PFP treatments, respectively (Table 3.5). Over the 85 d trial TFC showed an effect ($P < 0.001$) of treatment such that the heifers receiving the PFP diet had a lower TFC than heifers receiving the PLM+PFP ($P < 0.001$) and CON diets ($P = 0.001$) (Table 3.5). Heifers receiving the CON diet tended to have a lower ($P = 0.052$) TFC than the PLM+PFP diet (Table 3.5). Least square means for COG were $1.20, $1.51, and $1.11 for the heifers

receiving the CON, PLM+PFP, and PFP treatments, respectively (Table 3.5). Over the 85 d feeding period, COG tended ($P = 0.094$) to be increased for heifers receiving the PLM+PFP diet when compared to heifers receiving the PFP diet. However, no differences ($P > 0.094$) in COG were observed between heifers receiving the CON diet and heifers receiving the PLM+PFP or PFP diets.

DISCUSSION

The present study aimed to determine whether inclusion of fractionated alfalfa in the diet of developing heifers impacts performance. The nutrient composition of the alfalfa plant is variable, and by fractionating the plant into the leaf and stem portions, this variability can be decreased and a more consistent TMR can be produced and fed. Inclusion of alfalfa leaf products, such as alfalfa leaf meal (ALM), in the diet of livestock animals provides many benefits. Alfalfa leaf meal has an energy content that is similar to a high-quality hay or small grain silage (DiCostanzo et al., 1999) and a crude protein content of 22-28% (Jorgensen et al., 1997; DiCostanzo et al., 1999), which is two to three times the crude protein content of alfalfa stems (Mowat et al., 1965; Mowat and Wilton, 1984; Albrecht et al., 1987). However, alfalfa stems can also be utilized to improve livestock diets. In contrast to alfalfa leaves, alfalfa stems have a high fiber content, similar to that of straw, but have twice the crude protein content of straw (Su et al., 2017). As such, alfalfa stems could be used to replace straw in a livestock diet while providing more protein, which is especially important for growing animals.

Upon completion of the feeding trial, the three different treatment diets were analyzed. All treatment diets were balanced to be isocaloric and isonitrogenous, however, post-trial analyses showed slight differences in nutrient content between the different

treatment diets. The differences observed in the nutrient compositions between the treatment rations is most likely due to the variability in nutrient content between the different forage sources (Table 3.1). The CON diet had less ADF and NDF when compared to the other two treatment diets. The variations that were present between the different treatment diets could have influenced the results in the present study.

To the authors' knowledge, this is the first study that has investigated the effects of feeding fractionated alfalfa to developing heifers. Previously, our group published a paper that examined the effects of feeding fractionated alfalfa on growth and performance of finishing beef steers (Motsinger et al., 2021). Several other studies have looked at the effects of feeding alfalfa leaf meal (ALM) or alfalfa stem haylage and, as such, the results of the present study will be compared to these studies.

In the present study, decreased weight gain, HH and WH was observed in heifers that received the PFP diet when analyzed over time as a repeated measure. No differences in total weight gain or ADG between the different treatment groups were observed. However, Su et al. (2017) performed a similar study investigating the effects of feeding alfalfa stem haylage on performance of Holstein dairy heifers. In their study, Su et al. (2017) fed three different treatments (corn silage and alfalfa haylage; corn silage, alfalfa haylage, and alfalfa stem haylage; or corn silage, alfalfa haylage, and wheat straw) to Holstein heifers and measured weight gain and growth (as measured by HH, WH, heart girth, and body condition score). Similar to the results of the present study, Su et al. (2017) observed decreased weight gain and decreased growth parameters (HH, WH, heart girth, and body condition score) when alfalfa stem haylage was included in the diet. In contrast to the results of the present study, in a similar study conducted by our research

group with finishing beef steers, increased body weight gain and ADG were observed in steers receiving the PFP diet when compared to the steers receiving the PLM+PFP diet (Motsinger et al., 2021). However, the treatment diets in the present study utilized higher concentrations of PLM and PFP in the treatment diets than those used by Motsinger et al. (2021). In the present study, the PLM+PFP diet consisted of 22.4% PLM and 12.1% PFP and the PFP diet consisted of 24.7% PFP on a dry matter basis, while in the study completed by Motsinger et al. (2021), the PLM + PFP diet consisted of 13.8% PLM and 5.8% PFP and the PFP diet consisted of 14.0% PFP on a dry matter basis. The results of the present study also contrasted those of Gossett and Riggs (1956) whom observed improved weight gain and daily weight gains in steers that consumed a finishing beef steer diet consisting of low-quality prairie hay, cottonseed meal, and ground milo grain that was supplemented with varying amounts (7, 14, or 21% of the diet DM) of ALM when compared to the diet without ALM supplementation. Of note, all diets fed in the study completed by Gossett and Riggs (1956) were, overall, formulated to be isocaloric and isonitrogenous. However, unlike the present study, Gossett and Riggs (1956) did not include alfalfa in their control diet. In the present study, including PLM in the diet did not improve heifer growth, however, findings from previous studies indicate that supplementation of alfalfa leaf products has the potential to improve weight gain and growth in cattle. As such, additional research needs to be completed to determine the effects of different alfalfa leaf products and inclusion rates on heifer growth.

Examination of DMI showed a treatment x time effect when analyzed as repeated measures. Overall, heifers receiving the PFP diet had decreased DMI when compared to heifers receiving the CON and PLM+PFP diets throughout the 85 d feeding period,

however, there were no differences in G:F between the different treatment groups. The decreased DMI observed in heifers consuming the PFP diet could be explained by the greater fiber content in the PFP diet when compared to the other two treatment diets. Furthermore, the decreased DMI observed in heifers receiving the PFP diet could be responsible for the decreased weight gain and growth observed in heifers receiving this same treatment diet. In contrast to the present study, a previous study conducted by our research group in finishing beef steers found that steers receiving the PFP diet had the highest DMI after d 42 and the highest weight gain throughout the feeding trial when compared to the other two treatments (CON and PLM+PFP), but there were no differences in G:F between the different treatments (Motsinger et al., 2021). However, Gossett and Riggs (1956) observed improved feed efficiency in steers that received a diet that was supplemented with ALM when compared to the steers that received no supplemental ALM. In the present study, PLM did not result in improved DMI when compared to the control, however, DiCostanzo et al. (1999) demonstrated that ALM has the potential to improve DMI of finishing beef steers when constituting 12% (DM) of the diet. An additional study analyzed the effects of including alfalfa stem haylage in the ration and found no differences in DMI of Holstein dairy heifers when alfalfa stem haylage was used to dilute a basal diet that consisted of corn silage and alfalfa haylage (Su et al., 2017). Additionally, in beef heifers fed a corn-based diet that replaced soybean meal with ALM, no differences in DMI were observed (Zehnder et al., 2010). Previous studies indicate that including alfalfa leaf products (ALM or PLM) in the diet of finishing beef steers results in improved DMI (DiCostanzo et al., 1999; Motsinger et al., 2021) and subsequent weight gain (Motsinger et al., 2021). However, in the present study when

PLM was included in the diet for developing dairy heifers, improvements in DMI or growth were not observed. Additionally, feeding PFP in the diets of developing dairy heifers results in decreased DMI and growth. As such, further research is needed to determine the ideal concentrations of PLM and PFP in the diet for developing dairy heifers.

Assessment of BUN concentration is used to measure protein status of the animal (Hammond, 1997) and lean tissue anabolism (Smith and Johnson, 2020). Generally, if cattle are consuming diets that are isonitrogenous at similar rates, decreased serum urea nitrogen is an indication that protein is being incorporated into lean tissue (Smith and Johnson, 2020). In the present study, analysis of BUN concentration as a repeated measure demonstrated that BUN decreased over time in heifers that received the PFP diet. However, no differences were present in crude protein content between the different diets when analyzed at a commercial laboratory and, as such, the decreased BUN over time in heifers that received the PFP diet was not the result of lower CP in the diet. However, these heifers exhibited the lowest weight gain throughout the feeding trial when compared to heifers receiving the CON and PLM+PFP diets which could indicate that heifers metabolized the diets differently, resulting in alterations in circulating metabolites, which could have impacted growth, or a higher protein excretion rate. Furthermore, heifers receiving the PFP diet may have been accreting protein into something other than lean tissue. Blood urea nitrogen concentrations can also affect reproductive performance, such that a plasma urea nitrogen over 19 mg/dL in cows and heifers can decrease conception (Butler et al., 1996). However, in the present study, no BUN concentrations that neared 19 mg/dL were observed. Additionally, no differences in

conception rates were observed between treatment groups in the present study. However, the sample size in the present study was not sufficient for assessing conception rate and as such, these results should be interpreted with caution.

The economic results demonstrate that, as was expected after analyzing the DMI, potential cost saving can be expected through feeding PFP in the diet when compared to a diet with traditional alfalfa (CON). However, cost per kg of weight gain for heifers receiving the PFP diet was not different from heifers receiving the CON diet and tended to be decreased when compared to heifers receiving the PLM+PFP diet. These results demonstrate that inclusion of PFP, rather than whole alfalfa, in a developing dairy heifer diet decreased DMI, weight gain, HH, WH, while having no effect on reproductive performance. As such, if PFP can be procured at a price significantly less than traditional alfalfa, cost savings can be expected in feed costs per head, but DMI and growth may be decreased if PFP is included in the diet. Additionally, COG will not be decreased when feeding PFP to developing dairy heifers when compared to feeding a traditional alfalfa-based diet. However, the \$25.99 difference in expected feed costs ($TFC_{CON} - TFC_{PFP}$) has the potential to have a large impact on a dairy producer profits. Given a 1,000-cow herd with a 33% turnover rate, approximately 330 replacements would be required per year. Thus, based on the data from this study, a dairy of this size may expect total cost savings of \$8,576.70 per year when feeding the PFP diet as compared to the CON when considering raising replacement heifers. Overall, additional research needs to be completed to determine the optimal concentrations of fractionated alfalfa to include in a developing dairy heifer diet that could result in decreased TFC, decreased COG, and unaffected or improved growth.

CONCLUSION

Feed costs account for the majority of input costs required for raising dairy heifers (Zwald, 2007) and, therefore, it is necessary to maximize efficiency of dairy heifer production. Through improved understanding of nutritional management practices and their impacts, we can enhance efficiency, productivity, and profitability of developing heifers (Akins, 2016), whom will ultimately replace older and less productive cows in the milking herd. Overall, the findings of the present study demonstrate that inclusion of PFP, which can be purchased at a lower price than alfalfa hay or PLM, in a developing dairy heifer diet has the potential to decrease TFC but does not affect COG and may result in decreased weight gain and growth parameters, while having no impact on reproductive performance. Through inclusion of PFP in a developing dairy heifer diet, dairy heifer producers may be able to lower their input costs and increase profitability of their operation. However, more research on a larger number of animals is needed to determine the optimal concentrations of fractionated alfalfa to include in diets for developing dairy heifers. Additionally, research on lactating dairy cows is needed to further investigate the effects of feeding fractionated alfalfa on dairy production.

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Table 3.1. Nutrient composition of forage sources[1]

	Forage source					
Item	Alfalfa hay	PLM	PFP	Corn silage	Oat hay	Barley straw
DM, %	91.30	89.85	88.52	29.20	93.30	92.80
Analysis, DM basis						
Crude protein, %	13.40	24.05	12.07	8.00	9.90	3.60
ADF, %	40.80	26.40	50.05	29.20	36.30	54.40
aNDF, %	50.10	30.20	59.58	48.00	57.90	77.60
NFC, %	27.50	30.95	22.70	36.10	20.20	8.20
TDN, %	53.30	65.35	49.94	65.50	56.60	48.30
NE_m, Mcal/kg	0.22	0.30	0.22	0.31	0.24	0.19
NE_g, Mcal/kg	0.11	0.18	0.10	0.19	0.13	0.08
Ash, %	9.08	13.20	6.38	6.58	12.00	10.61
Calcium, %	1.11	2.17	0.70	0.22	0.32	0.24
Phosphorus, %	0.26	0.33	0.24	0.22	0.30	0.07
Magnesium, %	0.36	0.35	0.22	0.16	0.17	0.12
Potassium, %	2.57	3.25	2.26	1.43	3.03	1.53
Sodium, %	0.03	0.10	0.16	0.01	0.37	0.02
Iron, mg/kg	166.00	627.50	94.00	104.00	208.00	116.00
Manganese, mg/kg	25.00	57.00	17.50	68.00	82.00	39.00
Zinc, mg/kg	16.00	24.00	16.17	27.00	26.00	25.00
Copper, mg/kg	7.00	9.00	9.33	6.00	4.00	7.00

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE); ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, Non-fiber carbohydrates; TDN, total digestible nutrients; NE_m, net energy for maintenance; NE_g, net energy for gain; Mcal, megacalorie

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to developing dairy heifers for 85 d.

Table 3.2. Composition and nutrient density of treatment diets[1]

	Treatment		
Item	CON	PLM+PFP	PFP
Composition of treatment diets			
Feed, % DM			
Alfalfa hay	27.98	-	-
PLM	-	22.41	-
PFP	-	12.07	24.70
Oat hay	18.29	-	11.02
Barley straw	0.12	13.08	-
Corn silage	37.19	33.77	40.50
Steam flaked corn	14.60	16.88	21.37
Heifer supplement[2]	1.82	1.78	1.82
Urea	-	-	0.61
Nutrient density of treatment diets			
DM, %	58.55	58.50	56.87
Analysis, DM basis			
Crude protein, %	11.30	11.20	11.42
ADF, %	27.35	28.00	29.37
aNDF, %	39.45	40.22	42.07
NFC, %	38.94	37.74	37.71
TDN, %	64.48	63.88	64.48
NE_m, Mcal/kg	0.30	0.30	0.30
NE_g, Mcal/kg	0.18	0.17	0.18
Ash, %	8.52	9.06	7.19

DM, dry matter; PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE); ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, Non-fiber carbohydrates; TDN, total digestible nutrients; NE_m, net energy for maintenance; NE_g, net energy for gain; Mcal, megacalorie

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to developing dairy heifers for 85 d.

[2]The guaranteed analysis for the heifer supplement is as follows: 4.6% crude protein, 1.0% crude fat, 2.6% crude fiber, 8.7% calcium, 0.3% phosphorus, 180 mg/kg copper, 11 mg/kg selenium, 425 mg/kg zinc, and 326.6 mg/kg Monensin.

Table 3.3. Effects of feeding fractionated alfalfa on heifer body weights and feed efficiency over the 85 d feeding trial

	Weight[2] (kg)									
Treatment[1]	d 0	d 14	d 28	d 42	d 56	d 70	d 85[4]	Gain	ADG	G:F
CON	345	361	368	398	408	425	438[a]	104	1.2	0.11
PLM+PFP	339	353	364	385	397	408	427[b]	100	1.2	0.12
PFP	335	355	354	378	387	400	417[b]	91	1.1	0.12
SEM	19.7	19.7	19.7	19.7	19.7	19.7	19.7	8.0	0.1	0.01
Treatment x time[3]							*P = 0.98*	-	-	-
Treatment[3]							*P < 0.001*	*P = 0.49*	*P = 0.49*	*P = 0.82*
Time[3]							*P < 0.001*	-	-	-

PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE), ADG, average daily gain; G:F, gain to feed

[a,b]Means that have a different superscript represent differences ($P < 0.05$) between treatments over the 84 d feeding period.

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to developing dairy heifers for 85 d.

[2]Values within columns represent LSM.

[3]P-values for Treatment x Time, Time, and Treatment when heifer body weights were analyzed over time with repeated measures.

[4]Means that have a different superscript represent differences ($P < 0.05$) between treatments over the 85 d feeding period when analyzed as a repeated measure over time.

Table 3.4. Effects of feeding fractionated alfalfa on hip height and wither height over the 85 d feeding trial

	Hip height[2] (cm)						
Treatment[1]	d 0	d 14	d 28	d 42	d 56	d 70	d 85[4]
CON	135.1	136.0	137.7	138.7	138.5	140.8	140.8[ab]
PLM+PFP	138.5	136.7	137.9	139.2	138.9	139.6	141.1[a]
PFP	135.4	135.3	135.4	137.1	137.9	139.7	138.3[b]
SEM	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Treatment x time[3]							*P = 0.87*
Treatment[3]							*P = 0.009*
Time[3]							*P < 0.001*
	Wither height[2] (cm)						
Treatment[1]	d 0	d 14	d 28	d 42	d 56	d 70	d 85[4]
CON	-	130.2	131.4	132.9	132.1	133.9	134.9[a]
PLM+PFP	-	129.9	132.8	134.1	133.3	133.8	134.1[a]
PFP	-	128.9	129.8	131.2	131.6	133.1	133.8[b]
SEM	-	2.2	2.2	2.2	2.2	2.2	2.2
Treatment x time[3]							*P = 0.80*
Treatment[3]							*P = 0.003*
Time[3]							*P < 0.001*

PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE)

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to developing dairy heifers for 85 d.

[2]Values within columns represent LSM ± SEM

[3]P-values for Treatment x Time, Time, and Treatment when heifer hip heights and wither heights were analyzed over time with repeated measures.

[4]Means that have a different superscript represent differences ($P < 0.05$) between treatments over the 85 d feeding period when analyzed as a repeated measure over time.

Table 3.5. Effects of feeding fractionated alfalfa on total feed costs and cost of gain of developing dairy heifers

	Treatment[1]				
Item	CON	PLM+PFP	PFP	SEM	P-value
TFC[2]	$123.26[a]	$136.52[a]	$97.27[b]	6.68	*P < 0.001*
COG[3]	$1.20[a]	$1.51[a]	$1.11[a]	0.13	*P = 0.094*

PLM, ProLEAF MAX (Scoular, Omaha, NE); PFP, ProFiber Plus (Scoular, Omaha, NE); TFC, total feed costs; COG, cost of gain

[a,b]Means that have a different superscript represent differences (*P < 0.05*) between treatments throughout the 84 d feeding period.

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (CON; n=8), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=8), or alfalfa stems (PFP; n=8) and were fed to developing dairy heifers for 85 d. Values represent the least square mean ± SEM.

[2]TFC ($) is the total cost associated with feeding each treatment for the 85 d feeding period.

[3]COG is equal to the TFC/total weight gain.

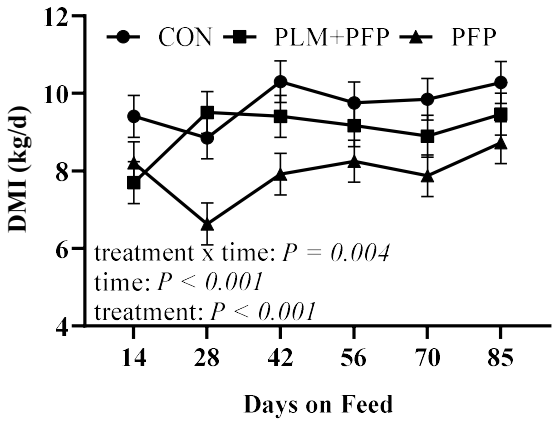

Figure 3.1. Average daily dry matter intake (DMI) of heifers fed developing heifer diets consisting of oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (control; CON; n = 8), alfalfa leaf pellets [ProLEAF MAX (Scoular, Omaha, NE)] and alfalfa stems [ProFiber Plus (Scoular, Omaha, NE)] (PLM+PFP; n = 8), or alfalfa stems (PFP; n = 8) for 85 d. P-values represent the effects of treatment x time, time, and treatment when analyzed with time as a repeated measure and values represent the least square mean ± SEM.

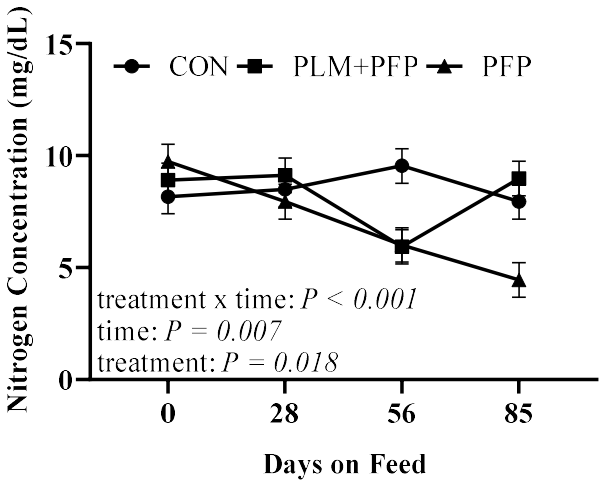

Figure 3.2. Average blood urea nitrogen of heifers fed developing heifer diets consisting of oat hay, barley straw, corn silage, steam flaked corn, a heifer supplement, and either alfalfa hay (control; CON; n = 8), alfalfa leaf pellets [ProLEAF MAX (Scoular, Omaha, NE)] and alfalfa stems [ProFiber Plus (Scoular, Omaha, NE)] (PLM+PFP; n = 8), or alfalfa stems (PFP; n = 8) for 85 d. P-values represent the effects of treatment x time, time, and treatment when analyzed with time as a repeated measure and values represent the least square mean ± SEM.

CHAPTER IV

INCLUSION OF A NOVEL ALFALFA LEAF PELLET PRODUCT (PROLEAF MAX™) AND/OR ALFALFA STEMS (PROFIBER PLUS™) IN THE DIET OF LACTATING DAIRY COWS ALTERS PRODUCTION

INTERPRETIVE SUMMARY

Alfalfa is a forage grown in the Intermountain west region of the United States and is commonly included in lactating dairy cow diets. The present study examined the effects of feeding fractionated alfalfa (stems and leaves) on dry matter intake, milk yield, milk components, body weight, rumination, and somatic cell count of lactating cows. Results indicated that feeding alfalfa stems decreased dry matter intake and feed costs, but improved milk fat. Feeding alfalfa leaf pellets increased milk yield, milk protein, and income over feed cost. Ultimately, dairy producers have the potential to increase production and revenue by feeding fractionated alfalfa.

ABSTRACT

Alfalfa is a commonly grown forage in the Intermountain west and is often included in diets for lactating dairy cows. This study investigated the effects of including novel alfalfa products: ProLEAF MAX™ (PLM), an alfalfa leaf pellet; and ProFiber Plus™ (PFP), alfalfa stems, in the diet of lactating dairy cows on dry matter intake (DMI), milk yield, milk components, body weight (BW), rumination, and somatic cell count (SCC). Holstein cows were housed in a freestall barn and milked in a free-flow automatic milking system (Lely Astronaut 4). All cows were fed each treatment for 21-day periods, then switched to the next treatment utilizing a crossover experimental

design. The five different treatment groups were: control (CON, typical diet that included alfalfa hay); low-quality alfalfa hay (LQ+PLM, alfalfa hay replaced with low-quality alfalfa hay and PLM); PLM+PFP (alfalfa hay replaced with PLM and PFP); PLM (alfalfa hay replaced with PLM); and PFP (alfalfa hay replaced with PFP). Cows were group fed a partial mixed ration balanced for 40.8 kg milk, 3.9% milkfat and 3.3% milk protein and a robot provided pellet based on milk production. Individual milk yield, milk fat, milk protein, BW, rumination, and SCC were recorded daily. Daily pen-level DMI was also recorded, as well as individual robot pellet intake. When fed the PFP diet, cows had decreased DMI compared to feeding the other diets. Milk yield per DMI was increased when cows were fed the PLM diet when compared to feeding the CON, LQ+PLM, and PLM+PFP diets. Milk yield, 150-day adjusted milk yield, and energy corrected milk were increased when cows received the PLM diet compared to the other diets. Feeding the PFP and PLM+PFP diets resulted in increased milk fat and adjusted milk fat when compared to the other diets. Analysis of milk protein yield (kg) and percent showed that feeding the PFP diet resulted in decreased milk protein and adjusted milk protein when compared to other diets. Average BW was increased when cows were fed the LQ+PLM diet when compared to feeding the PLM and PFP diets. Average rumination minutes per day were increased when cows were fed the PFP diet compared to feeding the PLM diet. No differences were observed in SCC between the different diets. Income over feed cost (IOFC) was increased when cows were fed the PLM diet. These data indicate that including PLM in lactating dairy cow diets has the potential to increase milk yield, milk protein, and IOFC while inclusion of PFP may result in decreased DMI, reduced feed costs, and increased milk fat.

INTRODUCTION

Milk production is greatly impacted by nutrition of dairy cattle (NRC, 2001). To optimize the synthesis of milk yield and milk components, adequate nutrients must be provided to the cows (NRC, 2001). To meet energy and protein requirements of lactating dairy cows, a variety of different feed sources can be included in the diet. However, balancing a diet for lactating dairy cattle can prove to be complex. Diets must be formulated to achieve optimum milk yield, milk protein, and milk fat, while meeting the nutrient requirements of the animal (Erickson and Kalscheur, 2020). Additionally, different stages of lactation have differing nutrient requirements. In early lactation, dairy cows are in a negative energy balance due to their energy requirements for maintenance and lactation exceeding their DM and energy intake (Bauman and Bruce Currie, 1980, Vazquez-Añon et al., 1997). In mid to late lactation, dietary energy intake often exceeds energy requirements for maintenance and lactation (Vazquez-Añon et al., 1997). During mid to late lactation, cows need to improve their body condition score (BCS) for the next lactation (Moe, 1981), while avoiding over conditioning. Ensuring proper nutrition throughout the different stages of lactation, while minimizing feed costs, is essential for maximizing efficiency, production, and profitability in dairy cattle operations.

Alfalfa is commonly included in lactating dairy cow diets. Protein and fiber content of the alfalfa plant can vary depending on the stage of growth that it is cut (Balliette and Torell, 2015). The protein and fiber variability in the alfalfa plant can lead to difficulty in developing TMR for lactating dairy cows. Alfalfa leaves tend to have less variability in their nutrient content than alfalfa stems (Popovic et al., 2001). Additionally, the leaf portion of the alfalfa plant has increased protein and decreased fiber content

when compared to alfalfa stems (Popovic et al., 2001). However, little research has been done on the effects of feeding fractionated alfalfa, either the leaves or the stems, in the diets of lactating dairy cows. Fractionation during harvest is possible and may be a good option to help dairy producers refine and control the nutrient composition of the diet their cows are receiving. Therefore, it is important to investigate the effects of including fractionated alfalfa in the diet of lactating dairy cows. The objective of this study was to examine the effects of including a novel alfalfa leaf pellet product (ProLEAF MAX™; PLM) and alfalfa stems (ProFiber Plus™, PFP) in the diet on milk yield and milk components of lactating dairy cows when compared to lactating dairy cows fed a typical lactating cow diet.

MATERIALS AND METHODS

Harvest and Preparation of Fractionated Alfalfa Products

A leaf combine (Pratt and Jackson, 2018), which was carried on a self-propelled vehicle, was used to fractionate the alfalfa plant into PLM and PFP. The combine strips the alfalfa leaves from the standing alfalfa plant and the leaves are conveyed onto a trailer. The alfalfa leaf fraction was then transported by truck to a drying facility for curing and processing into pellets. The stem alfalfa fraction was cut, conditioned, windrowed, and baled when dry.

Cows

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Utah State University, approval number ACUC-2821, and animals were cared for in accordance with the Live Animal Use guidelines (FASS, 1999). Sixty to sixty-five lactating Holstein cows from the Utah State University Caine Dairy Farm were

group housed in a freestall barn with free choice access to water. Cows were milked using a Lely Astronaut 4 automatic milking system (AMS; Maassluis, Netherlands). The partial mixed ration (PMR) was mixed each day and fed once daily at 0700 h.

Treatments

All cows were fed one of the five different treatment PMR diets for 21 days each (average cows per treatment ranged from 60 to 65). Treatments were fed from February 3, 2020, to May 19, 2020, to avoid months where heat stress would typically take place. The PMR was balanced for 40.8 kg milk, 3.9% milkfat and 3.3% milk protein using the AMTS ration balancing program (vs. 4.11.0; Groton, N.Y.). In addition, a robot provided pellet was fed to each cow based on daily milk production. The combined PMR for each treatment was designed to be isonitrogenous and isocaloric. The first 11 days of feeding each treatment diet was used as a "washout period" to allow cows to adapt to the new diet. Treatment diets included corn silage, oat hay, barley straw, beet pulp shred, canola meal, steam flaked corn, concentrate pellets (via the robotic milking system), a premixed supplement and either alfalfa hay (control; CON; n=65), low-quality alfalfa hay and alfalfa leaf pellets (low-quality alfalfa hay + ProLEAF MAX™; LQ + PLM; n=62), alfalfa leaf pellets and alfalfa stems (ProLEAF MAX™ + ProFiber Plus™; PLM + PFP; n=65), alfalfa leaf pellets (PLM; n=62), or alfalfa stems (PFP; n=60). The ingredients and nutrient compositions of the treatment diets are shown in Table 4.1.

Feed Sample Analysis

Samples of alfalfa hay, oat hay, barley straw, corn silage, steam flaked corn, canola meal, beet pulp shred, PLM, and PFP were collected pre-trial and analyzed at a commercial laboratory (Dairyland Laboratories, Inc., Arcadia, WI). Throughout the trial,

a sample of the TMR was collected every other day and a composite sample from each week was analyzed. All TMR samples were stored at -20°C and analyzed after completion of the feeding trial.

Data Collection

Individual cow data were collected from the AMS on a daily basis. Daily milk yield, fat and protein percent, SCC, body weight, rumination, and total pellet programmed and fed were each downloaded into an Excel spreadsheet. Daily robot-level data such as number of cows, total milk, percent time free, milk speed, failures, refusals, and average electrical conductivity were also downloaded into an Excel spreadsheet. Additional values such as pounds of fat and protein, energy-corrected milk and adjusted milk were calculated based on the downloaded values. Dry matter intake of the PMR was calculated using the average daily DMI for the entire pen (n=1) for the last 10 d of each treatment and then dividing by the number of animals in the pen. Total feed intake was calculated as the sum of the PMR, DMI and the robot provided pellet DMI. Average DIM varied between treatments as follows: CON: 150.7 ± 12.1; LQ+PLM: 140.3 ± 12.1; PLM+PFP: 129.6 ± 12.1; PLM: 129.6 ± 12.1; PFP: 137.0 ± 12.1). As such, adjusted DIM was calculated by adjusting DIM to 150 days by subtracting the DIM from 150, multiplying by 0.1 lb of milk, then adding that number to the ECM (Ferguson et al., 2000). The 0.1 lb of milk is the average change in milk per day for herds of this production level in the Rocky Mountain DHIA affiliate (RMDHIA, 2021).

Statistical Analyses

All cows on a single automatic milking system were used in a crossover design. The last 10 d of each treatment were analyzed using the Mixed models procedure of

SAS® (version 9.4; SAS Institute Inc., Cary, NC). The fixed effect of treatment was determined for each variable and the animal was the random effect. The model included animal as a repeated function. Each animal served as its own control and, therefore, the autoregressive function covariance structure was utilized. Degrees of freedom were determined by the Kenward-Roger method. Tukey's means comparison test was used to determine significant differences between treatments. A *P*-value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

To the best of our knowledge, our studies are the first to investigate the effects of pelletized alfalfa leaves and alfalfa stems on production of lactating dairy cows. However, other studies have examined the effects of similar products, such as alfalfa leaf meal (ALM), on performance of other livestock species. Alfalfa leaf meal, similar to the alfalfa leaf pellet product used in the present study, has a similar energy content to that of small grain silage or a high quality hay (DiCostanzo et al., 1999) and has a crude protein content of 22 to 28% (Jorgensen et al., 1997, DiCostanzo et al., 1999), which is two to three times the crude protein content of alfalfa stems (Mowat et al., 1965, Mowat and Wilton, 1984, Albrecht et al., 1987). Additionally, ALM is known to have an increased digestibility and decreased fiber content when compared to whole alfalfa or alfalfa stems (Collins, 1988, Buxton and Brasche, 1991, Titgemeyer et al., 1992).

Of note, the five different treatment diets were balanced to be isocaloric and isonitrogenous; however, post-trial analysis showed some small differences between the treatment diets (Table 4.1). The PFP diet had slightly increased ADF and NDF and the PLM+PFP diet had slightly increased starch percent when compared to the other

treatment diets. Additionally, the LQ+PLM diet had increased CP when compared to the other treatment diets. These slight differences in nutrient composition between the different treatment diets could have influenced the observed results.

Milk Yield

Analysis of milk yield showed that feeding the PLM diet resulted in increased ($P < 0.01$) milk yield, ECM and adjusted milk yield when compared to the other treatment diets. Feeding the PLM+PFP diet resulted in increased ($P < 0.01$) milk yield, ECM and adjusted milk yields compared to feeding the CON and LQ+PLM diets (Table 4.2). These data show that feeding the PLM diet has the potential to improve milk yield, possibly due to increased digestibility. Beauchemin (1991) fed lactating dairy cows diets with varying NDF concentrations and alfalfa cuttings and observed decreased milk yield as NDF concentration increased from 31 to 37%. Beauchemin (1991) found that as NDF concentration in the diet increased, milk fat content increased. In the present study, the PFP diet had increased NDF concentration and milk fat percent (Table 4.2); however, the present study did not observe decreased milk yield when compared with feeding the CON diet. Oba and Allen (1999) observed increased milk yield and DMI as NDF digestibility of the diet increased; therefore, in the present study, it is likely that the PLM treatment diet had improved NDF digestibility compared to the other treatment diets. Interestingly, feeding the PFP treatment decreased both milk yield and DMI, yet resulted in a milk/DMI similar to when feeding the PLM treatment diet. While not ideal, the decreased feed costs by feeding PFP, without sacrificing efficiency, may be of interest to some producers.

Milk Components

Milk fat and protein are important to the dairy industry because most dairy producers are paid based on the yield of milk components. Analysis of milk components demonstrated that feeding the PLM+PFP or PFP diets increased ($P < 0.05$) milk fat percent when compared to feeding the other diets (Table 4.2). Similar to the present study, Beauchemin (1991) observed increased milk fat as NDF concentration in the diet increased. In the present study, the PFP diet had increased NDF. However, the PLM+PFP diet did not have increased NDF concentration. Results of the present study also showed that feeding the LQ+PLM diet resulted in increased ($P < 0.01$) milk protein percent when compared to the other diets and feeding the PFP diet resulted in decreased ($P < 0.05$) milk protein percent compared to the other diets (Table 4.2). Milk protein yield was increased ($P < 0.05$) when cows were fed the PLM diet compared to the other diets (Table 4.2). In a study by Broderick (2003), lactating dairy cows fed various levels of energy and protein in the diet displayed increased milk protein as dietary CP and energy were increased. In the present study, the LQ+PLM and PLM treatments had increased CP when compared with the other treatments. These data suggest that inclusion of PLM in the diet of lactating dairy cows has the greatest potential to increase milk fat and protein yield and inclusion of PFP in the diet has the potential to increase milk fat yield. Dairy cows need sufficient NDF in their diets to maintain rumen health and optimum milk yield and milk components, however, if dietary NDF is too undigestible, DMI can be limited due to physical fill of the rumen (Oba and Allen, 1999). The results from the present study demonstrated improvement in milk fat and protein yield when cows consumed the PLM diet, which had the decreased undegradable neutral detergent fiber (uNDF) compared to the other treatment diets (Table 4.1). Additionally, the PFP treatment diet had increased

uNDF compared to the other treatment diets, which likely contributed to the decreased DMI and milk protein percent.

DMI

Insufficient DMI can result in low milk yields, excessive loss of body weight, and poor reproductive performance (Bernard and Montgomery, 2015). As such, it is important to ensure that DMI during times of peak milk production is maximized. In the present study, there were no differences ($P > 0.05$) in intake of the concentrate pellet provided by the AMS between treatment groups (Table 4.3). Treatment had an effect on PMR intake such that feeding the PFP treatment diet resulted in decreased ($P < 0.01$) PMR intake compared to the other treatment diets (Table 4.3). Total DMI (concentrate pellet + PMR intake) showed that feeding the PFP treatment diet resulted in decreased ($P < 0.01$) total DMI when compared to the other treatment diets (Table 4.3) due to PMR intake. In contrast to the present study, Su et al. (2017) did not observe decreased total DMI in Holstein heifers when alfalfa stem haylage was used to dilute a basal diet that consisted of corn silage and alfalfa haylage when compared to dilution with wheat straw. Zehnder et al. (2010) did not observe improved DMI in beef heifers that were offered a diet that replaced soybean meal with ALM in a corn-based diet, which is similar to the results that were observed in the present study. However, a study completed by DiCostanzo et al. (1999) investigated the effects of feeding finishing steers a diet that substituted ALM for hay and soybean meal and found that DMI could potentially be improved when ALM was included in the diet.

Feeding the PLM treatment diet resulted in increased ($P < 0.01$) milk per DMI when compared to feeding the CON, LQ+PLM and PLM+PFP treatment diets, but was

not different (P > 0.10) than feeding the PFP diet (Table 4.3). Oba and Allen (1999) observed that DMI and milk yield were increased as NDF digestibility of the forage increased. In the present study, the PFP treatment diet had decreased NDF content compared to the other treatment diets and, most likely, decreased digestibility compared with the other treatment diets (Table 4.1). This could be the cause of the observed decreased DMI. Overall, these data demonstrate that feeding PLM in the diet has the potential to increase milk/DMI and feeding PFP in the diet may decrease DMI and milk yield but result in milk/DMI similar to when feeding PLM.

BW

In the present study, analysis of average BW showed that feeding the LQ+PLM treatment diet resulted in increased (P < 0.05) BW compared to when feeding the PLM and PFP treatment diets (Table 4.2). Broderick (2003) and Su et al. (2017) fed various levels of protein and energy in the diets of lactation dairy cows and found that BW gain increased as dietary energy increased. In the present study, however, the LQ+PLM treatment diet did not have increased energy content when compared to the other treatment diets; therefore, energy content was likely not a factor. Additionally, in the present study, treatment diets were balanced to be isocaloric and energy content between the different treatment diets only varied from 63.8% to 67.3% TDN, while the energy content of the treatment diets in the previous studies were much more variable and ranged from 63-69% TDN (Broderick, 2003) and 59.1-65.4% TDN (Su et al., 2017).

While no previous studies have investigated the effects of feeding alfalfa leaf pellets and alfalfa stems to lactating dairy cows, our group has previously examined the effects of replacing alfalfa hay with PLM+PFP or PFP in the diet on growth and carcass

characteristics of finishing beef steers (Motsinger et al., 2021) and growth and reproductive performance of developing dairy heifers, L. A. Motsinger (Utah State University, Logan, UT, unpublished data). In finishing feedlot steers, we found that replacing alfalfa hay with PFP in the TMR resulted in increased DMI and weight gain; however, no differences in feed efficiency (FE) or carcass characteristics were observed (Motsinger et al., 2021). In developing dairy heifers, we observed decreased DMI, weight gain, HH, and WH in heifers that received a TMR that included PFP in place of alfalfa hay, L. A. Motsinger (Utah State University, Logan, UT, unpublished data). Additionally, by replacing alfalfa hay with PFP in the diet of developing dairy heifers, total feed cost was decreased, L. A. Motsinger (Utah State University, Logan, UT, unpublished data). Replacing alfalfa hay with fractionated alfalfa products in the TMR of developing dairy heifers had no impact on reproductive performance of the heifers, L. A. Motsinger (Utah State University, Logan, UT, unpublished data). Overall, inclusion of PFP in a developing dairy heifer diet decreases growth, L. A. Motsinger (Utah State University, Logan, UT, unpublished data), which will likely increase the age at which heifers enter the milking herd. Additionally, delayed growth of developing dairy heifers may have adverse effects on future milk production.

Rumination

Rumination is important for maintaining sufficient levels of DMI, optimum digestion of consumed feeds, reducing feed particle size, and minimizing risk of rumen acidosis (Beauchemin, 2018). Previous studies have demonstrated that increasing particle size of the diet and increasing the forage to concentrate ratio of the diet are factors that promote rumination, and therefore, promote overall rumen health (Yang and

Beauchemin, 2007, 2009, Beauchemin, 2018). In the present study, feeding the PFP treatment diet resulted in increased ($P < 0.05$) rumination minutes when compared to the PLM treatment diet (Table 4.2). The PLM treatment diet had decreased NDF% and uNDF% when compared to the other treatment diets which may be related to the decreased number of rumination minutes per day that were observed when feeding the PLM treatment diet. Overall, these data show that feeding all treatment diets resulted in rumination minutes that were over 400 minutes per day, which indicates good rumen health (Adin et al., 2009).

Other Measurements

In the present study, no differences ($P > 0.05$) in SCC were found between the different treatment diets (Table 4.2). Of note, none of the treatment diets resulted in SCC above 200,000 cells/mL, which is indicative of mastitis (Harmon, 1994).

Refusals are a measure of the average number of times per day that a cow comes to the robot but is not milked; usually because enough time has not elapsed since the last milking. The PFP treatment had a greater ($P < 0.05$) number of refusals than all other treatments (Table 4.2). However, the number was not considered excessive.

Economic Analysis

The treatments were compared economically by calculating the expected income over feed cost (IOFC) per cow per day for each treatment. The IOFC estimated only considered expected revenue from milk sales and expected feed costs while holding all other budget items constant across treatments. The IOFC were calculated as the difference between the milk revenue and feed expenses. Milk revenue was estimated based on historical milk fat, milk protein, and other milk solids price data from Northern

Utah. The 5-year average price values for milk fat, milk protein, and other milk solids together with the average milk fat and milk protein content observed for each treatment were used (Table 4.2) to estimate gross revenue from milk production for each treatment. The feed costs for each treatment were calculated as the summed product of daily quantity (kg as-fed) and cost ($/kg) of each individual feed component. Five-year historical average prices were used for all feed components other than the alfalfa leaf pellets, alfalfa stems, robot pellet, and premixed supplement. For the alfalfa leaf pellets and stems, actual prices were used. The average price for the robot pellet was over the past three years as that is all that was available from the dairy. The premixed supplement price was based on a current national average for mineral supplement for dairy cows. The milk revenues, feed costs, and expected IOFC for each treatment are summarized in Table 4.4. Income over feed cost is increased when feeding the PLM treatment compared to the other treatment diets (Table 4.4). All treatment diets, other than the PFP diet, had increased estimated feed cost when compared to feeding the CON treatment diet (Table 4.4). Yet, all treatment diets, other than the LQ+PLM diet, are expected to have an increased IOFC when compared to feeding the CON treatment diet (Table 4.4). These data demonstrate that feeding the PFP diet may reduce feed costs, while feeding the PLM diet may increase feed costs but result in increased IOFC (Table 4.4).

CONCLUSION

This study was conducted to determine the effects of including novel fractionated alfalfa products in the diet of lactating dairy cows. Inclusion of alfalfa stems (PFP) in the diet resulted in decreased feed costs and DMI; however, it also resulted in increased milk fat. Inclusion of alfalfa leaf pellets (PLM) in the diet resulted in increased milk yield,

ECM, adjusted milk yield, milk yield per DMI, milk protein, and slightly decreased rumination. Additionally, inclusion of PLM in the diet resulted in increased IOFC compared to the other treatment diets. These results suggest that inclusion of fractionated alfalfa products in the diet of lactating dairy cows has the potential to improve milk yield and milk components, which will ultimately result in dairy producers receiving increased returns for their milk.

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Table 4.1. Treatment diets.[1]

	CON	LQ+PLM	PLM+PFP	PLM	PFP
Feed, % of diet DM					
Alfalfa hay	19.8	-	-	-	-
Low-quality alfalfa hay	-	13.1	-	-	-
ProLEAF MAX™	-	7.4	13.1	19.6	-
ProFiber Plus™	-	-	6.6	-	19.5
Corn silage	23.1	22.9	22.9	22.8	22.7
Oat hay	4.1	4.1	4.1	4.1	4.1
Barley straw	3.3	3.3	3.3	5.7	-
Beet pulp shreds	9.9	9.8	9.8	9.8	9.7
Canola meal expelled	8.3	8.2	9.0	9.0	12.2
Steam flaked corn	14.9	10.8	14.7	12.6	15.4
Robot pellet	11.6	11.5	11.5	11.4	11.4
Premixed supplement	5.1	5.1	5.1	5.1	5.0
Analysis, DM basis					
DM, %	45.3	45.5	45.6	47.0	42.4
Crude protein, %	16.8	17.5	16.7	17.2	16.8
NFC, %	36.7	35.4	37.7	37.4	34.3
TDN, %	65.9	66.1	66.8	67.3	63.8
Ne_l (Mcal/kg)	1.50	1.50	1.52	1.53	1.44
Ne_m (Mcal/kg)	0.98	0.99	1.00	1.03	0.91
Starch, %	16.4	16.2	19.1	18.6	16.7
ADF, %	27.3	26.3	25.5	24.6	28.8
NDF, %	35.5	35.1	34.6	33.3	38.5
NDFDom, 240 h	61.5	62.8	61.6	64.2	57.3
uNDF, 240 h	12.9	12.4	12.6	11.2	15.7
Lignin, %	5.3	5.1	5.0	4.9	5.8
Ether extract, %	4.8	5.2	4.9	5.5	4.3
Ash, %	10.0	10.5	9.8	10.4	9.9
Calcium, %	1.6	1.5	1.4	1.7	1.1
Phosphorus, %	0.4	0.4	0.4	0.4	0.4
Magnesium, %	0.3	0.3	0.2	0.3	0.3
Potassium, %	1.4	1.4	1.2	1.2	1.1
Sulfur, %	0.3	0.3	0.3	0.3	0.3
Sodium, %	0.6	0.6	0.6	0.6	0.5
Chlorine, %	0.5	0.6	0.5	0.5	0.4
Iron (mg/kg)	408.7	450.7	377.5	588.0	299.0
Zinc (mg/kg)	113.0	170.5	116.3	117.0	88.5
Copper (mg/kg)	28.0	31.5	23.5	24.5	18.0
Manganese (mg/kg)	110.5	131.3	101.5	123.5	83.5

[1]Treatment diets were balanced for a Holstein cow producing 40.8 kg milk, 3.9% milk fat and 3.30% milk protein. The treatments were: dairy quality alfalfa hay (CON; n=65), low-quality alfalfa hay and alfalfa leaf pellets (LQ+PLM; n=62), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=65), alfalfa leaf pellets (PLM; n=62), or alfalfa stems (PFP; n=60) and were fed to lactating dairy cows for 21 d each. Data reflect the last 10 d of each treatment period.

Table 4.2. Effect of feeding fractionated alfalfa on daily average production values of lactating Holstein cows.

	Treatment[1]						
Item	CON	LQ+ PLM	PLM +PFP	PLM	PFP	SEM	Significance
Milk yield (kg)	33.8^{cd}	34.3^{cd}	35.9^{b}	38.3^{a}	33.9^{d}	1.02	P < 0.01
ECM (kg)	35.0^{c}	35.0^{c}	37.8^{b}	40.1^{a}	36.0^{c}	0.86	P < 0.01
Adjusted milk yield (kg)[2]	35.1^{d}	34.7^{d}	37.0^{bc}	39.4^{a}	35.6^{cd}	0.80	P < 0.01
Milk fat (%)	3.85^{b}	3.71^{c}	4.08^{a}	3.95^{b}	4.11^{a}	0.07	P < 0.01
Milk fat (kg)	1.24^{c}	1.20^{c}	1.37^{b}	1.44^{a}	1.36^{b}	0.03	P < 0.01
Milk protein (%)	3.29^{b}	3.40^{a}	3.29^{b}	3.27^{b}	3.05^{c}	0.03	P < 0.01
Milk protein (kg)	1.08^{c}	1.14^{b}	1.14^{b}	1.22^{a}	1.00^{d}	0.03	P < 0.01
SCC (x1000 cells/mL)	135	119	118	166	136	22.2	P = 0.12
Rumination (min)	472^{ab}	470^{ab}	467^{abc}	453^{bcd}	484^{a}	4.93	P < 0.01
BW (kg)	733.4^{abc}	738.6^{a}	731.8^{ab}	723.0^{c}	723.3^{bc}	9.2	P < 0.01
Refusals	0.9^{b}	0.5^{b}	0.4^{b}	0.7^{b}	1.6^{a}	0.13	P < 0.01

[a-d]Least square mean values in the same row with different superscripts differ (P < 0.05).

[1]Treatment diets consisted of the following ingredients: corn silage, oat hay, barley straw, beet pulp shred, canola meal, steam flaked corn, concentrate pellets (via the automatic milking system), a premixed supplement, and either alfalfa hay (CON; n=65), low-quality alfalfa hay and alfalfa leaf pellets (LQ+PLM; n=62), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=65), alfalfa leaf pellets (PLM; n=62), or alfalfa stems (PFP; n=60) and were fed to lactating dairy cows for 21 d each. Data reflect the last 10 d of each treatment period.

[2]Adjusted milk yield is a measure of energy ECM adjusted for DIM. It is calculated by subtracting the actual DIM from 150 and multiplying by 0.1. That value is then added to ECM.

Table 4.3. Effects of feeding fractionated alfalfa on dry matter intake of lactating dairy cows.

	Treatment[1]						
Item	CON	LQ+PLM	PLM+PFP	PLM	PFP	SEM	Significance
Robot pellet (kg)	4.8	4.9	4.9	5.0	4.9	0.06	$P < 0.01$
PMR (kg)[2]	21.2[a]	21.2[a]	20.6[ab]	20.2[b]	19.2[c]	0.22	$P < 0.01$
Total intake (kg)[3]	26.0[ab]	26.1[a]	25.5[ab]	25.1[b]	24.1[c]	0.22	$P < 0.01$
Milk/DMI	1.36[c]	1.42[bc]	1.44[bc]	1.55[a]	1.51[ab]	0.02	$P < 0.01$

[a-d]Least square mean values in the same row with different superscripts differ ($P < 0.05$).

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, oat hay, barley straw, beet pulp shred, canola meal, steam flaked corn, concentrate pellets (via the automatic milking system), a premixed supplement, and either dairy quality alfalfa hay (CON; n=65), low-quality alfalfa hay and alfalfa leaf pellets (LQ+PLM; n=62), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=65), alfalfa leaf pellets (PLM; n=62), or alfalfa stems (PFP; n=60) and were fed to lactating dairy cows for 21 d each. Data reflect the last 10 d of each treatment period.

[2]Partial mixed ration (PMR) is based on the daily total amount fed to the group divided by the number of cows in the pen and averaged over the last 10 days of the treatment period.

[3]Total intake is the sum of the daily average group PMR and the daily average individual intake of robot pellet per cow.

Table 4.4. Estimated treatment milk revenues, feed costs, and income over feed costs per cow per day.

	Treatments[1]				
Budget Item	CON	LQ+PLM	PLM+PFP	PLM	PFP
Milk Revenues	$18.33	$18.45	$19.31	$20.09	$18.50
Feed Costs					
Alfalfa hay	$1.08				
Low-quality alfalfa hay		$0.69			
ProLEAF MAX™		$0.61	$1.00	$1.47	
ProFiber Plus™			$0.29		$0.79
Corn silage	$0.96	$1.00	$0.93	$0.90	$0.86
Oat hay	$0.15	$0.16	$0.15	$0.15	$0.14
Barley straw	$0.08	$0.08	$0.07	$0.12	
Beet pulp shreds	$0.66	$0.68	$0.63	$0.62	$0.58
Canola meal expelled	$0.75	$0.78	$0.79	$0.78	$1.00
Steam flaked corn	$0.98	$0.74	$0.94	$0.79	$0.92
Robot pellet	$2.42	$2.47	$2.47	$2.52	$2.47
Premixed supplement	$0.86	$0.89	$0.83	$0.81	$0.76
Total Feed Costs	$7.94	$8.10	$8.11	$8.16	$7.52
IOFC[2]	$10.39	$10.35	$11.20	$11.93	$10.98

[1]Treatment diets consisted of the following ingredients as a percent of dry matter: corn silage, oat hay, barley straw, beet pulp shred, canola meal, steam flaked corn, concentrate pellets (via the automatic milking system), a premixed supplement, and either dairy quality alfalfa hay (CON; n=65), low-quality alfalfa hay and alfalfa leaf pellets (LQ+PLM; n=62), alfalfa leaf pellets (PLM) and alfalfa stems (PFP) (PLM+PFP; n=65), alfalfa leaf pellets (PLM; n=62), or alfalfa stems (PFP; n=60) and were fed to lactating dairy cows for 21 d each. Data reflect the last 10 d of each treatment period.

[2]Income over feed cost (IOFC) is the difference between milk revenue and feed cost.

CHAPTER V

UNDERSTANDING THE EFFECTS OF TRENBOLONE ACETATE, POLYAMINE PRECURSORS, AND POLYAMINES ON PROLIFERATION RATES AND ABUNDANCE OF GENES INVOLVED IN MYOBLAST GROWTH AND POLYAMINE BIOSYNTHESIS IN MURINE MYOBLASTS

Abstract

Androgens increase skeletal muscle growth, but the mechanism(s) through which this occurs is unknown. Recent research suggests androgens may increase skeletal muscle growth by modulating the polyamine biosynthetic pathway. As such, the objectives of this study were to investigate the effects of trenbolone acetate (TBA), polyamine precursors (methionine (Met) and ornithine (Orn)), and polyamines (putrescine (Put), spermidine (Spd) and spermine (Spe)) on proliferation rate and mRNA abundance of genes involved in polyamine biosynthesis (ornithine decarboxylase (*ODC*) and S-adenosylmethionine decarboxylase (*AMD1*)) and growth (paired box transcription factor 7 (*Pax7*), Sprouty1 (*Spry1*), and mitogen activated protein kinase (*MapK*)) in C2C12 and Sol8 cells. Cultures were treated with 1% fetal bovine serum and/or 10 nM TBA, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Messenger RNA was isolated at 0.5, 12, and 24 h post-treatment. The effects of cell type and treatment on proliferation and mRNA abundance at each time point were assessed. Cell type had no effect ($P > 0.10$) on proliferation rate or mRNA abundance of any gene assessed at any time point. Each treatment resulted in an increased ($P < 0.01$) proliferation rate compared to control cultures. Relative mRNA abundance of *ODC* was increased 0.5 h and 24 h after treatment with Spe ($P = 0.02$) or Spd ($P = 0.04$), respectively, compared to control cultures. No

differences ($P > 0.10$) in relative mRNA abundance of *AMD1* were observed due to treatment. Relative mRNA abundance of *Pax7* was increased 12 h and 24 h after treatment with Spd ($P = 0.03$) or Put ($P < 0.01$), respectively, compared to control cultures. Sprouty1 tended ($P = 0.09$) to increase 12 h after treatment with Put and did increase ($P = 0.03$) 24 h after treatment with Orn compared to control cultures. Abundance of *MapK* was decreased ($P = 0.03$) 0.5 h after treatment with Met and increased ($P = 0.02$) 24 h after treatment with Met ($P = 0.02$) or Put ($P = 0.02$) compared to control cultures. These results demonstrate that C2C12 and Sol8 clonal lines of mouse myoblasts do not have different proliferation rates or mRNA abundance of the genes measured at the time points assessed. Furthermore, TBA, polyamines and polyamine precursors increase proliferation of murine myoblasts. Polyamines also impact abundance of mRNA involved in the polyamine biosynthetic pathway and polyamines and their precursors alter abundance of mRNA involved in myoblast growth.

1. Introduction

Androgens have become molecules of interest relative to skeletal muscle growth in both humans and livestock species. In humans, testosterone can be used as a remedy for conditions such as sarcopenia, which is muscle atrophy associated with natural aging processes [1-3]. In livestock species, trenbolone acetate (a testosterone analog, TBA) is commonly administered to beef animals in the form of anabolic implants to improve growth and efficiency [4]. Studies have established that administration of androgens results in increased skeletal muscle growth in many species including humans [5, 6], mice [7], and cattle [8], to name a few. For post-natal hypertrophy of mammalian muscle to occur, additional nuclei must be obtained from satellite cells [9-11]. However, the observed effects of androgens on proliferation, differentiation, and protein synthesis are inconsistent throughout the literature. Androgens have been shown to both stimulate [12] and have no effect [13] on proliferation of C2C12 murine myoblast cells and increase proliferation of bovine satellite cells [14, 15]. Additionally, previous research has demonstrated that treatment of C2C12 cells with androgens has no effect on protein synthesis [13], but increases differentiation [12] and treatment of bovine satellite cells with androgens results in increased protein synthesis [8, 16]. As such, this research study was to determine how androgens impact growth of skeletal muscle. Past research suggests that androgens, such as testosterone or TBA, likely interact with the polyamine biosynthetic pathway [15, 17-22]. This may be one mechanism through which androgens are able to enhance skeletal muscle growth.

In the polyamine biosynthetic pathway (Fig. 5.1), methionine (Met), ornithine (Orn), and arginine (Arg) are utilized as substrates to produce putrescine (Put),

spermidine (Spd), and spermine (Spe), which are the three polyamines found in mammalian cells [17, 23, 24]. Polyamines are naturally occurring amino acid derivatives with bioactivities that are essential for growth, cell proliferation, cell differentiation, and protein synthesis [23, 25-28]. In skeletal muscle, polyamine biosynthesis increases during hypertrophy and decreases during atrophy [29]. Androgens are thought to regulate polyamine biosynthesis by increasing expression of two enzymes that are involved in the polyamine biosynthetic pathway, ornithine decarboxylase (*ODC*) and S-adenosylmethionine decarboxylase (*AMD1*) [30, 31]. However, the role of TBA in the polyamine biosynthetic pathway has not been well-characterized in skeletal muscle cells.

An improved understanding of the interaction between anabolic hormones, the polyamine biosynthetic pathway, and growth is essential for development of alternative remedies for muscle atrophy in humans and for the development of alternative growth promoting technologies in cattle. As such, the goal of the present research was to better understand the effects of TBA, polyamines, and polyamine precursors on proliferation and mRNA abundance of genes involved in the polyamine biosynthetic pathway and skeletal muscle growth in proliferating murine myoblasts. We hypothesized that TBA, polyamines, and polyamine precursors would each increases proliferation and mRNA abundance and that TBA would increase abundance of *ODC* and *AMD1*.

2. Materials and methods

2.1 Culture of murine myoblasts

Sol8 and C2C12 mouse myoblast cell lines were obtained from American Type Culture Collections® and grown according to manufacturer specifications. Cells remained stored in liquid nitrogen until resurrection. Cells were cultured in a growth

medium that consisted of phenol-red free Dulbecco's Modified Eagle Medium (DMEM) [32] with 10% fetal bovine serum (FBS) in 75 mL flasks and incubated at 37°C with 5% CO_2 in a water saturated environment [33]. Every 48 h, cells were washed twice with DMEM and new growth medium was added.

2.2 Treatment of myoblast cultures

Cells were plated into 96-well plates for analysis of proliferation rates or into 12-well plates for mRNA isolation at a density of 11,200 cells/cm^2 with fresh growth medium and allowed 24 h to establish. Once cultures reached 70% confluency, they were washed twice with DMEM and then treated with DMEM containing 1% FBS and 10 nM TBA, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Control cultures were treated with DMEM containing 1% FBS. Treatment concentrations for analysis of proliferation rates in the present study were based off of previous work from our laboratory group that demonstrated increased proliferation in bovine satellite cells [15]. Three separate replicates were completed for each cell type.

2.3 Analysis of proliferation rates

Proliferation assays were performed 21 h after treatment using a commercially available proliferation kit (DELFIA, PerkinElmer, Waltham, MA) and following the manufacturer specifications and previously published methods [15]. In short, bromodeoxyuridine (BrdU) was diluted with DMEM to 1:100 and added to cultures. Cultures were incubated with BrdU for 3 h to allow for proliferating cells to be labeled. After incubation, cells were fixed to the plate using fix solution and anti-BrdU was added to the cultures followed by a 1 h incubation period. Results of the proliferation assay were analyzed via fluorescent detection on a BioTek Synergy H1 plate reader using the

all-in-one microplate reader software, Gen 5 2.09 (BioTek Instruments, Winooski, VT). Proliferation assays were run congruently with mRNA isolation to establish the relationship between mRNA abundance and proliferation rate.

2.4 mRNA isolation, quantification, and cDNA synthesis

An Absolutely RNA Microprep Kit (Agilent Technologies, Cedar Creek, TX) was utilized to isolate total RNA from cultures following the manufacturer's protocol. Briefly, cell lysate was collected at three different time points (0.5, 12 and 24 h) post-treatment. Prior to each lysate collection, cells were washed once with phosphate-buffered saline (PBS) solution and cell lysis buffer was added to the cultures. Cell scrapers were used to lyse cells. Cell lysate samples were stored at -80°C until mRNA isolation was performed. Cell lysate samples were vortexed and received an equal volume of 70% ethanol. Each sample was then centrifuged, filtered, subjected to a series of wash buffers, and mRNA was then eluted. All RNA samples were stored at -80°C until quantification. Quantification of mRNA was performed using a Take3 plate on a BioTek Synergy H1 plate reader using the Gen 5 2.09 all-in-one microplate reader software (BioTek Instruments, Winooski, VT). Quality of mRNA was determined by analyzing the 260/280 ratio and all samples that had a ratio greater than 2.0 were considered acceptable. Acceptable RNA samples were deoxyribonuclease (Ambion, Foster City, CA) treated and then converted into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystem, Foster City, CA) as per the manufacturer's protocol.

2.5 Quantitative real-time PCR

Real-time PCR quantification and the TaqMan MGB primer/probe system was used following previously described protocols [34, 35] to evaluate mRNA abundance.

Primers and probes for the genes that were investigated are shown in Table 5.1 and were designed by our laboratory using the Primer Express 3.0 software (Applied Biosystems, Waltham, MA) [15]. Relative mRNA abundance of ribosomal 18S (*18S*, housekeeping gene [34, 35]), *ODC*, *AMD1*, paired box transcription factor 7 (*Pax7*), sprouty1 (*Spry1*), and mitogen activated protein kinase (*MapK*), and were evaluated using an ABI 7500 real-time PCR system (Applied Biosystems, Waltham, MA).

2.6 Statistical analysis

The effect of cell type on proliferation rate and mRNA abundance at each specific time point (0.5, 12 or 24 h) was assessed using the MIXED procedure of SAS® (version 9.4; SAS Institute Inc., Cary, NC) where cell type, cell type^{x}treatment and treatment served as a fixed effect and plate and experiment number were included as random effects in the model. No effects ($P > 0.10$) of cell type^{x}treatment were found relative to proliferation rate or mRNA abundance. As such, the main effects of cell type and treatment were analyzed separately. In addition, no effects ($P > 0.10$) of cell type were observed with any gene at any time point and, as such, cell type was included as a random variable in statistical analyses analyzing the effects of treatment. The effect of each treatment relative to the control on proliferation rate and mRNA abundance at each specific time point (0.5, 12 and 24 h) was analyzed using a series of contrast statements within the mixed procedure of SAS with each treatment serving as the fixed effect and plate, experiment number and cell type as random effects. All proliferation rate data are displayed as the least squares mean ± SEM with values representing the fold change of treated cultures relative to the control cultures (with a set value of 1.0). Gene expression data is presented as the relative mRNA abundance of each sample (calculated as $2^{-\text{relative}}$

threshold cycle (ΔCt)) relative to the control value. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered a tendency for significance.

3. Results

3.1 Effects of cell type on proliferation and relative mRNA abundance

Cell type had no effect ($P > 0.10$) on proliferation rates (Fig. 5.2A). Analysis of cell type on relative mRNA abundance of genes involved in polyamine biosynthesis (*ODC* and *AMD1*) and genes involved in skeletal muscle growth (*Pax7*, *Spry1*, or *MapK*) showed that cell type had no effect ($P > 0.10$) on mRNA expression at 0.5, 12, and 24 h post-treatment (Table 5.2).

3.2 Effect of treatment on proliferation rates of murine myoblasts

Treatment of C2C12 and Sol8 cells with 10 nM TBA, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe each resulted in an increased ($P < 0.010$) proliferation rate when compared to control cultures (Fig. 5.2B).

3.3 Effect of treatment on relative mRNA abundance of genes involved in polyamine biosynthesis

Analysis of treatment on relative mRNA abundance of *ODC* showed that Spe increased ($P = 0.015$) relative mRNA abundance of *ODC* 0.5 h post-treatment when compared to control cultures; however, no differences ($P > 0.10$) were observed 12 and 24 h post-treatment between control cultures and treated cultures (Table 5.3). Relative mRNA abundance of *ODC* was increased ($P = 0.045$) 12 h post-treatment with Spd, but no differences ($P > 0.10$) in *ODC* expression were observed 0.5 and 24 h post-treatment when compared to control cultures (Table 5.3). No differences ($P > 0.10$) in relative mRNA abundance of *ODC* were observed 0.5, 12, or 24 h post-treatment when cells were

treated with TBA, Met, Orn, or Put when compared to control cultures (Table 5.3). Analysis of the effect of treatment on relative mRNA abundance of *AMD1* showed no differences ($P > 0.10$) between the control cultures and the different treatments at 0.5, 12, or 24 h post-treatment (Table 5.3).

3.4 Effect of treatment on relative mRNA abundance of genes involved in skeletal muscle growth

Treatment of C2C12 and Sol8 cells with Spd resulted in increased ($P = 0.031$) relative mRNA abundance of *Pax7* 12 h post-treatment when compared to control cultures; however, no differences ($P > 0.10$) in *Pax7* expression were observed 0.5 and 24 h post-treatment when compared to control cultures (Table 5.4). Additionally, relative mRNA abundance of *Pax7* was increased ($P = 0.001$) 24 h post-treatment when cells were treated with Put when compared to control cultures, but no differences ($P > 0.10$) in *Pax7* expression 0.5 and 12 h post-treatment were observed when compared to control cultures (Table 5.4). No differences ($P > 0.10$) in relative mRNA abundance of *Pax7* were observed at 0.5, 12, or 24 h post-treatment when cells were treated with TBA, Met, Orn, and Spe when compared to control cultures (Table 5.4). Analysis of relative mRNA abundance of *Spry1* showed that cells treated with Put tended to have increased ($P = 0.093$) relative mRNA abundance of *Spry1* 12 h post-treatment when compared to control cultures (Table 5.4). However, treatment with Put had no effect ($P > 0.10$) on *Spry1* expression 0.5 and 24 h post-treatment when compared to control cultures (Table 5.4). Relative abundance of *Spry1* was also increased ($P = 0.028$) 24 h after treatment with Orn when compared to control cultures, but no differences ($P > 0.10$) in *Spry1* expression were observed 0.5 or 12 h post-treatment when compared to control cultures (Table 5.4).

Relative mRNA abundance of *Spry1* at 0.5, 12, or 24 h post-treatment was not different ($P > 0.10$) in cultures treated with TBA, Met, Spd, and Spe when compared to control cultures (Table 5.4). Treatment of cells with Met resulted in decreased ($P = 0.034$) relative mRNA abundance of *MapK* 0.5 h after treatment and increased ($P = 0.021$) relative mRNA abundance of *MapK* 24 h after treatment when compared to control cultures; however, no differences ($P > 0.10$) were observed 12 h post-treatment when compared to control cultures (Table 5.4). Relative mRNA abundance of *MapK* was increased ($P = 0.020$) 24 h after treatment with Put when compared to control cultures (Table 5.4), but no differences ($P > 0.10$) were observed 0.5 and 12 h post-treatment when compared to control cultures (Table 5.4). No differences ($P > 0.10$) in relative mRNA abundance of *MapK* at 0.5, 12, or 24 h post-treatment were observed when cells were treated with TBA, Orn, Spd, or Spe when compared to control cultures (Table 5.4).

4. Discussion

Androgens are potent stimulators of skeletal muscle growth and are known to stimulate cell proliferation [12, 14], differentiation [12], and protein synthesis [8, 16]. Androgens have become hormones of interest in both humans and livestock species, such as cattle, due to their known positive impacts on skeletal muscle growth. In humans, testosterone has been utilized as a treatment for conditions resulting in muscle atrophy, such as sarcopenia [1, 2]. Trenbolone acetate is an androgenic compound that has 3-5 times the androgenic activity and 5-8 times the anabolic activity of testosterone [36] and is widely used in anabolic implants for cattle to improve growth and efficiency [4]. However, the exact mechanisms through which androgens improve skeletal muscle growth has not been fully elucidated [1, 6]. In addition, over 50% of the consumer

population is concerned with exogenous hormones being provided to beef cattle [37]. Previous research in mice [17], chickens [18], and rats [19-21] has shown that androgens interact with the polyamine biosynthetic pathway. As such, an improved understanding of the interaction between androgens, the polyamine biosynthetic pathway, and growth, is necessary for the development of alternative ways to increase skeletal muscle growth. In the present study, the relationship between TBA, polyamines and polyamine precursors on mRNA abundance of genes involved in the polyamine biosynthesis pathway and skeletal muscle growth were examined in proliferating C2C12 and Sol8 murine myoblast cultures.

To the best of the authors' knowledge, no other studies have examined the differences in proliferation or mRNA abundance of the genes measured between C2C12 and Sol8 murine myoblast cells. C2C12 cells are immortalized non-cancerous murine myoblast cells obtained from a muscle sarcoma of adult mice and Sol8 cells are myoblast cells obtained from the skeletal muscle of 4-week-old mice. The present study demonstrates that C2C12 and Sol8 cells respond similarly when treated with TBA, polyamine precursors, or polyamines. As such, utilization of both cell types in future studies may help to improve power of cell culture experiments by examining two different clonal lines.

Increased proliferation rates were observed in murine myoblasts treated with TBA, polyamine precursors, or polyamines when compared to control cultures. Polyamines are naturally occurring amino acid derivatives that are important modulators of growth, cell proliferation, and cell differentiation [17, 23-27]. Putrescine, Spd, and Spe are produced from Met, Orn, and Arg through the polyamine biosynthetic pathway (Fig.

5.1) [17, 23, 24]. Androgenic compounds, such as TBA, are thought to regulate polyamine biosynthesis through increasing expression of *ODC* and *AMD1* [30, 31]. The positive effect of TBA on proliferation rate of bovine satellite cells in culture has been well-established [14, 15, 33]. However, past work in C2C12 cells contrasts from results observed in the present study and demonstrates that treatment with testosterone or dihydrotestosterone has no effect on proliferation rates [13, 38], likely because C2C12 cells only express 0.1% of the androgen receptor mRNA than that found in the skeletal muscle of adult mice [38]. As such, the increased proliferation rates observed in C2C12 cells treated with TBA in the present study was likely due to TBA having increased androgenic and anabolic activity when compared to testosterone [36]. Additionally, effects of polyamines on proliferation rates of C2C12 cells from previous studies are inconsistent. Contrary to the observed results in the present study, a recent study found that treatment of C2C12 cells with putrescine had no effect on proliferation rates 24, 48, 72, or 96 h post-treatment, but increased differentiation [39], while another study observed similar results to the present study and found that treatment of C2C12 cells with concentrations of spermidine that were much less than used in the present study (200, 600, and 2,000 nM) increased proliferation rates 48 h post-treatment [40], indicating that spermidine has a potent effect on proliferation. Additionally, polyamine depletion of mouse fibroblasts cells results in an arrest of cell proliferation [41], demonstrating the importance of polyamines for proliferation. This study supports previous studies and suggests that TBA, polyamine precursors, and polyamines improve skeletal muscle growth by stimulating proliferation of muscle precursor cells.

Ornithine decarboxylase is an enzyme involved in polyamine biosynthesis [30, 31] through the production of Put from Orn [17] (Fig. 5.1). Androgens are thought to directly modulate the polyamine biosynthesis pathway through upregulation of *ODC* [30]. The present study analyzed the effects of TBA, polyamines, and polyamine precursors on mRNA abundance of *ODC* and found that treatment of cultures with TBA had no effect on mRNA abundance of *ODC* when compared to control cultures. The *ODC* gene promotor contains an androgen response element [42] and when the androgen receptor is knocked out in mice, *ODC* mRNA abundance is decreased [43]. Contrary to the results observed in the present study, previous research in mouse skeletal muscle tissue shows that treatment with a selective androgen receptor modulator, a therapeutic compound that has anabolic effects similar to that of anabolic steroids without having the androgenic characteristics, results in increased mRNA expression of *ODC* 14 d after treatment [40], which provides further evidence that an interaction between androgens and the polyamine biosynthetic pathway exists through *ODC*. Abundance of *ODC* was unaffected by treatment with polyamine precursors and was increased when cells were treated with Spe and Spd 0.5 and 12 h after treatment, respectively, when compared to control cultures. In human colon adenocarcinoma cells and intestinal crypt cells from rats, *ODC* activity was decreased after treatment with polyamine precursors [44] and polyamines [45], respectively. These data suggest that treatment of murine myoblasts with polyamines increases *ODC* expression, which may play a role in the increased proliferation rates observed in cells that were treated with polyamines, but treatment with TBA does not impact mRNA abundance of *ODC* at the time points measured.

Another important enzyme involved in the polyamine biosynthetic pathway is *AMD1* [30, 31], which is involved in the production of decarboxylated S adenosylmethionine from adenosylmethionine [17] (Fig. 5.1). Decarboxylated S adenosylmethionine can then stimulate production of Spd and Spe through spermidine synthase and spermine synthase, respectively [23]. In the present study, expression of *AMD1* was unaffected by any of the treatments given. Previous studies have found that *AMD1* is likely a direct target gene of the androgen receptor [42] and when the androgen receptor is knocked out in mice, *AMD1* expression is decreased [43]. To the best of the authors' knowledge, no other published studies have reported the effects of polyamines and their precursors on *AMD1* expression in murine myoblast cells. Overall, the results of the present study indicate that treatment of murine myoblasts with TBA, polyamines, and polyamine precursors does not affect *AMD1* expression at the time points measures. As such, additional research, perhaps at time points different from those assessed in the present study, is needed to further explore the effects of these compounds on *AMD1* expression in murine myoblasts.

Quiescent muscle satellite cells express *Pax7* [10] and, once activated and committed to the myogenic lineage, *Pax7* decreases. Past work has shown that C2C12 cells still express *Pax7*, but at lower abundance than satellite cells [46]. In the present study, treatment of C2C12 and Sol8 cells with Spd or Put resulted in increased relative mRNA abundance of *Pax7* 12 and 24 h post-treatment, respectively. Expression of *Pax7* was unaffected by treatment with TBA, Met, Orn, and Spe. To the best of the authors' knowledge, the present study is the first to investigate the effects of TBA, polyamines, and polyamine precursors on Pax7 expression of proliferating C2C12 and Sol8 murine

myoblasts. However, previous research has assessed the effects of androgens and polyamines on differentiation of C2C12 cells [12, 47]. Past work has demonstrated that treatment of C2C12 cells with the androgenic compound, dihydrotestosterone, results in increased proliferation and differentiation (as measured by creatine kinase activity) as well as increased *Pax7* protein expression in differentiating cells 2 and 4 d after treatment and decreased *Pax7* expression 6 and 8 d after treatment [12], indicating that as C2C12 cells differentiate, *Pax7* expression decreases. Previous research has also demonstrated that proliferating C2C12 cells treated with an *ODC* inhibitor to achieve depletion of cellular polyamines are unable to differentiate and the downregulation of *Pax7* mRNA expression, which is necessary for cell differentiation, is impaired, suggesting that polyamines are important in the modulation of skeletal muscle cell differentiation [47]. Furthermore, the addition of Spd to polyamine depleted C2C12 cells reversed the effects that were previously discussed and resulted in cell differentiation and a more rapid downregulation of *Pax7* mRNA abundance [47]. In contrast to the results of the present study, a previous study found that *Pax7* expression was not affected when proliferating bovine satellite cells were treated with polyamines, but observed increased *Pax7* expression when satellite cells were treated with Met [15]. The increased *Pax7* abundance observed in myoblast cells treated with Spd and Put in the present study demonstrates that more myoblasts may be present or that these myoblasts may be proliferating to replenish the satellite cell pool. However, more research needs to be completed to determine how increased *Pax7* abundance in proliferating myoblasts impacts future growth potential.

Sprouty1 is necessary for renewal of the quiescent satellite cell population and is a marker of decreased satellite cell proliferation [10, 48]. To further examine the effects of TBA, polyamines, and polyamine precursors on genes related to cell growth, expression of *Spry1* was also analyzed. Analysis of *Spry1* abundance demonstrated that treatment of cells with Put tended to increase *Spry1* expression 12 h after treatment. Additionally, expression of *Spry1* was increased when cells were treated with Orn 24 h after treatment. Expression of *Spry1* was unaffected by treatment with TBA, Met, Spd, and Spe when compared to control cultures. To the best of the authors' knowledge, no other studies have investigated the effects of TBA, polyamine precursors, and polyamines on *Spry1* abundance in C2C12 or Sol8 myoblasts. However, recent work completed in bovine satellite cells observed that *Spry1* expression was increased 12 h post-treatment with TBA when compared to control cultures [15], suggesting that treatment of bovine satellite cells with TBA results in decreased proliferation of satellite cells and the return of satellite cells to the quiescent state 12 h after treatment. The results observed in the present study combined with the results from past work suggest that TBA stimulates the return of activated satellite cells to quiescence 12 h after treatment, but likely results in satellite cell activation and progression down the myogenic lineage prior to 12 h post-treatment. Overall, these results, which align with the findings from analysis of *Pax7* expression, suggest that adequate concentrations of polyamines and their precursors in the cell may promote increased *Spry1* expression to perhaps, prevent myoblasts from differentiating and rather proliferate instead.

Myoblasts are capable of proliferating, as marked by increased expression of mitogen activated protein kinase (*MapK*) [49] and can eventually fuse into existing

muscle fibers to increase muscle fiber hypertrophy and, ultimately, promote skeletal muscle growth [50, 51]. In the present study, analysis of *MapK* expression showed that treatment of cells with Met decreased *MapK* expression 0.5 h after treatment and increased expression of *MapK* 24 h after treatment when compared to control cultures. Expression of *MapK* was increased 24 h after treatment with Put and was not affected by treatment with TBA, Orn, Spd, or Spe. These results indicate that an increase in *MapK* abundance plays a key role in the increased proliferation rates of cells treated with Met and Put, however, the response of cells to these molecules is delayed until 24 h after treatment. Of note, the primary regulation of MapK activity is through phosphorylation and dephosphorylation of the protein [52], which was not assessed in the present study. In contrast to the observed results in the present study, previous research has shown that treatment of C2C12 cells with 10 nM TBA promoted the MapK pathway through increased extracellular signal-regulated kinase 1/2 (*ERK1/2*) phosphorylation [53]. Furthermore, treatment of L6 rat myoblast cells with testosterone increases proliferation through the MapK pathway by increasing *ERK* activity 5 to 20 min post-treatment, but *ERK* activity was shown to decline after 20 min post-treatment [54]. Together, results from previous studies suggest that one of the mechanisms through which androgens increase cell proliferation is through non-genomic mechanisms involving the MAPK/ERK pathway. Previous research has also found that the MapK pathway may be involved in polyamine biosynthesis, as demonstrated by a decrease in *ODC* mRNA expression when leukemia L1210 cells are treated with an inhibitor of the MapK pathway and when L1210 cells are grown in the presence of Put and Spd, phosphorylation of MapK is increased [55]. The increased proliferation rates observed in cells treated with

Orn, Spd, and Spe must occur through mechanisms other than upregulation of *MapK* or different time points need to be investigated to determine the time frame at which this happens.

5. Conclusions

Overall, the present study found treatment with TBA, polyamine precursors, and polyamines increase proliferation rate of murine myoblasts. Furthermore, these results demonstrate that treatment of murine myoblasts with polyamines increases mRNA expression of genes involved polyamine biosynthesis and growth and polyamines and their precursors may function as signaling molecules to prevent the differentiation of myoblasts and alternatively promote proliferation. Expression of genes involved in polyamine biosynthesis showed that treatment with polyamines increased *ODC* abundance, while *AMD1* abundance was unaffected by treatment. Analysis of genes involved in skeletal muscle growth demonstrated that treatment of cultures with polyamines resulted in increased abundance of *Pax7* and *MapK* and tended to increase *Spry1* expression and treatment of cultures with polyamine precursors resulted in increased expression of *Spry1* and both increased and decreased *MapK* expression depending on the time point. Treatment with TBA had no effect on mRNA abundance of genes involved in polyamine biosynthesis or skeletal muscle growth. Ultimately, additional research is needed to determine the effects of TBA, polyamine precursors, and polyamines on a wider array of mRNA targets and at more time points, as well as analyzing the impacts of these molecules at other stages of muscle growth, such as differentiation or during protein synthesis. An improved understanding of androgens and

their effect on the polyamine biosynthetic pathway and growth is essential for the development of natural alternatives to improve and/or enhance skeletal muscle growth.

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Table 5.1. Primer and Probe Sequences used in Real-Time qPCR for analysis of mRNA expression in proliferating murine myoblasts

Messenger RNA	GBA Number	Primer and Probe Sequences (5'-3')
Ribosomal 18S (*18S*)	AF243428	FP: CCACGCGAGATTGAGCAAT RP: GCAGCCCCGGACATCTAA TP: ACAGGTCTGTGATGCC
Ornithine decarboxylase (*ODC*)	NM_013614.2	FP: CCTGAGCGGATGAGCATTATAG RP: CGACAGACAGCTTTGGAATCA TP: AGGTTGGTTCTACGGATTGCCACT
S-adenosylmethionine decarboxylase (*AMD1*)	NM_009665.5	FP: CTACTTGTCCTACCGTCAGCTG RP: CAGAATATTGCGCCGTTCCATC TP: CAGGTTACTCAGCCAGATAGTGAA
Paired box transcription factor 7 (*Pax7*)	XM_616352.4	FP: TTGTACCCCGCCCTCTCTTA RP: GGCTCAGCAATCCGTTTCC TP: AGCTGGGTCTTTTG
Sprouty 1 (*Spry1*)	NM_001099366.1	FP: TCACAGGAAGACAGCAAAGA RP: GCAAACAGGAAGACACGAC TP: TGCTTCTTAGAAGCTGGAGAGCA
Mitogen activated protein kinase (*MapK*)	NM_001038663.1	FP: CCACCCATACCTGGAGCAGTA RP: CAAACTTGAATGGCGCTTCA TP: CCCAAGTGATGAGCCCA

GBA, gene bank accession; FP, forward primer; RP, reverse primer; TP, TaqMan probe

Table 5.2. Relative mRNA abundance of *ODC*, *AMD1*, *Pax7*, *Spry1*, and *MapK* between C2C12 and Sol8 cells at 0.5, 12 or 24 h after treatment[a]

Time (h)	C2C12	Sol8	SEM	P-value
ODC				
0.5	1.23	0.96	0.19	*P = 0.32*
12	0.97	1.13	0.23	*P = 0.63*
24	1.04	0.86	0.37	*P = 0.56*
AMD1				
0.5	1.38	1.54	0.43	*P = 0.79*
12	1.03	1.02	0.18	*P = 0.95*
24	1.22	1.01	0.27	*P = 0.57*
Pax7				
0.5	1.10	0.84	0.30	*P = 0.52*
12	1.08	0.74	0.15	*P = 0.10*
24	1.37	0.88	0.25	*P = 0.14*
Spry1				
0.5	1.13	1.10	0.24	*P = 0.91*
12	0.99	0.92	0.21	*P = 0.89*
24	1.43	0.85	0.30	*P = 0.14*
MapK				
0.5	0.96	0.78	0.09	*P = 0.17*
12	0.96	0.92	0.21	*P = 0.89*
24	1.43	0.85	0.30	*P = 0.14*

[a]Relative mRNA abundance of two genes, ornithine decarboxylase (*ODC*), S-adenosylmethionine decarboxylase (*AMD1*), involved in polyamine synthesis and three genes, paired box transcription factor 7 (*Pax7*), Sprouty1 (*Spry1*), and mitogen activated protein kinase (*MapK*), involved in skeletal muscle growth in C2C12 and Sol8 murine myoblast cells after treatment with 1% fetal bovine serum (FBS, control, Con), 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). This figure demonstrates differences in mRNA abundance between the two cell types, regardless of treatment. Data represent relative mRNA abundance from C2C12 (n=3) and Sol8 (n=3) cultures.

Table 5.3. Effect of treatment on relative mRNA abundance of genes involved in polyamine biosynthesis[a]

	Treatment							
Time (h)	Con	TBA	Met	Orn	Put	Spd	Spe	SEM
ODC								
0.5	0.96	0.79	0.77	1.13	0.83	0.74	**1.96***	0.54
12	0.96	0.74	0.90	0.72	1.06	**2.09***	1.42	0.50
24	0.95	0.96	1.07	1.06	0.76	0.35	1.34	0.80
AMD1								
0.5	1.10	1.24	1.89	1.50	1.82	1.12	1.85	0.62
12	1.00	1.51	1.15	1.20	1.19	0.76	0.61	0.27
24	0.97	1.13	1.42	1.15	1.66	1.14	0.96	0.44

[a]Relative mRNA abundance of two genes, ornithine decarboxylase (*ODC*) and S-adenosylmethionine decarboxylase (*AMD1*), involved in polyamine biosynthesis in C2C12 and Sol8 murine myoblast cells after treatment with 1% fetal bovine serum (FBS, control, Con), 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cultures were grown in DMEM + 10% FBS until they reached approximately 70% confluency, plated into 96-well plates, and allowed 24 h to establish prior to treatment as described in the Materials and Methods. After 24 h, cultures were washed twice with Dulbecco's Modified Eagle Medium (DMEM) and then treated with DMEM/1% FBS and/or one of the following: 10 nM TBA, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Messenger RNA abundance was measured 0.5 h, 12 h, and 24 h after treatment as described in the Materials and Methods. Values represent the relative mRNA abundance of *ODC* and *AMD1* normalized against ribosomal 18S mRNA abundance and are presented as the least square mean ± SEM from three separate replicates of C2C12 cultures and three separate replicates of Sol8 cultures. Values that are bolded with a star (*) next to them indicate differences ($P \leq 0.05$) in relative mRNA abundance when compared to control cultures at the same time point.

Table 5.4. Effect of treatment on relative mRNA abundance of genes involved in growth[a]

	Treatment							
Time (h)	Con	TBA	Met	Orn	Put	Spd	Spe	SEM
Pax7								
0.5	0.91	1.02	1.64	0.57	0.58	1.02	1.17	0.66
12	0.97	0.83	1.09	0.98	0.56	**1.68***	0.67	0.29
24	0.96	0.96	1.59	0.98	**2.94***	0.71	1.27	0.46
Spry1								
0.5	1.00	1.22	0.78	1.00	1.05	1.74	1.26	0.83
12	0.98	1.74	1.01	1.34	**1.81***	0.42	0.70	0.44
24	1.00	1.55	1.27	**2.08***	1.00	0.71	0.63	0.69
MapK								
0.5	1.00	0.84	**0.20***	0.75	0.66	0.96	1.03	0.53
12	1.00	0.70	1.32	0.86	1.08	0.62	0.76	0.33
24	0.93	1.08	**1.96***	0.53	**2.09***	0.71	1.49	0.47

[a]Relative mRNA abundance of three genes, paired box transcription factor 7 (*Pax7*), Sprouty1 (Spry1), and mitogen activated protein kinase (*MapK*), involved in skeletal muscle growth in C2C12 and Sol8 murine myoblast cells after treatment with 1% fetal bovine serum (FBS, control, Con), 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cultures were grown in DMEM + 10% FBS until they reached approximately 70% confluency, plated into 96-well plates, and allowed 24 h to establish prior to treatment as described in the Materials and Methods. After 24 h, cultures were washed twice with Dulbecco's Modified Eagle Medium (DMEM) and then treated with DMEM/1% FBS and/or one of the following: 10 nM TBA, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Messenger RNA abundance was measured 0.5 h, 12 h, and 24 h after treatment as described in the Materials and Methods. Values represent the relative mRNA abundance of *Pax7*, *Spry1*, and *MapK* normalized against ribosomal 18S mRNA abundance and are presented as the least square mean ± SEM from three separate replicates of C2C12 cultures and three separate replicates of Sol8 cultures. Values that are bolded with a star (*) next to them indicate differences ($P \leq 0.05$) in relative mRNA abundance when compared to control cultures at the same time point.

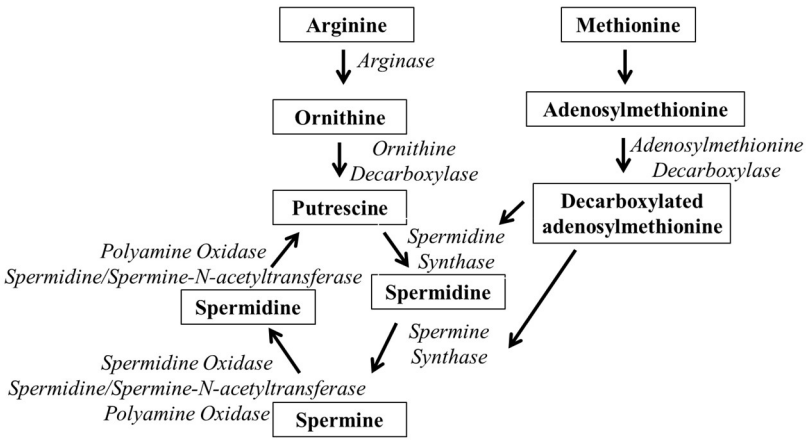

Figure 5.1. Polyamine biosynthesis and interconversion pathway. Polyamines (putrescine, spermidine, and spermine) and their precursors are shown in bold font and enzymes are shown in italic font. Figure adapted from Pegg and McCann [23].

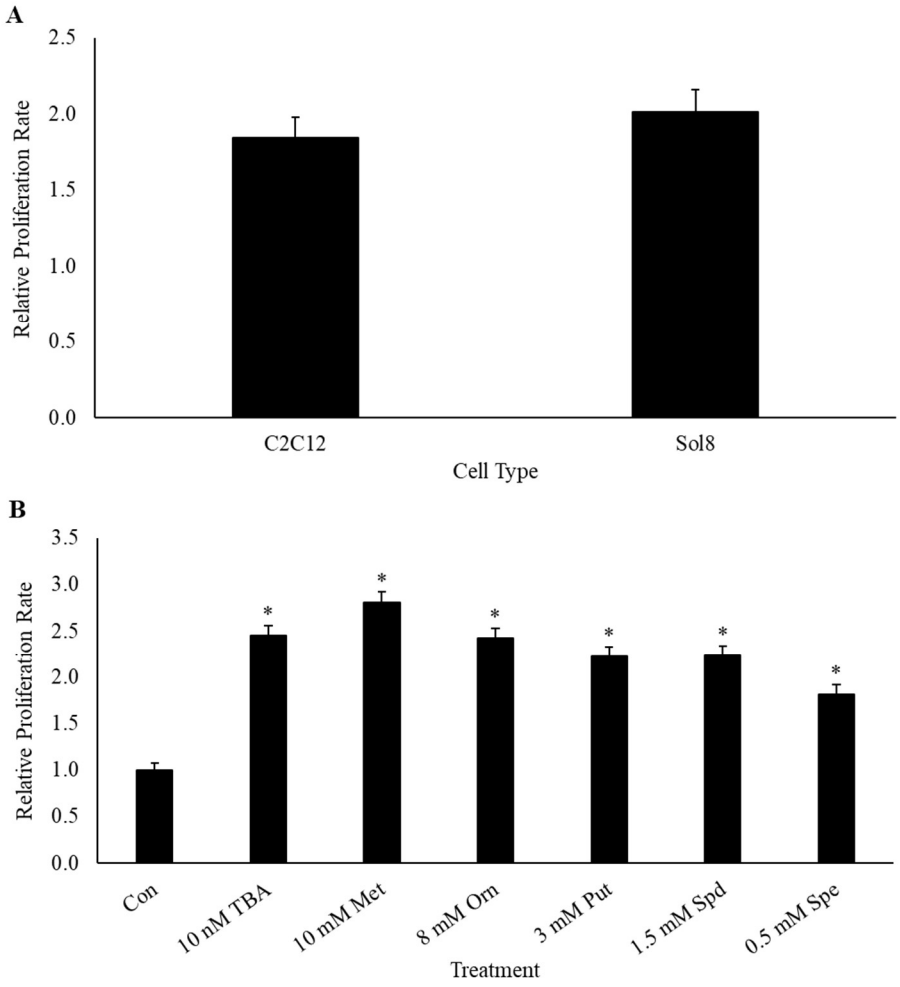

Figure 5.2. Effect of cell type (C2C12 or Sol8) or treatment on proliferation rates. Panel A demonstrates that there is no difference (*P = 0.38*) in protein synthesis rates between cell types. Panel B shows the effect of treatment on proliferation rates of combined data from both C2C12 and Sol8 cells in the presence of 1% fetal bovine serum (FBS, control, Con), 10 nM trenbolone acetate (TBA), 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Cultures were grown in DMEM + 10% FBS until they reached approximately 70% confluency, plated into 96-well plates, and allowed 24 h to establish prior to treatment as described in the Materials and Methods. After 24 h, cultures were washed twice with Dulbecco's

Modified Eagle Medium (DMEM) and then treated with DMEM/1% FBS and/or one of the following: 10 nM TBA, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Incorporation of bromodeoxyuridine was measured as described in the Materials and Methods. Values represent the relative proliferation rate compared to the control and are presented as the least square mean ± SEM from three separate replicates of C2C12 cultures and three separate replicates of Sol8 cultures. Treatments with a star (*) indicate differences ($P \leq 0.05$) in proliferation rates when compared to control cultures.

CHAPTER VI

IMPACTS OF ANABOLIC HORMONES, POLYAMINE PRECURSORS, AND POLYAMINES ON PROTEIN SYNTHESIS RATES AND ABUNDANCE OF GENES INVOLVED IN PROTEIN SYNTHESIS AND POLYAMINE BIOSYNTHESIS IN MURINE MYOBLASTS

Simple Summary

Anabolic hormones are commonly administered to cattle to improve skeletal muscle growth and feed efficiency and used to treat muscle wasting conditions in humans. However, over 50% of beef consumers are concerned about hormones being provided to beef cattle and anabolic hormones can cause off-target side effects in humans when used to treat muscle wasting. As such, natural growth-promoting alternatives to anabolic hormones are needed. Skeletal muscle growth is achieved when muscle protein synthesis exceeds protein degradation. Previous research suggests that one mechanism through which anabolic hormones improve skeletal muscle protein synthesis is through modulation of the polyamine biosynthetic pathway. As such, the purpose of this study was to examine the effects of anabolic hormones, polyamine precursors, and polyamines, on protein synthesis rates and messenger RNA expression of murine myoblasts. The results demonstrate that anabolic hormones increase protein synthesis rates and polyamines and their precursors alter expression of genes involved in polyamine biosynthesis and protein synthesis. However, additional research is needed to further investigate the relationship between anabolic hormones and polyamines relative to skeletal muscle growth to determine if polyamines and their precursors can be utilized as natural growth-promoting alternatives to anabolic hormones.

Abstract

Anabolic hormones promote muscle growth by modulating the polyamine biosynthetic pathway. The objectives of this study were to examine the effects of anabolic hormones, polyamine precursors, and polyamines on protein synthesis and mRNA abundance of genes involved in polyamine biosynthesis and protein synthesis in both C2C12 and Sol8 cells. Fused cultures were treated in serum-free media with 10 nM trenbolone acetate, 10 nM estradiol-17β, 10 nM trenbolone acetate + 10 nM estradiol-17β, 10 mM methionine, 8 mM ornithine, 3 mM putrescine, 1.5 mM spermidine, or 0.5 mM spermine. Protein synthesis was assessed 3 h after treatment and mRNA was isolated 1, 12, and 24 h post-treatment. The effects of cell type and treatment on protein synthesis and mRNA abundance were evaluated. Cell type had no effect ($P > 0.10$) on protein synthesis or mRNA expression of any gene at any time point. Anabolic hormones increased ($P = 0.04$) protein synthesis. Polyamines and their precursors impacted ($P < 0.05$) expression of genes involved in polyamine biosynthesis and protein synthesis. These data demonstrate that anabolic hormones increase protein synthesis and polyamines and polyamine precursors alter mRNA expression of genes involved in polyamine biosynthesis and protein synthesis.

1. Introduction

Muscle fiber number in mammals is predominately fixed at birth and, therefore, post-natal muscle growth occurs almost exclusively through hypertrophy of existing muscle fibers [1]. In times of muscle growth, injury, or regeneration, quiescent muscle satellite cells become activated and differentiate into myoblasts [2, 3]. Myoblasts fuse with existing muscle fibers to support hypertrophy during post-natal growth [2, 3]. Muscle is a highly plastic tissue that is continually being remodeled through a balance of both protein synthesis and protein degradation [4]. Muscle protein turnover is necessary to maintain muscle mass [4]. Ultimately, for skeletal muscle growth to occur, muscle protein synthesis, especially synthesis of the contractile myofibrillar protein fraction, must be greater than muscle protein degradation [4], which is marked by increased abundance of mammalian target of rapamycin (*mTOR*) [5] and eukaryotic translation initiation factor 2B subunit epsilon (*eIF-2Bε*) [6], among other genes.

Administration of exogenous anabolic hormones, such as estrogens and androgens, results in increased skeletal muscle growth in many species including humans [7, 8] mice [9], and cattle [10]. Estrogens and androgens stimulate cell proliferation in murine satellite cells [9] and bovine satellite cells [11, 12], both stimulate [13] and have no effect [14] on proliferation rates of C2C12 cells, increase differentiation in murine C2C12 cells [13] and rat L6 myoblasts [15], and increase protein synthesis in bovine satellite cells [10, 12, 16], but have no effect on protein synthesis in C2C12 cells [14]. However, the exact mechanisms through which these anabolic hormones accelerate skeletal muscle growth has not been well characterized. Past research suggests that one mechanism by which androgens, such as trenbolone acetate (TBA), and estrogens, such

as estradiol-17β (E2), may improve growth is through their interaction with the polyamine biosynthetic pathway [17-25].

Methionine (Met), ornithine (Orn), and arginine (Arg) are the main substrates utilized for synthesis of the polyamines, putrescine (Put), spermidine (Spd), and spermine (Spe) in the polyamine biosynthetic pathway (Figure 6.1) [17, 26, 27]. Polyamines are essential for growth, cell proliferation, cell differentiation, and protein synthesis [26, 28-31] and polyamine biosynthesis increases during times of skeletal muscle hypertrophy and decreases during muscle atrophy [32]. Additionally, anabolic hormones, which are known to increase growth upon administration, have been shown to interact with the polyamine biosynthetic pathway. Androgens, such as testosterone and TBA, increase expression of ornithine decarboxylase (*ODC*) and S-adenosylmethionine decarboxylase (*AMD1*) [33, 34], two enzymes involved the biosynthesis of Put from Orn and the production of decarboxylated S-adenosylmethionine, respectively [17]. Estradiol-17β has also been shown to increase *ODC* and *AMD1* activity, which, subsequently, leads to increases in Put, Spd, and Spe concentrations [22].

Anabolic hormones, such as TBA and E2, are used to improve growth in many species, ranging from anabolic implants to improve growth of beef cattle to treating muscle wasting conditions in humans [35-38]. However, the mechanisms through which these steroids improve growth has yet to be entirely elucidated. Past studies have shown that anabolic hormones are involved in modulation of the polyamine biosynthetic pathway. Through an improved understanding of how anabolic hormones regulate the polyamine biosynthetic pathway, alternative routes for improving muscle growth, aside from administration of hormones, could potentially be identified. As such, the goal of this

study was to examine the effects of TBA, E2, polyamine precursors, and polyamines on protein synthesis rates of murine myoblasts and mRNA expression of genes involved in polyamine biosynthesis and growth.

2. Materials and Methods

2.1 Culture of murine myoblasts

Murine myoblast cell lines, C2C12 and Sol8 cells, were obtained from American Type Culture Collections® and grown according to manufacturer specifications. Prior to resurrection, cells were stored in liquid nitrogen. Cells were cultured in a growth medium containing phenol-red free Dulbecco's Modified Eagle Medium (DMEM) [39] with 10% fetal bovine serum (FBS) in 75 mL flasks and incubated at 37°C with 5% CO_2 in a water saturated environment [40]. Cells were washed twice with DMEM and provided fresh growth medium every 48 h.

2.2 Treatment of myoblast cultures

Cultures were grown to approximately 70% confluency, plated, and given 24 h to establish. Cultures for analysis of protein synthesis were plated into 96-well plates and cultures for mRNA isolation were plated into 12-well plates at a density of 11,200 cells/cm^2. Cultures were subsequently grown to approximately 80% confluency, washed twice with DMEM, and induced to differentiate in DMEM containing 3% horse serum and 1.5% bovine serum albumin-linoleic acid (BSA-LA). Cytosine arabinoside was added to cultures 24 h after the addition of differentiation media and remained on cultures for a minimum of 18 h to ensure that all proliferating cells were eliminated, resulting in a pure culture of myotubes. Cultures were then treated with serum-free media (SFM) containing DMEM, 10^{-8} M insulin from bovine pancreas, 250 µg fetuin from fetal bovine

serum/mL, 100 μg BSA-LA/mL and/or one of the following: 10 nM TBA, 10 nM E2, 10 nM TBA + 10 nM E2, 10 mM Met, 8 mM Orn, 3 mM Put, 1.5 mM Spd, or 0.5 mM Spe. Control cultures were treated with SFM. Treatment concentrations used in the analysis of protein synthesis were selected based upon results from past studies completed by our laboratory that impacted growth of bovine satellite cells [24, 25].

2.3 Analysis of protein synthesis rates

Protein synthesis assays were performed 3 h after treatment using a commercially available protein synthesis kit (Click-iT Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit, Invitrogen, Waltham, MA, USA) and following the manufacturer specifications. In short, O-propargyl-puromycin (OPP) was diluted with DMEM to 1:100 and added to cultures. Cultures were then incubated for 3 h to allow for newly synthesized proteins to be labeled with OPP. After incubation, cultures were fixed to the plate with 3.7% formaldehyde and permeabilized with 0.5% ethanol. Cultures were then dyed with NuclearMask™ Blue Stain and incubated for 30 min. Results of the protein synthesis assay were analyzed by fluorescence detection on a BioTek Synergy H1 plate reader using all-in-one microplate reader software, Gen 5 2.09 (BioTek Instruments, Winooski, VT, USA). Protein synthesis assays were run congruently with collection of RNA to ensure that cultures used for RNA isolation were synthesizing proteins.

2.4 mRNA isolation, quantification, and cDNA synthesis

Total RNA was extracted from cultures using an Absolutely RNA Microprep Kit (Agilent Technologies, Cedar Creek, TX, USA) following the manufacturer's protocol. In short, cell lysate was collected at three different time points, 1, 12, and 24 h after treatment. Before lysate collection, cells were washed once with phosphate-buffered

saline (PBS), lysis buffer was added to cultures, cells were scraped to complete cell lysing. All cell lysate samples were stored at -80°C until RNA isolation. Cell lysate samples were vortexed and then received an equal volume of 70% ethanol. Samples were then centrifuged and filtered, subjected to a series of wash buffers, and RNA was eluted in the provided buffer. All RNA samples were stored at -80°C until quantification. Briefly, quantification of isolated RNA was performed using a Take3 plate on a BioTek Synergy H1 plate reader using the Gen 5 2.09 all-in-one microplate reader software (BioTek Instruments, Winooski, VT, USA). Quality of RNA was established using the 260/280 ratio. All samples that had a ratio greater than 2.0 were considered acceptable. The RNA samples that were deemed acceptable received a deoxyribonuclease (Ambion, Foster City, CA, USA) treatment and then a high-capacity cDNA reverse transcription kit (Applied Biosystem, Foster City, CA, USA) was used for synthesis of cDNA from the acceptable RNA samples following the manufacturer's protocol.

2.5 Quantitative real-time PCR

To evaluate mRNA expression, real-time PCR quantification utilizing TaqMan MGB primer/probe system was used following procedures that have been described previously [41, 42]. Primers and probes for the genes that were examined are shown in Table 6.1 and were designed using the Primer Express 3.0 software (Applied Biosystems, Waltham, MA, USA) [24]. Relative mRNA abundance of ribosomal 18S (*18S*, housekeeping gene [41, 42]), *ODC*, *AMD1*, eukaryotic translation initiation factor subunit 2B epsilon (*eIF-2Bε*), and mammalian target of rapamycin (*mTOR*) were assessed with an ABI 7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA).

2.6 Statistical analysis

Analysis of cell type on protein synthesis rate and mRNA expression at three time points (1, 12, or 24 h) was completed using the MIXED procedure of SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA) where cell type, cell type^{x}treatment, and treatment were included as fixed effects and plate and experiment number served as random effects in the model. Analysis of cell type^{x}treatment showed no effects ($P > 0.10$) relative to protein synthesis rates or mRNA expression. As such, the main effects of cell type and treatment were analyzed separately. Cell type had no effect ($P > 0.10$) on protein synthesis rates and only showed a tendency ($P = 0.06$) for C2C12 cells to have increased *AMD1* expression 1 h post-treatment when compared to Sol8 cells and a tendency ($P = 0.10$) for *eIF-2Bε* to be increased in Sol8 cells 24 h post-treatment when compared to C2C12 cells. Consequently, analysis of the effects of treatment was completed with treatment serving as a fixed effect and cell type, plate, and experiment number included as random effects in the model. Contrast statements were used to control for multiple comparisons so that each treatment was only compared to the control cultures. Protein synthesis rates are presented as the least square mean ± SEM with values representing the fold change of treated cultures relative to the control cultures (with a set value of 1.0). Messenger RNA expression is presented as the relative mRNA abundance of each sample (calculated as $2^{-\text{relative threshold cycle }(\Delta Ct)}$) relative to the control value. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered a tendency for significance.

3. Results

3.1 Effects of cell type on protein synthesis rates and relative mRNA abundance

Cell type had no effect ($P > 0.10$) on protein synthesis rates (Figure 6.2A). Further, cell type had no effect ($P > 0.10$) on expression of *ODC*, or *mTOR* (Table 6.2). Expression of *AMD1* tended ($P = 0.06$) to be increased in C2C12 cells 1 h after treatment when compared to Sol8 cells; however, no differences ($P > 0.10$) in *AMD1* expression were observed between the different cell types at 12 or 24 h post-treatment (Table 6.2). Abundance of *eIF-2Bε* tended ($P = 0.10$) to be increased in Sol8 cells 24 h after treatment when compared to C2C12 cells (Table 6.2). No differences ($P > 0.10$) in *eIF-2Bε* expression were observed between the different cell types at 1 or 12 h post-treatment (Table 6.2).

3.2 Effects of different treatments on protein synthesis rates

Treatment of cells with TBA + E2 increased ($P = 0.04$) protein synthesis rate compared to control cultures (Figure 6.2B). However, treatment of cells with TBA, E2, Met, Orn, Put, Spd, or Spe had no effect ($P > 0.10$) on protein synthesis rate when compared to control cultures (Figure 6.2B).

3.3 Effects of treatment on relative mRNA abundance of genes involved in polyamine biosynthesis

Treatment of cells with Orn tended ($P = 0.06$) to decrease *ODC* expression 1 h post-treatment when compared to control cultures; however, no differences ($P > 0.10$) in *ODC* expression were observed between control and treatment cultures 12 and 24 h post-treatment (Figure 6.3A). Additionally, *ODC* expression was decreased ($P = 0.044$) 1 h post-treatment in cultures treated with Spe when compared to control cultures, but *ODC* expression 12 and 24 h post-treatment was not different ($P > 0.05$) from control cultures (Figure 6.3A). Treatment of cells with TBA, E2, TBA + E2, Met, Put, or Spd had no

effect (*P > 0.05*) on *ODC* expression when compared to control cultures at 1, 12, or 24 h post-treatment (Figure 6.3A). Analysis of *AMD1* expression showed that treatment of cells with TBA tended (*P = 0.09*) to increase *AMD1* expression 12 h post-treatment when compared to control cultures; however, no differences (*P > 0.10*) between treatment and control cultures were observed 1 or 24 h post-treatment (Figure 6.3B). Treatment of cultures with Put resulted in increased (*P = 0.002*) *AMD1* expression 12 h after treatment when compared to control cultures (Figure 6.3B). No differences (*P > 0.10*) in *AMD1* expression between control cultures and treatment cultures were observed 1 or 24 h post-treatment with Put (Figure 6.3B). Treatment of cells with E2, TBA + E2, Met, Orn, Spd, or Spe had no effect (*P > 0.05*) on *AMD1* expression when compared to control cultures at 1, 12, or 24 h post-treatment (Figure 6.3B).

3.4 Effects of treatment on relative mRNA abundance of genes involved in skeletal muscle protein synthesis

Expression of *mTOR* tended (*P = 0.08*) to be decreased 1 h after treatment in cultures treated with E2 compared to control cultures; however, no differences (*P > 0.10*) were observed between treatment cultures and control cultures 12 or 24 h post-treatment (Figure 6.4A). Treatment of cultures with Orn decreased (*P = 0.008*) *mTOR* expression 1 h after treatment compared to control cultures (Figure 6.4A). No differences (*P > 0.10*) in *mTOR* expression were observed between control cultures and cultures treated with Orn 12 or 24 h post-treatment when compared to control cultures (Figure 6.4A). Expression of *mTOR* was increased (*P = 0.007*) 12 h post-treatment with Put relative to control cultures; however, no differences (*P > 0.10*) were observed 1 or 24 h after treatment with Put when compared to control cultures (Figure 6.4A). Treatment of cultures with Spd

resulted in increased (*P = 0.004*) *mTOR* expression 12 h post-treatment when compared to control cultures, but no differences (*P > 0.10*) were observed between treated and control cultures 1 and 24 h post-treatment (Figure 6.4A). Relative mRNA abundance of *mTOR* tended (*P = 0.07*) to be decreased 12 h after treatment in cultures treated with Spe compared to control cultures (Figure 6.4A). However, treatment of cultures with Spe had no effect (*P > 0.10*) on *mTOR* mRNA abundance 1 or 24 h post-treatment when compared to control cultures (Figure 6.4A). No differences (*P > 0.10*) in *mTOR* expression between control cultures and cultures treated with TBA, TBA + E2, or Met were observed 1, 12, or 24 h post-treatment (Figure 6.4A).

Analysis of *eIF-2Bε* expression showed that treatment of cultures with Put increased (*P = 0.01*) *eIF-2Bε* abundance 12 h post-treatment relative to control cultures; however, no differences (*P > 0.10*) were observed 1 and 24 h post-treatment when compared to control cultures (Figure 6.4B). Additionally, treatment of cultures with Spe tended (*P = 0.07*) to increased *eIF-2Bε* expression 12 h post-treatment but had no effects (*P > 0.10*) on *eIF-2Bε* expression 1 or 24 h post-treatment when compared to control cultures (Figure 6.4B). No differences (*P > 0.10*) in *eIF-2Bε* expression were observed 1, 12, or 24 h after treatment with TBA, E2, TBA + E2, Met, Orn, or Spd when compared to control cultures (Figure 6.4B).

4. Discussion

Anabolic hormones, such as TBA and E2, stimulate muscle growth in humans [7, 8] mice [9], and cattle [10]. As such, anabolic hormones are widely used in the United States for improving growth and feed efficiency in feedlot cattle [10] and can be used to improve muscle mass and strength in people that suffer from some muscle wasting

conditions, such as sarcopenia [43]. However, the exact mechanism(s) through which anabolic hormones improve skeletal muscle growth are not well understood. Previous research suggest that androgens and estrogens interact with the polyamine biosynthetic pathway through increasing the expression of two enzymes involved in polyamine biosynthesis, *ODC* and *AMD1*, resulting in increased polyamine biosynthesis in muscle tissue of mice [44] and the uterus of rats [22]. Polyamines are amino acid derivatives with unique bioactivities that are required for protein synthesis and growth [31] through their function in regulating translation during both initiation and elongation [45]. These molecules can be synthesized through the polyamine biosynthetic pathway from their precursors, arginine, Met, and Orn via *ODC* and *AMD1* [17, 26, 27] (Figure 6.1). Polyamines can be acquired from the diet and are found in high concentrations in many food sources including potatoes, tomatoes, most meats, and matured cheeses [46]. With over 50% of beef consumers concerned about hormones being provided to beef cattle [47] and anabolic hormones having off-target side effects when given to humans to treat muscle wasting [48], polyamines and their precursors could serve as alternatives to exogenous hormone administration to improve/remedy skeletal muscle growth. As such, the present study examines the effect of TBA, E2, polyamines, and polyamine precursors on protein synthesis and mRNA expression of genes involved in polyamine biosynthesis and protein synthesis in two different clonal lines of murine myoblasts (C2C12 and Sol8).

To the best of the authors' knowledge, the present study is the first to report the differences in protein synthesis rates and mRNA abundance between C2C12 and Sol8 murine myoblast cell lines. In the present study, cell type had no effect on protein synthesis rates and expression of *ODC* or *mTOR*. Relative mRNA abundance of *AMD1*

tended to be increased in C2C12 cultures 1 h after treatment when compared to Sol8 cultures and expression of *eIF-2Bε* tended to be increased in Sol8 cells 24 h after treatment when compared to C2C12 cells. Although similar, C2C12 and Sol8 myoblast cells differ such that C2C12 cells are non-cancerous cells that are obtained from sarcomas of adult mice and Sol8 cells are obtained from the skeletal muscle of 4-week-old mice. Overall, these results show that C2C12 and Sol8 cells respond similarly when in the presence of TBA, E2, polyamines, or polyamine precursors.

Growth of skeletal muscle occurs when protein synthesis rates exceed protein degradation rates [4]. In the present study, treatment of C2C12 and Sol8 cells with TBA+E2 increased protein synthesis; however, treatment with TBA, E2, Met, Orn, Put, Spd, or Spe had no effect on protein synthesis rate relative to control cultures. In contrast to the results of the present study, previous research has shown that treatment of C2C12 cells with 10 nM TBA results in increased protein synthesis rates [49]. Similar to the results observed in the present study, previous research has also demonstrated that treatment of C2C12 cells with testosterone, dihydrotestosterone, or estradiol does not affect protein synthesis rates [14]. Androgen treatment alone likely does not affect protein synthesis rates of C2C12 cells because they express androgen receptor mRNA at only 0.1% of the level found in muscle from adult mice [50]. However, previous work suggests that C2C12 cells express the estrogen receptor at levels similar to that of the uterus and ovary [51]. The positive effects of anabolic hormones on protein synthesis rates in culture has been well-characterized in bovine satellite cells and demonstrates that treatment with 10 nM TBA or 10 nM E2, the same concentrations used in the present study, results in increased protein synthesis rates [12, 16], which was contrary to the

results observed in the present study. To the best of the authors' knowledge, the present study is the first to examine the effects of polyamines and their precursors on protein synthesis rates of C2C12 and Sol8 murine myoblasts. Although treatment of C2C12 and Sol8 myoblasts with polyamines and polyamine precursors did not result in increased protein synthesis rates in the present study, others have shown that depletion of polyamines with L-α-Difluoromethylornithine, an irreversible suicide inhibitor of *ODC* [52], results in decreased protein synthesis in NIH3T3 mouse fibroblasts through inhibition of translation initiation [45], further highlighting the importance of polyamines for protein synthesis. Perhaps, additional time points post-treatment of fused cultures with polyamines and their precursors need to be examined to determine whether protein synthesis rate is impacted by these treatments. The results of the present study demonstrate that anabolic hormones increase protein synthesis rates in both bovine satellite cells and murine myoblasts. Further, polyamines and their precursors do not alter protein synthesis in C2C12 and Sol8 cells, but polyamines play a role in protein synthesis through regulation of translation initiation and elongation, as demonstrated by others [45].

Ornithine decarboxylase is an enzyme involved in the polyamine biosynthetic pathway that is responsible for synthesis of the polyamine Put from its precursor Orn [17, 26] (Figure 6.1). The polyamines Spd and Spe can then be synthesized from Put in the presence of Spd synthase and Spe synthase, respectively (Figure 6.1) [26]. In the present study, the effects of anabolic hormones, polyamine precursors, and polyamines on *ODC* abundance were assessed. Treatment of cultures with TBA, E2, or TBA + E2 had no effect on *ODC* expression. Contrary to the results observed in the present study, other

studies have found that treating castrated mice with testosterone results in increased *ODC* expression in the kidneys [53] and treatment of ovariectomized rats with E2 results in increased *ODC* abundance in the uterus and kidneys [54]. Furthermore, the *ODC* gene promotor has been found to contain an androgen response element [55] and *ODC* mRNA expression is decreased in the absence of the androgen receptor in mice [44]. In the present study, it is possible that *ODC* mRNA expression was unaffected by treatment with anabolic hormones because ODC protein expression was already high, however, the present study did not assess ODC protein expression or *ODC* enzyme activity. Treatment of cultures with polyamine precursors showed that *ODC* expression tended to be decreased 1 h after treatment with Orn compared to control cultures, however, treatment with Met did not affect *ODC* abundance. Results from the present study contrast with those reported in human colon adenocarcinoma cells that showed decreased *ODC* activity 4 h after treatment with 10 mM of the polyamine precursors Met or arginine [56]. Ornithine decarboxylase expression was also decreased 1 h after treatment with the polyamine Spe, but treatment with Put or Spd had no effect on *ODC* abundance. To the best of the authors' knowledge, the present study is the first to assess mRNA abundance of *ODC* in murine myoblast cultures treated with polyamines and their precursors. As such, the results of the present study will be compared to results from studies that have assessed *ODC* enzyme activity. Work completed in intestinal epithelial crypt (IEC-6) cells from rats found that polyamines have an inhibitory effect on *ODC* activity [57], which is in agreement with the results observed in the present study. Overall, these data suggest that treatment of murine myoblasts with anabolic hormones does not impact *ODC* mRNA abundance; however, treatment with polyamines and their precursors may

have an inhibitory effect on *ODC*, as treatment with these molecules have the potential to in decrease *ODC* expression. However additional research needs to be done investigating mRNA expression at different time points, protein/enzyme quantification, enzyme activity, and substrate availability/utilization.

Another enzyme involved in polyamine biosynthesis is *AMD1*, which catalyzes the synthesis of decarboxylated S adenosylmethionine from adenosylmethionine (Figure 6.1) [17, 26]. The polyamines Spd and Spe can then be synthesized from decarboxylated adenosylmethionine through Spd synthase and Spe synthase, respectively (Figure 6.1) [26]. The present study analyzed the effects of anabolic hormones, polyamine precursors, and polyamines on *AMD1* expression. Treatment of cultures with TBA tended to increase *AMD1* expression 12 h after treatment; however, treatment with E2 or TBA + E2 did not affect *AMD1* abundance. Previous work demonstrated the relationship between androgens and the polyamine biosynthetic pathway and found that *AMD1* is a direct target of the androgen receptor [55] and *AMD1* expression is decreased in androgen receptor knock out mice [44]. In contrast to the observed results of the present study, previous work found that administration of E2 to ovariectomized rats results in increased *AMD1* abundance in the uterus [54]. To the best of the authors' knowledge, the present study is the first to examine the effects of polyamines and their precursors on *AMD1* abundance in C2C12 and Sol8 myoblast cells. This study indicates that treatment of myoblasts with polyamine precursors had no effect on *AMD1* expression. However, treatment of cultures with Put resulted in increased *AMD1* expression 12 h after treatment. These data suggest that treatment of murine myoblasts with TBA and Put have the potential to increase *AMD1* expression 12 h after treatment, but polyamine precursors

have no effect on *AMD1* abundance. As such, further research is needed to determine if *AMD1* expression in murine myoblasts differs at different time points other than those analyzed in the present study.

Mammalian target of rapamycin is a serine/threonine kinase that modulates protein synthesis through activation of S6 kinase and inhibition of 4E-binding protein 1 [5, 58]. The present study examined the effects of anabolic hormones, polyamine precursors, and polyamines on abundance of *mTOR*. The present study found abundance of *mTOR* tended to be decreased 1 h after treatment with E2; however, no differences were observed in cultures treated with TBA or TBA+E2. In contrast to the results of the present study, previous studies have observed increased mammalian target of rapamycin complex 1 (*mTORC1*) activity and *mTOR* phosphorylation after C2C12 cells were treated with testosterone and TBA, respectively [59]. The analysis of polyamine precursors and their effects on *mTOR* expression demonstrated that treatment with Orn decreases *mTOR* expression 1 h after treatment, but *mTOR* expression was unaffected by treatment with Met, which contrasted from previous work that observed increased *mTORC1* activity in murine embryonic fibroblasts and human embryonic kidney 293A cells 15 min after treatment with Met [60]. The differences observed between results of the present study and those from past work may be due to differences in the timepoints analyzed, differences in treatment concentrations, and that the other studies analyzed protein expression. Additional research is needed prior to 1 h post-treatment to further elucidate the effects of polyamine precursors on *mTOR* expression of murine myoblasts. Treatment of cultures with polyamines resulted in increased *mTOR* expression 12 h after treatment with Put and Spd and a tendency for *mTOR* abundance to be increased 12 h after

treatment with Spe. In agreement with results observed in the present study, previous studies have shown that Spd and Spe stimulate *mTORC1* activity in rat intestinal epithelial cells [61]. Overall, these results indicate that treatment of murine myoblasts with E2 or Orn may decrease *mTOR* expression, while treatment with polyamines increases *mTOR* abundance. As such, further research is needed to determine the exact effects of alterations in *mTOR* expression from treatment with these molecules.

Eukaryotic initiation translation factor 2B subunit epsilon is another gene that is important in protein synthesis [62, 63]. In an anabolic state (i.e. during protein synthesis) expression of *eIF-2Bε* is increased in rat fibroblast cells and rat skeletal muscle [64-67]. Eukaryotic initiation translation factor 2B subunit epsilon initiates translation through the protein kinase B (AKT/PKB) pathway and independent of *mTOR* [62]. In short, AKT/PKB phosphorylates glycogen synthase kinase-3 (GSK-3), inhibiting GSK-3 from phosphorylating (inhibiting) *eIF-2Bε* [62, 68]. In the present study, the effects of anabolic hormones, polyamine precursors, and polyamines on *eIF-2Bε* were assessed. Treatment with anabolic hormones had no effect on *eIF-2Bε* expression. Abundance of *eIF-2Bε* was unaffected by treatment with polyamine precursors. Treatment with Put increased expression of *eIF-2Bε* and treatment with Spe tended to increase *eIF-2Bε* abundance 12 h after treatment. To the best of the authors' knowledge, the present study is the first to examine the effects of these compounds on *eIF-2Bε* abundance. Although treatment of cultures with Met in the present study did not result in increased *eIF-2Bε* expression, eukaryotic translation initiation factor 2B is known to play a role in mRNA translation and, in the presence of amino acids, has been shown to promote skeletal muscle hypertrophy in human embryonic kidney cells through repression of *eIF-2Bε*

phosphorylation [62, 69]. Together, these data suggest that polyamines may interact with the AKT/PKB pathway to increase *eIF-2Bε* abundance. However, it is important to note that eIF-2Bε is also largely regulated at the translational and post-translational levels through phosphorylation of the protein [70]. When the eIF-2Bε protein is phosphorylated, the guanine nucleotide exchange factor activity of eIF2B is altered and initiation of translation is inhibited [71-73]. As such, further research that examines how the treatments used in the present study affect eIF-2Bε protein expression is warranted.

5. Conclusions

This study demonstrates that treatment of murine myoblasts with anabolic hormones increases protein synthesis, while treatment with polyamines and their precursors has no effect on protein synthesis at the time points assessed. Additionally, these data demonstrate that anabolic hormones, polyamines, and polyamine precursors alter mRNA abundance of genes involved in polyamine biosynthesis and protein synthesis. Analysis of genes involved in polyamine biosynthesis demonstrated that *ODC* abundance was decreased after treatment with polyamines, tended to be decreased after treatment with polyamine precursors, and was unaffected by treatment with anabolic hormones, while *AMD1* expression was increased after treatment with polyamines, tended to be increased after treatment with anabolic hormones, and was not affected by treatment with polyamine precursors. Assessment of genes involved in protein synthesis showed that *mTOR* abundance was decreased after treatment with anabolic hormones and polyamine precursors and increased after treatment with polyamines, while *eIF-2Bε* expression was increased after treatment with polyamines and unaffected by treatment with polyamine precursors and anabolic hormones. Additional research at different time

points is needed to further elucidate the effects of anabolic hormones, polyamine precursors, and polyamines on protein synthesis rates and mRNA abundance of genes involved in polyamine biosynthesis and protein synthesis. Furthermore, exploring the effects of these molecules *in vivo* is essential for the development of alternative technologies to improve skeletal muscle growth.

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Table 6.1. Primer and Probe Sequences used in Real-Time qPCR for analysis of mRNA expression

Messenger RNA	GBA Number	Primer and Probe Sequences (5'-3')
Ribosomal 18S (*18S*)	AF243428	FP: CCACGCGAGATTGAGCAAT RP: GCAGCCCCGGACATCTAA TP: ACAGGTCTGTGATGCC
Ornithine decarboxylase (*ODC*)	NM_013614.2	FP: CCTGAGCGGATGAGCATTATAG RP: CGACAGACAGCTTTGGAATCA TP: AGGTTGGTTCTACGGATTGCCACT
S-adenosylmethionine decarboxylase (*AMD1*)	NM_009665.5	FP: CTACTTGTCCTACCGTCAGCTG RP: CAGAATATTGCGCCGTTCCATC TP: CAGGTTACTCAGCCAGATAGTGAA
Eukaryotic translation initiation factor 2B subunit epsilon (*eIF-2Bε*)	NM_172265.2	FP: CAAAGAGACACAACTGACGAAGG RP: GTTACGAGGACAGCCAATGAGA TP: CTGAGAGAGGCAGAAGAAGAGTC
Mammalian target of rapamycin (*mTOR*)	NM_020009.2	FP: CATCCCTCTGTCCACCAACTC RP: TGCTCAAACACCTCTACCTTCT TP: CGGGACTACAGAGAGAAGAAGAAG

GBA, gene bank accession; FP, forward primer; RP, reverse primer; TP, TaqMan probe

Table 6.2. Relative mRNA abundance of *ODC*, *AMD1*, *mTOR*, and *eIF-2Bε* between fused C2C12 and Sol8 cells at 1, 12, or 24 h after treatment[1]

Time (h)	C2C12	Sol8	SEM	P-value
ODC				
1	0.87	0.81	0.14	*P = 0.77*
12	1.13	1.30	0.15	*P = 0.42*
24	0.87	1.31	0.25	*P = 0.22*
AMD1				
1	1.10	0.65	0.17	*P = 0.06*
12	1.50	1.59	0.47	*P = 0.47*
24	0.85	0.96	0.15	*P = 0.61*
mTOR				
1	0.95	0.91	0.19	*P = 0.88*
12	1.05	1.28	0.13	*P = 0.20*
24	0.90	1.32	0.21	*P = 0.17*
eIF-2Bε				
1	0.87	0.68	0.11	*P = 0.22*
12	1.29	1.41	0.22	*P = 0.70*
24	0.88	1.38	0.21	*P = 0.10*

[1]Relative mRNA abundance of two genes, ornithine decarboxylase (*ODC*) and s-adenosylmethionine (*AMD1*), involved in polyamine biosynthesis and two genes, mammalian target of rapamycin (*mTOR*) and eukaryotic translation initiation factor 2B subunit epsilon (*eIF-2Bε*), involved in skeletal muscle protein synthesis were quantified in fused C2C12 and Sol8 murine myoblast cells. This table demonstrates differences in mRNA abundance between two cell types, regardless of treatment. Data represent least squares means ± standard error of the mean (SEM) of relative mRNA abundance in C2C12 (n=5) and Sol8 (n=5) cultures.

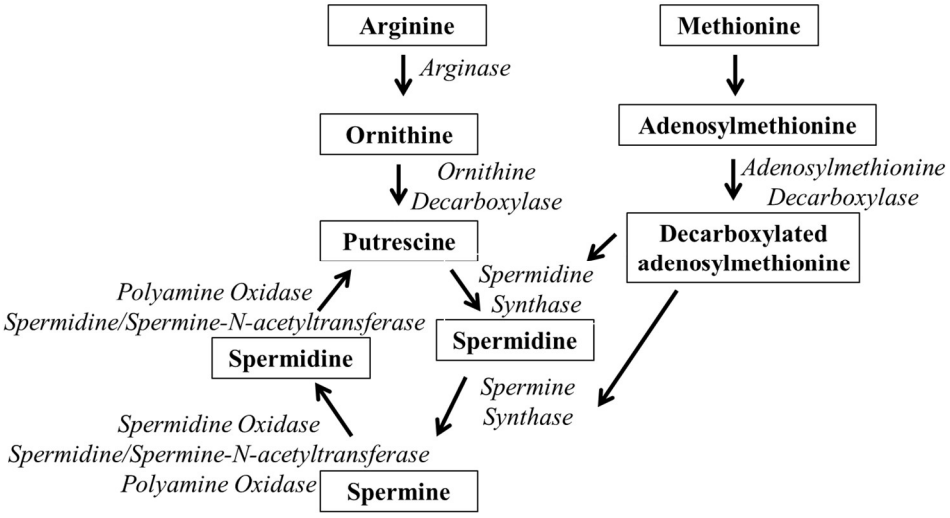

Figure 6.1. Polyamine biosynthesis and interconversion pathway. Polyamines (putrescine, spermidine, and spermine) and their precursors are displayed in bold font and enzymes are displayed in italic font. Figure adapted from Pegg and McCann [26].

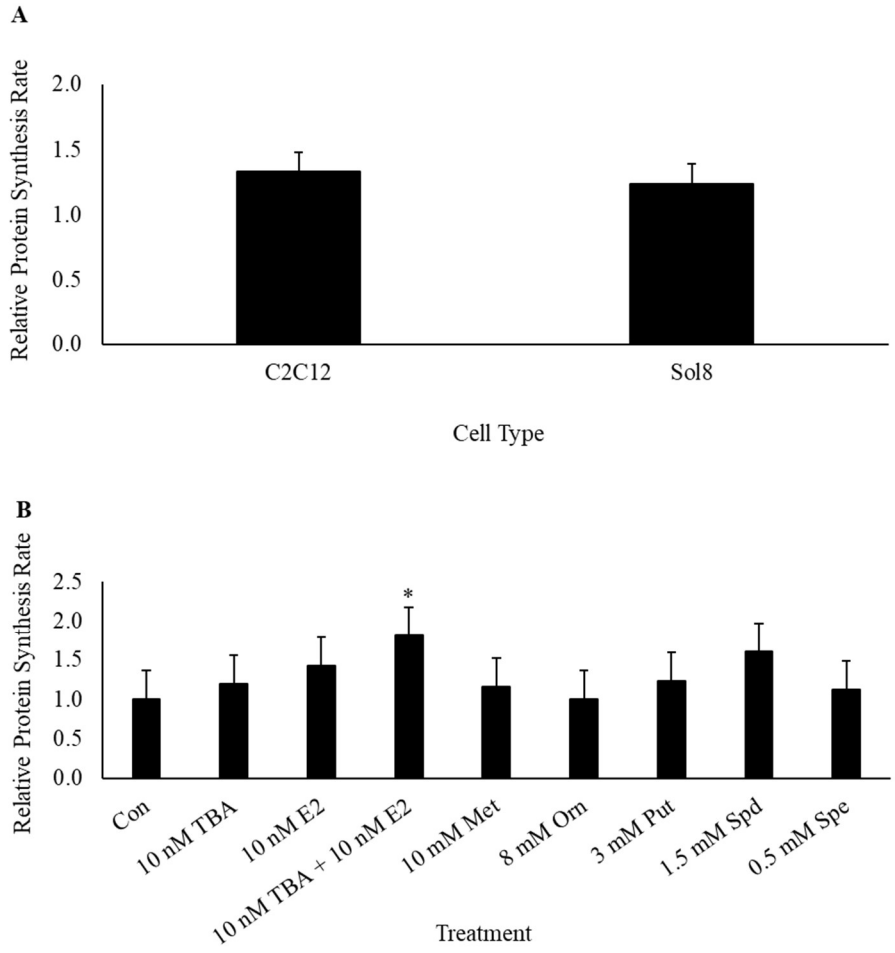

Figure 6.2. Effect of cell type (C2C12 or Sol8) or treatment on protein synthesis rates. Panel A demonstrates there is no difference (*P = 0.67*) in protein synthesis rate between cell types. Panel B shows the effect of treatment on protein synthesis rates of combined data from both C2C12 and Sol8 cells in the presence of serum free media (SFM, control, Con), 10 nM trenbolone acetate (TBA), 10 nM estradiol (E2), 10 nM TBA + 10 nM E2, (TBA+E2) 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Values represent the relative protein synthesis rate of each treatment compared to control cultures and are presented as the least square mean ± SEM from 5 separate replicates of C2C12 cultures and 5 separate replicates

of Sol8 cultures. Time points with a star (*) indicate differences ($P \leq 0.05$) in protein synthesis rates between that treatment and control cultures.

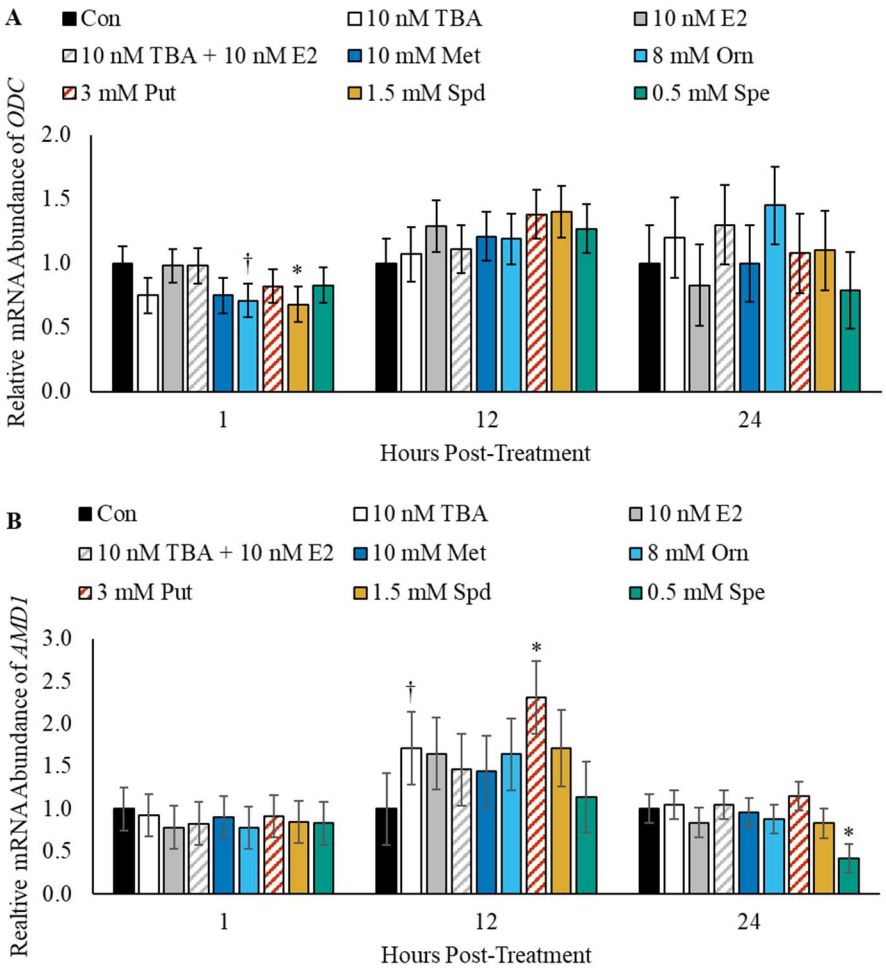

Figure 6.3. Relative mRNA abundance of two genes, (A) ornithine decarboxylase (ODC) or (B) S-adenosylmethionine decarboxylase (AMD1), involved in polyamine biosynthesis in fused C2C12 and Sol8 murine myoblast cells after treatment with serum free media (SFM, control, Con), 10 nM trenbolone acetate (TBA), 10 nM estradiol (E2), 10 nM TBA + 10 nM E2, (TBA+E2), 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Messenger RNA abundance was measured 1, 12, and 24 h after treatment as described in the Materials and Methods. Values represent the relative mRNA abundance and are presented as the least square mean ± SEM from 5 separate replicates of C2C12 cultures and 5 separate replicates of Sol8

cultures. Time points with a star (*) indicate that relative mRNA abundance of treatment cultures differed ($P \leq 0.05$) from control cultures and time points with a cross (†) indicate a tendency ($0.05 < P \leq 0.10$) for relative mRNA abundance of treatment cultures to be different from the control cultures.

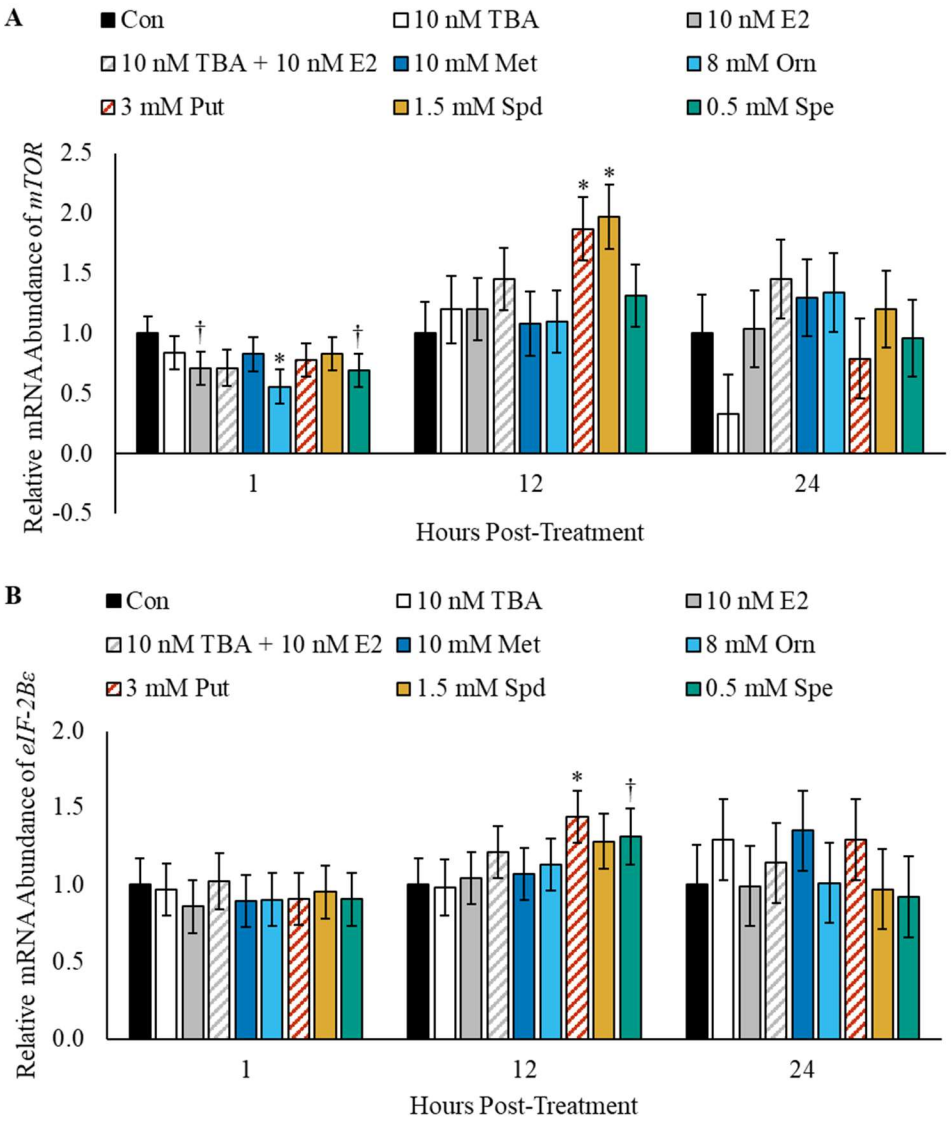

Figure 6.4. Relative mRNA abundance of two genes, (A) mammalian target of rapamycin (mTOR) or (B) eukaryotic translation initiation factor 2B subunit epsilon (eIF-2Bε), involved in skeletal muscle protein synthesis in fused C2C12 and Sol8 murine myoblasts after treatment with serum free media (SFM, control, Con), 10 nM trenbolone acetate (TBA), 10 nM estradiol (E2), 10 nM TBA + 10 nM E2, (TBA+E2), 10 mM methionine (Met), 8 nM ornithine (Orn), 3 mM putrescine (Put), 1.5 mM spermidine (Spd), or 0.5 mM spermine (Spe). Messenger RNA abundance was measured 1, 12, and 24 h after treatment

as described in the Materials and Methods. Values represent relative mRNA abundance and are presented as the least square mean ± SEM from 5 separate replicates of C2C12 cultures and 5 separate replicates of Sol8 cultures. Time points with a star (*) indicate that relative mRNA abundance of treatment cultures differed ($P \leq 0.05$) from control cultures and time points with a cross (†) indicate a tendency ($0.05 < P \leq 0.10$) for relative mRNA abundance of treatment cultures to be different from the control cultures.

CHAPTER VII

CONCLUSIONS

The population is increasing at an exponential rate and is expected to reach upwards of 9 billion by 2050 [1]. As the population continues to increase, land that is available for animal agriculture and food production is decreasing at a rapid rate [1, 2]. Therefore, livestock producers must adopt practices that maximize feed efficiency because feed comprises the largest part of input costs in a livestock operation [3]. Novel feedstuffs, exogenous compounds, and anabolic hormones can be utilized to maximize feed efficiency by decreasing feed costs, while maximizing skeletal muscle accretion, which becomes the consumable product at harvest. However, the exact effects of feeding some of the recently developed novel feedstuffs and the mechanisms through which exogenous compounds and hormones affect skeletal muscle growth remain largely unknown. This led to our hypothesis that through nutrition and supplementation of exogenous compounds or anabolic hormones, feed efficiency and growth can be improved. As such, the goal of this research was to gain an improved understanding of how novel fractionated alfalfa products affect growth, feed efficiency, and production of cattle and to determine the effects of polyamines, polyamine precursors, and anabolic hormones on skeletal muscle growth *in vitro* through assessment of proliferation, protein synthesis, and mRNA expression.

Chapters I through IV investigate the effects of feeding novel fractionated alfalfa products, alfalfa leaf pellets (PLM) and alfalfa stems (PFP), to finishing beef steers, developing dairy heifers, and lactating dairy cows. While others have investigated the effects of feeding alfalfa leaf meal [4-8] and alfalfa stem haylage [9] to cattle, we are the

first to examine the effects of feeding PLM and PFP to cattle. In finishing beef steers, inclusion of PFP in the diet results in decreased cost per kg of weight gain (COG), while having no adverse effects on growth or carcass characteristics. Inclusion of fractionated alfalfa in the diet of developing dairy heifers demonstrated that inclusion of PFP in the diet of developing dairy heifers results in decreased total feed costs, weight gain, hip height, wither height, and DMI, while having no effect on reproductive performance. In lactating dairy cows, inclusion of PFP in the diet resulted in decreased DMI and increased milk fat and rumination while feeding PLM in the diet resulted in increased milk yield, feed efficiency, and income over feed cost. Additionally, when both PFP and PLM are included in the diet of lactating dairy cows, milk fat is increased. Together, these results indicate that PFP has the potential to decrease feed costs without impacting growth of finishing beef steers but may not be an ideal feedstuff to include in diets for developing dairy heifers as it could decrease growth. Furthermore, inclusion of fractionated alfalfa in the diet of lactating dairy cows has the potential to improve milk yield and milk components. Additional research with more animals is needed to determine the optimum concentrations of these products in the diets for cattle.

To further investigate how molecules involved in growth and growth-promoting compounds affect growth, chapters V and VI take a mechanistic approach at examining the effects of anabolic hormones, polyamines, and polyamine precursors on skeletal muscle growth. Previous studies have investigated the effects of these compounds in bovine satellite cells [10, 11], and the effects of anabolic hormones in culture have been well-characterized in bovine satellite cells [12, 13], murine satellite cells [14], C2C12 murine myoblasts [15], and L6 rat myoblast cells [16]; however, no other studies have

examined the effects of anabolic hormones, polyamines, and polyamine precursors on proliferation, protein synthesis, and changes in mRNA abundance over time of genes involved in polyamine biosynthesis, protein synthesis, and growth in two different cell types (C2C12 and Sol8 murine myoblast cells). Results indicate that treatment of C2C12 and Sol8 cells with anabolic hormones results in increased proliferation and protein synthesis rates and treatment with polyamines and their precursors results in increased proliferation rates but has no effect on protein synthesis rates. Additionally, treatment of proliferating cells with polyamines increased mRNA expression of genes involved in polyamine biosynthesis and growth and treatment with polyamine precursors resulted in increased mRNA abundance of genes involved in growth; however, treatment with anabolic hormones had no effect on mRNA abundance of genes involved in polyamine biosynthesis or growth. Treatment of fused cells with anabolic hormones, polyamines, and polyamine precursors resulted in changes in mRNA expression such that treatment with anabolic hormones tended to increase expression of genes involved in polyamine biosynthesis and tended to decrease genes involved in protein synthesis and treatment with polyamines and their precursors both increased and decreased expression of genes involved in polyamine biosynthesis and growth. Overall, these results indicate that anabolic hormones, polyamines, and polyamine precursors have the ability to improve growth of skeletal muscle cells through increased proliferation and protein synthesis and alterations in mRNA expression of genes involved in polyamine biosynthesis, protein synthesis, and growth. However, additional research to assess mRNA expression of more genes and at additional time points is needed to determine the exact effects of these

compounds on skeletal muscle growth. Additionally, the effects of these compounds in differentiating cells should be explored to determine how they affect differentiation.

As with any study, the research presented in this dissertation had several limitations that need to be acknowledged. In chapters II and III, eight steers and heifers per treatment, respectively, were utilized in the experimental designs because this was the capacity of the university beef research facility at that time. Perhaps with more animals, more differences between the different treatment diets would have been observed. Another limitation is presented in chapter IV, where individual DMI was not recorded because the university dairy research unit is comprised of robotic milkers and, as such, DMI of the entire pen was recorded instead. If individual DMI could have been recorded, this may have impacted the observed results. In chapters II, III, and IV, diets were balanced to be isocaloric and isonitrogenous, however, post-trial feed analyses showed slight fiber and protein composition differences which could have influenced the observed results. In chapters V and VI, limitations included the number of timepoints (three) and genes (five in chapter V and four in chapter VI) that were analyzed. Perhaps, if more genes were analyzed at more than three timepoints, the observed results could have told a more complete story of how these compounds affect growth. Additionally, another approach to examining cell activity in chapters V and VI would be to assess protein expression instead of mRNA abundance because the rate of transcription and transcript stability can influence transcription [17]; however, our research group and others [12, 13, 18] have previously analyzed mRNA abundance to assess skeletal muscle cell activity.

In conclusion, this research provided an improved understanding of how feeding fractionated alfalfa to cattle affects growth, performance, and carcass characteristics of beef cattle, growth, development, and reproductive performance of developing dairy heifers, and milk yield and milk components of lactating dairy cows. Furthermore, this research elucidates the mechanisms through which anabolic hormones, polyamines, and polyamine precursors function to improve skeletal muscle growth. Limitations included a low number of experimental units in chapters II and III, the inability to calculate individual DMI in chapter IV, nutrient composition differences between the different treatment diets in chapters II, III, and IV, limited genes and timepoints analyzed in chapter V and VI, and analysis of mRNA expression to assess cell activity in chapters V and VI. Future studies should focus on optimizing concentrations of PLM and/or PFP in the diets of cattle to determine ideal inclusion rates for improving growth and feed efficiency at different stages of production in both beef and dairy cattle. Additionally, future studies should investigate the effects of anabolic hormones, polyamines, and polyamine precursors, on protein abundance at time points different from those analyzed in the present study to gain a further understanding of how these compounds improve skeletal muscle growth.

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12. Kamanga-Sollo, E., M. E. White, M. R. Hathaway, W. J. Weber, and W. R. Dayton. Effect of Estradiol-17β on protein synthesis and degradation rates in fused bovine satellite cell cultures. Domest Anim Endocrinol. 2010;39(1):54-62.

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associated with a modulation of myostatin and Pax7 expression. J Mol Endocrinol. 2008;40(5):231-241.

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CURRICULUM VITAE

Laura Ann Motsinger

Education

- 2018-2021 Ph.D. Utah State University, Logan, UT
  Animal Nutrition and Growth Biology; GPA: 3.61
- 2015-2017 B.S. Northwest Missouri State University, Maryville, MO
  Agricultural Business; GPA: 3.69
- 2015-2017 B.S. Northwest Missouri State University, Maryville, MO
  Animal Science; GPA: 3.92
- 2013-2015 A.S. Northeast Community College, Norfolk, NE
  Agriculture; GPA: 3.63

Professional Experience

- 4/20-Present Ph.D. Candidate, Utah State University, College of Agricultural and Applied Sciences, Department of Animal, Dairy and Veterinary Sciences
- 01/18-4/20 Graduate Research Assistant, Utah State University, College of Agricultural and Applied Sciences, Department of Animal, Dairy and Veterinary Sciences
- 06/17-08/17 Intern, Seaboard Foods, Guymon, OK
- 02/10-05/17 Farm Manager & Artificial Insemination Technician, Smith Farms, Kennard, NE
- 05/16-08/16 Intern, Missouri Department of Agriculture – State Milk Board, Jefferson City, MO

Professional and Academic Honors

- 2021 Recipient of the Mr. & Mrs. William W. Owens Scholarship ($688 award)
- 2021 Recipient of the Ogden Area Beef Feeders Scholarship ($818 award)
- 2020 1st Place in the ADVS Student Research Symposium Ph.D. and DVM Mini Presentation Competition, Utah State University ($200 award)
- 2020 Recipient of the Cache Valley Cooperative Dairy Scholarship ($319 award)
- 2017 Graduated Cum Laude (Fall), Northwest Missouri State University
- 2017 Academic Honor Roll (Fall), Northwest Missouri State University
- 2017 President's Honor Roll (Spring), Northwest Missouri State University
- 2016 Academic Honor Roll (Fall), Northwest Missouri State University
- 2016 Academic Honor Roll (Spring), Northwest Missouri State University
- 2015 President's Honor List (Spring), Northeast Community College
- 2015 American FFA Degree, National FFA Organization
- 2014 Dean's List (Fall), Northeast Community College

Research Support

Competitive Grant Awards

Internal

- 10/19-10/20 Determining the effects of zinc and manganese in the presence of trenbolone aetate and/or estradiol on proliferation rates of bovine satellite cells
    - Project Director: Laura A. Motsinger
    - ADVS Graduate Research Opportunity (AGRO) Grant Program
    - $4,000 awarded
    - Goal: to refine the roles of zinc and manganese in support of bovine muscle growth.

External

- Not funded Examining the effects of various trace mineral supplementation strategies on growth and reproductive performance of developing beef heifers
    - Project Director: Laura A. Motsinger
    - USDA-NIFA Education and Workforce Development Predoctoral Fellowship Program
    - $119,621.54 requested
    - Goal: to improve our understanding of the effects of trace mineral supplementation on performance of developing beef heifers.

Publications

Submitted or in Preparation for Submission

- **L. A. Motsinger**, A. Y. Young, R. Feuz, R. Larsen, T. J. Brady, R. K. Briggs, C. C. Reichhardt, C. Pratt, K. J. Thornton. Replacing alfalfa hay with a novel alfalfa leaf pellet product (ProLEAF MAX) and/or alfalfa stems (ProFiber Plus) in the diet of developing dairy heifers influences growth and development while having no impact on reproductive performance. In preparation for submission to Translational Animal Science.

- **L. A. Motsinger**, A. Y. Young, R. Feuz, R. Larsen, C. Pratt, K. J. Thornton. Inclusion of a novel alfalfa leaf pellet product (ProLEAF MAX™) and/or alfalfa stems (ProFiber Plus™) in the diet of lactating dairy cows alters production. In preparation for submission Journal of Dairy Science.

- **L. A. Motsinger**, N. E. Ineck, B. A. Udy, C. L. Erickson, L. L. Okamoto, C. C. Reichhardt, G.K. Murdoch, K. J. Thornton. Understanding the effects of trenbolone acetate, polyamine precursors, and polyamines on proliferation rates and abundance of genes involved in myoblast growth and polyamine biosynthesis in murine myoblasts. In preparation for submission to Domestic Animal Endocrinology.

- **L. A. Motsinger**, L. L. Okamoto, C. C. Reichhardt, Y. Harraq, G. K. Murdoch, K. J. Thornton. Impacts of anabolic hormones, polyamine precursors, and polyamines on protein synthesis rates and abundance of genes involved in protein synthesis and polyamine biosynthesis in murine myoblasts. In preparation for submission to Animals.

- C. C. Reichhardt, L. L. Okamoto, S. P. Lopez, A. F. Alberto, R. K. Briggs, **L. A. Motsinger**, F. Batistel, K. J. Thornton. The influence of fish oil supplementation and muscle and adipose location on transcriptional markers of inflammation and growth of skeletal muscle and subcutaneous adipose in weaned piglets. In preparation for submission.

Published Articles

- **L. A. Motsinger**, A.Y. Young, R. Feuz, R. Larsen, T. J. Brady, R. K. Briggs, B. Bowman, C. Pratt, K. J. Thornton. 2021. Effects of feeding a novel alfalfa leaf pellet product (ProLEAF MAX) and alfalfa stems (ProFiber Plus) on performance in the feedlot and carcass quality of beef steers. Translational Animal Science. 5(3). doi: 10.1093/tas.txab098.

- C. C. Reichhardt, E. M. Messersmith, T. J. Brady, **L. A. Motsinger**, R. K. Briggs, B. R. Bowman, S. L. Hansen, K. J. Thornton. 2021. Anabolic implants varying in hormone type and concentration influence performance, feeding behavior, carcass characteristics, plasma trace mineral concentrations, and liver trace mineral concentrations of Angus sired steers. Animals. 11(7), 1964. doi: 10.3390/ani11071964.

- C. C. Reichhardt, R. Feuz, T. J. Brady, **L. A. Motsinger,** R. K. Briggs, B. R. Bowman, M. D. Garcia, R. Larsen, K. J. Thornton. 2021. Interactions between cattle breed type and anabolic implant strategy impact circulating serum metabolites, feedlot performance, feeding behavior, carcass characteristics, and economic return in beef steers. Domestic Animal Endocrinology. 77(106633). doi: 10.1016/j.domaniend.2021.106633.

- C. C. Reichhardt, L. L. Okamoto, **L. A. Motsinger**, B. P. Griffin, G. K. Murdoch, K. J. Thornton. 2021. The impact of polyamine precursors, polyamines, and steroidal hormones on temporal messenger RNA abundance in bovine satellite cells induced to differentiate. Animals. 11(3), 764. doi: 10.3390/ani11030764.

<u>Oral Symposium Presentations</u>

- **L. A. Smith**, A. Y. Young, R. K. Briggs, T. J. Brady, C. C. Reichhardt, K. J. Thornton. Effects of feeding a novel alfalfa leaf pellet (ProLEAF MAX™) and an alfalfa stem byproduct (ProFiber Plus™) on growth and conception rates of developing dairy heifers. ADVS Student Research Symposium, August 2020.

<u>Conference and Symposium Poster Presentations</u>

- **L. A. Smith**, A. Y. Young, C. Pratt, K. J. Thornton. Effects of feeding a novel alfalfa leaf pellet product (ProLEAF MAX™) and alfalfa stems (ProFiber Plus™) on production of lactating dairy cows. American Society of Animal Science/Canadian Society of Animal Science/Southern Section – American Society of Animal Science Annual Meeting and Trade Show Annual Meeting and Trade Show, July 2021, Louisville, KY.

- **L. A. Smith**, N. E. Ineck, B. A. Udy, C. L. Erickson, K. J. Thornton. PSIV-9 Effects of polyamines and trenbolone acetate on proliferation rates of murine myoblasts. American Society of Animal Science/Canadian Society of Animal Science/Western Section – American Society of Animal Science Virtual Annual Meeting and Trade Show, July 2020.

- **L. A. Smith**, A. Y. Young, R. K. Briggs, T. J. Brady, C. C. Reichhardt, K. J. Thornton. PSVI-6 Effects of feeding a novel alfalfa leaf pellet (ProLEAF MAX™) and an alfalfa stem byproduct (ProFiber Plus™) on growth and conception rates of developing dairy heifers. American Society of Animal Science/Canadian Society of Animal Science/Western Section – American Society of Animal Science Virtual Annual Meeting and Trade Show, July 2020.

- **L. A. Smith**, N. E. Ineck, B. A. Udy, C. L. Erickson, K. J. Thornton. Effects of polyamines and trenbolone acetate on proliferation rates of murine myoblasts. ADVS Student Research Symposium, August 2019, Logan, UT.

- **L. A. Smith**, N. E. Ineck, B. A. Udy, C. L. Erickson, K. J. Thornton. PSII-8 Effects of polyamines and trenbolone acetate on proliferation rates of murine myoblasts. American Society of Animal Science and Canadian Society of Animal Science Annual Meeting and Trade Show, July 2019, Austin, TX.

- **L. A. Smith**, A. Y. Young, R. K. Briggs, T. J. Brady, K. J. Thornton. Effects of feeding a novel alfalfa product on performance of feedlot steers. ADVS Student Research Symposium, August 2018, Logan, UT.

Undergraduate Students Mentored

- 2021-present Bailee Brown; Animal, Dairy and Veterinary Sciences Undergraduate Student, Utah State University
- 2019-present Lillian Okamoto; Animal, Dairy and Veterinary Sciences Undergraduate Student, Utah State University
- 2021 Youssef Harraq, SURE Student, Snow College
- 2021 Alanna Stramm; Animal, Dairy and Veterinary Sciences Undergraduate Student, Utah State University
- 2020 Sierra Lopez; Animal, Dairy and Veterinary Sciences Summer Intern, Utah State University
- 2019 Abbey Spencer; Animal, Dairy and Veterinary Sciences Lab Volunteer, Utah State University

- 2018 Khloe Tebbs; Animal, Dairy and Veterinary Sciences Lab Volunteer, Utah State University
- 2019 Emily Esposito; Animal, Dairy and Veterinary Sciences Summer Intern, Dixie State University
- 2019 Tanya Adamson; Animal, Dairy and Veterinary Sciences Summer Intern, Brigham Young University-Idaho
- 2019 Ian Sroufe; Animal, Dairy and Veterinary Sciences Summer Intern, Utah State University
- 2019 Jefawn Eskeets; Native American STEM Mentorship Program, Utah State University Eastern-Blanding
- 2019 Hannah Begay; Native American STEM Mentorship Program, Utah State University Eastern-Blanding
- 2019 Shania Whitney; Native American STEM Mentorship Program, Utah State University Eastern-Blanding
- 2018 Chris Erickson; SURE Student, Utah State University Eastern
- 2018 Brynne Udy; Animal, Dairy and Veterinary Sciences Summer Intern, University of Utah

Teaching Activities

Department of Animal, Dairy and Veterinary Science, Utah State University

Teaching Assistantship (TA Positions)

- Fall 2018, 2019, 2020: ADVS 2500, Feeds and Feeding, Instructor: Dr. Kara J. Thornton

Delivered Lectures

- Guest panel for ADVS 1110 Introduction to Animal Science, Instructor: Dr. Chad Page. Careers in Animal Science Guest Panel. Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah, October 2021
- Feeds and Feeding ADVS 2500 (Laboratory Session), Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, Utah, 2018-2020
- Guest lecture for Senior Ag Capstone. Instructor: Tyler Schindler. Animal Production Enterprises/Animal Management Systems Informational Session. Omaha Bryan High School, Omaha, Nebraska, October 2019
- Guest lecture for AGRI 03460 Advanced Animal Nutrition, Instructor: Dr. M. McGee. Insight to graduate school. School of Agricultural Sciences, Northwest Missouri State University, Maryville, Missouri, March 2019

Professional Development

Professional Meetings (Attended and Presented)

- 10/21 Attended NC1184: Molecular Mechanisms Regulating Skeletal Muscle Growth and Differentiation Multistate Annual Meeting
- 07/21 Attended ASAS-CSAS-SSASAS Annual Meeting and Trade Show, Louisville, KY
- 08/20 Attended Virtual ADVS Student Research Symposium, Utah State University

- 07/20 Attended ASAS-CSAS-WSASAS Virtual Annual Meeting and Trade Show
- 08/19 Attended ADVS Student Research Symposium, Utah State University, Logan, UT
- 07/19 Attended ASAS-CSAS Annual Meeting and Trade Show, Austin, TX
- 08/18 Attended ADVS Student Research Symposium, Utah State University, Logan, UT

Memberships in Professional Societies & Organizations

- 2019-present American Society of Animal Science and Canadian Society of Animal Science
- 2015-present Phi Theta Kappa Honor Society
- 2016-2017 National Society of Leadership and Success
- 2016-2017 Sigma Alpha Professional Agricultural Sorority
- 2016-2017 Block and Bridle
- 2015-2017 Delta Tau Alpha Honor Society
- 2015-2017 Collegiate Farm Bureau
- 2015-2017 ATA/CFFA/PAS
- 2015 Agricultural Communications Team

Workshops

- 08/20 Empowering Teaching Excellence Conference, Utah State University, Logan, UT
- 07/20 USDA Pre-/Post-doctoral Fellowship Writing Workshop, ASAS-CSAS-WSASAS Virtual Annual Meeting and Trade Show
- 11/19 Powerful Publishable Plots, Utah State University, Logan, UT
- 09/19 Planning and Writing Successful Proposals: Getting Started, Utah State University, Logan, UT
- 06/19 CAAS Grantsmanship Training Series: Developing Budgets for Grant Proposals, Utah State University, Logan, UT
- 06/19 CAAS Summer Professional Development Workshop Series for Research Trainees: Orientation to Scientific Scholarly Literature, Utah State University, Logan, UT
- 04/19 SAS Workshop, Utah State University, Logan, UT
- 08/18 Empowering Teaching Excellence Conference, Utah State University, Logan, UT
- 07/18 Effective Research Presentations, Utah State University, Logan, UT
- 06/18 Managing Your Research Data, Utah State University, Logan, UT

Service

- 04/19 Graduate Enhancement Award Review Panel, Utah State University, Logan, UT
- 04/19 Research Judge, Student Research Symposium, Utah State University, Logan, UT

Other

- 04/19 Cultural Competence Course, Utah State University, Logan, UT