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Genomic investigation of beta agonist supplementation and heat stress in livestock species

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GENOMIC INVESTIGATION OF BETA AGONIST SUPPLEMENTATION AND HEAT STRESS IN LIVESTOCK SPECIES

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β-adrenergic agonists (β-AA), commonly fed to cattle during the last 20-40 days of the finishing period, improve muscle growth by decreasing adipose deposition and increasing muscle accretion. In most cases, final live weights, hot carcass weight and average daily gain have all been shown to increase when β-AA are feed while fed intake, back fat, and marbling all decrease. Two β-AA, Ractopamine HCl (β1-AA) and Zilpaterol HCl (β2-AA) are currently approved for use in beef cattle in the United States. Converse to the beneficial effects of β-AA, heat stress in livestock decreases production efficiency and growth. There have also been reports that β-AA supplementation during times of stress, including heat stress, can cause mobility issues and even death. Little research has been done investigation the interaction between these two factors. The purpose of these studies was to investigate β-AA, heat stress and the interaction between them with respect to transcript expression in different skeletal muscles of cattle and lambs. RNA was isolated from the biceps femoris and longissimus dorsi of cattle supplemented with Ziplaterol and the semitendinosus of lambs supplemented with either Ractopamine or Zilpaterol subjected to either an ambient or heat stressed environment. Two to five million reads per sample was obtained using 3' QuantSeq and were aligned to either the UMD3.1 bovine transcriptome or the Oar_v4.0 ovine transcriptome. Reads aligned at a rate of 50-60% and represented 10,000 to 13,000 transcripts. No interaction was found between β-AA and heat stress. Between the cattle and sheep studies, β-AA altered cyclic AMP signaling by decreasing proteolysis. Novel mechanisms were also identified relating to exercise and the callipyge phenotype. Heat stress increase the oxidative stress response along with increasing other stress related responses. Based on these results, β-AA do not induce an increased stress risk in livestock species.

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CHAPTER I: A LITERATURE REVIEW

Introduction

As the world's population continues to increase, the demand for food becomes greater. This comes at a time when agricultural land is decreasing, regulations have become more strict and the cost to maintain animals, including feed costs, is increasing (Lake et al., 2012). To keep up with rising demands, new strategies and techniques must be applied to production agriculture systems. Furthermore, it is necessary that advances in animal agriculture also consider animal wellbeing. The purpose of this work is to investigate how the genetic and genomic response of livestock contribute to both growth and the animals' response to environmental stress. An improved understanding of these aspects of livestock production have the potential to contribute significantly toward the goal of meeting the growing consumer needs.

Beta-Adrenergic Agonists

The supplementation of livestock with synthetic compounds known as beta-adrenergic agonists (β-AA) help to achieve more efficient muscle growth (Johnson et al., 2014). These compounds have been of interest to researchers for the past two decades as a method to improve efficiency in livestock production (Montgomery et al., 2009). They are proposed to achieve this outcome by decreasing adipose accretion and increasing protein accretion, thereby resulting in a leaner carcass. Understanding the mechanism by which this occurs is important to improving the efficiency in the livestock industry.

Mechanisms of Action

β-AA interact with cells by binding to beta-adrenergic receptors (β-AR), G-protein coupled receptors, located on the plasma membrane (Mills and Mersmann, 1995). In addition to synthetic β-AA, the catecholamines, epinephrine and norepinephrine, naturally compete to bind β-ARs (Mersmann, 1998). β-AR contain over 400 amino acids with different models indicating that the β-AR have seven hydrophobic transmembrane domains which attach the receptor to the plasma membrane (Mersmann, 1998). The ligand binding site is located in the center of the seven transmembrane domains (Mills and Mersmann, 1995). When the β-AR is bound, adenylate cyclase is stimulated through

the stimulatory G-protein system, increasing cyclic adenosine monophosphate (cAMP) (Liggett and Raymond, 1993). When the cAMP concentration is increased, it binds to a regulatory subunit of protein kinase A causing the phosphorylation of serine residues of metabolic hormones. This phosphorylation results in the partial hydrolysis of triacylglycerol (TAG) (Ricks et al., 1984b). The phosphorylation of acetyl-CoA carboxylase inhibits de novo biosynthesis of fatty acids. Therefore the response of adipose tissue to β-AA administration is to depress TAG accumulation and release fatty acids (Oscar, 1995; Mersmann, 1998; Johnson et al., 2014). Based on this proposed mechanism, it has been hypothesized that β-AA can reduce adipose tissue accretion by inhibiting de novo fatty acid biosynthesis and stimulating TAG hydrolysis of lipolysis (Mersmann, 2002). Further, inhibiting protein turnover and promoting myofibrillar protein synthesis increases muscle mass (Ricks et al., 1984a).

Subtypes of Beta-Receptors

Different subtypes of β-AR have been identified and include β1-AR, β2-AR and β3-AR. The β1-AR and β2-AR are the most well characterized and most abundant in mammalian cells (Mersmann, 1998). Across species, the orthology is over 70% for a specific subtype while the homology within a species is between 45 and 60% (Mersmann, 2002). Of the three identified subtypes, the β1-AR subtype is the largest with approximately 460 amino acids, β2-AR has 420 amino acids and β3-AR is the smallest being composed of 410 amino acids (McNeel and Mersmann, 1999). The function of these receptors is similar, only differing in the compounds they bind. Studies using norepinephrine, epinephrine and other synthetic antagonists and agonists along with ligand binding showed that within a tissue, one receptor type predominates (Mersmann, 2002). For example, rat heart contains over 90% β1-AR, while guinea pig tracheal muscle contains over 85% β2-AR, and rat adipocytes contains over 90% β3-AR (Mersmann, 1998; Mersmann, 2002). Ligand binding studies have shown that bovine and ovine skeletal muscle and adipocytes contain primarily β2-AR (Johnson et al., 2014). Although β3-AR have been found in rat adipocytes, none have been found in cattle adipocytes (Mersmann, 1998). There have been reports of β3-AR found on porcine adipocytes with an estimate that porcine adipocytes contained 7% β3-AR (McNeel and Mersmann, 1999).

Types of Beta-Agonists

Clenbuterol and cimaterol were the first β-AA investigated as potential means of increasing production efficiency in livestock species including cattle and sheep (Warriss et al., 1989). Although they were effective, the improvements they made were not significant enough to account for their cost (Warriss et al., 1989; Koohmaraie et al., 1991; Pringle et al., 1993). During the 1990s and early 2000s, two new β-AA were introduced to the market; at the current time, these are the only approved for use in cattle in the United States. Ractopamine Hydrochloride (RAC) is produced by Elanco Animal Health and sold under the trade name Optaflexx for cattle, Paylean for swine and Topmax for turkeys. Zilpaterol Hydrochloride (ZH) is produced by Merck Animal Health and sold under the trade name Zilmax. RAC is a β1-AA where ZH is β2-AA. While both β-AA have been approved by the FDA, animal wellbeing concerns related to feeding ZH resulted in its removal from production systems, therefore RAC is the primary β-AA used in cattle production (AVMA, 2014). At the current time, neither β-AAs are approved in sheep or chickens, although research continues to gain approval for these species (AMSA, 2015).

Benefits of Beta-Agonists

In livestock, β-AAs are typically fed during the last 20 to 40 days of the finishing period. The amount of β-AA fed can affect the amount of growth, as too much or two little can have a negative effect (O'Connor et al., 1991; Smith and Paulson, 1997; Avendaño-Reyes et al., 2006; Johnson et al., 2014). Improving efficiency is an important reason why β-AA are being used today. Producers and consumers can be positively impacted with an improved efficiency by increasing gains and decreasing feed intake (Johnson et al., 2014). There are many performance, carcass and physiological traits that are affected when β-AA are fed including feed intake, gain to feed ratios, average daily gain and final body weights (Vasconcelos et al., 2008; Elam et al., 2009; Montgomery et al., 2009). Different carcass traits including hot carcass weight, dressing percentage, marbling, ribeye area and yield grade are also affected by β-AA (Vogel et al., 2005; Strydom et al., 2009; Strydom et al., 2011; Hales et al., 2014).

Feed intake and efficiency are two performance traits that are affected by feeding β-AA and are of high importance to producers. Reports of the impact of β-AA on dry matter intake (DMI) vary from suggesting that they have no impact (Vogel et al., 2005; Gruber et al., 2007; Elam et al., 2009; Robles-Estrada et al., 2009; Lopez-Carlos et al., 2011) to showing as much as a 7.4% decrease (Scramlin et al., 2010; Hales et al., 2014). Gain to feed ratios (G:F), indicating a more efficient animal, have been reported to increase from 15-30% when ZH is fed to cattle (Vasconcelos et al., 2008; Montgomery et al., 2009; Hales et al., 2014). RAC has also been shown to increase G:F ratios by 20.5% (Abney et al., 2007). In addition to feed efficiency average daily gain (ADG) is increased up to 74% due to ZH supplementation (Montgomery et al., 2009; Hales et al., 2014). RAC is reported to have a similar effect with an increase of 17.9% when fed at 200 mg per head per day (Vogel et al., 2005). Final live weight is increased as seen by a 5% improvement in cattle fed ZH (Montgomery et al., 2009; Robles-Estrada et al., 2009) as well and an increase when RAC is fed (Vogel et al., 2005; Lopez-Carlos et al., 2011).

Compared to final live weight, hot carcass weight (HCW), the weight of an animal's muscle, fat and bone after slaughter once the head, hide, intestinal tract and internal organs have been removed (Knight, 2014), is a better indicator of muscle growth of an animal. From the HCW, yield grade and dressing percentage can be assessed. A majority of reports state that HCW is increased from 5 to 16.4 kg when β-AA are fed to different livestock species (Vogel et al., 2005; Gruber et al., 2007; Vasconcelos et al., 2008; Elam et al., 2009; Montgomery et al., 2009; Hales et al., 2014; Van Donkersgoed et al., 2014; Boyd et al., 2015). With dressing percentage being directly correlated to HCW, increase in HCW will be accompanied by an increase in dressing percentage (Vasconcelos et al., 2008; Elam et al., 2009; Montgomery et al., 2009; Strydom et al., 2009; Scramlin et al., 2010; Hales et al., 2014). In another trial, cattle fed 300 mg of RAC per head per day had an increase in final live weights by 22 lbs. and hot carcass weight by 20 lbs. (Elanco, 2017). Another study found that heifers fed 300 mg of RAC had a greater increase in final body weight and hot carcass weight than those fed 200 mg (Edenburn et al., 2016). An increase in longissimus muscle area as well as dressing percentage were observed in heifers fed 300 mg (Edenburn et al., 2016). Contrary to

these findings, Gruber et al (2007) reported no difference in dressing percentage between cattle fed RAC and those fed a control diet, although an increase in HCW was observed.

Along with altering the efficiency of muscle growth, β-AA are hypothesized to decrease lipogenesis and adipose deposition throughout the body (Mersmann, 1998). Multiple studies have reported that 12th rib fat was decreased when ZH (Vasconcelos et al., 2008; Elam et al., 2009) or RAC (Vogel et al., 2005) was fed to cattle. However, β-AA effects on internal kidney-pelvic-heart (KPH) fat are varied. Some reports state that KPH fat is decreased after feeding β-AA (Vasconcelos et al., 2008; Elam et al., 2009) while others report that it is unchanged (Vogel et al., 2005; Montgomery et al., 2009). Although decreased fat deposition is one benefit of β-AAs, it can cause a decrease in marbling score, or the amount of fat present in the muscle (Knight, 2014). A decrease in marbling score is often accompanied by a decrease in tenderness as fat in the muscle increases tenderness (Knight, 2014). The impact of β-AA on fat deposition is supported by data showing both ZH and RAC decrease marbling score (Vogel et al., 2005; Gruber et al., 2007; Elam et al., 2009; Montgomery et al., 2009; Hales et al., 2014). Zilpaterol Hydrochloride has been shown to be more effective than other β-AA when considering growth and carcass characteristics and profitability. Cattle fed ZH had approximately a 2% higher dressing percentage than cattle fed RAC (Scramlin et al., 2010). An increase in profit makes ZH more favorable than RAC for producers and consumers (Schroeder and Tonsor, 2011).

Consumption Safety

Zilpaterol Hydrochloride has a minimum three day withdrawal period before slaughter to ensure residues in the muscle tissue are eliminated before consumption (Merck, 2017). Conversely, RAC has not been shown to leave residues after feeding so a withdrawal period is not necessary (Elanco, 2017). Shelver and Smith (2006) showed that up to 95% of Zilpaterol residue was absent from muscle tissue on day two of the withdrawal period. Another study reported that only 6% of Zilpaterol residues found on day one of withdrawal were detectable on day two in skeletal muscle and liver tissue (Stachel et al., 2003). These studies support the three day withdrawal period as sufficient in removing residues from muscle making it safe for human consumption (Shelver and

Smith, 2006). Public perception of ZH compared to RAC has been damaged because of isolated instances of illness following consumption of meat from animals fed ZH (Kuiper et al., 1998; Mazzanti et al., 2003). Showing that ZH fed animals are safe to consume is important to keep these products available to producers.

Potential Adverse Effects of Beta-Agonists

Numerous studies have been conducted to evaluate the effects of β-AA in livestock species. Many studies report the benefits β-AA have on animals and their positive impact on the industry. However, some studies report unwanted effects of β-AA. As noted previously, cattle fed the β1-AA clenbuterol had decreased marbling scores and tenderness compared to cattle fed a control diet (Schiavetta et al., 1990). This is similar to Montgomery et al. (2009), who additionally showed a decrease in carcass quality. While these carcass characteristics are decreased, flavor and palatability are not. Merck Animal Health reported that although tenderness is decreased, flavor is unaffected when cattle are fed ZH (Merck, 2017). Another study states that sensory panel tasting scores were no different between control fed and ZH fed cattle (Weber et al., 2013).

Physiological Response to Beta-Agonists

Very little literature exists looking at the effects of β-AA on respiration rates and rectal temperatures, both measures of stress in livestock. Hales et al (2014) was one of the first to look at the effects of β-AA on respiration rate. It was reported that, as days fed ZH increased from 20 to 40, respiration rate increased linearly. Although an increase was seen, it is unclear whether the increase was due to feeding the β-AA or an increase in animal weight (Hales et al., 2014). Another study examined respiration rate and rectal temperature of cattle housed in shaded or unshaded housing fed either ZH or a control diet. ZH was associated with increased respiration rate and decreased body temperature irrespective of the housing condition (Boyd et al., 2015). To account for this change, Mersmann (1998) suggested that when β-AA are fed, blood flow may increase to the muscles, leading to the hypothesis that with blood flow away from the core may have cooling effects on the body. However additional research needs to be conducted to fully understand this and other physiological effects of β-AAs.

Genes Affected by Beta-Agonists

Calpains and calpastatins play a role in meat quality and tenderness. Calpastatin is positively correlated with meat tenderness where calpains inhibit calpastatin (Du Toit and Oguttu, 2013). Both are altered during the administration of β-AA (Parr et al., 1992) although reports conflict on the specifics of this dysregulation. Some studies suggest they decrease (Brooks et al., 2009; Strydom et al., 2009; Strydom et al., 2011), while Parr et al. (1992) shows an increase in both calpain and calpastatin protein expression by 27 and 76%, respectively (Parr et al., 1992). Although, this result is conflicting to the described relationship between calpains and calpistatins. An increase in calpastatin and/or a decrease in calpains could have negative effects on meat quality as they could contribute to decreased tenderness (Koohmaraie et al., 2002) as was also found in Nellore cattle, where ZH increased calpastatin activity and tenderness was decreased (Cônsolo et al., 2016).

Different genes related to muscle characteristics have been investigated for changes due to β-AA. Evaluation of myosin heavy chain (MyHC) isoforms are a means to determine which fiber types are present in muscle (Gunawan et al., 2007; Kellermeier et al., 2009). In swine, *MYH-1* was not altered after RAC was fed, while *MYH-2A* expression decreased after one week and remained lower than controls through week two; by weeks two and four, *MYH-2X* expression was decreased (Gunawan et al., 2007). Similar effects are seen when ZH was fed to steers: *MYH-1* remained unchanged, *MYH-2A* decreased and *MYH-2X* increased (Baxa et al., 2010). Differences seen in *MYH-2X* expression when RAC and ZH are fed could account for differences in muscle growth between the two. *MYH-2X* gives rise to the largest diameter fibers which are also fast and glycolytic. An increase in *MYH-2X* expression could explain a larger increase in muscle seen in ZH fed animals. A comprehensive study of altered gene expression is necessary to fully understand the effects of β-AA.

Zilmax Controversy

In 2013, there were instances of increased mobility issues and mortality in feedlots using ZH and as a result, concerns arose about the wellbeing of cattle being fed ZH (Loneragan et al., 2014). Anecdotal claims attributed these issues to feeding ZH,

causing Tyson and many other packing plants to stop accepting cattle fed this supplement (Sorensen, 2016). Soon after, Merck Animal Health removed ZH from the market in the United States, and as a consequence its use in the United States has dramatically decreased. Regardless of the true cause of the animal wellbeing issues observed in the initial complaints, the public's perception of ZH is negative (Sorensen, 2016). Regaining a positive perception of ZH is key to getting it back in the hands of producers. Toward that end, mobility issues of cattle fed ZH and a control diet were compared showing that as time on fed increased, mobility decreased for all animals, including controls (Boyd et al., 2015). This work suggested that ZH was not the cause of mobility issues but could be attributed to the increase in muscle mass as cattle age and grow. Further, transport and standing on concrete floors at the packing plants were also hypothesized to contribute to mobility problems (Boyd et al., 2015). This research agrees with many other studies, which are finding that ZH has no measurable negative effect on animal wellbeing (Hales et al., 2014; Boyd et al., 2015; Sorensen, 2016)

Heat Stress

A major concern surrounding the livestock industry today is heat stress, which is defined as when an animal's body temperature rises above their thermo-neutral zone and the heat load exceeds the capacity for heat dissipation (Bernabucci et al., 2010). An animal's thermo-neutral zone is characterized as when heat production and heat loss are equal (Srikandakumar et al., 2003). Heat stress is more pronounced when surface temperatures and humidity are increased. Both these factors, high temperature and humidity, along with alterations in precipitation and atmospheric greenhouse gases, are affected by a changing climate (Archana et al., 2017). It has been predicted that by 2100, the average surface temperature will be 1.8 º C to 4 º C higher than it is at the current time (Renaudeau et al., 2012). Accompanied with an increase in average temperatures, there has been an increase in sporadic heat wave events across the United States and other parts of the world. These heat wave events are characterized as periods of above average temperatures for days, weeks, or months and can be accompanied high humidity with little to no rain (Renaudeau et al., 2012). Over the next few decades, the number of these heat wave incidents is predicted to increase in number and intensity (Renaudeau et

al., 2012). The increase in temperatures has led to a heightened concern for the wellbeing of animals living in these conditions.

Temperature-Humidity Index (THI)

A strategy to monitor heat stress in livestock, and predict times of extreme danger to an animal's health is the use of the temperature-humidity index (THI) (Silanikove, 2000). Various means are used for the calculation of the THI (Silanikove, 2000), but commonly it is determined as: THI = (Dry bulb temperature ºC) + (0.36 x dew point temperature ºC) + 41.2 with dry bulb temperature being the temperature and dew point temperature being the relative humidity (St-Pierre et al., 2003). The interpretation of THI values also vary between species depending on their specific thermo-tolerance. Sheep tend to have a higher tolerance to heat stress and can maintain thermoregulatory functions at a higher THI value (Srikandakumar et al., 2003). For cattle, values under 70 are comfortable, values between 75-78 are stressful and values above 78 are considered dangerous and can cause distress (Silanikove, 2000). Figure 1 shows a standard THI chart used in the livestock industry.

Animal Wellbeing

Within the livestock industry, animal wellbeing is of utmost concern. Animal wellbeing can be defined as an individuals' ability to cope with the environment in which it lives (Silanikove, 2000). Additionally, an animal's wellbeing is directly connected to its productivity. When an animal's wellbeing is not optimal, its productivity, including growth rates and carcass characteristics, is lowered (Silanikove, 2000) (IFC, 2014). Along with production, increased mobility issues and mortality are also concerns. As an example, during a severe heat wave in 2006, more than 25,000 cattle and 700,000 poultry were lost due to heat stress in California (Renaudeau et al., 2012; Belhadj Slimen et al., 2016). Understanding heat stress at a physiological level and being able to identify the signs are key to maintaining animal wellbeing and productivity.

Signs of Heat Stress

Heat stress can have many effects on an animal including increased rectal temperature, respiration rate, panting and open mouth breathing, water consumption and

a decrease in feed intake (Marai et al., 2007). Rectal temperature is commonly used as indicator of an animal's core body temperature (Schmidt-Nielsen, 1997). As homeotherms, livestock generally maintain their core temperature even under changing environmental conditions by dissipating excess heat from the body (Abd-El-Samee and Marai, 1997). In sheep, a normal range for rectal temperature is between 38.3 and 39.9 º C. When rectal temperatures reach above 42 ºC, it is considered to be dangerous for the animal and can cause damage (Thwaites, 1985). Cattle have a normal temperature range from 36.7 to 39.1º C (Merck, 2017). Rectal temperature varies only slightly depending on the season and the time of day (Marai et al., 2007). In lambs, rectal temperature was notably lower in the morning hours than the afternoon and evening hours (Shalaby, 1985). This could be attributed to heat dissipation overnight, which helps to control the heat load on the animal and helps maintain normal eating behaviors (Mader et al., 2006). Coat color and type can also affect the heat load on the animal. Cattle with a slick coat have the ability to dissipate heat better than those with a rough coat (Hammond et al., 1998). Similarly, those with a lighter colored coat were found to have a lower body temperature than those with a darker coat (Olson et al., 2003). All of these factors can play a role in an animal's ability to control its body temperature.

Increased respiration rate is another indicator of heat stress in animals. Respiration is used to prevent hyperthermia by the evaporation of moisture from the respiratory tract (Phillips and Piggins, 1992). When high temperatures are paired with high humidity, respiration rate is increased even further (Marai et al., 2002). Walking has also been shown to increase respiration rate especially during the summer months (Khan and Ghosh, 1989). An increased respiration rate can lead to panting and open mouth breathing, which helps to dissipate heat more effectively when the heat load becomes too great for the primary methods of evaporative mechanisms (Hagenmaier et al., 2016). Once open mouth breathing is observed, respiration rates and rectal temperatures will continue to increase as environmental temperatures remain high (Johnson et al., 2014; Hagenmaier et al., 2016).

Animals under heat stress conditions have decreased feed intake and increased water consumption (Marai et al., 2007). During heat stress, animals will shift their

feeding to the morning hours instead of in the afternoon and evening (Blackshaw and Blackshaw, 1994). Converse to feed intake, water intake increased in the morning hours and decreased in the afternoon to evening (Marai et al., 2000). Breed can also effect feeding and water consumption during heat stress. Comparisons between *Bos indicus* and *Bos taurus* concluded that feed intake for *B. taurus* was significantly decreased during heat stress, while *B. indicus* showed no significant change (Beatty et al., 2006). Desert and mountain sheep breeds have an increased water turnover compared to arid sheep breeds (Marai et al., 2007). There have been reports that water temperature has an effect on water consumption. One study concluded that dairy cows drank more warm water than chilled water during heat stress conditions (Wilks et al., 1990). Although dairy cows tended to drink warmer water, if the water temperature was too high, it had a negative effect on water consumption (Wilks et al., 1990).

Strategies to Combat Heat Stress

Finding ways to combat heat stress is a priority of producers as heat stress reduces production, which in turn reduces profit (Renaudeau et al., 2012). It is therefore in the producer's best interest to find ways to minimize heat stress. There are many different methods that are used to combat heat stress. One of the most popular methods to alleviate heat stress is shade (Mitlöhner et al., 2002). Other methods include altering feeding times, water availability, monitoring rectal temperatures and installing water sprayers (Beatty et al., 2006; Renaudeau et al., 2012). Some of these methods are harder to accomplish than others because they require frequent handling of the animals, which itself can also add to stress. Alleviating heat stress while minimizing other stressors is key in keeping production levels up during periods of increased heat.

Providing shade is one of the simplest methods to alleviate heat stress in livestock animals as it is relatively inexpensive and hands off. Shade can either be manmade (i.e. sheds) or natural (i.e. trees and bushes). Trees are an excellent example of natural shading because they are able to provide both shade and a cooling effect as moisture evaporates from the leaves (Renaudeau et al., 2012). Shade structures made of wood, metal slates or snow fence are less affective at alleviating heat stress than shade structures made with aluminum or galvanized white roofs (Renaudeau et al., 2012). In an experiment by

Mitlohner et al (2002), ADG and final body weight were higher in cattle provided shade than those left unshaded. Many reports state that time of feeding plays a role in how much feed is consumed during heat stress (Beatty et al., 2006). Adjusting feeding times could help keep intake and energy levels up. Increasing energy content in the diet can also help to maintain production (Renaudeau et al., 2012). Water consumption must increase to make up for requirements needed in heat stressed animals (Renaudeau et al., 2012). One strategy used to maintain these requirements is an ab libitum supply of water available to the animals especially during the hottest time of the day. Misters and sprayers are two systems that are used to alleviate heat stress. Spraying and misting systems use the energy from the air to evaporate water and reduce air temperatures (Mitlöhner et al., 2001; Mitlöhner et al., 2002). These systems tend to work better in dry arid environments because in more humid environments the water droplets may be too large and will not evaporate fully, which can cause feed and bedding to become wet (Renaudeau et al., 2012). The results of studies looking at misters and sprayer have been somewhat varied. Some studies have reported that sprayers have the ability to increase performance in finishing pigs by up to 10% (Renaudeau et al., 2012)

Physiological Response

Originally, it was believed that the heat stress response was an intracellular response not associated with an extracellular response, meaning the physiological and genetic responses were not connected (Collier et al., 2008). Further research has concluded that the heat stress response is integrated with the physiological stress response as part of a gene network in a variety of tissues and cells (Collier et al., 2008). At the cellular level, heat stress produces changes in the cellular function, which can induce oxidative cell damage, disrupt cellular function and lead to apoptosis and necrosis of tissue (Du et al., 2008). All of these changes in cellular response can be characterized by transcriptional gene changes and altered protein synthesis (Collier et al., 2008). Variation between thermal tolerance has been seen amongst different cell and tissue types (Collier et al., 2008). Cellular functions altered by heat stress include the inhibition of DNA synthesis, transcription, RNA processing and translation, inhibition of the progression through the cell cycle, denaturation of proteins, and degradation of proteins through

lysosomal and proteasomal pathways (Archana et al., 2017). There is also a disruption of components of the cytoskeleton, reduction in cellular ATP due to altered metabolism, and changes in membrane permeability, which lead to increased intracellular levels of Na^+, H^+ and Ca^{2+} (Belhadj Slimen et al., 2016). One of the first cellular components affected by heat stress is the mitochondria. Altered morphology, histological differences, and even necrosis in the mitochondria have been reported in the skeletal muscles of animals during heat stress (Belhadj Slimen et al., 2016). As a result, normal cellular functions are inhibited.

Heat Shock Proteins

Heat shock proteins (HSPs) are activated by heat and other stressors. They act as molecular chaperons which provide the cell's ability to survive injury and oxidative stress (Collier et al., 2008). During stress conditions, HSPs interact with denatured proteins by inhibiting cytotoxic protein aggregates to form which helps maintain homeostasis within the cell (Mayer, 2005). HSPs can also provide protection against other stressors such as bacteria by signaling the immune system to increase the number of neutrophils and macrophages to combat pathogenic bacteria in the body (De, 1999).When heat stress occurs, the expression of these proteins is increased considerably (De, 1999). HSPs lack introns, which in part may explain their rapid expression due to stressors (Sonna et al., 2002).

HSPs are classified based on their biological function and molecular weight. Within livestock species the most studied HSPs include HSP70 and HSP90. Both HSP70 and HSP90 are found in low levels under normal conditions but they increase considerable when stress is introduced (Feder and Hofmann, 1999; Hue et al., 2013). There are reports that HSP70 is the most abundant and heat sensitive (Feder and Hofmann, 1999). HSP70, HSP90 and HSP27 have been found to be anti-apoptotic in mammalian cells (Garrido et al., 2001). In cattle, HSP70 has a cytoprotective function in the intestine, kidney and embryo (Bhat et al., 2016). An improvement in heat tolerance in cattle has also been associated with SNPs within the 5'-UTR region of HSP70 (Silva et al., 2013). Additionally, the expression of HSP27 is increased in the muscle of beef cattle under stress conditions (Shibata et al., 2014).

Genetic Response

Identifying genes related to heat stress and heat tolerance is of interest to the industry as that understanding can be incorporated into management and breeding decisions. Rimoldi et al (2015) examined the expression of stress-related genes in growing broiler strains of chicken. The CAT (catalase) mRNA was upregulated in the liver of broilers, while CASP6 (caspase 6) expression was downregulated in broilers exposed to heat stress (Rimoldi et al., 2015). Another gene under investigation to elucidate its role in heat stress response is that responsible for slick hair in Senepol cattle. Evidence suggests that dairy cattle with the dominant slick hair allele have lower rectal temperatures and respiration rates than those without (Olson et al., 2003). This is an example where if the role of a particular gene is understood, it can be incorporated into additional populations allowing them to become more successful under heat stress conditions. However, the use of a single locus for selection should proceed with caution; therefore, the impact of this variant on carcass traits and growth performance is under investigation (Olson et al., 2003).

Interaction of β-AA and Heat Stress

The individual effects of β-AA and heat stress on livestock species have been studied but little research has been performed to examine the interaction between the two. Some data suggest that feeding a β-AA during hot conditions increases the symptoms of heat stress. Associations without supported causation include that cattle fed ZH during the summer months have a larger drop-off in feed intake than those fed ZH during cooler months (Allen, 2017); however there is no consensus among reports regarding the impact of heat stress during β-AA supplementation (Hagenmaier et al., 2016); Allen, 2017; Grandin, 2013). Little to no research has specifically examined the interaction between β-AA and heat stress and its effect on growth traits and carcass characteristics. This is an area of research that is timely given concerns with climate change as well as consumer perception of regarding natural practices of animal production.

Marcias-Cruz et al (2010) represents one of a few studies examining changes to growth traits and carcass characteristics during heat stress conditions while being fed a β-AA. Ewe lambs were fed a control diet or ZH during heat stress conditions with an

average ambient temperature of 34.1º C and an average of 50.4% humidity. For the traits of final live weight, ADG, feed intake, feed conversion, and G:F, there was no difference between controls and those fed ZH. Under normal conditions, all but feed intake increased with ZH. These data suggest that the effect of heat stress on performance traits is diminished during times of heat stress. When carcass traits were analyzed, there was a significant increase in HCW, cold carcass weight (CCW) and dressing percentage when lambs were fed ZH consistent with the effects seen from ZH under normal conditions (Macías-Cruz et al., 2010). These data suggest there are no consequential interactions between β-AA and heat stress. However, additional research must be conducted to fully understand what is happening when β-AA are fed during heat stress conditions.

RNA-sequencing

RNA-sequencing (RNA-Seq) is a relatively new strategy, which uses deep-sequencing technologies to quantify transcript expression (Wang et al., 2009a). For this method, a population of RNA is converted to a cDNA library with adaptors attached. This library is then sequenced to obtain short (30 to 400bp), single- or paired-end reads, (Wang et al., 2009a). More recently, 3' QuantSeq has gained popularity as it generates low-noise and low-cost gene expression data (Moll et al., 2014). This method generates a single library molecule per transcript which is complementary to the 3' end. Generally the libraries are sequenced with 100bp, single-end reads (Asmann et al., 2009). For projects only interested in differential expression, 3' QuantSeq will provide enough information for these analyses (Moll et al., 2014).

Benefits of RNA-Seq

Unlike microarrays, which assay only the transcripts included on the platform, RNA-Seq allows for the investigation of known and novel transcripts. This is ideal for discovery-based experiments where little to no information is available on the molecular mechanisms underlying a condition of interest (Moll et al., 2014). Further, as new genome annotations are released, data from RNA-Seq can be reevaluated with the new genome (Tachibana, 2015). RNA-Seq also allows for the analysis of a wide range of variables other than differential expression. It allows for the examination SNPs, insertions, deletions, and alternative splice variations, which can be achieved from one

data set instead of multiple sets individually. Analysis of miRNA, mRNAs, siRNAs, and lnRNAs are also all possible with RNA-Seq (Wang et al., 2009b). RNA-Seq has also presented itself to have a beneficial application in the clinical setting. For example, scientists are using this technology to identify drug targets, disease biomarkers and even how individuals may respond to drug treatments (Tachibana, 2015)

Drawbacks of RNA-Seq

Although there are many benefits to RNA-Seq, there are still draw backs. One is the price of sequencing large data sets. It can cost roughly $2000 per sample depending on how many reads wanted and if the sequencing is single end or pair end (Wang et al., 2009a). If the goal of the project is differentially expression, this can be accomplished at a cheaper price using 3' Quant Seq (Moll et al., 2014). Another drawback is performing an experiment on a species with a poorly annotated genome or transcriptome, which could result in a misinterpretation of data, missing, or excluded information. Lastly, RNA-Seq is unable to distinguish the difference between two genes that have overlapping transcripts. (Hirsch et al., 2015). Although there are drawbacks, the benefits greatly outweigh them. RNA-Seq has become the standard in majority of genomics-based projects today.

Conclusion

Heat stress and β-AA have been studied individually, but little is known about the interaction. By gaining a further understanding of the mechanisms of each individually and their interaction, we can improve growth and efficiency along with animal welfare. This researched aimed to identify the mechanisms why which they work and identified ones that could be used to further improve production. It also aimed to study the interaction between the two and identify methods to improve animal welfare if needed.

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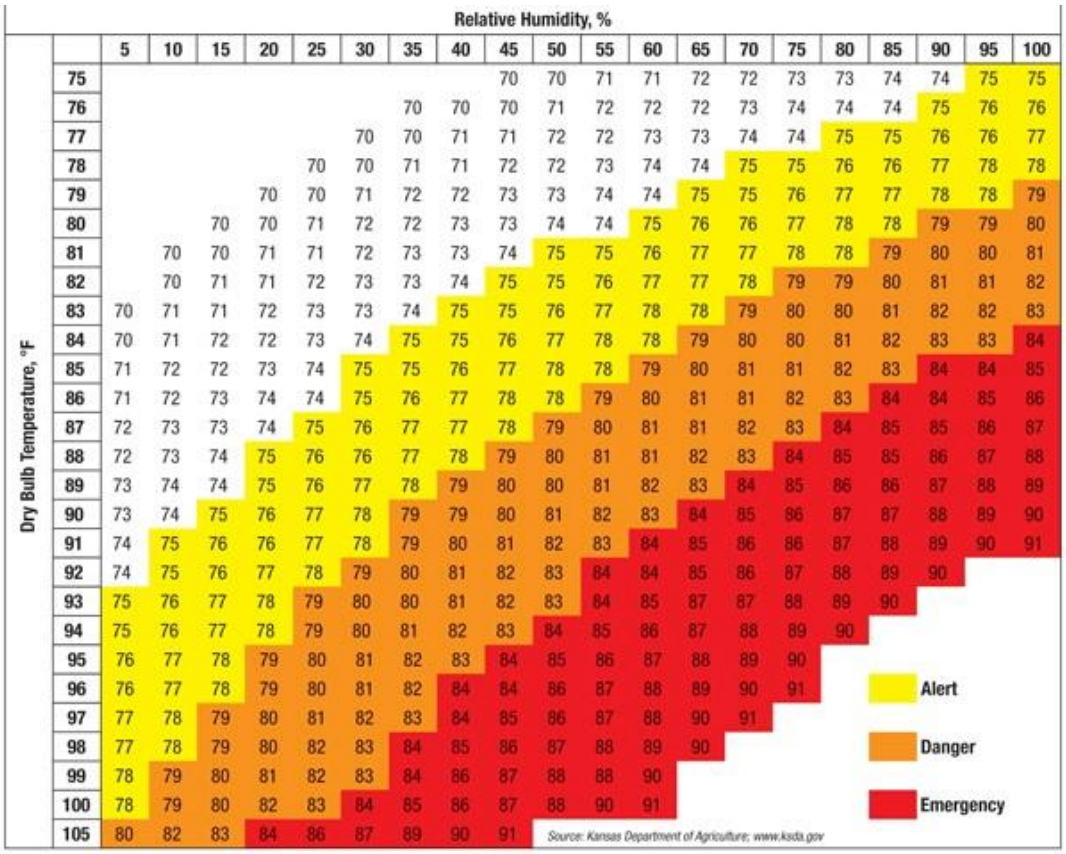

Dry Bulb Temperature, °F \ Relative Humidity, %	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
75									70	70	71	71	72	72	73	73	74	74	75	75
76							70	70	70	71	72	72	72	73	74	74	74	75	76	76
77						70	70	71	71	72	72	73	73	74	74	75	75	76	76	77
78					70	70	71	71	72	72	73	74	74	75	75	76	76	77	78	78
79				70	70	71	72	72	73	73	74	74	75	75	76	77	77	78	78	79
80			70	70	71	72	72	73	73	74	74	75	76	76	77	78	78	79	79	80
81		70	70	71	71	72	73	73	74	75	75	76	77	77	78	78	79	80	80	81
82		70	71	71	72	73	73	74	75	75	76	77	77	78	79	79	80	81	81	82
83	70	71	71	72	73	73	74	75	75	76	77	78	78	79	80	80	81	82	82	83
84	70	71	72	72	73	74	75	75	76	77	78	78	79	80	80	81	82	83	83	84
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88	72	73	74	75	76	76	77	78	79	80	81	81	82	83	84	85	85	86	87	88
89	73	74	74	75	76	77	78	79	80	80	81	82	83	84	85	86	86	87	88	89
90	73	74	75	76	77	78	79	79	80	81	82	83	84	85	86	87	87	88	89	90
91	74	75	76	76	77	78	79	80	81	82	83	84	85	86	86	87	88	89	90	91
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94	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90				
95	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90					
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97	77	78	79	80	81	82	83	84	85	86	87	88	90	91						
98	77	78	79	80	82	83	84	85	86	87	88	89	90							
99	78	79	80	81	82	83	84	86	87	88	88	90								
100	78	79	80	82	83	84	85	86	87	88	90	91								
105	80	82	83	84	86	87	89	90	91											

Alert
Danger
Emergency

Source: Kansas Department of Agriculture; www.ksda.gov

Figure 1. This figure shows a standard THI for livestock species including cattle, swine and sheep. (Source: Kansas Department of Agriculture, http://agriculture.ks.gov/)

CHAPTER II: INVESTIGATION OF DIFFERENTIALLY EXPRESSED TRANSCRIPTS IN CATTLE SUPPLEMENTED WITH ZILPATEROL HYDROCHLORIDE

Introduction

As the worlds' population increases, the need for food increases. The land we have to grow crops and raise livestock is decreasing, therefore more efficient crops and livestock are needed (Lake et al., 2012). Increasing amount of product produced per head of livestock is one necessary means by which to meet these needs. While this increase is beneficial to the consumer, it can also benefit the producer. If growth can be increased while time on feed can be decreased, the enterprise is more profitable. Many different variables alter growth rate in livestock, including nutrition, genetics, environment, and management techniques.

Growth differences, including hot carcass weight, average daily gain and amount of fat deposition, have been documented between *Bos indicus* and *Bos taurus* (Marshall, 1994) as well as differences in breeds within *B. taurus* cattle (Greiner, 2005). Environment can also cause changes in growth with animals in hotter environments having slower growth (Marai et al., 2007). In addition, natural genetic variation can alter growth composition. One example is double muscling in both Belgian Blues and Piedmontese caused by a mutation in the myostatin gene that causes a reduction or elimination of the myostatin protein, which determines muscle fiber number at birth (McPherron and Lee, 1997). When this determining factor is eliminated, cattle have an increase in both hyperplasia and hypertrophy (McPherron and Lee, 1997). Another example of natural increased muscle growth is the callipyge phenotype in sheep. Callipyge is caused by a single point mutation in the *DLK1* and *MEG3* gene region; those with the phenotype have an increase in muscle in the hind region (Koohmaraie et al., 1995; Yu et al., 2018).

As one means to increase growth efficiency, β-Adrenergic agonists (β-AA) are fed to livestock during the last 20-40 days of the finishing period. These supplements increase muscle protein accretion and decrease adipose deposition (Johnson et al., 2014), by acting through specific 7-transmembrane β-adrenoreceptors. Final live weight, hot

carcass weight, average daily gain and gain to feed increase during supplementation of β2-AA while dry matter intake and fat secretion decrease (Elam et al., 2009). The two types of β-AA approved for use in the United States are classified by the receptor isoform to which they primarily bind (Mersmann, 1998). Ractopamine Hydrochloride (RAC, binds to β1 receptor) and Zilpaterol Hydrochloride (ZH, binds to β2 receptor) (Johnson et al., 2014) are those that are, or have been respectively used in finishing cattle. Skeletal muscle contains proportionally more β2 than β1 receptors which has led to the hypothesis that β2-AA are more effective than β1 in terms of muscle growth and final gains (Scramlin et al., 2010). One study states that cattle fed ZH had an increased hot carcass weight, cold carcass weight and dressing percentage when compared to those fed RAC (Lopez-Carlos et al., 2011). The mechanism by which β-AA work to increase lean composition is not fully understood. However, β-AA signal through cyclic adenosine monophosphate (cAMP) (Baviera et al., 2010). In turn, cAMP signals through several downstream effectors; two altered by β-AA are the ubiquitin proteasome pathway and the calcium dependent pathway (Mersmann, 1998; Baviera et al., 2010; Figure 1). When these systems are decreased, muscle growth is increased, due to a decrease in proteolysis. The purpose of this research is to identify genes and pathways that are altered due to β-AA and to understand the mechanism by which they act to increase muscle deposition in finishing livestock.

Materials/Methods

Animal Model

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee at the University of Nebraska (IACUC #902).

Twenty ovariectomized cross-bred heifers were assigned to one of two treatment groups: a finishing diet without supplementation (CON; n =10) or the same finishing diet including supplementation of ZH (ZH; n =10) at 8.33 mg/kg body weight (BW) on a dry matter (DM) basis. In ZH fed heifers, 5% of the high-moisture corn in the diet was replaced with 4.99% fine ground corn and 0.015% ZH. Heifers on the control diet also received 5% fine ground corn in place of the normal diet to ensure similarity between the

two treatments. All heifers were fed daily at 0800 h for twenty d. On d 24, following a mandatory three day withdrawal, all feed was removed and at 1000 h each heifer received an intravenous bolus of bovine CRH and arginine VP, 0.3 ug/kg BW and 1.0 ug/kg BW respectively. The catheters were removed at 1800 h and the daily allotment of feed was delivered. During the withdrawal period, both groups had the 5% high-moisture corn returned to their diets (Buntyn et al., 2016). On d 25, 26 and 27, all heifers were harvested at the Loeffel Meat Lab at the University of Nebraska – Lincoln. Harvest order was randomly assigned based on treatment, therefore some heifers experienced a longer withdrawal period from ZH. At harvest, muscle samples were taken from the longissimus dorsi (LD) and the biceps femoris (BF), flash frozen in liquid nitrogen and stored at −80°C until further analysis. Additional details can be found in Buntyn et al (2015).

RNA Isolation/RNA-Sequencing

RNA was isolated from the BF and LD muscles from 11 heifers (CON n = 5; ZH n = 6) using the Direct-zol RNA MiniPrep Plus kit (Zymo Research) with modifications. After the tissue was homogenized in Tri-zol, a chloroform precipitation was performed. Roughly 100 mg of tissue was weighed and minced quickly before being placed in 600 uL of Trizol. Sample was left in Trizol for approximately 5 min then homogenized with an addition of 400 uL Trizol. The homogenized tissue was incubated at room temperature for 5 min after which 200 uL of chloroform was added, vortexed for 30 sec and incubated at room temperature for 3 min. Following this incubation, samples were centrifuged for 15 min at 11,900 rpm and 4 ºC. The top aqueous layer was removed and placed in 600 uL of 100% ethanol and mixed. This mixture was placed on a spin column and washed twice using 400 uL of RNA pre-wash buffer. A 15 min DNase treatment was then performed using 75 uL of DNA digestion buffer and 5 uL of 6U/uL of DNase per sample. This treatment was followed by three washes with 700 uL of RNA wash buffer. RNA was eluted in 50 uL of RNase free water. Once isolated, RNA was quantified and integrity analyzed using the Agilent Bioanalyzer. RNA Integrity Number (RIN) scores above 7 were considered adequate and sent to the University of California Davis' Genome center (Davis, CA) for QuantSeq 3' mRNA library prep and 100 bp, single-end sequencing on an Illumina HiSeq 4000.

Bioinformatics Analyses

Sequence quality was assessed on all samples through FASTQC (Andrews, 2010). Poly-A ends and adapters were trimmed using bbmap (k=13 ktrim=r forcetrimleft=11 useshortkmers=t mink=5 qtrim=t trimq=10 minlength=20; Bushnell, 2014) then reevaluated with FASTQC. All reads were mapped to the Bovine UMD3.1 transcriptome with STAR alignment (Dobin et al., 2013). Bam files were created and Integrative Genomics Viewer (IGV) (Thorvaldsdóttir et al., 2013) was used to visualize the data. The trimmed sequences were then pseudo aligned to the transcriptome incorporating the flag –noLengthCorrection and quantified using Salmon Quantification (Patro et al., 2015). A read count matrix was constructed using the generated counts from Salmon. Differential expression analysis was performed in R (RStudio, v1.1.442) using limma-voom (Law et al., 2014; Ritchie et al., 2015). The counts were trimmed removing those with eight or less observations within treatment groups. Transcripts with an adjusted P-value less than 0.1 were considered significant and analyzed for inclusion in known molecular pathways through Ingenuity Pathway Analysis (IPA; Krämer et al., 2013). As IPA only recognizes human, mouse and rat genes, human or mouse orthologous gene names were used in place of bovine locus identifiers when needed.

Results

An average of 4.4 million reads (min=3.6 million, max=5.2 million) were obtained per sample. The cattle genome contains 22,915 annotated transcripts; after trimming lowly expressed transcripts, 10,295 transcripts were observed across the samples. Three genes were differentially expressed (AdjP < 0.1) in ZH compared to the control in the LD whereas 39 were differentially expressed in the BF. The three DE genes in the LD were also DE in the BF were: calmodulin (*CALM1*), Solute Carrier Family 25 Member 25 (*SLC25A25*), and Solute Carrier Family 9 Member A2 (*SLC9A2*). The significantly DE genes from each comparison can be found in Tables 1 and 2.

In the BF, 22 pathways were altered due to ZH and 42 were altered in the LD. Pathways were considered significant with a p value < 0.05 and a –log (p-value) > 1. One pathway altered in both muscles was nNOS signaling in skeletal muscle (p-value = 0.006 (LD) and 0.002 (BF)). Also altered in the BF were glycolysis (p-value = 0.001) and fatty

acid β-oxidation (p-value = 0.002). All significant pathways altered in the BF and LD can be found in tables 3 and 4, respectively.

Discussion

The purpose of this study was to identify genes and pathways affected by β-AA supplementation. This information is valuable because it allows for a better understand of how β-AA work. In this study, along with β-AA supplementation, all heifers received a CRH/VP bolus. Transportation of cattle before slaughter has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis. The CRH/VP challenge has been shown to elicit a similar endocrine response to transportation (Carroll et al., 2007), therefore it was given to mimic this response. Since all heifers received the same bolus, any alterations in the transcriptome due to the bolus are expected to be similar across all animals; therefore all significant effects are assumed to be due to ZH.

In both muscles, the most noticeable change in gene expression was calmodulin 1 (*CALM1*). *CALM1*, is a calcium binding protein which plays an important role in many cellular processes. An upregulation of *CALM1* and other genes within the calmodulin pathway have been implicated in muscle growth. McKinsey et al. (2002) described that *CALM1* leads to activation of myocyte enhancer factor-2 (*MEF2*). The *MEF2* transcription factors interact with myogenic regulatory factors which lead to an activation of muscle specific genes. Additionally one cellular process controlled partly by *CALM1* is muscle contraction (McGivney et al., 2009). Smooth muscle contraction is signaled by the phosphorylation of myosin light chain (MLC). MLC is phosphorylated by MLC kinase which is activated by calmodulin bound by calcium (Tansey et al., 1994). Similarly, in broiler chickens fed phytase, a supplement known to increase final live weights and improve feed conversion, *CALM1* was also upregulated (Schmeisser et al., 2017) as was the case in lambs fed ZH (R.M. Kubik, unpublished). This and the prior work noted, indicate that the upregulation in *CALM1* in these heifers is contributing to muscle growth (Friday et al., 2000; McKinsey et al., 2002).

As previously identified, β-AA signal through cAMP. A decrease in calcium dependent proteolysis, the breakdown of protein dependent on calcium and calpain, is decreased due to β-AA supplementation. An increase in calmodulin has been shown to

decrease calpain activity, in turn decreasing proteolysis (Tremper-Wells and Vallano, 2005). Along with *CALM1*, salt inducible kinase 1 (*SIK1*) is involved in cAMP signaling. *SIK1* is a transcriptional target of cAMP response element-binding protein (*CREB*) and has been shown to promote *MEF2* activity in myocytes through the phosphorylation of histone deacetylase proteins (HDACs) (Stewart et al., 2013). As previously stated, *MEF2* activates multiple muscle specific genes (Figure 2). Stewart et al. (2013) found that when *SIK1* was decreased in primary muscle precursor cells, *MEF2* protein accumulation and myogenic differentiation were decreased. It was also found that *SIK1* transcript levels increase when protein kinase A (PKA) activity increases (Stewart et al., 2013). These findings support a model in which *SIK1* integrates cAMP signaling with myogenesis to correctly identify the timing of differentiation.

Another gene with a possible role in cAMP signaling and muscle growth is *SLC25A25*. *SLC25A25*, a mitochondrial membrane solute transporter that possibly controls ATP homeostasis as a calcium regulated shuttle, has been hypothesized to play a role in metabolic efficiency linked to muscle function (Anunciado-Koza et al., 2011). In mice lacking *SLC25A25* that were subjected to exercise on a treadmill, muscle function was compromised along with lowered exercise capability. They also had a reduced calcium flux an ATP content. In the wild type mice, *SLC25A25* was upregulated during exercise. (Anunciado-Koza et al., 2011). Another study in mice injected with formoterol, a β2-AA, there was an increase in *SLC25A25* at one h post injection (Pearen et al., 2009). In the current study, heifers fed ZH had a 2.5 true fold increase in *SLC25A25* similar to work in a lamb model (R.M. Kubik, unpublished) and in bovine myoblast cell culture (J.L. Petersen, unpublished). The link of *SLC25A25* to calcium can also lead to the hypothesis of its involvement in cAMP signaling. These data point to *SLC25A25* having an important role in muscle function and growth.

Pathway analysis of the differentially expressed genes predicted the up- and down-regulation of glycolysis and fatty acid β-oxidation with β-AA supplementation, respectively (Figures 3 and 4). An increase in glucose along with a corresponding increase in glucose induced insulin secretion have been attributed to β-AA, especially ZH, in skeletal muscle (Lacey et al., 1990). In a study by Barnes et al. (2017), glucose

oxidation was increased in the flexor digitorum superficialis muscle in lambs fed ZH. An increase in both glucose and glucose oxidation indicate an increase in glycolysis, which was predicted to be upregulated in both heifers and lambs fed ZH. Fatty acid β-oxidation is negatively correlated with glucose levels (Muoio and Newgard, 2008). A decrease in fatty acid β-oxidation had also been linked to an increase in carbohydrate breakdown (Muoio and Newgard, 2008) and ultimately a decrease in adipose deposition (Devarshi, 2017). β-AA decrease fat deposition, which could be done though a decrease in fatty acid β-oxidation. These pathways together indicate a mechanism by which β-AA work to increase lean muscle accretion and decease adipose deposition.

Based upon the DE of transcripts *CALM1* and *RYR1*, IPA analyses predicted neuronal nitric oxide synthase (nNOS) signaling to be increased in both the BF and LD. An increase in nNOS signaling has multiple effects on muscle development and function along with mitochondrial bioenergetics (Stamler and Meissner, 2001). Further, nitric oxide (NO) appears to have an important role in muscle repair as defects in nNOS signaling are found in many skeletal muscle diseases in which there is a dysfunction in repair (Brenman et al., 1995). As an example, myogenic precursor cell homeostasis was altered in mouse skeletal muscle with an impairment in nNOS signaling. This deficiency causes stunted muscle fiber growth and subsequently a decrease in muscle performance (De Palma et al., 2014). Based on these data, supplementation with ZH leads to an increase in nNOS signaling which ultimately contributes to the observed increases in muscle growth and performance.

Conclusion

β-AA supplementation is common in livestock production to increase muscle growth and efficiency. Both the consumer and the producer benefit from more efficient animals. Although the exact mechanism by which β-AA work, they have been shown to signal through cAMP and different secondary messengers. A decrease in calcium dependent proteolysis along with a decrease in ubiquitin proteolysis were predicted with β-AA supplementation. This decreased breakdown of protein can lead to muscle growth. An increase in glycolysis along with a decrease in fatty acid β oxidation linked to glucose concentration is also a possible mechanism through which β-AA act. Increased glucose

oxidation can lead to muscle growth whereas a decrease in fatty acid β oxidation can decrease adipose deposition, both of which are observed in β-AA fed animals. These data indicate that β2-AA are working through multiple pathways to achieve the desired effects. By gaining a greater understanding of how β-AA work, we can further improve these methods. It can help us create target these specific pathways to create a better, more efficient supplement.

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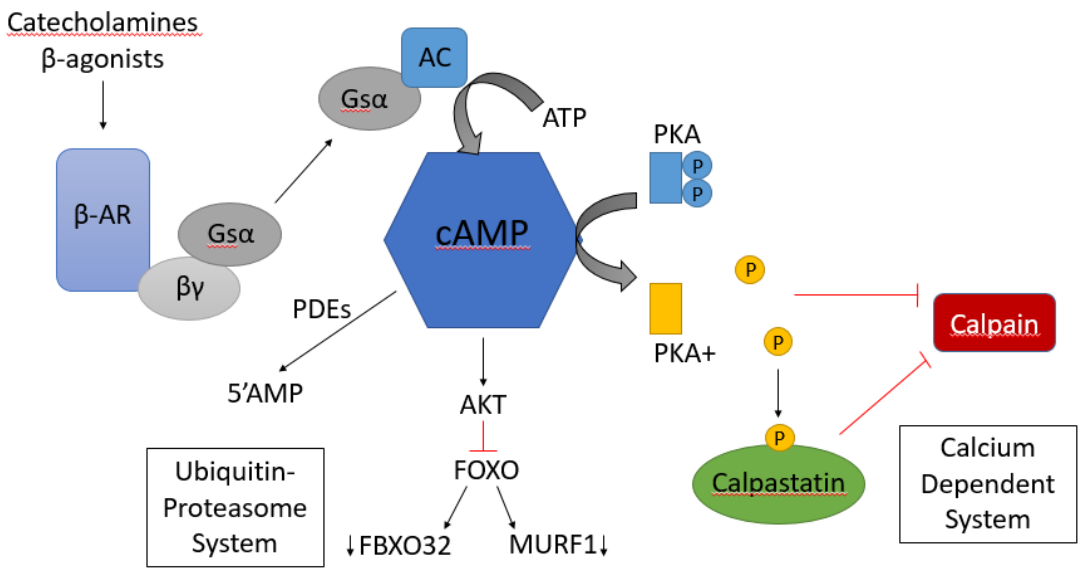

Figure 1. cAMP signaling pathway. β-AA act as artificial catcholamines and bind to the β-adrenoceptor (β-AR). This causes a conformational change in Gαs to activate adenylate cyclase which causes an increase in cAMP. Proteolysis can occur through two systems downstream of the secondary messenger, cAMP. The first being the calcium dependent system (right). cAMP dephosphorylates protein kinase A (PKA) which can inhibit calpains or bind with calpastatin subsequently inhibiting calpains. This pathway is calcium dependent meaning when calcium levels are increased, this pathway is decreased and calpains are inhibited. The second system is the ubiquitin proteasome proteolytic pathway. AKT is inhibited when can lead to the phosphorylation of FOXO. This phosphorylation can decrease both FBXO32 and MURF1 suppressing this system. PDEs are responsible for the breakdown of cAMP to 5' AMP. The 5' AMP can then be used as 5' adenosine monophosphate activated protein kinase (AMPK).

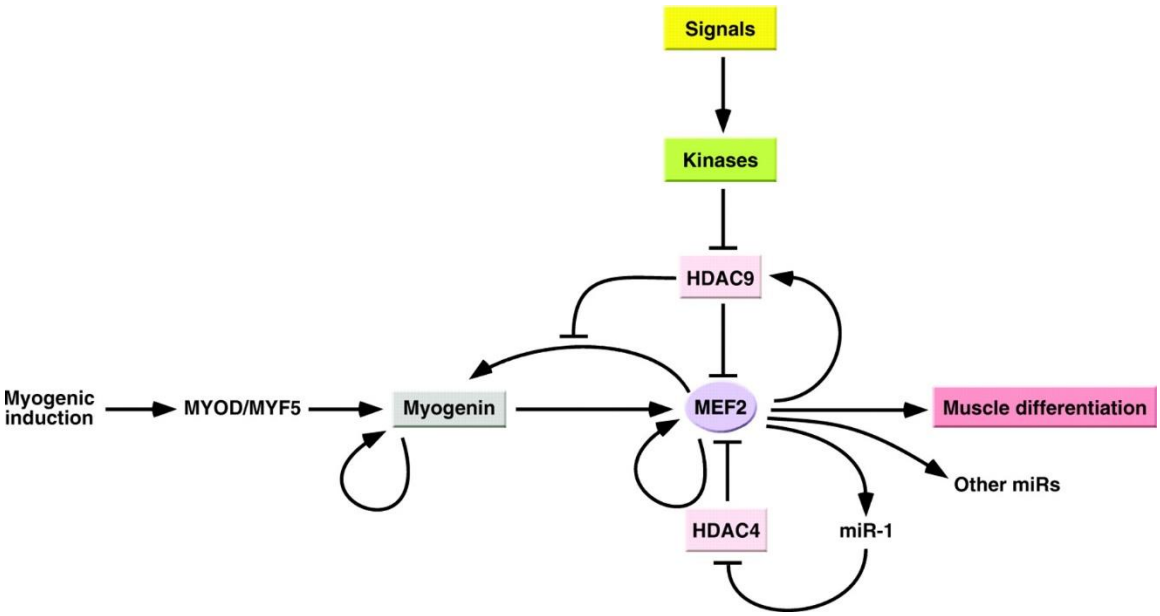

Figure 2. MEF2 signaling. Myogenic inductive signals activate MYOD and MYF5 which activate myogenin in skeletal muscle cells. This myogenin activates MEF2 which will feed back to the myogenin promotor and amplify its expression. MEF2 will then activate genes that are involved in muscle differentiation. MEF2 also activates HDAC9, a negative feedback loop that regulates its phosphorylation. MEF2 can also repress HDAC4 through miR-1.

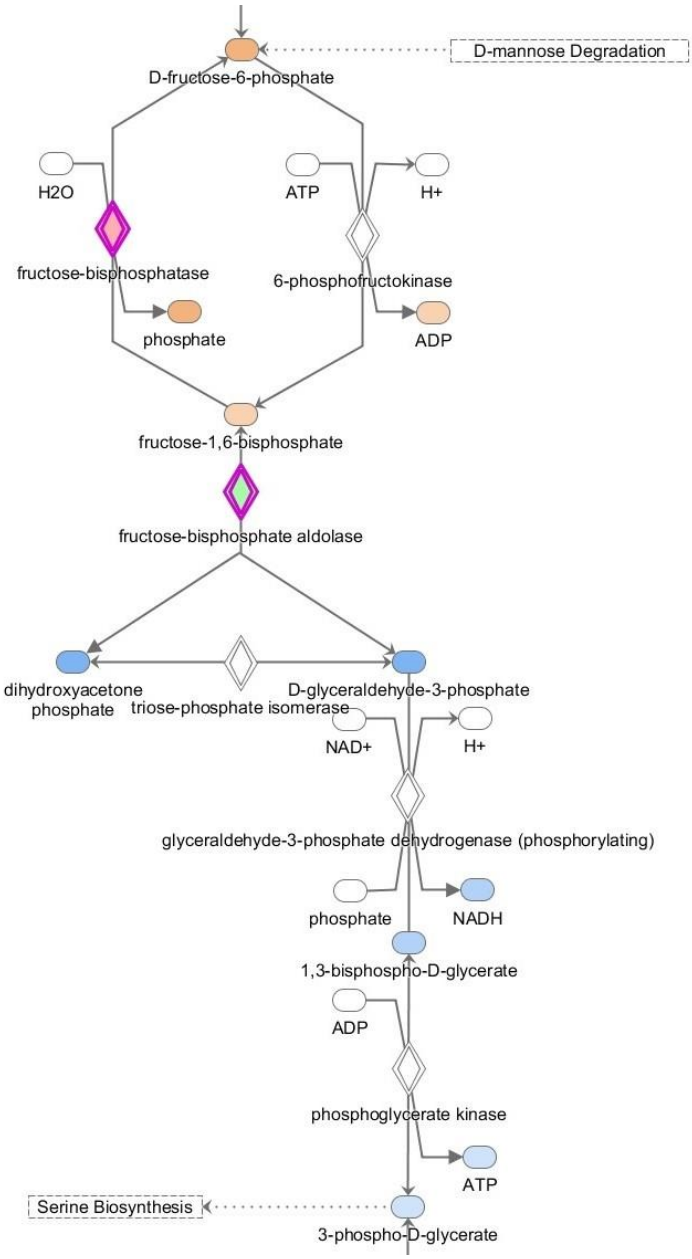

Figure 3. Glycolysis pathway (IPA). Glycolysis was predicted to be upregulated in heifers fed β2-AA. Those colored genes green represent an observed increase and blue a predicted increase. Red represents an observed decrease and orange a predicted decrease. An increase in fructose-bisphosphate aldolase, and 3-phospho-D-glycerate have been observed during an increase in glycolysis (Jenkins et al., 2014). An increase in glucose oxidation has been attributed to muscle growth (Barnes et al., 2017).

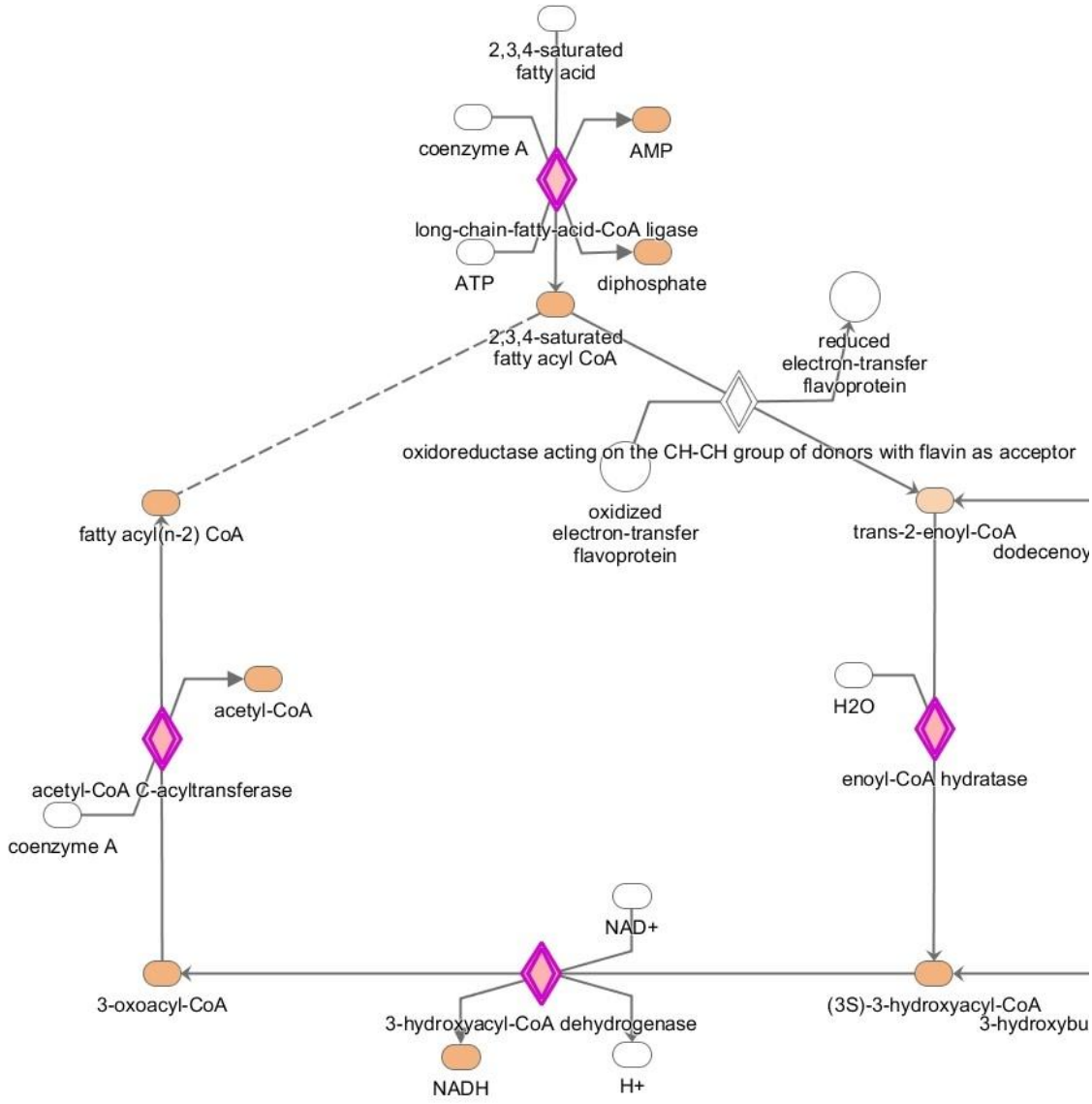

Figure 4. Fatty acid β-oxidation pathway. A decrease in fatty acid β-oxidation was observed when ZH was fed. Those in green represent an observed increase and blue a predicted increase. Red represents an observed decrease and orange a predicted decrease. This entire pathway was predicted to be decreased along with a few enzymes observed to be decreased. A decrease in fatty acid β-oxidation can lead to a decrease in adipose deposition (Devarshi et al., 2017).

Table 1. Transcripts found to be differentially expressed due to β2-AA (adj P-value < 0.1) in the LD. logFC is base 2.

Gene Name	logFC	adj.P.Val
CALM1	0.987	0.009
SLC9A2	-1.129	0.052
SLC25A25	1.316	0.053

Table 2. Transcripts found to be differentially expressed due to β2-AA (adj P-value < 0.1) in the BF. logFC is base 2.

Gene Name	logFC	adj.P.Val
CALM1	1.001	0.001
ASCC1	1.611	0.030
CREM	2.247	0.030
SLC9A2	0.854	0.043
PGAP2	-1.417	0.043
SIK1	-0.817	0.043
COL8A1	-1.024	0.043
PSMB10	1.015	0.043
TXNIP	2.165	0.043
CCDC88C	-0.835	0.043
SLC25A25	1.027	0.043
SYT4	1.807	0.054
GTF2E2	-0.631	0.054
ATXN10	-0.713	0.060
HADHB	-0.545	0.060
TRMT1L	-0.620	0.060
WDR1	-0.756	0.060
TP53BP1	-0.442	0.060
BTBD10	0.832	0.060
JMJD1C	0.904	0.067
OPTN	-0.597	0.067
ARPP21	0.915	0.069
KDELR1	0.685	0.069
RYR1	-0.680	0.069
PDLIM3	-0.725	0.069
ACSL3	-1.141	0.069
EXOC1	0.393	0.069
BAG2	-0.501	0.080
RSPO2	1.048	0.080
FBP2	0.853	0.091
ETS2	-0.617	0.092
ALDOA	1.753	0.092
RELT	1.235	0.092
PPM1A	-0.665	0.092
MAP1A	-1.455	0.094
APOD	1.484	0.094
PAOX	-1.651	0.094
NPEPPS	0.560	0.099
PHIP	-0.420	0.099

Table 3. Top canonical pathways altered in the BF due to β2-AA (p-value < 0.05, -log(p-value) > 1).

Canonical Pathways	**P-value**
Glycolysis I	0.001
Gluconeogenesis I	0.001
Fatty Acid β-oxidation I	0.001
nNOS Signaling in Skeletal Muscle Cells	0.002
Protein Kinase A Signaling	0.005
Spermine and Spermidine Degradation I	0.007
Sucrose Degradation V (Mammalian)	0.016
Ketogenesis	0.018
Ketolysis	0.018
Fatty Acid Activation	0.023
Mevalonate Pathway I	0.023
GP6 Signaling Pathway	0.024
Isoleucine Degradation I	0.025
Androgen Signaling	0.025
Glutaryl-CoA Degradation	0.028
γ-linolenate Biosynthesis II (Animals)	0.030
Mitochondrial L-carnitine Shuttle Pathway	0.030
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	0.030
Valine Degradation I	0.032
Dopamine-DARPP32 Feedback in cAMP Signaling	0.035
Tryptophan Degradation III (Eukaryotic)	0.044
Superpathway of Cholesterol Biosynthesis	0.049

Table 4. Top pathways altered in the LD due to β2-AA (p-value< 0.05, -log(p-value)> 1).

Canonical Pathways	P-value
nNOS Signaling in Skeletal Muscle Cells	0.006
iNOS Signaling	0.006
nNOS Signaling in Neurons	0.006
Glutamate Receptor Signaling	0.008
Nur77 Signaling in T Lymphocytes	0.008
Calcium-induced T Lymphocyte Apoptosis	0.009
Chemokine Signaling	0.010
Melatonin Signaling	0.010
Regulation of IL-2 Expression in Activated T Lymphocytes	0.011
α-Adrenergic Signaling	0.012
CCR5 Signaling in Macrophages	0.013
RANK Signaling in Osteoclasts	0.014
T Cell Receptor Signaling	0.015
Nitric Oxide Signaling in the Cardiovascular System	0.015
Glioma Signaling	0.016
Synaptic Long Term Potentiation	0.017
fMLP Signaling in Neutrophils	0.017
iCOS-iCOSL Signaling in T Helper Cells	0.017
Sperm Motility	0.017
CCR3 Signaling in Eosinophils	0.018
PI3K Signaling in B Lymphocytes	0.018
Cellular Effects of Sildenafil (Viagra)	0.018
CD28 Signaling in T Helper Cells	0.018
GP6 Signaling Pathway	0.018
Androgen Signaling	0.019
Corticotropin Releasing Hormone Signaling	0.019
Gαq Signaling	0.022
Dopamine-DARPP32 Feedback in cAMP Signaling	0.022
GNRH Signaling	0.023
eNOS Signaling	0.023
Role of NFAT in Regulation of the Immune Response	0.025
B Cell Receptor Signaling	0.026
Breast Cancer Regulation by Stathmin1	0.028
Calcium Signaling	0.028
CREB Signaling in Neurons	0.029
Role of NFAT in Cardiac Hypertrophy	0.030
cAMP-mediated signaling	0.031
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.032
Cardiac Hypertrophy Signaling	0.032
Phospholipase C Signaling	0.033
Opioid Signaling Pathway	0.033
Role of Macrophages, Fibroblasts and Endothelial Cells in Arthritis	0.042

CHAPTER III: INVESTIGATION OF THE SKELETAL MUSCLE TRANSCRIPTOME IN LAMBS FED BETA AGONISTS AND SUBJECTED TO HEAT STRESS FOR 21 DAYS

Introduction

An animal's growth and efficiency are important aspects of the livestock industry. If animals grow more quickly, or can do so utilizing less fed, the producer will ultimately profit. One strategy to increase the efficiency of growth is the supplementation of livestock with dietary β-adrenergic agonists (β-AA). These compounds are used to increase muscle protein accretion and decrease adipose deposition, thereby increasing the efficiency of the animal and resulting in a leaner carcass (Elam et al., 2009). Two β-AA are approved for use in beef cattle in the US: Ractopamine HCl (RAC, β1 agonist) and Zilpaterol HCl (ZH, β2 agonist) (Johnson et al., 2014). Average daily gain (ADG) has been reported to increase by 17.9% in cattle fed RAC (Vogel et al., 2005) and up to 74% in cattle fed ZH (Montgomery et al., 2009; Hales et al., 2014). Additionally, live weights can improve by 5% when feeding ZH (Montgomery et al., 2009; Robles-Estrada et al., 2009) while hot carcass weight (HCW) can increase anywhere from 5 to 16 kg in different livestock species (Vogel et al., 2005; Gruber et al., 2007; Vasconcelos et al., 2008; Elam et al., 2009; Montgomery et al., 2009; Hales et al., 2014; Van Donkersgoed et al., 2014; Boyd et al., 2015). An increase in all of these growth traits indicate a more effective animal. Slightly contrary to the cattle studies, sheep fed RAC, had an increase in final gains and HCW weight but no difference was seen in ADG (Romero-Maya et al., 2013).

Although β-AA provide a positive impact on the industry, some studies indicate it may be doing harm on supplemented animals. Reports have suggested that feeding β-AA while animals are subjected to other stressors such as heat or handling stress, can harm to the animals. In 2018, Dr. Temple Grandin stated that there was a higher incidence of death in cattle fed β-AA during the summer months (Grandin, 2018). Additionally, in an analysis of feedlots across the United States, 40 to 50% of deaths were attributed to β-AA administration (Loneragan et al., 2014). Mobility issues have been seen in some cattle fed β-AA although no data can attribute the issues directly to the β-AA itself. In 4,300 heat of

cattle at JBS facilities in 2013, 28% were considered difficult to move and of that 92% were fed β-AA (Vance, 2013). Contrary to the suggested negative impact of β-AA in that study, rams fed ZH under heat stress had no negative effects on health (Dávila-Ramírez et al., 2014). In a study by Boyd et al. (2015) cattle were fed either a control or ZH and house in either open pens or shaded pens during summer months. On average, those fed ZH in open pen had the lowest body temperatures. It was also stated that all animals regardless of housing condition or supplement became less mobile as time went on. This was attributed to muscle growth, the bigger an animal became, the less mobile it was (Boyd et al., 2015; Hagenmaier et al., 2017). There are many conflicting reports relating β-AA and heat stress. Understanding heat stress is key to determining if there is an interaction between these variables.

Heat stress has long been a major concern in the livestock industry. Heat stress occurs when an animal's body temperature rises above its thermoneutral zone, at which point the heat load exceeds the animal's capacity for heat dissipation (Bernabucci et al., 2010), resulting in decreased feed intake and poor performance (Marai et al., 2007; Guo et al., 2018; Johnson, 2018). Therefore, growth and production decreases during heat stress, affecting economically important carcass and reproductive traits as well as morbidity. Additionally, feed intake is decreased in heat stressed cattle (Beatty et al., 2006) along with a decrease in ADG and final weights (Mitlöhner et al., 2002; Blaine and Nsahlai, 2011). As a result, millions of dollars are lost each year due to heat stress (Renaudeau et al., 2012).

Individually, heat stress and β-AA supplementation have antagonistic effects on muscle growth. However, there is a gap in understanding of the genomic mechanisms through which animals respond to these factors individually and in concert. The purpose of this study is to investigate the effects of β-AA, heat stress, and their interaction on skeletal muscle using transcriptomic analyses.

Materials and Methods

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal and Care and Use Committee at the University of Nebraska - Lincoln (IACUC

protocol #1300). Forty-nine crossbred wether lambs (53.26 ± 3.68 kg) were utilized in a 2X3 factorial design. Upon arrival, lambs were stratified by body weight and divided into two replicates (replication one = 39.99 ±1.92 kg; replication two = 37.35 ± 1.92 kg) to accommodate limitation of thermal chambers (12 stalls).

Daily Observations

At 0600, 1400, and 2000 h each d beginning four days prior to the start of the trial supplementation, respiration rates, body temperature and appearance were observed. Respiration rates were recorded pen-side by counting how many breaths were taken in a 15 sec interval multiplied by four to determine respiration rate per min. Body temperature was recorded by a rectal thermometer (ReliOn, Bentonville, AR) and individual water consumption was recorded to the closest half liter with water buckets filled to 15 L with fresh water after each observation. Appearance was observed and recorded as active and alert (AA), lethargic (L), depression (D) or Severe-Intervention (SI). Also during these checkpoints, ambient temperature and humidity were manually recorded. The ambient temperature and humidity were also recorded at two locations in each room every 15 min by HOBO data loggers (Onset Computer Corporation, Bourne, MA).

Environmental Conditions

The lambs were housed in one of two environmental conditions. The thermal neutral condition was approximately 21 ºC and 25% humidity over the 21 d study period. Heat stress was achieved by maintaining a temperature of approximately 34 ºC and 35% humidity from 0800 to 2000 h, and 29 ºC between 2000 and 0800 with temperature changing over a period of 2 h at the beginning and end of each heat cycle. In both environmental conditions, the light was from 0630 to 2045 h.

Supplementation and Feed

Lambs assigned to replicate one were transitioned to a 90% concentrate finishing diet (49% SweetBran®; 37.8% dry-rolled corn; 8.3% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidiostat (20 g/ton) over a period of 21 d. Lambs assigned to replication two were held on a maintained diet (54.8% SweetBran®; 41.1% chopped alfalfa hay; 4% mineral supplementation; dosage of coccidostat (20 g/ton) at 2% of BW

for 41 d prior to 21 d transition to 90% concentrate finishing diet with the addition of ammonium chloride (10 mg/hd/d).

Lambs were subjected to one of three supplemental treatments. Ractopamine hydrochloride was supplemented at 18.14 g/ton (40 mg/kg) and zilpaterol hydrochloride was supplemented at 5.44 g/ton (2.5 mg/kg). The supplement was mixed into 200 g of fine ground corn with the control receiving 200 g of fine ground corn containing no supplement. β-AA were supplemented into the diet beginning on d1 and mixed into 907.19 g of feed. The remaining amount of regular feed was given around 1400 h.

Within replicate, lambs were stratified by body weight and randomly assigned to one of six treatment groups: control/thermoneutral (CON/TN), Ractopamine HCl/thermoneutral (RAC/TN), Zilpaterol HCl/termoneutral (ZH/TN), control/heat stress (CON/HS), Ractopamine HCl/heat stress (RAC/HS), and Zilpaterol HCl/heat stress (ZH/HS). Table 1 lists replicate, supplement and temperature for each individual lamb. A timeline of supplementation and all experimental procedures can be found in Figure 1.

Skeletal Muscle Biopsy

A biopsy of the semitendinosus muscle from the right hind limb was taken three days into the dietary supplementation. The samples were taken by creating an approximately 2 cm incision and the biopsy retrieved with an 8 mm biopsy punch following local anesthetic (1-3 mL to effect). The wound was closed with 2-3 staples and monitored for any signs of infections. The tissue was placed in a 2 mL tube, flash frozen in liquid nitrogen and stored at -80°C until further analysis.

Necropsy

All lambs were harvested on trial d 21. The harvest order was randomly assigned and 12 were harvested each d via captive bolt followed by exsanguination. Hot carcass weight was recorded and the head, hooves and pelt were removed and weighed. Five different skeletal muscles (semitendinosus, vastus lateralus, biceps femoris, longissimus dorsi, and the flexor digitorum superficialus) were collected from the right hind limb of the animal. The flexor digitorum superficialus was removed in its entirety, tendon to tendon and weighed; in addition to cryopreservation, this sample was utilized for glucose

oxidation studies (Barnes et al., (2017). All organs were then removed and weights were taken on the GI tract, liver with gallbladder attached, heart, lungs, spleen, kidneys, and rumen. Tissue samples were taken from the five muscles previously stated, heart, liver, lungs, kidney, pancreas, diaphragm, ileum, rumen, cecum, adrenal gland, kidney pelvic heart fat and tail head fat. All tissue samples collected were placed in 2 mL collection tubes and flash frozen in liquid nitrogen. Cecum and rumen contents were and flash frozen. Tissue samples from the five muscles, diaphragm, pancreas, liver, spleen, duodenum, adrenal glands, and kidney pelvic heart fat were also fixed in paraformaldehyde for histologically analyses.

RNA Isolation from Muscle

RNA was isolated from the semitendinosus muscle taken both at biopsy and harvest following the Direct-zol RNA MiniPrep Plus kit (Zymo Research) with the addition of chloroform precipitation after homogenization in Trizol. Roughly 100 mg of tissue was weighed and minced quickly before being placed in 600 uL of Trizol. Sample was left in Trizol for approximately 5 min then homogenized with an addition of 400 uL Trizol. The homogenized tissue was incubated at room temperature for 5 min after which 200 uL of chloroform was added, vortexed for 30 sec and incubated at room temperature for 3 min. Following this incubation, samples were centrifuged for 15 min at 11,900 rpm and 4 ºC. The top aqueous layer was removed and placed in 600 uL of 100% ethanol and mixed. This mixture was placed on a spin column and washed twice using 400 uL of RNA pre-wash buffer. A 15 min DNase treatment was then performed using 75 uL of DNA digestion buffer and 5 uL of 6U/uL of DNase per sample. This treatment was followed by three washes with 700 uL of RNA wash buffer. RNA was eluted in 50 uL of RNase free water. Once isolated, RNA was quantified and integrity analyzed using the Agilent Bioanalyzer. RNA Integrity Number (RIN) scores above 7 were considered adequate and sent to the University of California Davis' Genome center (Davis, CA) for QuantSeq 3' mRNA library prep and 100 bp, single-end sequencing on an Illumina HiSeq 4000. Table 2 lists all samples and their accompanying concentrations and RIN scores.

Bioinformatic Analysis

Sequence quality was accessed (FASTQC) (Andrews, 2010) and poly-A tails and adapters trimmed (bbmap) (Bushnell, 2014). The trimmed sequences were pseudo-aligned to both the Ovine Oar_v3.1 and Oar_v4.0 reference genomes and quantified in STAR. After the removal of lowly expressed transcripts (count < 8), differential expression (DE) analysis was performed in R using limma-voom (Law et al., 2014; Ritchie et al., 2015). Transcripts with an adjusted $P \leq 0.05$ were considered significant and analyzed using Ingenuity Pathway Analysis (QIAGEN Inc.; IPA) to predict pathways altered. For transcripts without annotated gene IDs in the ovine genome, human or mouse orthologs were input when possible.

Results

An average of 1.9 million reads (min=1.2 million, max=2.8 million) were obtained per sample. Samples were mapped to both the Oar_v3.1 and Oar_v4.0 transcriptomes. To version 3.1, mapping rate averaged 35%. When mapped to version 4.0, mapping rate increased to 62% for all samples. Due to a failure to gain weight throughout the study, data from one lamb (32) was removed from analysis. After quality control and trimming, 13,862 transcripts were observed. No interaction was found between temperature and supplement, therefore only main effects (HS vs TN; RAC, ZH vs control) were evaluated. Analyses showed 326 transcripts were differentially expressed (Adj $P \leq 0.05$) between HS and TN lambs at biopsy (Table 1), and 42 were differentially expressed (Adj $P \leq 0.05$) between ZH and control lambs at biopsy (Table 2). No transcripts were found to be differentially between RAC and control lambs at biopsy or harvest. At harvest, 177 transcripts were differentially expressed between HS and TN, whereas only 8 genes were differentially expressed between ZH and control. At biopsy, 70 pathways were altered due to heat stress and 12 due to ZH. At harvest both numbers drop to 63 and 2, respectively.

Discussion

The purpose of this study was to investigate the interaction between heat stress and β-AA supplementation and to identify genes and pathways altered by each. This

could be accomplished through differential expression analysis therefore 3' QuantSeq was used. This is a low cost method that provides sufficient power for differential expression analysis (Moll et al., 2014). Understanding the interaction between this supplementation and heat stress can help producers manage the animals in a manner to promote both efficiency and animal well-being. Due to a poor mapping rate to Oar_v3.1, results from the mapping of Oar_v4.0 were used. We predict these differences were due to a poor annotation of the transcriptome in Oar_v3.1 and fewer gaps in the Oar_v4.0 assembly. No interaction was found between β-AA and heat stress which was contrary to what we hypothesized. Due to no interaction, only main effects are discussed.

Notably, RAC was found to have no effect on the skeletal muscle transcriptome, which was also unexpected given its ultimate impact on carcass composition. This is also contrary to prior studies such as that of Gunawan et al (2007), in which swine fed RAC for 4 weeks showed decreased and an increased between Type IIA and type IIX MyHC expression in skeletal muscle (Gunawan et al., 2007). Further, in adipose tissue of swine fed RAC for 42 days, *GLUT4* expression was decreased along with *FAS* and *SREBP-1* (Halsey et al., 2011). One possible reason no difference was seen could be due to the transcriptome annotation, those for which the 3' end of the gene is not well annotated will not show up in analysis, or a possible physiological difference between swine and ruminant livestock. These results could also suggest that skeletal muscle is not significantly impacted by β1-AA which would suggest that an increase in lean composition is due to activity in another tissue/tissues. Adipose tissue was taken in the current study but has yet to be analyzed. Upon analysis, it could provide an insight into where RAC may be working in the body if not in the skeletal muscle.

Heat Stress

One interesting gene differentially expressed at biopsy due to heat stress was *AHSA1*. *AHSA1*, activator of *HSP90* ATPase activity, along with *HSP90AA1* were increased in heat stressed lambs. *HSP90AA1* is a chaperone that is involved in structure maintenance, maturation and regulation of target proteins involved in cell cycle control (Zuehlke et al., 2015). *AHSA1* has been shown to increase 3-fold in human K562 erythroleukemia cultured cells exposed to heat stress (Vihervaara et al., 2013). This is

consistent with that found in the current study. *AHSA1* along with many other heat shock proteins were differentially expressed in heat stressed lambs.

Oxidative Stress

Heat stress is a cause of oxidative stress (Akbarian et al., 2016; Alemu et al., 2018). Oxidative stress, in turn can impair or delay protein recovery (Adachi et al., 2009). The IPA analysis of samples from lambs subjected to heat stress compared to those in the ambient room indicated an upregulation of the NRF2-mediated oxidative stress response at both biopsy and at harvest. Many different genes involved in eliminating and detoxifying reactive oxidants are included in this pathway which is activated when oxidative stress is present (Nguyen et al., 2009). When bovine granulosa cells were exposed to heat stress, there was an increase in this response (Alemu et al., 2018). In the present study, the biopsy of lambs exposed to heat stress showed an increase (z-score = 1.667) in this response whereas the sample from harvest shows a decrease (z-score = -0.3). Tissue at biopsy has been exposed to heat stress for 72 h, similar to that in the Alemu et al. (2018) study. This short exposure could be triggering an increase to limit stress whereas over time, this response could be desensitized or damaged. Specific genes included in this pathway (multiple *DNAJ* genes, *ACTG1*, and *GAB1*) are also decreased after 21 d of heat exposure. It is also possible that expression in transcripts associated with this pathway were altered during the period of time the lambs were held in the abitoir awaiting harvest (at ambient condition). If the former is true, it appears the body is able to combat short exposure to heat stress but overtime it is not able to keep up with the demand. Alternatively, the lambs may have become acclimated to the thermal stress environment, resulting in a decreased need for the response.

Another pathway relating to oxidative stress found to be altered was the HIPPO signaling pathway. At biopsy, it was upregulated in heat stressed lambs, supported by the up/down regulation of *YWHAZ, YWHAH,* and *PARD3.* At harvest there was no alteration to this pathway which suggests that it was an acute response to heat stress. This pathway has been implicated in the response to oxidative stress, mechanic stress and DNA damage (Mao et al., 2014). It helps maintain homeostasis at a cellular level by regulating cell proliferation, differentiation and stress induced apoptosis (Mao et al., 2014). The HIPPO

signaling pathway has also been linked to the MST-FOXO signaling pathway which is involved in mediating oxidative stress (Lehtinen et al., 2006; Mao et al., 2014). It can be hypothesized based on its part in relieving oxidative stress, HIPPO signaling plays a role in mediating heat stress.

Protein Ubiquitination

Due to heat stress, protein ubiquitination was predicted to be upregulated at biopsy and downregulated at harvest. During this process, ubiquitin is attached to the lysine residue of a protein, marking it for degradation and/or preventing its activity in various interactions (Lecker et al., 2006). Ubiquitination can be monoubiquitious, in which the ubiquitin is ligated to the protein itself or polyubiquitious if the ubiquitin is attached to another already ligated to the protein (Sadowski and Sarcevic, 2010). Heat stress causes protein damage (Schröder et al., 1993), therefore an increase in ubiquitination is expected given this increase in damage. After 72 h of heat exposure, both polyubiquitination and monoubiquitination were upregulated in the semitendinosus of the lamb. At harvest, both pathways were predicted to be downregulated. The cause of change in expression is not fully understood but it could be similar to those hypothesized in the oxidative stress pathway. This pathway could be damaged, or the body could be desensitized to the damage.

Cholesterol Biosynthesis

Significantly downregulated at biopsy in the lambs under heat stress, but not harvest, was the cholesterol biosynthesis super pathway. In mice exposed to heat stress for 48 h, corticosterone levels were increased (Ippolito et al., 2014), which would suggest an increase in cholesterol metabolism and steroidogenesis (Ippolito et al., 2014). In contrast, in the follicular fluid of Egyptian buffalo, cholesterol levels were significantly decreased when subjected to heat stress (Hozyen et al., 2016). In the present study, plasma cholesterol and dHDL cholesterol were tested. By d three and nine, levels were significantly less than those observed prior to the start of the study. By d eighteen, both measures had returned to normal levels. This decrease in plasma cholesterol can be linked to the predicted down-regulation in cholesterol biosynthesis. By day eighteen, the return

to normal levels could explain why no alterations were seen in the genes involved in this pathway at harvest. It could also signify an acclimation to the environment.

EIF2 Signaling

The pathway predicted to be most significantly (p-value = 3.14E-18) altered due to heat stress at harvest was the EIF2 signaling pathway, which was upregulated (z-score=3.838). However, it was not predicted to be altered at biopsy, based on our samples. The EIF2 signaling pathway is upregulated in response to stress (Shrestha et al., 2012) and along with the alpha subunit, the initiation factor eIF2 can be phosphorylated by various protein kinases that are activated by cellular stress (Clemens, 2001; Shrestha et al., 2012). In yeast that was exposed to prolonged heat stress, eIF2 was induced and upregulated (Groušl et al., 2009). In the present study, sheep were exposed to heat for 21 d. Denaturation, apoptosis and heat shock are all associated with prolonged exposure to heat stress, therefore an increase at harvest instead of biopsy fits these prior observations. One hypothesis for why EIF2 signaling was not altered at biopsy was the limited heat exposure. The samples at biopsy had only been exposed to heat for 72 h which may not be enough to elicit this response.

Beta-Agonist

In the eighteen d period between biopsy and harvest, the number of genes differentially expressed between β-AA and control dropped from 42 to eight. Of the eight differentially expressed at harvest, only two were also differentially expressed at biopsy, *ENHO* and *BEX2*. Little data is present on *ENHO* and its role in muscle growth and development. *BEX2* however appears to play a role in skeletal muscle regeneration-; *BEX1* was found to interact with *CALM1* in a calcium dependent manner (Koo et al., 2007). Both genes were also found to be upregulated during skeletal muscle regeneration (Koo et al., 2007). Both *BEX2* and *CALM1* were upregulated in the current data which could suggest an increase in muscle regeneration could play a role in muscle growth.

cAMP Signaling

β-AA signal through the secondary messenger cAMP (Mersmann, 1998; Johnson, 2014). The mechanism by which cAMP signals can be found in Figure 1. Two of these

systems related to β-AA and regulated through cAMP signaling are the ubiquitin proteasome and calcium dependent systems, both of which are involved in protein breakdown (Baviera et al., 2010). When there is an increase in cAMP, both systems are decreased (Baviera et al., 2010). If there is less protein degradation, more muscle growth is possible. Multiple genes along with the signaling pathway itself were found to be altered during β-AA supplementation. Figure 3 shows the prediction of the upregulated pathway in IPA with respect to the individual transcripts observed in our data. An upregulation of *CALM1, PDE4B* and a downregulation of *AKAP6* lead to this observed upregulation. Another downstream gene not found in this specific pathway but a known downstream messenger of cAMP was *FBXO32*. This decrease plays a part in the decrease of ubiquitin proteolysis.

Calmodulin kinase (CaMK) was predicted to be increased in the cAMP signaling pathway. CaMKII is of interest due to its activity in skeletal muscle (Al-Shanti and Stewart, 2009). One important role CaMKII plays is in the activation of *MEF2*. *MEF2* is a muscle enriched nuclear factor and is highly expressed in skeletal muscle (Al-Shanti and Stewart, 2009). It has an important role in muscle differentiation, growth and hypertrophy (Nakagawa et al., 2005). When intracellular Ca^{2+}/calmodulin levels increase, CaMKII is activated and translocates into the nucleus where it phosphorylates and deactivates *HDAC4*. This deactivation of *HDAC4* allows for it to dissociate from the binding domain of *MEF2* which allows *MEF2* to bind its DNA-binding domain to active transcription of MyoD and myogenin which both play important roles in muscle hypertrophy. MyoD and myogenin are also MEF2-dependent target gene products of *MEF2* (Molkentin et al., 1995). This pathway can be observed in Figure 4. These observed increases in *CALM1* and CaMKs could lead to an increase in muscle growth.

Callipyge Muscle Hypertrophy

Sheep with the callipyge phenotype have an increase in muscle hypertrophy caused by a single point mutation (A>G) in the *DLK1* and *MEG3* region (Freking et al., 2002). In a study by Duckett et al. (2000) Longissimus and semimembranosus muscles were 40% heavier in callipyge sheep than wild type although carcass characteristics such as shear force (Duckett et al., 2000) is increased, marbling and fat thickness (Koohmaraie

et al., 1995) are decreased. β-AA similarly increase muscle mass and shear force while they decrease marbling and fat deposition (Martin et al., 2014). In our data, neither *DLK1* nor *MEG3* were found to be differentially expressed.

Recently, a study by Yu et al. (2018) identified five genes that were co-expressed with DLK1 and considered secondary effector genes. These genes include *METTL21E*, *PARK7, DNTTIP1, SLC22A3,* and *PDE4D*. All five of these genes, including *KCNN3*, were upregulated in hypertrophied muscle (Yu et al., 2018). *PARK7* was also found to play a role in muscle fiber switching (Yu et al., 2018). In our data, *METTL21E, PARK7*, and *KCNN3* were all increased. *SLC22A3* and *PDE4D* were not explicitly expressed, possibly due to a limited annotation, but *SLC25A25* and *PDE4B*, similar isoforms, were significant. *PDE4B* and *PDE4D* differ in the location in which they act, with *PDE4D* being globally available and *PDE4B* being localized to the plasma membrane (Blackman et al., 2011). *DNTTIP1* was increased but was not significant (Adj P = 0.33). With no change to *DLK1* but an increase in downstream genes, our data suggest β2-AA supplementation is acting through another mechanism to target this pathway. Thus far, the mechanism of callipyge muscle hypertrophy has not been investigated in the presence of β-AA.

Cellular Effects of Sildenafil

In the pathway analysis of the biopsy samples of lambs fed β-AA compared to those on the control supplement, the most significantly (p-value < 0.001) altered pathway was the cellular effects of Sildenafil (Viagra). Sildenafil is a known vasodilator as well as causing an increase in blood flow and muscle contraction (Sheffield-Moore et al., 2013). It has also been shown to cause a decrease in muscle fatigue (Sheffield-Moore et al., 2013). An increase in muscle contraction and a decrease in muscle fatigue are physiological consequences linked to exercise and muscle growth in humans (Tipton and Wolfe, 2001). When mice lacking the gene *SLC25A25* were subjected to exercise, they had decreased endurance along with decreased muscle capacity for work (Anunciado-Koza et al., 2011). In the same study, wild type mice subjected to exercise had an upregulation of *SLC25A25* (Anunciado-Koza et al., 2011). Similarly, in the current study, lambs fed ZH had an upregulation of *SLC25A25* (logFC=1.001).

β-AA also caused an increase heart rate and therefore vasodilation in both sheep and steers (Frese et al., 2016; Hatefi et al., 2017). Although heart rate was not monitored in the present study, the Sildenafil pathway was increased. Genes in the pathway that were increased include *CALM1*, *PDE4B,* and *KCNN3*, while *MYH3* was decreased. An increase in vasodilation and blood flow can lead to an increase in waste removal and nutrient delivery. These properties could contribute to altered muscle metabolism (Barnes et al., 2017). If β-AA are able to elicit a similar response in terms of increased blood flow, muscle contraction and decreased muscle fatigue, it could cause an increase in hypertrophy.

Conclusion

β-AA supplementation is common in livestock production. Further, heat stress is both costly to the industry and impacts animal wellbeing. While no interaction between environment and supplement was identified, differentially expressed transcripts due to heat stress or to ZH supplementation revealed potential mechanisms through which animals respond to these treatments. Surprisingly, no impact of RAC was observed, indicating it has an alternative mode of action compared to ZH and reinforcing the need for additional molecular studies to elucidate its specific impacts. Many of the transcripts and pathways identified to be altered due to supplementation are associated with the secondary messenger, cAMP. However, these data also reveal several transcripts and possible pathways not previously associated with supplementation. A greater understanding of how animals respond to these supplements and environmental conditions has the potential to improve management practices and may lead to means in which to select for more efficient animals.

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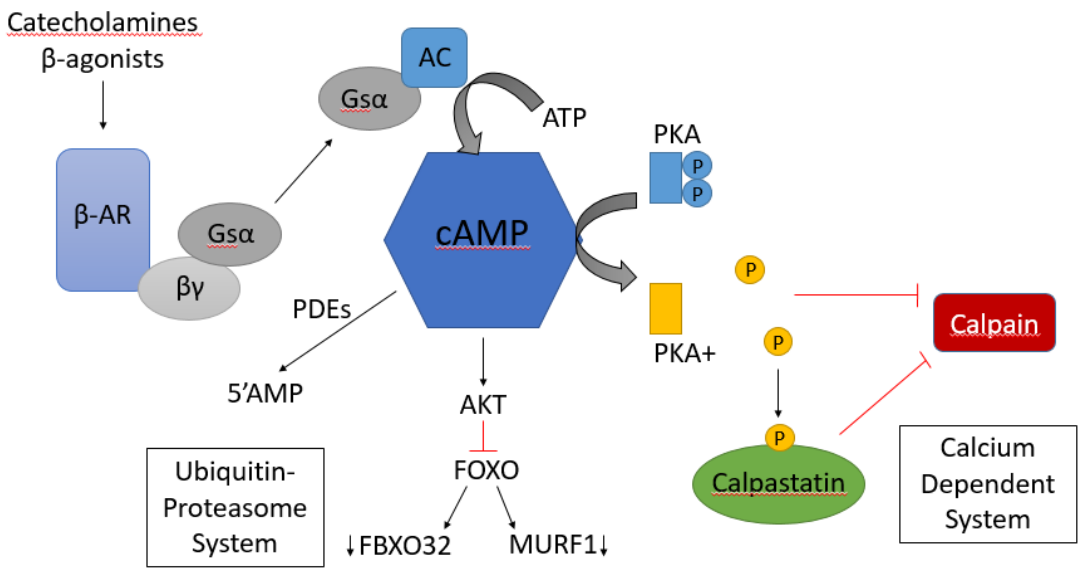

Figure 1. cAMP signaling pathway. β-AA act as artificial catcholamines and bind to the β-adrenoceptor (β-AR). This causes a conformational change in Gαs to activate adenylate cyclase which causes an increase in cAMP. Proteolysis can occur through two systems downstream of the secondary messenger, cAMP. The first being the calcium dependent system (right). cAMP dephosphorylates protein kinase A (PKA) which can inhibit calpains or bind with calpastatin subsequently inhibiting calpains. This pathway is calcium dependent meaning when calcium levels are increased, this pathway is decreased and calpains are inhibited. The second system is the ubiquitin proteasome proteolytic pathway. AKT is inhibited when can lead to the phosphorylation of FOXO. This phosphorylation can decrease both FBXO32 and MURF1 suppressing this system. PDEs are responsible for the breakdown of cAMP to 5' AMP. The 5' AMP can then be used as 5' adenosine monophosphate activated protein kinase (AMPK).

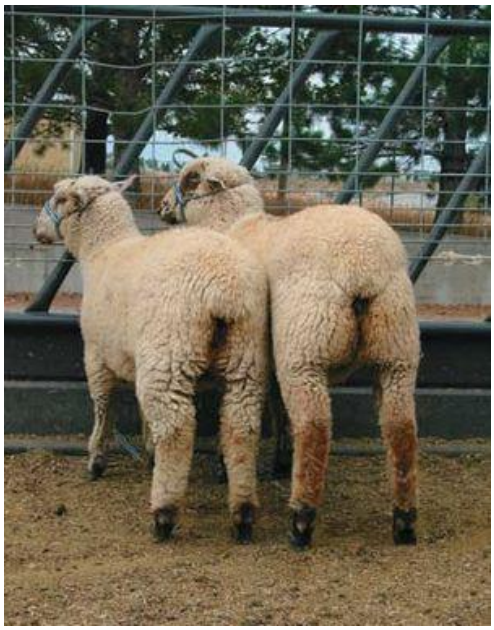

Figure 2. Picture of a normal sheep (left) and one with the callipyge phenotype (right). An increase in muscle in the hind region can be observed.

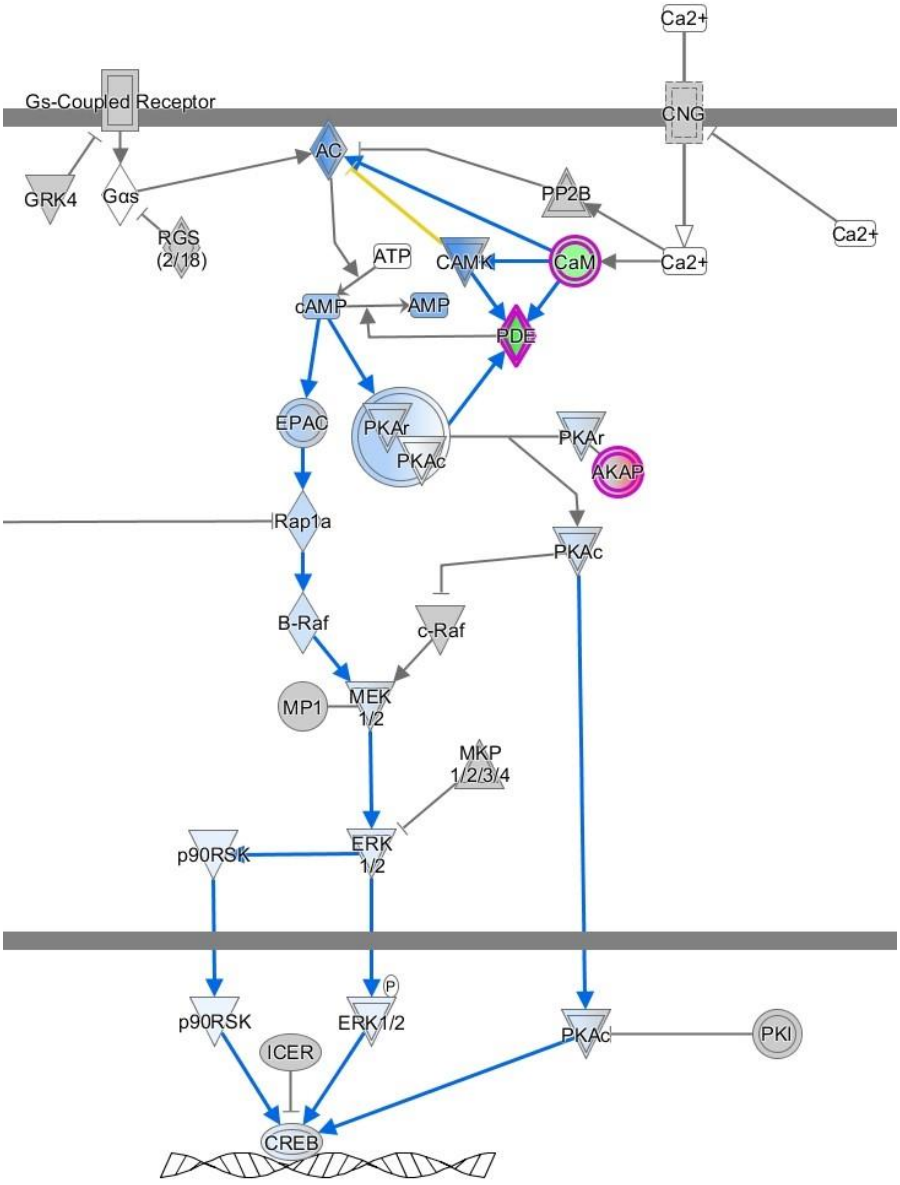

Figure 3. cAMP-mediated signaling (IPA). cAMP-mediated signaling was predicted to be upregulated in lambs fed β2-AA. Genes colored green represent an observed increase and blue a predicted increase. Red represents an observed decrease and orange a predicted decrease. Increases in *CALM1* and *PDE4B* along with a decrease in *AKAP6* lead to an increase in cAMP signaling. These increases correlate with those indicated in Figure 1.

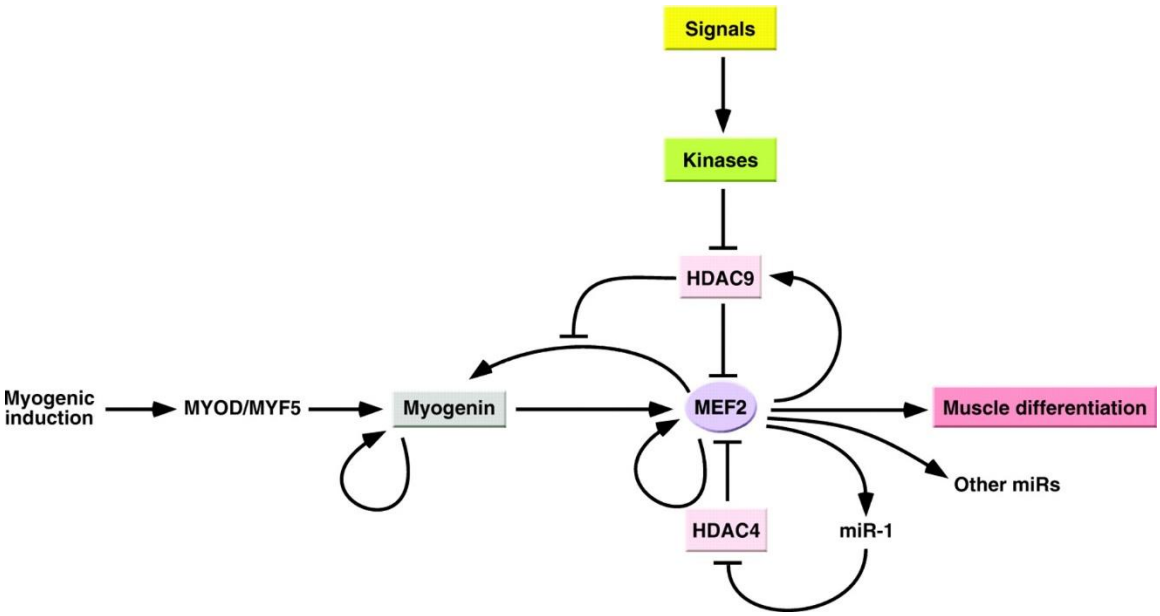

Figure 4. MEF2 signaling. Myogenic inductive signals activate MYOD and MYF5 which activate myogenin in skeletal muscle cells. This myogenin activates MEF2 which will feed back to the myogenin promotor and amplify its expression. MEF2 will then activate genes that are involved in muscle differentiation. MEF2 also activates HDAC9, a negative feedback loop that regulates its phosphorylation. MEF2 can also repress HDAC4 through miR-1.

Table 1. Sheep ID, supplement type (RAC=Ractopamine HCl, ZH=Zilpaterol HCl, CON=Control), environment (HS=Heat stress, TN=Thermalneutral), concentrations (ng/uL) at biopsy (bio) and harvest (harv), as well as RIN scores from each sample.

ID	Supp.	Temp.	Rep	Bio conc	Harv conc	Bio RIN	Harv RIN
1	RAC	HS	1	187	387	8.8	9.4
2	ZH	TN	2	110	463	8.1	8.7
3	ZH	HS	1	202	107	7.9	9.1
4	RAC	TN	2	200	253	7.4	8.2
5	CON	HS	1	192	134	8.5	9.6
6	CON	HS	1	287	169	8.7	8.5
7	CON	TN	2	212	446	8.3	9.1
8	ZH	TN	1	255	263	7.3	8.8
9	ZH	HS	2	142	248	7.9	8.7
10	RAC	HS	2	262	245	8	9.4
11	CON	HS	2	145	245	8	9.7
12	CON	TN	1	186	149	7.9	8.8
13	ZH	HS	2	297	81	8.1	9.3
14	CON	HS	2	293	275	7.9	7.3
15	RAC	HS	2	318	164	8.6	9.1
16	CON	TN	2	238	287	8.7	9.7
17	RAC	TN	2	176	265	7.7	8.6
19	ZH	TN	1	240	346	8	9.1
20	RAC	TN	1	164	556	8.3	8.3
21	ZH	HS	1	170	412	8.5	8.9
23	RAC	HS	1	299	354	8.6	9.1
24	CON	HS	2	206	132	8.2	8.6
25	ZH	HS	2	138	325	8.4	9
26	CON	TN	1	252	118	8.6	8.8
27	ZH	TN	2	150	573	8.4	8.5
28	RAC	TN	2	194	384	-	8.1
29	CON	HS	1	144	155	8.5	8.7
30	RAC	TN	1	141	434	7.9	8.7
32	ZH	TN	2	89	250	7.6	8.6
33	RAC	TN	1	186	203	8.1	7.4
34	RAC	HS	2	166	468	8.1	8.6
36	RAC	TN	1	279	247	7.5	8.9
37	ZH	TN	1	160	219	9	8.7
38	RAC	HS	2	226	216	8.4	8.7
39	CON	TN	1	172	201	7.5	8.9
40	RAC	TN	2	193	192	8.4	9.1
42	ZH	HS	2	160	317	8.4	8.6
43	CON	HS	2	336	400	-	8.7
44	CON	TN	1	146	276	8.3	7.9
45	CON	TN	2	370	330	8.9	9

46	ZH	TN	2	192	254	7.6	9.5
47	CON	HS	1	166	215	8	9
48	ZH	HS	1	286	197	8.6	8.3
49	ZH	HS	1	292	153	9	8.7
50	CON	TN	2	308	401	8.5	9
51	ZH	TN	1	108	689	7.9	8.5
52	RAC	HS	1	292	401	8.4	8.3
53	RAC	HS	1	158	209	7.7	8.7
54	CON	TN	2	192	357	8.5	8.9

Table 2. Transcripts differentially expressed at biopsy due to heat stress (adj P-value < 0.05). Some transcripts, not annotated in the sheep, were identified by using LOC numbers to find a human, mouse or cow ortholog (when possible).

Gene Name	logFC	adj.P.Val
RBM3	-1.181	0.000
POLR1D	0.562	0.000
CENPF	-1.292	0.000
AHSA1	0.587	0.000
CYP51A1	-0.940	0.000
CCDC117	0.575	0.000
HBB	-1.953	0.000
CDC6	-1.647	0.000
NUSAP1	-1.220	0.000
DBI	-0.461	0.000
MCM4	-0.830	0.000
TTC9	0.632	0.000
TOP2A	-1.176	0.000
NUF2	-1.522	0.000
HMGCS1	-0.532	0.000
COX6A1	-0.586	0.000
ACOT9	-0.812	0.000
DDX5	-0.377	0.000
REPIN1	-0.908	0.000
GYPC	0.591	0.000
YWHAZ	-0.541	0.000
ALAS2	-1.715	0.000
AURKB	-1.595	0.000
RQCD1	0.465	0.000
ACLY	-0.911	0.001
RHBDL2	-1.455	0.001
RPP25L	0.440	0.001
CBFA2T3	0.483	0.001
WIPF1	-0.523	0.001
LEAP2	1.140	0.001
ZBED5	-0.509	0.001
PLCD4	0.590	0.001
PARL	-0.520	0.001
HSP90AA1	0.454	0.001
HBA1	-1.894	0.001
STMN1	-0.578	0.001
DTNA	0.342	0.001
GSTM1	0.629	0.001
SQLE	-0.947	0.002
GBX1	0.962	0.002
MAPK12	0.528	0.002
COX7A2	-0.573	0.002
DNAJB2	0.585	0.002
HBB	-2.088	0.002
ATP1A1	-0.617	0.002
FGL2	0.548	0.003
KIAA0101	-1.308	0.003
KCNIP2	0.673	0.003
TIMM17A	-0.597	0.003
CDIP1	0.437	0.003
MYOZ1	0.429	0.003
NMRK2	-0.908	0.003
CATSPER4	-1.272	0.004
OBSCN	0.514	0.004
PPM1J	0.521	0.004
CENPE	-1.131	0.004
SREBF2	-0.475	0.004
BRI3BP	-0.766	0.004
CARNMT1	-0.656	0.004
HSP90AB1	0.359	0.004
DLGAP5	-1.215	0.004
BZW1	-0.437	0.004
HBM	-1.545	0.004
TPM4	-0.633	0.004
AGAP3	0.510	0.005
DNAJB1	0.548	0.005
LAMA2	0.543	0.005
CREBRF	0.382	0.006
ISOC1	-0.449	0.006
DYNLL1	0.547	0.006
PTBP1	-0.394	0.007
ID2	-0.657	0.007
FKBP2	-0.441	0.007
RORA	0.520	0.007
ASF1B	-1.237	0.007
SIK3	0.444	0.007
HDAC1	-0.392	0.007
SLC25A16	-0.503	0.008
CCDC82	0.410	0.008
NUDT12	0.515	0.008
SHPK	1.178	0.009

EPHX2	-0.655	0.009
STIP1	0.670	0.009
CIRBP	-0.518	0.009
PPIA	-0.469	0.009
TMEM185A	0.503	0.009
GPAM	-0.813	0.009
QPRT	-0.910	0.009
TEAD1	0.343	0.009
HIGD1A	-0.428	0.009
BAK1	-0.619	0.009
FAM209A	-1.158	0.009
ELOVL5	-0.506	0.009
SEC23B	-0.513	0.010
P4HB	-0.232	0.010
SMC2	-0.629	0.010
KLHL23	0.650	0.010
VPS4A	0.354	0.010
PCDH12	-0.776	0.011
KIF1C	0.361	0.011
PTGS1	0.557	0.011
TMCC1	0.407	0.011
SLC29A2	0.833	0.011
SNRPN	0.471	0.011
LDLR	-0.782	0.011
ESCO1	0.458	0.011
AGPAT2	-0.953	0.012
PTOV1	0.448	0.012
VHL	-0.579	0.012
MEOX1	-0.792	0.012
FRMD3	0.530	0.012
PARD3	0.347	0.012
ST13	0.317	0.013
SRGAP1	-0.527	0.013
SS18L2	0.372	0.013
HSPH1	0.698	0.013
AACS	-0.715	0.013
SFXN5	-0.765	0.013
ABCD2	-1.242	0.013
OCRL	0.527	0.013
GGTA2P	0.263	0.013
IMMT	-0.356	0.013
PRND	-1.545	0.013
LXN	-0.789	0.013
MND1	-1.205	0.013
ATXN7L1	0.581	0.013

GSTA4	-0.497	0.013
GLB1	-0.462	0.013
TFRC	-0.759	0.014
COL15A1	-0.385	0.014
JADE1	0.398	0.014
ASB2	0.608	0.015
ATXN7L3B	0.251	0.015
CEP85L	0.435	0.015
ST3GAL6	0.865	0.015
FASN	-1.029	0.015
SFPQ	-0.332	0.015
C1H1orf56	1.164	0.015
TRIM28	0.484	0.016
SRSF12	1.296	0.016
PRPF3	0.327	0.016
PRKRA	-0.519	0.016
PPM1A	0.320	0.016
TAX1BP3	-0.410	0.016
KIF20B	-0.480	0.016
FER1L5	0.494	0.016
NHP2	0.360	0.017
JPH1	0.347	0.018
SYNPO	0.580	0.018
THRSP	-1.049	0.018
USP43	1.215	0.018
ACSL3	-0.430	0.019
CTBP2	0.332	0.019
TTK	-1.286	0.019
TMOD4	0.384	0.019
DHRS4	-0.469	0.019
SDR39U1	0.330	0.019
DPY19L1	-0.423	0.019
PGP	-0.396	0.020
DNAJA1	0.645	0.020
GOT1	-0.506	0.020
ZBTB16	1.183	0.020
TSTD3	-0.587	0.020
THOC5	0.480	0.020
AOX1	0.507	0.020
FAM64A	-1.290	0.020
DHX36	-0.352	0.020
ELOVL6	-1.079	0.020
EFCAB2	0.523	0.020
PITPNB	-0.348	0.021
MICAL3	0.498	0.022

GSTA1	0.673	0.022
MYOZ3	0.431	0.022
FAM83D	-1.262	0.022
HSPB1	0.528	0.022
MPP6	0.490	0.023
PAPD5	0.363	0.023
DGAT2	-1.013	0.023
SCARB1	-0.635	0.023
PRTFDC1	-0.574	0.023
KRAS	-0.253	0.023
LRP8	-0.907	0.023
ZMAT2	0.243	0.023
KIF1B	0.296	0.023
ATP5G3	-0.492	0.023
SLC43A3	-0.620	0.023
ARHGAP11A	-1.085	0.023
IGFBP2	-1.022	0.023
RAD23B	0.277	0.023
LOC106990101	-0.495	0.023
FRAT2	0.471	0.024
ACTR3	-0.464	0.024
CENPA	-1.248	0.024
MFF	-0.247	0.024
MAP1B	-0.510	0.024
LIG1	-1.022	0.024
FAM101B	-0.719	0.025
MED31	0.371	0.025
ADAMTS9	-0.649	0.026
RIMS1	1.277	0.026
TOMM5	-0.371	0.026
CNTFR	1.177	0.026
PRCP	-0.556	0.026
TLCD2	-0.606	0.026
RCN1	-0.455	0.027
SNTG1	1.117	0.027
ZFAND3	0.419	0.028
SRI	-0.322	0.029
ZNF331	0.744	0.029
YWHAQ	-0.383	0.029
CAPN3	0.366	0.029
LOC443471	-0.531	0.029
CCNF	-1.079	0.029
FTH1	0.436	0.029
LRRC51	0.854	0.029
TMEM187	-0.613	0.029

CHAMP1	0.330	0.029
LMNTD1	-0.872	0.029
UBQLN4	0.412	0.030
ACSS2	-0.662	0.030
SMTNL2	0.467	0.030
ACTG1	-0.414	0.030
PAQR4	-0.794	0.030
GADL1	-0.705	0.030
LIN7B	0.558	0.030
LEMD2	0.298	0.031
CYP4B1	1.259	0.031
DEAF1	0.429	0.031
CREBBP	0.220	0.031
NICN1	-0.519	0.031
RFC4	0.398	0.031
C1QTNF3	-0.666	0.032
BMP1	-0.488	0.032
EIF4B	0.354	0.033
C1H1orf54	-0.400	0.035
KISS1R	1.088	0.035
HIST2H2BE	-0.846	0.035
EEF1A2	0.400	0.035
LYZ	-0.831	0.035
NFX1	0.644	0.036
ANKRD29	-1.060	0.036
C1H3orf33	-0.492	0.036
CRLF1	-0.950	0.036
FIS1	0.372	0.036
NUMA1	0.332	0.036
DHCR7	-0.693	0.036
FAM229B	0.399	0.036
KIF11	-1.178	0.037
LRRC71	-1.076	0.037
PRKAR2A	0.284	0.037
COL18A1	-0.490	0.037
PDE4A	0.562	0.037
ACSL1	-0.482	0.038
PHF3	0.197	0.038
UBB	0.309	0.038
LRRC40	-0.351	0.038
KPNB1	0.231	0.039
BANF1	0.279	0.039
SIGLEC10	-0.400	0.039
NEK2	-0.894	0.039
MCPH1	0.625	0.039

SS18	-0.379	0.039
CYSLTR2	1.034	0.039
SLC25A27	0.893	0.039
CCT3	0.336	0.039
WASF3	0.300	0.039
RPA3	0.325	0.040
SMIM14	-0.288	0.041
BHLHE40	0.675	0.041
RGN	-0.948	0.041
CIDEA	-0.907	0.041
SLC7A10	0.513	0.041
TRIM2	0.388	0.041
MLPH	1.142	0.041
A1BG	1.056	0.041
PF4	-0.981	0.041
TMEM164	0.521	0.041
NDUFA5	-0.396	0.041
EPHB4	-0.519	0.041
CPPED1	-0.844	0.043
RING1	0.367	0.044
NOP10	0.282	0.044
LZTS3	0.487	0.044
FKBP1A	-0.799	0.044
MRPL9	0.334	0.044
IBTK	-0.262	0.044
GFPT2	0.677	0.044
RCOR2	-0.698	0.044
DAZAP2	-0.243	0.044
GLRX5	-0.607	0.044
VSIG2	0.898	0.044
SYNM	0.305	0.044

FEM1A	0.299	0.044
YPEL2	0.346	0.044
KDELR2	-0.225	0.044
MGEA5	0.298	0.044
OVGP1	0.799	0.044
ATP5C1	-0.268	0.045
LYRM1	0.438	0.045
PDHB	-0.338	0.045
SF3B5	0.394	0.045
CCDC50	-0.282	0.045
FUT2	-1.052	0.045
GUSB	-0.535	0.045
FSD1L	-0.396	0.045
FSCN1	-0.431	0.045
TAF6	0.573	0.045
DYNLL2	0.328	0.045
SLC19A3	0.738	0.046
ACTN1	-0.396	0.046
DYNLL1	0.450	0.047
LARS2	0.920	0.047
CA3	-0.911	0.047
MYL6	-0.246	0.047
POU6F1	1.026	0.047
DEPDC1B	-1.034	0.047
RIMKLA	-1.036	0.047
FANCD2	-1.123	0.047
TXLNB	0.403	0.048
MSMO1	-0.438	0.049
RAP1B	-0.222	0.049
TNFRSF21	-0.453	0.049

Table 3. Transcripts differentially expressed at biopsy due to ZH (adj P-value < 0.05). Some transcripts, not annotated in the sheep, were identified by using LOC numbers to find a human, mouse or cow ortholog (when possible).

Gene Name	logFC	adj.P.Val
ADCK3	-1.106	0.000
METTL21E	1.541	0.000
MCHR1	1.883	0.000
ENHO	1.353	0.000
PALLD	0.689	0.000
SMTNL1	-1.187	0.000
SLC25A25	1.001	0.000
AKAP6	-0.772	0.001
TTC7A	1.069	0.001
PDE4B	1.231	0.001
CXADR	0.721	0.001
HDAC1	0.656	0.001
ART5	-0.939	0.006
GSTM1	0.829	0.006
SPHK1	0.622	0.006
FAM71E1	1.295	0.008
CTNNBIP1	0.584	0.008
RNF149	0.480	0.008
FBXO32	-0.917	0.008
DDC	1.328	0.009
MYH3	-1.020	0.009
FHL1	-0.648	0.009
EGLN3	-0.933	0.011
LOC101109633	-1.263	0.013
SSH2	-0.414	0.014
NCAPD2	1.008	0.017
BEX2	0.548	0.019
NRAP	-0.718	0.019
GDAP1	0.680	0.019
PCYOX1	-0.503	0.022
TRAF3IP3	0.656	0.023
KCNN3	0.580	0.023
S100A16	0.542	0.027
NFX1	0.879	0.027
ART5	-0.920	0.027
GSTM1	0.656	0.029
PGK1	0.429	0.032
SERPINB11	1.324	0.032
RPS15A	0.466	0.032
NRIP1	0.562	0.036
VAMP5	-0.717	0.039
ME2	-1.012	0.048

Table 4. Transcripts differentially expressed at harvest due to heat stress (adj P-value < 0.05). Some transcripts, not annotated in the sheep, were identified by using LOC numbers to find a human, mouse or cow ortholog (when possible).

Gene Name	logFC	adj.P.Val
HSPA1A	-1.766	0.005
HIST1H1D	-0.999	0.009
COL4A1	-0.592	0.009
GYPC	0.544	0.009
CDK6	-0.460	0.009
SLC30A1	-0.565	0.009
HSP90B1	-0.576	0.010
HNRNPDL	0.358	0.010
DYNLL1	-0.642	0.010
DNAJA1	-0.795	0.010
SNRNP35	0.728	0.010
DNAJA1	-0.691	0.011
HFE2	0.441	0.013
TMOD3	-0.554	0.013
RPS7	0.345	0.014
RPS12	0.424	0.017
CAPN3	0.446	0.017
FAM101B	-0.961	0.017
FAM198B	-0.662	0.017
SEC24A	-0.581	0.017
RABEPK	0.600	0.017
PELI2	-0.660	0.017
YWHAH	-0.363	0.017
HSPH1	-0.727	0.017
TTC9	0.447	0.017
RPL26	0.364	0.017
P4HA1	-0.538	0.018
NFKBIB	0.625	0.018
MBNL2	-0.396	0.018
MANF	-0.597	0.019
AMOTL2	-0.830	0.019
UBE2C	-1.146	0.019
ARHGEF10L	0.408	0.020
NELL1	-1.353	0.020
SPG21	-0.621	0.020
NDUFA10	-0.736	0.020
PRICKLE4	0.293	0.020
HSPA1B	-1.337	0.020
EFHD2	-0.662	0.020
POMT2	0.627	0.020
FAM13A	-0.682	0.020
RPS24	0.367	0.020
RGP1	0.419	0.020
RPS3A	0.310	0.020
AHCYL1	0.364	0.020
CCAR2	-0.717	0.021
LRRC8A	-0.731	0.021
SPARC	-0.706	0.022
KPNA2	-0.415	0.022
KITLG	-0.665	0.024
RBM39	-0.398	0.024
DNAJB4	-0.686	0.025
BORCS6	0.475	0.025
EEF2KMT	-0.987	0.025
HNRNPDL	0.447	0.025
ATG10	0.426	0.025
HIST2H2BF	-0.869	0.026
NOTCH1	-0.791	0.027
IFRG15	0.368	0.028
RPL26	0.331	0.028
URAH	1.152	0.028
ABCF1	0.400	0.028
NSG1	-0.811	0.028
RAP2C	0.360	0.028
NUSAP1	-0.771	0.028
SGPL1	-0.632	0.028
MYO1B	-0.516	0.029
RASAL2	-0.580	0.029
SURF6	0.396	0.030
ZC3H15	0.348	0.030
ETNK1	-0.517	0.030
PSPC1	0.291	0.030
RPL27A	0.415	0.030
REM1	-0.707	0.030
ZNF346	0.756	0.030

ADAM10	-0.479	0.030
CDK17	-0.875	0.030
MRPL20	0.323	0.030
CRIM1	-0.673	0.030
ZNF366	-0.752	0.030
PGRMC2	0.394	0.032
ASAP2	-0.484	0.032
MRPL55	0.486	0.032
MTFR1	-0.695	0.032
MTIF2	-0.620	0.032
KDM7A	-0.516	0.032
GLP1R	1.405	0.032
RPS4X	0.346	0.032
EEF1B2	0.415	0.033
MPST	0.547	0.033
TMTC2	-0.835	0.033
CDK2AP2	-0.591	0.033
TMEM126B	0.440	0.033
KIAA0319L	0.466	0.033
MFSD4	-1.237	0.033
INTS4	-0.639	0.034
NCOA4	0.295	0.034
CLEC2H	-1.019	0.034
NT5C2	0.430	0.034
CDH5	-0.676	0.034
RPL34	0.296	0.034
FAM219A	0.440	0.034
CXCL1	-0.897	0.034
RPL7A	0.391	0.034
CDKN2AIPNL	-0.413	0.035
FAU	0.322	0.035
FUS	0.356	0.035
SYNCRIP	0.240	0.035
RPL37AB	0.353	0.035
UQCC3	-1.024	0.035
TSC22D3	-0.795	0.036
DZIP3	0.417	0.036
FJX1	-1.144	0.036
RPL4	0.249	0.036
CCNB1	-1.238	0.036
C20H6orf47	0.534	0.036

NOV	-1.208	0.036
PLK2	-0.536	0.036
EIF3D	0.373	0.036
PDPR	-0.391	0.037
ZBTB20	-0.238	0.037
VASP	-0.545	0.037
RPS27A	0.341	0.037
LRRC41	-0.956	0.037
RPL10A	0.305	0.038
IKZF4	0.667	0.038
TTC4	0.356	0.038
COA5	0.406	0.038
DHRS3	0.379	0.038
CCR2	-1.076	0.038
MPDU1	0.531	0.038
STMN2	-1.569	0.038
NMT1	0.503	0.039
GGA2	0.323	0.040
L3MBTL3	-0.665	0.040
DIS3L	0.410	0.040
ATP2C1	-0.366	0.040
MMRN2	-0.479	0.040
TMEM138	-0.843	0.040
GSTA1	1.196	0.040
CCDC117	-0.323	0.040
RPL17	0.345	0.040
RFX3	-0.715	0.040
PSMC5	0.447	0.040
DNAJC15	0.387	0.040
ZBTB21	-0.612	0.040
PSME3	0.331	0.040
CYP2J2	-1.334	0.040
COQ10B	-0.469	0.040
LOC105609992	1.029	0.040
KPTN	0.581	0.040
LHPP	0.457	0.041
PLPP2	0.569	0.041
NT5DC3	-0.886	0.041
PFDN5	0.390	0.041
MTMR10	0.488	0.041
ANKMY2	0.379	0.041

PRMT9	-0.449	0.042
TNKS	-0.314	0.043
WRB	-0.617	0.043
PPP1R27	-1.126	0.043
CEP83	0.392	0.043
PCDHGC4	-0.507	0.044
PHTF2	0.416	0.045
NADK2	0.316	0.045
RBSN	0.329	0.045
TRMT10B	0.545	0.047
GPS2	0.350	0.047
LINGO1	0.455	0.047
ART1	0.744	0.047
ATF3	-1.106	0.047
TXN	-0.886	0.047
PREX2	-0.397	0.047
LRRFIP2	0.274	0.047
GPATCH3	0.512	0.047
LTBP4	-1.000	0.049
VCL	-0.396	0.049
FAM124B	-1.346	0.050
TJP2	-0.610	0.050
FKBPL	0.732	0.050

Table 5. Transcripts differentially expressed at harvest due to ZH (adj P-value < 0.05). Some transcripts, not annotated in the sheep, were identified by using LOC numbers to find a human, mouse or cow ortholog (when possible).

Gene Name	logFC	adj.P.Val
SIK1	-1.496	0.014
URB2	1.147	0.014
BEX2	0.635	0.014
ATP1B4	0.748	0.017
SPIDR	-1.192	0.018
MID1IP1	0.940	0.033
ENHO	1.148	0.033
RUFY3	0.541	0.044

Table 6. Canonical pathways altered due to HS at biopsy (p-value < 0.05, -log(p-value) > 1).

Canonical Pathways	P-value
NRF2-mediated Oxidative Stress Response	0.000
Cholesterol Biosynthesis I	0.000
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	0.000
Cholesterol Biosynthesis III (via Desmosterol)	0.000
Superpathway of Cholesterol Biosynthesis	0.000
HIPPO signaling	0.000
Cell Cycle Control of Chromosomal Replication	0.001
Hypoxia Signaling in the Cardiovascular System	0.001
Palmitate Biosynthesis I (Animals)	0.001
Fatty Acid Biosynthesis Initiation II	0.001
Stearate Biosynthesis I (Animals)	0.001
Aldosterone Signaling in Epithelial Cells	0.001
Protein Ubiquitination Pathway	0.001
PI3K/AKT Signaling	0.001
Mitochondrial Dysfunction	0.001
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.002
Protein Kinase A Signaling	0.003
TR/RXR Activation	0.003
ATM Signaling	0.003
Acetate Conversion to Acetyl-CoA	0.004
ERK/MAPK Signaling	0.005
2-ketoglutarate Dehydrogenase Complex	0.006
Mitotic Roles of Polo-Like Kinase	0.007
ERK5 Signaling	0.007
P2Y Purigenic Receptor Signaling Pathway	0.007
Agrin Interactions at Neuromuscular Junction	0.008
Myc Mediated Apoptosis Signaling	0.009
Zymosterol Biosynthesis	0.009
Amyloid Processing	0.009
Integrin Signaling	0.010
Ephrin B Signaling	0.011
Epithelial Adherens Junction Signaling	0.012
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	0.012
Signaling by Rho Family GTPases	0.013
BMP signaling pathway	0.013
Actin Nucleation by ARP-WASP Complex	0.014
Ephrin Receptor Signaling	0.014
Breast Cancer Regulation by Stathmin1	0.016
Sirtuin Signaling Pathway	0.018
IGF-1 Signaling	0.019
Renal Cell Carcinoma Signaling	0.019

Leukocyte Extravasation Signaling	0.019
p70S6K Signaling	0.020
Glutathione Redox Reactions I	0.022
Axonal Guidance Signaling	0.023
FLT3 Signaling in Hematopoietic Progenitor Cells	0.023
Paxillin Signaling	0.025
Acetyl-CoA Biosynthesis III (from Citrate)	0.025
Lanosterol Biosynthesis	0.025
Corticotropin Releasing Hormone Signaling	0.026
Triacylglycerol Biosynthesis	0.029
ILK Signaling	0.029
Hereditary Breast Cancer Signaling	0.031
PTEN Signaling	0.032
NGF Signaling	0.035
Sumoylation Pathway	0.036
Acyl-CoA Hydrolysis	0.036
Synaptic Long Term Potentiation	0.036
Prostate Cancer Signaling	0.037
Cardiac Hypertrophy Signaling	0.038
Melanocyte Development and Pigmentation Signaling	0.039
Clathrin-mediated Endocytosis Signaling	0.039
CDK5 Signaling	0.040
Fatty Acid Activation	0.042
Glutathione-mediated Detoxification	0.043
Estrogen Receptor Signaling	0.045
Neurotrophin/TRK Signaling	0.046
Relaxin Signaling	0.049
14-3-3-mediated Signaling	0.049
Cellular Effects of Sildenafil (Viagra)	0.049

Table 7. Canonical pathways altered due to ZH at biopsy (p-value < 0.1, -log(p-value) > 1).

Canonical Pathways	**P-value**
Cellular Effects of Sildenafil (Viagra)	0.000
Glutaryl-CoA Degradation	0.001
Tryptophan Degradation III (Eukaryotic)	0.001
GluCONeogenesis I	0.001
Catecholamine Biosynthesis	0.009
Serotonin and Melatonin Biosynthesis	0.013
cAMP-mediated signaling	0.013
Glioma Signaling	0.025
Sperm Motility	0.030
Parkinson's Signaling	0.034
Aryl Hydrocarbon Receptor Signaling	0.037
Cardiac β-adrenergic Signaling	0.037

Table 8. Canonical pathways altered due to HS at harvest (p-value < 0.1, -log(p-value) > 1).

Canonical Pathways	P-value
EIF2 Signaling	0.000
Protein Ubiquitination Pathway	0.000
Regulation of eIF4 and p70S6K Signaling	0.000
Unfolded protein response	0.000
mTOR Signaling	0.000
Aldosterone Signaling in Epithelial Cells	0.001
Glucocorticoid Receptor Signaling	0.001
Leukocyte Extravasation Signaling	0.002
Hypoxia Signaling in the Cardiovascular System	0.002
Integrin Signaling	0.002
eNOS Signaling	0.003
Small Cell Lung Cancer Signaling	0.004
Relaxin Signaling	0.004
Mitotic Roles of Polo-Like Kinase	0.005
Nitric Oxide Signaling in the Cardiovascular System	0.005
Molecular Mechanisms of Cancer	0.006
Tight Junction Signaling	0.006
Death Receptor Signaling	0.006
Semaphorin Signaling in Neurons	0.008
FAK Signaling	0.009
PI3K/AKT Signaling	0.010
Sertoli Cell-Sertoli Cell Junction Signaling	0.010
Sirtuin Signaling Pathway	0.010
Cell Cycle CONtrol of Chromosomal Replication	0.010
IL-17A Signaling in Airway Cells	0.010
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	0.011
PI3K Signaling in B Lymphocytes	0.012
Polyamine Regulation in Colon Cancer	0.014
B Cell Receptor Signaling	0.016
NRF2-mediated Oxidative Stress Response	0.017
NF-κB Activation by Viruses	0.017
Gap Junction Signaling	0.018
iNOS Signaling	0.021
Estrogen-mediated S-phase Entry	0.023
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.023
PTEN Signaling	0.023
Role of IL-17A in Arthritis	0.023
Glutamate Removal from Folates	0.024
Caveolar-mediated Endocytosis Signaling	0.026
iCOS-iCOSL Signaling in T Helper Cells	0.027
Prostate Cancer Signaling	0.027

Guanosine Nucleotides Degradation III	0.031
G-Protein Coupled Receptor Signaling	0.032
TNFR2 Signaling	0.033
RANK Signaling in Osteoclasts	0.034
Urate Biosynthesis/Inosine 5'-phosphate Degradation	0.036
Angiopoietin Signaling	0.037
4-1BB Signaling in T Lymphocytes	0.039
Chronic Myeloid Leukemia Signaling	0.040
Agranulocyte Adhesion and Diapedesis	0.040
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.041
Adrenomedullin signaling pathway	0.041
Regulation of Cellular Mechanics by Calpain Protease	0.054
Virus Entry via Endocytic Pathways	0.046
Spliceosomal Cycle	0.047
Epoxysqualene Biosynthesis	0.047
L-cysteine Degradation III	0.047
ERK/MAPK Signaling	0.048
TWEAK Signaling	0.049
MIF-mediated Glucocorticoid Regulation	0.049
IL-17A Signaling in Fibroblasts	0.049
Aryl Hydrocarbon Receptor Signaling	0.049
Insulin Receptor Signaling	0.049

Table 9. Canonical pathways altered due to ZH at harvest (p-value < 0.1, -log(p-value) > 1)

Canonical Pathways	P-value
Pyruvate Fermentation to Lactate	0.004
Hypoxia Signaling in the Cardiovascular System	0.044

APPENDIX A: UNDERSTANDING THE EFFECTS OF RACTOPAMINE HYDROCHLORIDE SUPPLEMENTATION ON THE BLOOD TRANSCRIPTOME IN FINISHING CATTLE

Introduction

β-adrenergic agonists (β-AA) are synthetic compounds fed to livestock to increase muscle protein accretion and decrease adipose deposition (Johnson et al., 2014). Different effects of β-AA as well as proposed mechanisms of action were described in previous chapters. A majority of previous work in livestock species has examined the effects of β-AA in skeletal muscle. To study skeletal muscle, the animal is usually scarified, meaning a sample from only one time point can be taken. A biopsy could be taken to look at the effect throughout the study, but this is rarely feasible in a feedlot setting. Furthers, obtaining samples is difficult at a commercial slaughter house. Alternative methods to study animals within a commercial feedlot setting are needed. One such method would be to use blood to investigate the effects of β-AA. The purpose of this study is to examine effects of Ractopamine Hydrochloride (RAC) on the blood transcriptome of finishing cattle.

Materials/Methods

Animal Model

Sixteen Angus-based cattle (8 steers, 8 heifers) were utilized for this study. The cattle were being fed a finishing diet at the University of Nebraska Eastern Nebraska Research and Extension Center near Mead, Nebraska. Whole blood was collected the day prior to the start of supplementation with Ractopamine HCl (300 mg/hd/d) using Tempus collection tubes (Thermo Fisher Scientific). Tempus tubes contain 3 mL of RNA stabilization solution. Upon collection, the blood was shaken vigorously for 15 sec to ensure stabilization of the RNA. A second blood collection was performed on the same animals 35 days after the start of supplementation before shipping to the abbitoir.

RNA Isolation/RNA-Sequencing

RNA was isolated from all Tempus tubes using the following protocol. Tube contents (~12 mL) were added to 3 mL of 1X PBS and vortexed for 30 sec. Samples were then centrifuged at 3,000 x g for 30 min. Supernatant was poured off leaving a clear RNA pellet which was re-suspended in 400 uL of RNA Purification Resuspension solution. To a purification filter from the kit, 100 uL of RNA Purification wash solution 1 was added. The re-suspended RNA was then added and centrifuged for 30 sec. Flow through was discarded, 500 uL of RNA Purification wash solution 1 was added, then centrifuged again. Next, 500 uL of RNA Purification wash solution 2 was added and centrifuged for 1 min. A DNase treatment was then preformed using 100 uL of Absolute RNA wash solution and incubated at room temperature for 15 min. A second incubation was performed for 5 min using 500 uL RNA Purification wash solution 2. Samples were centrifuged again for 30 sec followed by another wash using 500 uL of RNA Purification wash solution. RNA was then eluted using 100 uL of Nucleic Acid Purification elution solution following a 3 min incubation at 70 º C (Thermo Fisher Scientific). Once isolated, RNA was quantified and integrity analyzed using the Agilent Bioanalyzer. RNA Integrity Number (RIN) scores above 7 were considered adequate and sent to the University of California Davis' Genome center (Davis, CA) for QuantSeq 3' mRNA library prep and 100 bp, single-end sequencing on an Illumina HiSeq 4000.

Bioinformatics Analyses

Sequence quality was assessed on all samples through FASTQC (Andrews, 2010). Poly-A ends and adapters were trimmed using bbmap (k=13 ktrim=r forcetrimleft=11 useshortkmers=t mink=5 qtrim=t trimq=10 minlength=20) (Bushnell, 2014) then reevaluated with FASTQC. All reads were mapped to the Bovine UMD3.1 transcriptome with STAR alignment (Dobin et al., 2013). Bam files were created and Integrative Genomics Viewer (IGV) (Thorvaldsdóttir et al., 2013) was used to visualize the data. The trimmed sequences were then pseudo aligned to the transcriptome incorporating the flag –noLengthCorrection and quantified using Salmon Quantification (Patro et al., 2015). A read count matrix was constructed using the generated counts from Salmon. Differential expression analysis was performed in R (RStudio, v1.1.442) using limma-voom (Law et

al., 2014; Ritchie et al., 2015). The counts were trimmed removing those with eight or less observations within treatment groups. Transcripts with an adjusted P-value less than 0.1 were considered significant and analyzed for inclusion in known molecular pathways through Ingenuity Pathway Analysis (IPA) (Krämer et al., 2013). As IPA only recognizes human, mouse and rat genes, human or mouse orthologous gene names were used in place of bovine locus identifiers when needed.

Results and Discussion

An average of 3.6 million reads (min=3.0 million, max=4.6 million) were obtained per sample. The cattle genome contains 22,915 annotated transcripts; after trimming lowly expressed transcripts, 10,370 transcripts were observed across the samples. Zero transcripts were found to be differentially expressed (AdjP < 0.1) when comparing samples after RAC supplementation to before. In the study described in Chapter 3, RAC had no effect on the skeletal muscle transcriptome of sheep. β1-AA are known to bind to β1-ARs which are found in higher proportions in adipose tissue than skeletal muscle tissue. This could be one explanation as to why no alterations were seen in the skeletal muscle or blood. We hypothesized we might see changes due to inflammatory signaling via macrophages which would systemic in the blood, but this was not seen in the transcriptome.

Conclusion

Over 80% of fed lot cattle are fed some sort of β-AA. Different mechanisms through which these supplements work to increase muscle growth have been proposed although the exact mechanism is still being investigated. Skeletal muscle is normally used to study β-AA although this can be difficult in certain settings. Muscle samples can generally only be taken at harvest unless a biopsy is taken. This singular sample can cause difficulty in understanding the changes that may be happening due to the supplement. As shown in the study described in Chapter 3, samples from biopsy contained more information on how β-AA may be working compared to samples taken at harvest. Blood has been proposed as another method to study the effects of β-AA. Samples can be taken as often as the researcher likes as well as representing multiple time points throughout the study. Based on the current study, the blood transcriptome

does not appear to be an appropriate useful method to study β-AA in terms of muscle growth, specifically β1-AA as used in the study. Blood could still be used as an indicator of systemic issues that could be occurring. Blood metabolites like glucose, calcium and cholesterol, which have all been linked to muscle growth as indicated in the previous studies (Barnes et al., 2017; Chapter 3), can all be studied. Although blood doesn't appear to be an appropriate method to study muscle growth due to β-AA, it still presents itself as a powerful method to understand different effects of the supplement.

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APPENDIX B: INVESTIGATION OF CANDIDATE GENES RELATED TO MUSCLE GROWTH AND FUNCTION USING DROPLET DIGITAL PCR TECHNOLOGIES

Introduction

Droplet digital polymerase chain reaction (ddPCR) is a new technology that allows for the quantification of nucleic acids (Taylor et al., 2015). This can be done from a wide variety of samples with an increased sensitivity and precision compared to other PCR methods (Taylor et al., 2015). This method is performed by fractioning the sample into 20,000 droplets using water-oil emulsion technology, PCR amplification is then performed within each individual droplet (Hindson et al., 2011). Droplet digital PCR allows for the absolute quantification of samples without the need of a standard curve or the need to run duplicates or triplicates (Hindson et al., 2013). In the previous studies described in Chapters 2 and 3, candidate transcripts were identified as being dysregulated in the semitendinosus muscle of lambs as well at the bicecps femoris and longissimus dorsi of cattle due to supplementation with a β-adrenergic agonist (β-AA). The purpose of this work was to investigate whether these transcripts were also differently expressed in another skeletal muscle (flexor digitorum superficialis) from the lambs utilized in Chapter 3. We hypothesize a similar trend will be seen in both muscle types.

Materials/Methods

RNA was isolated from the flexor digitorum superficialis (FDS) taken at harvest from 24 of the lambs utilized in the study described in Chapter 3. RNA was isolated following the same protocol described in Chapters 2 and 3. The concentration and purity of the isolated RNA was quantified using an Epoch (BioTek). Each RNA sample was then converted to cDNA with the first strand synthesis kit (GE Healthcare). For the conversion, 500 ng of total RNA was needed therefore number of uL needed to reach this 500 ng was calculated. Water was then added to this sample to bring the volume to 8 uL. Samples were incubated at 65 º C for 1 h, then chilled on ice from 10 min. To each sample, 7 uL of master mix (5 uL Frist-strand reaction mix, 1 uL DTT solution, 0.5 uL NOT-I-d(T)18 primer, Pd(N)6 primer) was added and then incubated at 37 º C for 1 h. Samples were then stored at -20 º C until further analysis.

Candidate genes were selected for analysis using droplet digital polymerase chain reaction (ddPCR) based upon results of prior work (Chapters 2 and 3). The ddPCR reaction consisted of adding 1.1 uL of a forward and reverse strand primer (Table 1) to a mixture of H_2O (8.8 uL) and ddPCR Evagreen supermix (11 uL; BioRad). Then 17 uL of this mastermix was added to each well followed by an addition of 5 uL of sample. This mixture was vortexed and 20 uL transferred to a droplet generator cartridge. 70 uL of generator oil was added to another well of the cartridge then placed in the droplet generator machine run at manufacturer settings. Droplets were carefully transferred to a PCR plate and placed in a thermocycler with an annealing temperature of 62 °C. Finally, the plate was read on the droplet reader. An example of the output from the droplet reader can be found in Figure 1. A reference gene, B2M, was included to normalize each sample (Table 1). Once counts were calculated, statistical analysis was conducted using the proc glimmix function in SAS. Model included- β-AA, environment and β-AA by environment interactions.

Results

Candidate genes were selected based on those found significant and/or interesting in the study from Chapters 2 and 3. Those genes included *CALM1, SLC25A25, PALLD,* and *PDE4B. CALM1* and *SLC25A25* were found to be upregulated in both studies of cattle and sheep skeletal muscle where as *PDE4B* and *PALLD* were found to be upregulated in the study described in Chapter 3. Another gene investigated was *ADRB2* or the β2-AR to which β2-AA bind. This gene was selected to investigate whether or not this receptor expression is altered when β-AA are fed. Both *ADRB2* and *CALM1* were not found to have an interaction between heat stress and β-AA or significant main effects. An interaction was found significant for *SLC25A25*, *PDE4B*, and *PALLD*. Table 2 lists all genes along with p-values and estimates.

Discussion

Alterations in genes due to β-AA were identified in two previous studies by our lab. Some of these alterations were seen in both sheep semitendinosus muscle and cattle biceps femoris and longissimus dorsi. Some were just seen in the sheep muscle. We aimed to identify if these genes were also altered in another sheep muscle from the same

animals from which the differential expression analysis in Chapter 3 was derived. *CALM1* was upregulated due to supplementation with a β2-AA in both studies and all 3 muscle types although, in the FDS, there was no alteration due to supplementation. This lack of alteration could be due to the proportion of β2-AA receptors or muscle fiber type. BF along with ST have a higher proportion of type 2 fibers than the FDS which has more type 1 fibers (Hwang et al., 2013; Kim et al., 2018). This could explain the differences seen. *ADRB2* was not found to be altered in the previous studies as well as not being altered by supplementation in the FDS of sheep fed a β2-AA. This lack of an alteration shows that β-AA are not directly altering expression of β-AR but could alter it chemically or conformationally.

An interaction between heat stress and β-AA supplementation was observed for *SLC25A25, PDE4B* and *PALLD* (Table 3). Sheep fed RAC in the ambient condition had higher estimates on average than any other treatment group. These results were contrary to the previous study which found RAC had no effect on the ST muscle. These differences between studies could be due again to the muscle fiber type or proportion of receptors. *SLC25A25* and *PDE4B* could be acting similarly to that seen in other muscle due to β2-AA (Chapter 2 and 3). The differences between ambient and heat stressed could be due to the amount of feed consumed. Those in heat consumed less and gained less than those in the ambient condition. RAC could be having a greater effect because more nutrients are consumed along with the supplementation. Further studies into the effects of β1-AA on different types of skeletal muscle will help answer questions remaining to explain how skeletal muscle responds to these treatments.

Conclusion

Droplet digital PCR (ddPCR) has recently become an important method for gene expression analysis. It allows for researchers to perform these studies in their lab and receive results the same day, unlike sequencing methods which can take weeks to receive data. Depending on the method, to analyze many genes, multiple runs must be performed unlike sequencing which can give you results for all annotated genes. Candidate genes from two previous studies were identified for investigation using ddPCR. Differences were observed between the different muscles between the studies suggesting β-AA may

be working differently in different muscle types. The glycolytic or oxidative ability of different muscles could partially explain differences in their response to β-AA. By understanding these differences, we can gain a further understanding of how β-AA work to alter carcass composition in ruminant livestock.

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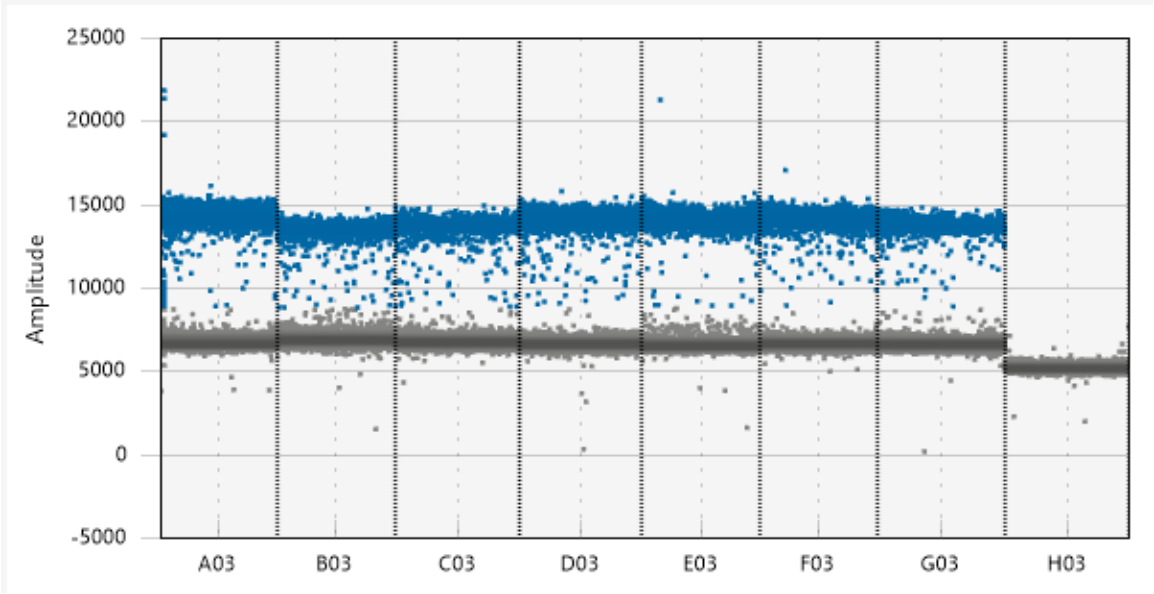

Figure 1. Output from ddPCR droplet reader. Those droplet in blue are considered positive, containing the transcript, and those in grey are negative, lacking the transcript. The first seven columns contained samples where the eight contained a negative control of water.

Table 1. Primer pairs used for ddPCR analysis.

Gene Name	Direction	Sequence (5' → 3')
ADRB2	Forward	CTGTCTTTCTGGGTGGGAGA
	Reverse	CAGGCCCATGACCAGGTC
CALM1	Forward	CAGCTGACCGAAGAGCAGAT
	Reverse	CCGAGTTCCTTGGTTGTGAT
PDE4B	Forward	ATCAGGGAACCAGGTGTCTG
	Reverse	CTGTCTTTCTGGGTGGGAGA
SLC25A25	Forward	TATCTGAGCAGCAGGCAGAA
	Reverse	ACTCGTTCCAGTCGATGGTC
PALLD	Forward	AGCCCCACCTTGTTCCTC
	Reverse	CCCTCAGACGCACAAATGTT

Table 2. Estimates and p-values for all genes included in the study generated in SAS using the PRCO GLIMMIX command.

Gene Name		TN				HS				P-Value		
	CON	RAC	ZH	SE		CON	RAC	ZH	SE	Supplement	Environment	Interaction
ADRB2	0.103	0.121	0.093	0.032		0.057	0.119	0.103	0.032	0.475	0.622	0.671
CALM1	0.836	4.9	0.815	1.712		2.154	0.989	0.693	1.712	0.445	0.526	0.312
PDE4B	0.348	2.011	0.332	0.417		1.483	0.586	0.228	0.417	(-)	(-)	**0.023**
SLC25A25	0.302	1.426	0.143	0.254		0.737	0.319	0.097	0.254	(-)	(-)	**0.021**
PALLD	0.242	1.739	0.314	0.333		0.561	0.288	0.17	0.333	(-)	(-)	**0.042**