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THE EFFECT OF HEAT STRESS AND BETA-ADRENERGIC AGONISTS ON FATTY ACID MOBILIZATION AND THEIR INDIVIDUAL AND INTERACTING IMPACT ON THE ADIPOSE TRANSCRIPTOME OF RUMINANT LIVESTOCK

by

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THE EFFECT OF HEAT STRESS AND BETA-ADRENERGIC AGONISTS ON FATTY ACID MOBILIZATION AND THEIR INDIVIDUAL AND INTERACTING IMPACT ON THE ADIPOSE TRANSCRIPTOME OF RUMINANT LIVESTOCK

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University of Nebraska, 2021

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Heat stress reduces livestock efficiency, induces inflammation, and alters adipose tissue metabolism through stress-induced epinephrine acting on beta-adrenoreceptors (β-AR). Supplementation of beta-adrenergic agonists (β-AA) improve livestock growth and carcass traits, also by acting on β-AR located in skeletal muscle and adipose tissue. The binding of β-AR in adipose tissue results in changes in adipose tissue metabolism including increased lipolysis and decreased fatty acid synthesis. Due to their similar effects on stress response pathways, the possible interaction of heat stress and β-AA could negatively impact animal well-being. The purpose of the first study was to understand how ractopamine (RAC), a beta-one adrenergic agonist, and heat stress alter the subcutaneous adipose tissue transcriptome in sheep, and to identify interaction between the two. Based on differential expression and pathway analysis, the interaction of RAC and heat stress was predicted to alter adipose tissue metabolism and inflammation, but physiological data did not show a negative impact due to interaction. The second study investigated possible interaction of zilpaterol hydrochloride (ZH), a

beta-two adrenergic agonist, and heat stress in subcutaneous adipose tissue transcriptome in beef cattle. Transcriptomic evidence suggests that ZH did not exacerbate the negative effects of heat stress and instead moderated heat stress-induced inflammation and oxidative stress. The purpose of the third study was to understand the effects of chronic heat stress and ZH supplementation on fatty acid mobilization in *ex vivo* visceral adipose tissue with and without stimulation of the adipose tissue with epinephrine. All treatment groups responded to stimulation by epinephrine, with no interaction of heat stress and ZH being apparent. Heat stress decreased fatty acid concentration while ZH increased it. Overall, these findings indicate that while the interaction of heat stress and β-AA in ruminant adipose may impact gene expression, there was no evidence of a detriment to animal well-being based on the parameters measured; in fact, β-AA supplementation may moderate many negative effects of heat stress.

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CHAPTER 1: LITERATURE REVIEW

Introduction

The health and productivity of livestock are vital to maintaining efficient food systems to keep up with the growing human population. Factors such as environment and feed supplement can improve or reduce animal health and productivity. These changes to biological function are due to changes in expression of genes which can be observed by measuring RNA transcripts. Understanding the effects of heat stress and beta-adrenergic agonists on the ruminant transcriptome may help to clarify how these factors alter animal production and health.

β-adrenergic agonists

β-adrenergic agonists (β-AA) are drugs that act on the adrenergic system of the body, similar to how epinephrine does. β-adrenergic agonists are frequently supplemented to cattle, sheep, and pigs due to their ability to improve growth traits and carcass traits. A commonly used beta-one adrenergic agonist (β_1-AA) in cattle is ractopamine hydrochloride (RAC); zilpaterol hydrochloride (ZH) is a beta-two adrenergic agonist (β_2-AA) that was often used in the United States prior to the manufacturer's voluntary removal of it from the market after concerns regarding its impact on animal well-being (Grandin, 2018). Both ZH and RAC alter growth parameters such as increased average daily gain, gain to feed ratio, and final body weight (Avendaño-Reyes et al., 2006; Brown et al., 2014). These β-AA are reported to improve

carcass traits including increased longissimus muscle area (LM area), hot carcass weight (HCW) and decreased overall carcass fatness (Elam et al., 2009; Brown et al., 2014). Though they work similarly, ractopamine tends to have a lesser effect on adipose and muscle in cattle than zilpaterol (Lean et al., 2014). Zilpaterol has been reported to induce a greater final body weight, gain to feed ratio, HCW, LM area, and dressed carcass yield than RAC in cattle (Avendaño-Reyes et al., 2006; Brown et al., 2014). Prior work in sheep found no differences in body weight, gain-to-feed ratios, or dry matter intake (DMI) between lambs supplemented with ractopamine or zilpaterol or the control group (Barnes et al., 2019). Unfortunately, the aforementioned effects of β-AA, especially ZH, are not consistent; ZH does not always impact final BW (Vasconcelos et al., 2008; Brown et al., 2014; Boyd et al., 2015) nor does it consistently decrease fat thickness and marbling across studies (Elam et al., 2009; Brown et al., 2014; Boyd et al., 2015).

Variation in the outcome of β-AA supplementation may be due in part to the distribution of beta-adrenoreceptors (β-AR) among tissue types and species. As there are beta-one adrenergic agonists and beta-two adrenergic agonists, there are also β-AR subtypes: β_1-AR, β_2-AR, and β_3-AR (Johnson et al., 2014). Beta-one adrenergic agonists tend to have a higher binding affinity for β_1-AR than β_2-AR, while β_2-AA have a higher binding affinity for β_2-AR than β_1-AR (Lodish et al., 2000). The proportion and distribution of β-AR subtypes varies based on livestock species and tissue. Cattle, for example, have almost exclusively β_2-AR in adipose tissue, while in pigs β_1-AR is the primary β-AR in adipose (Mersmann, 1998; Mills and Mersmann, 1995). Because of this, the effectiveness of different β-AA varies among livestock species. For example,

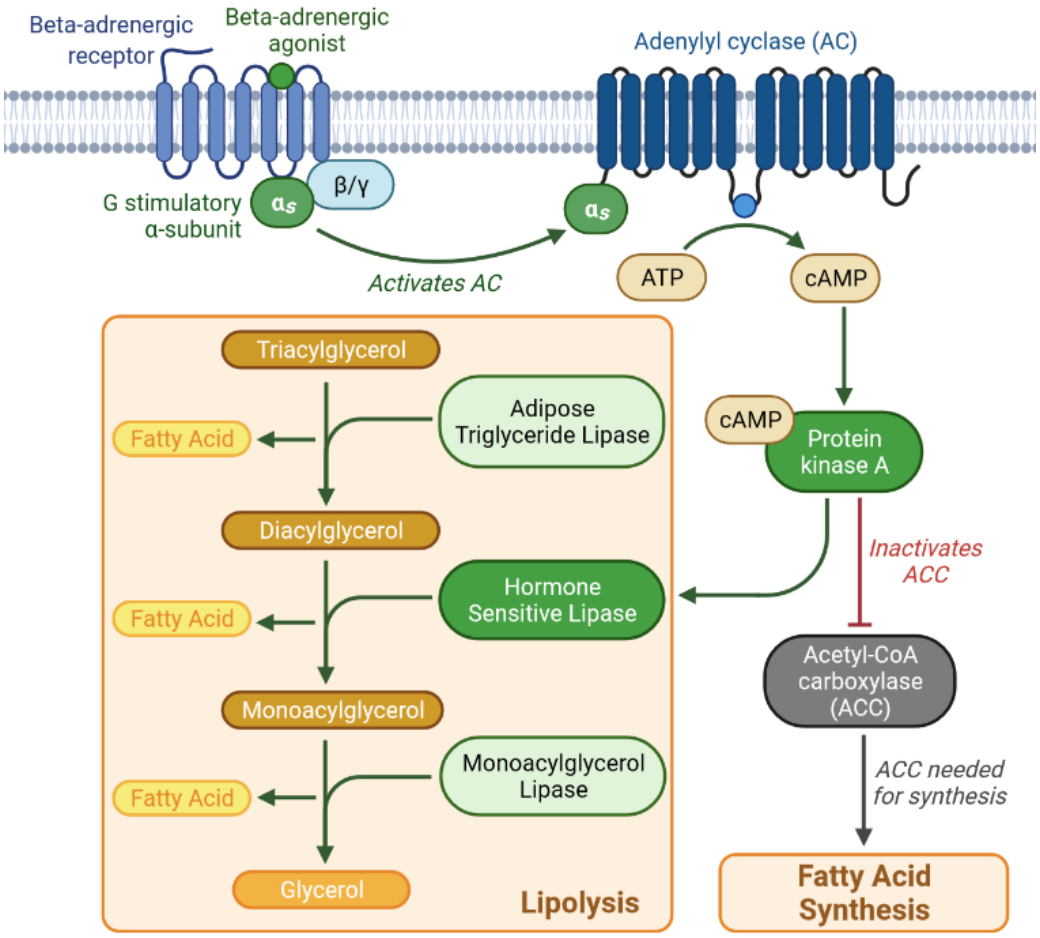
ractopamine increased longissimus muscle area in pigs, sheep, and cattle, but only increased HCW in pigs and cattle (Armstrong et al., 2004; López-Carlos et al., 2010; Hagenmaier et al., 2017). Some reported side effects of β-AA supplementation are tougher LM area, increased amount of liver abscesses, and increased respiration (Avendaño-Reyes et al., 2006; Elam et al., 2009; Rathmann et al., 2009; Boyd et al., 2015), though these effects are also not consistent.

While β-AA affect muscle, adipose tissue, and other organs, primarily the mechanisms within adipose tissue will be discussed. β-adrenergic agonists act on β-AR which are part of the G protein-coupled receptor (GPCR) family (Figure 1.1). The β-AA binds to the β-AR on the cell membrane of cells such as adipocytes and myocytes (Johnson et al., 2014). Beta-adrenoreceptors are coupled to G_s proteins that activate adenylyl cyclase (AC) leading to an increase in cyclic adenosine monophosphate (cAMP) levels in the cell (Lodish et al., 2000). Cyclic adenosine monophosphate binds to cAMP-dependent protein kinase A, which then phosphorylates several hormones including hormone sensitive lipase (HSL) and acetyl-CoA carboxylase (ACC) (Johnson et al., 2014). Acetyl-CoA carboxylase is the catalyst for the rate-limiting step in fatty acid synthesis, and when phosphorylated, acetyl-CoA carboxylase is inactivated, inhibiting fatty acid synthesis (Oh et al., 2005; Johnson et al., 2014). Phosphorylated HSL is active and partially hydrolyzes triacylglycerols, releasing fatty acids (Watt et al., 2006; Tsiloulis and Watt, 2015). Thus, β-AA binding to β-AR on adipose causes increased lipolysis and decreased lipogenesis (Kim et al., 2010; Johnson et al., 2014). However, β-AR become

desensitized due to chronic activation, thus supplementation is limited to the last 20-40 days of feeding (Re et al., 1997).

The effects of β-AA on fatty acid synthesis are not consistent as *ex vivo* studies observed ZH supplementation had little effect on fatty acid biosynthesis in bovine adipose tissue (Miller et al., 2012). Yet, the rate of *in vitro* fatty acid synthesis in adipose tissue from sheep can be altered due to supplementation of the β-AA L-644,969, depending on thermal environment and location of adipose tissue (Moibi et al., 2000).

Figure 1.1. Beta-adrenergic agonist effect on adipose



There have been welfare and health concerns over regarding the use of β-AA such as RAC and ZH, especially when paired with other stressors (Grandin, 2018). There is variation in the effects of β-AA and stress interaction on physiological parameters. Studies have reported increased death rates in cattle supplemented with RAC or ZH, though they conclude that increased stress from heat stress or handling may have exacerbated some effects of supplement (Loneragan et al., 2014). Indeed, results have shown that pigs fed 7.5 mg/kg of ractopamine compared to 5 mg/kg and 0 mg/kg had higher levels of plasma epinephrine and greater incidences of nonambulatory, noninjured pigs after handling (Peterson et al., 2015). Conversely, ZH-supplemented heifers challenged with corticotropin-releasing hormone and vasopressin had lower serum cortisol and epinephrine concentrations than the unsupplemented challenged heifers (Buntyn et al., 2016). Zilpaterol-supplemented lambs in heat stress had higher rectal temperatures than their unsupplmented counterparts, yet in another study, ractopamine lambs in heat stress had lower rectal temperatures than unsupplmented heat stressed lambs (Barnes et al., 2019; Swanson et al., 2020). Barnes observed lower respiratory rates in RAC supplemented lambs than unsupplemented heat stressed lambs, but Swanson reported no differences in respiratory rates between heart stressed RAC-supplemented lambs and heat stressed unsupplemented lambs (Barnes et al., 2019; Swanson et al., 2020). In both studies, it was concluded that supplementation did not negatively affect animal well-being based on the parameters measured, even in heat stress (Barnes et al., 2019; Swanson et al., 2020).

Heat Stress

As mentioned earlier, another factor that alters animal growth and health is heat stress. Heat stress decreases growth rates, DMI, and average daily gain of livestock, costing the US beef industry about $369 million per year (Mitlöhner et al., 2001; St-Pierre et al., 2003; Gaughan et al., 2010). Cattle in heat stress eat less to reduce the amount of heat produced during digestion (Basarab et al., 2003). They also tend to have lower hot carcass weight and less fat thickness, in part due to the decreased intake, as well as other stress effects (Mitlöhner et al., 2001; Gaughan et al., 2010; Edwards-Callaway et al., 2021). Heat-stressed animals still exhibit reduced rate of gain, muscle growth, and other stress effects even when differences in feed intake is accounted for with pair-feeding trials (Swanson et al., 2020).

The initial metabolic response to stress is the release of epinephrine from the adrenal medulla (Tai et al., 2007). Epinephrine regulates the animal's physiological response to heat stress in part by binding β-AR to activate downstream signaling pathways (Gonzalez-Rivas et al., 2020). Adipose tissue is altered by this acute heat stress signaling, which activates lipolysis and increases responsiveness of adipocytes to those signals (Campbell et al., 2009; Faylon et al., 2015). Lipolytic activity is decreased due to chronic heat stress, while lipogenesis and adipogenesis are upregulated (Qu et al., 2015; Qu and Ajuwon, 2018).

Another response to heat stress is the activation of the autonomic nervous system. The autonomic nervous system induces signaling pathways for immune response and thermoregulation (Gonzalez-Rivas et al., 2020). Rectal temperatures increase due to the

heat, so respiration rates increase to combat the rising body temperatures (Gaughan et al., 2010). Chronic heat stress induces an inflammatory response characterized by increased levels of inflammatory cytokines, TNF-α, and IL-6 (Min et al., 2016). Systemic inflammation reduces growth rates and muscle growth, evident by pair-fed trials where reduced feed intake was removed as a cause (Swanson et al., 2020). Heat stress can alter the immune system to the point of being susceptible to viruses (Jin et al., 2011), similarly to how heat stress is hypothesized to interact with β-AA to alter animal well-being. However, there is evidence that the effects of heat stress may be mediated by β-AA rather than exacerbated by it. As mentioned earlier, respiratory rates and rectal temperatures may be reduced in heat stressed animals supplemented with β-AA (Barnes et al., 2019; Swanson et al., 2020). β-adrenergic agonists can also reduce cardiac hypertrophy and improve impaired growth rates induced by heat stress (Swanson et al., 2020). These changes in growth and health mean that RNA expression is being altered in response to heat stress or β-AA, which can be investigated through RNA expression analysis.

Transcriptome

Analyzing RNA transcription can identify changes in gene expression due to different factors, and thus lead to the observed physiological outcomes. Heat stress and β-AA induce physiological changes by altering the transcriptomes of affected cells. Transcriptome analyses use RNA isolated from a specific tissue to quantify expression levels for all annotated transcripts for that species. For example, RNA from porcine skeletal muscle revealed that expression of heat shock protein and hypoxia genes were

upregulated by heat stress (Hao et al., 2016). They also found that heat stress alters expression of microRNAs, small single-stranded noncoding RNAs, that bind to RNA from genes important for muscle structure and function, therefore suppressing them (Hao et al., 2016). One way to count RNA transcripts is to use real-time PCR (RT-PCR) and qPCR, which quantify specific transcripts by reverse transcribing RNA into DNA then amplifying the DNA with PCR. The amplification can be tracked in real time, and the relative quantity is calculated using a control gene as reference. This allows gene expression from different treatment groups to be directly compared. For example, RT-PCR using RNA from porcine subcutaneous fat showed that heat stress increased expression of fatty acid synthase, lipoprotein lipase, fatty acid translocase 36, and several heat shock proteins (Qu et al., 2015). Rat osteoblast-like UMR106 cells incubated in a β_3AA exhibited increased expression of β_1AR and β_3AR, but decreased expression of β_2AR based on gel results from RT-PCR (Nuntapornsak et al., 2010). In that experiment, they also showed that β_3AR mRNA was greatly expressed in adipose, brain, and liver tissues and less so in jejunum and lung tissue (Nuntapornsak et al., 2010). The effect of varying concentrations of ZH in bovine myoblasts was compared with RT-PCR; 1 μM of ZH decreased β_1AR expression, 0.1 μM and 1 μM of ZH decreased β_2AR expression, while 1 μM of ZH increased IGF-1 expression (Miller et al., 2012). Real-time PCR can also allow expression comparisons across time points. A 23 d heat stress trial revealed differential expression patterns of IL-2 and IL-6 in cattle peripheral blood mononuclear cells (PBMC) throughout the 23 days (Bharati et al., 2017). As a result of these studies, the authors concluded that the increased expression of IL-2 and IL-6 in the first few days

compared to the latter half of the trial, were acute heat stress responses indicating possible heat stress tolerance over time (Bharati et al., 2017).

Differentially expressed loci can also be used for pathway analysis to predict biological changes. Liver mRNA analysis from heat stressed sheep found gene ontology (GO) terms associated with cellular metabolic processes, regulation of glucocorticoid receptor signaling pathways, and biosynthetic processes to be enriched (Li et al., 2019). I the same study, they observed differentially expressed long non-coding RNAs (lncRNA) near protein-coding genes associated with regulation of growth and triglyceride catabolic processes (Li et al., 2019). Previous work in lambs predicted oxidative stress response and protein ubiquination pathways to be altered in muscle due to heat stress (Kubik et al., 2018). From the same study, catecholamine biosynthesis and cardiac β-adrenergic signaling were predicted to be altered due to zilpaterol (Kubik et al., 2018). While these are only predictions based on RNA expression, they can be indicators of possible functional effects due to treatments.

Conclusion

Since both β-AA and heat stress alter adipose metabolism and function, transcriptomic evidence may elucidate how each factor individually and possibly interactively alter adipose tissue in ruminants. Pathway analysis may reveal the physiological effects of heat stress and β-AA on animal metabolism and health as well. Further, studying possible interactions could add to the growing literature that β-AA does

not exacerbate the negative effects of heat stress, but may mediate some of the effects. The studies in this thesis are outlined as follows: 1) effect of RAC and heat stress on ovine adipose tissue transcriptome, 2) effect of ZH and heat stress on bovine adipose tissue transcriptome, and 3) changes in fatty acid mobilization due to ZH and heat stress.

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CHAPTER 2: EFFECT OF 30 D OF RACTOPAMINE SUPPLEMENTATION AND HEAT STRESS ON THE ADIPOSE TISSUE TRANSCRIPTOME OF LAMBS

Introduction

Growth and feed efficiency of cattle is improved by supplementation with the beta-adrenergic agonists (β-AA) ractopamine hydrochloride (RAC; β_1-AA) or zilpaterol hydrochloride (ZH; β_2-AA) (Elam et al., 2009). Beta-adrenergic agonists also improve carcass traits including increasing longissimus muscle area (LM area), hot carcass weight (HCW), and decreasing overall carcass fatness and marbling (Elam et al., 2009; Brown et al., 2014). In sheep, ZH increases carcass weight, dressing percentage, and rib eye area, as does RAC though often to a lesser extent (López-Carlos et al., 2010; Macías-Cruz et al., 2010; Rojo-Rubio et al., 2018). Beta-adrenergic agonist supplementation alters adipose tissue deposition by inhibiting fatty acid biosynthesis and promoting lipolysis of stored triacylglycerols into free fatty acids (Johnson et al., 2014). However, beta-adrenoceptors (β-AR) desensitize with chronic activation (Mills et al., 1990; Re et al., 1997; Avendaño-Reyes et al., 2006); thus, supplementation is limited to the last 20-40 d of feeding.

The annual economic impact of heat stress on the US livestock industry was estimated to exceed $2.4 billion in 2003 (St-Pierre et al., 2003). Heat-stressed livestock have reduced growth rates, dry matter intake, and average daily gain (Mitlöhner et al., 2001; St-Pierre et al., 2003). Sheep in heat stress have increased rectal temperature,

respiration rates, plasma fatty acid concentration and reduced growth (Caroprese et al., 2012; Barnes et al., 2019). In response to acute stress, signaling pathways for lipolysis of circulating and stored triglycerides are activated, while chronic stress increases lipogenesis and adipogenesis (Campbell et al., 2009; Peckett et al., 2011). In cattle, heat stress also increases the responsiveness of adipocytes to further lipolytic signals, increasing lipolytic activity (Faylon et al., 2015). Liver RNA analyses from sheep in heat stress identified processes including fatty acid biosynthesis and metabolism, cellular metabolism, cellular response to stress, and fat metabolism to be enriched due to differentially expressed genes between the heat-stressed and thermoneutral groups (Li et al., 2019; Lu et al., 2019).

The objective of this study was to understand how heat stress and β-AA independently and interactively affect adipose tissue in ruminants. Lambs were used as a model for cattle due to similar digestive systems and response to β-AA supplementation (Johnson et al., 2014). Prior work identified minimal impact of RAC on metabolic properties (Barnes et al., 2019) and transcriptome of skeletal muscle (Kubik et al., 2018) of wether lambs. We therefore hypothesized that RAC may be primarily affecting adipose tissue to achieve its impact on altered carcass composition of ruminant livestock; specifically, that lipolytic activity is increased due to heat and β-AA in an additive fashion. We tested this hypothesis in RAC-supplemented lambs exposed to heat stress for 30 d.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln, which is accredited by AAALAC International.

Rambouillet-cross, wether lambs (average initial weight = 43 kg) were housed in group pens and acclimated for 21 d. After 21 d, lambs were stratified by body weight and randomly assigned to one of four treatment groups: not supplemented/thermoneutral (NSTN), ractopamine/thermoneutral (RHTN), not supplemented/heat stress (NSHS), and ractopamine/heat stress (RHHS). During the trial, the lambs were individually penned, but were in the same room as the other lambs of their environment group. A temperature humidity index (THI) was used to determine the room temperature and humidity. Lambs were housed for 30 d under thermoneutral (TN; THI=65; n=14) or heat stress (HS; THI=80; n=12) conditions and not supplemented with ractopamine HCl (NS) or supplemented with ractopamine HCl (RAC; 60mg/head/d), per industry standard, in a 2x2 factorial. Heat stress was achieved by maintaining a temperature of approximately 40°C and 35% humidity from 0800 to 2000 h, and 29°C between 2000 and 0800 with temperature changing over a period of 2 h at the beginning and end of each heat cycle. The thermoneutral room was maintained at 25°C with 15% humidity. In both environmental conditions, the light was on from 0630 to 2045 h.

Thermoneutral lambs were pair-fed to the average intake of HS lambs, which were fed Lamb Grower-Finisher Complete B30 (16% crude protein, 2% crude fat, 16% crude fiber; Purina) *ad libitum*. Orts, feed left over from the day before, were collected daily and recorded to evaluate average performance statistics (e.g., average daily gain,

gain: feed), to determine the amount of feed to offer the following day as well as that to offer the pair-fed counterparts. The HS lambs started the trial a day before the TN lambs to calculate daily intake; thus, the TN lambs were a day behind the HS lambs throughout the trial. On d 30, lambs were harvested by an overdose of pentobarbital. Subcutaneous adipose tissue was collected, flash-frozen, and stored at -80°C until isolation.

RNA from the subcutaneous adipose tissue was extracted using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), adopting protocol from Rneasy® Plus Mini Handbook. RNA was washed and eluted on columns (Direct-zol™ RNA MiniPrep Plus, Zymo, Irvine, CA) according to manufacturer's instructions. Quality of RNA was evaluated using the Pico chip on the Agilent Bioanalyzer 2100 (Santa Clara, CA, USA).

RNA was sent to the Michigan State University, Research Technology Support Facility for Poly-A^+ library preparation and sequencing using 150bp, paired-end reads to a targeted, minimum depth of 20 million reads/sample. Reads were trimmed with TrimGalore (Martin, 2011). Reads with a quality of less than 20 were removed with FastQC (Andrews, 2010). Transcripts as annotated in Oar_rambouillet_v1.0 were quantified (STAR; Dobin et al., 2013). Loci with less than 20 total reads across all samples were removed from analysis. Outliers were identified using principal component analysis and pheatmap (Kolde, 2019). Differential expression (DE) analyses were performed in DESeq2 (Love et al., 2014) with a significance threshold (FRD) of 0.05 testing, to correct for 5% of the results that are expected to be false positives. The statistical model included the main effects, environment and supplement, as well as the

interaction of thereof. Pathway analysis (IPA, Qiagen) was conducted on all loci with raw $P < 0.05$.

Results

The RNA integrity number (RIN) scores of the adipose tissue RNA ranged from 6.5 to 8.3. On average, 59.5 million reads were obtained per animal. An average of 82.2% of reads mapped to the genome. Out of annotated 31,902 transcripts, 20,023 had non-zero sum counts across all samples and were observed and kept for DESeq2 analysis. One animal from the RAC/HS group was identified as an outlier and removed from analyses.

Thirty-six loci were DE ($P_{adj}<0.05$) due to a RAC by HS interaction (Table 2.1), including *GPRC5A* (Figure 2.1). Three loci were DE due to HS alone: *GSTM1*, *GSTM1-like*, and *SLC6A17*. 119 loci were DE due to RAC alone (Table 2.2). Of the 36 loci DE due to RAC by HS interaction, 26 were also DE due to RAC alone.

HS was predicted to alter 45 pathways, including interleukin signaling, G protein-coupled receptor signaling, and acute phase response (Table 2.3). The only pathway predicted to be upregulated by HS was NRF2-mediated oxidative stress response. Ractopamine was predicted to alter 170 pathways including adipogenesis, fatty acid beta-oxidation, NRF2-mediated oxidative stress response, and G protein-coupled receptor signaling (Table 2.4). The treatment of RAC by HS interaction was predicted to alter 130 pathways ($P<0.05$), including several interleukin pathways and the acute phase response (Table 2.5).

After 30 d, there was no differences in DMI or average daily gain due to environment, supplement, or the combination thereof as reported in Swanson et al. (2020).

Discussion

We identified interacting and independent effects of HS and RAC supplementation on metabolism and the immune response in sheep, as indicated by differences in loci expression among treatments. Both RAC alone and the HS by RAC interaction were predicted to have altered G protein-coupled receptor signaling, and adipocyte metabolism. HS, RAC and their interaction also altered expression of loci included in inflammatory and immune pathways.

G protein-coupled receptor signaling

Several loci DE due to RAC and HS by RAC interaction are components of G protein-coupled receptor (GPCR) pathways, through which β-AA signal. Specifically, β-AA bind to beta-adrenoreceptors (β-AR), which are part of the GPCR family, categorized in class A, rhodopsin-like GPCRs, based on structure and function (Isberg et al., 2016; Kooistra et al., 2021). Ractopamine downregulated *SAA1*, *LTB4R, MRGPRF*, *CD55, NR4A1*, *GPRC5A*; all involved in the GPCR or GPCR signaling pathways. *GPRC5A* encodes retinoic acid-induced protein 3, a retinoic acid-inducible orphan GPCR involved in cell proliferation (Hirano et al., 2006), and is in class C of the GPCR family, glutamate

receptors (Isberg et al., 2016; Kooistra et al., 2021). This gene is induced by all-*trans*-retinoic acid and elevated cyclic adenosine monophosphate (cAMP) levels (Hirano et al., 2006). Overexpression of *GPRC5A* causes a decrease in intracellular cAMP, creating a negative feedback loop (Hirano et al., 2006). Beta-adrenergic agonists activate a pathway that increases cAMP levels (Johnson et al., 2014), yet *GPRC5A* was downregulated by RAC alone by 30 d. One possible explanation for this outcome is that *GPRC5A* had been previously upregulated due to supplement and was downregulated by the collection point, due to the effects of negative feedback. This cannot be confirmed as additional sampling would have increased costs and we were interested in the effects of chronic treatment on RNA at the time.

Pathway analysis provided additional evidence of changes related to *GPRC5A* expression as retinoic acid receptor (RAR) activation and retinol biosynthesis were predicted to be dysregulated due to supplement; retinol biosynthesis was predicted to be upregulated while RAR activation had no predicted direction of change, indicating the alteration of expression of loci in this pathway could not strongly support up or downregulation. The predicted dysregulation of GPCR signaling and upregulation of cAMP-mediated signaling due to RAC also support that RAC altered GPCR function. Based upon Hirano et. al (2006), the combination of elevated cAMP and retinol levels may have initially upregulated *GPRC5A* (Hirano et al., 2006), but this overexpression may have initiated the negative feedback loop, causing the downregulation predicted by our data. Another explanation is that the β-AR on the adipocytes have become desensitized due to chronic RAC supplementation, an effect observed for most β-AA

(Mills et al., 1990; Mersmann, 1998; Johnson et al., 2014). The chronic activation of β-AR would leave them inactive by 30 d, which would prevent them from further elevating cAMP levels to induce *GPRC5A*. Regardless, these data support the predicted impact of RAC on GPCRs in adipose tissue.

Conversely, the interaction of RAC and HS was predicted to have a different effect on GPCRs. Loci in the GPCR pathway that were DE (P_{adj}<0.05) due to interaction included *NR4A1, NGEF*, and *GPRC5A*; all were upregulated, having similar expression levels as the control group. This could be due to epinephrine, released in response to heat stress, binding to both α-AR and β-AR (Gonzalez-Rivas et al., 2020), resulting in a situation where these loci were upregulated despite chronic activation. While HS was predicted to dysregulate GPCR signaling, interaction of RAC by HS was not predicted to significantly alter the GPCR signaling pathway. The interaction of HS and RAC may have resulted in conflicting signals with the GPCR pathway that was not able to be elucidated by these data. While it is not certain what all the effects of RAC by HS interaction were on GPCR signaling or related pathways, the data suggests that HS altered the effect of RAC on GPCR function.

Metabolism

There were other metabolic loci and pathways altered due to RAC alone. RAC downregulated *S100A4* and *PFKP*, and upregulated *GPD2;* all are genes associated with cellular metabolism. *S100A4* has been implicated in controlling obesity, as overexpressed

S100A4 inhibited adipogenesis in mice while S100A4 deficient mice displayed obesity and inflammation (Hou et al., 2018). Based upon the other studies, the downregulation of *S100A4* suggests an inflammatory response and does not necessarily support a decrease in lipid accumulation although that was not explicitly studied. *PFKP* is the platelet isoform of phosphofructokinase, which regulates glycolysis and increases glucose uptake (Wang et al., 2021). *GPD2* codes for mitochondrial glycerol-3-phosphate dehydrogenase, which is involved in glycolysis, fatty acid metabolism, and oxidative phosphorylation (Mráček et al., 2013). The changes to *PFKP* indicate that RAC downregulated glycolysis indirectly, but glucose oxidation in skeletal muscle from this study was not altered by RAC supplementation (Swanson et al., 2020) though ZH supplementation increased glucose oxidation in a previous study (Barnes et al., 2019). Yet, the upregulation of *GPD2* is evidence that RAC was still inducing some metabolic pathways. Indeed, fatty acid β-oxidation and triacylglycerol biosynthesis were predicted to be upregulated due to RAC, while mitochondrial dysfunction and adipogenesis pathways were predicted to be dysregulated with no clear direction. Physical evidence supports this, as the RAC wethers had less back fat thickness at the beginning of the trial than unsupplemented wethers, but had no significant difference in back fat thickness after (Swanson et al., 2020). Ractopamine-supplemented wethers also had greater fat mass in their four-rib cut-out and a higher fat percentage in their longissimus dorsi than unsupplemented wethers (Swanson et al., 2020), possibly related to the downregulation of *S100A4*. This could be an effect from desensitization of the β-AR, resulting in no or few significant effects on adipose tissue traits due to RAC, an outcome observed in pigs and beef steers (Mills et al., 1990;

Avendaño-Reyes et al., 2006). Overall, evidence suggests that RAC altered adipose tissue metabolism, but not via a means that decreased fat deposition by 30 d.

There were few metabolic pathways predicted to be altered by HS alone. However, the combination of pathways, growth hormone signaling and leptin signaling in obesity, which were predicted to be downregulated due to HS, illustrate the chronic effect of HS on metabolism. Growth hormones (GH) control metabolism by inducing IGF-1 and promoting lipolysis and protein synthesis (Møller and Jørgensen, 2009; Jiang and Ge, 2014). Leptin is a hormone that suppresses hunger, IGF-1 and GH, and normally has increased expression under heat stress (Ajuwon et al., 2003; Archana et al., 2018; Qu and Ajuwon, 2018). Yet, HS was predicted to downregulate leptin signaling, which would be expected to increase appetite, an outcome that does not necessarily fit with the expectations of reduced intake due to HS. There were no differences in feed intake due to pair-feeding, but heat-stressed wethers had reduced weight gain, and loin growth (Swanson et al., 2020). Possible adaptation to HS would explain decreased leptin signaling at 30 d, but our evidence suggests HS continued to suppress growth pathways preventing normal growth.

The interaction of RAC and HS altered metabolic loci in adipose tissue that indicate the use of glucose for energy rather than adipose stores. Loci involved in the RAC by HS interaction included the upregulation of *S100A4*, *PFKP*, and *SFRP4*. Given that *S100A4* inhibits adipogenesis (Hou et al., 2018), *SFRP4* decreases adipocyte size (Zhang et al., 2020), and *PFKP* is necessary for glycolysis (Wang et al., 2021), the upregulation of those loci support the use of glucose instead of adipose for energy.

Pathway analysis however provided conflicting results. Apelin adipocyte signaling, which inhibits adipogenesis and lipolysis (Than et al., 2012), was predicted to be downregulated due the interaction of the treatments. Apelin uses the MAPK/ERK pathway to regulate adipogenesis and lipolysis (Than et al., 2012), which has no predicted direction of change in our data. Yet, another pathway induced by apelin, the white adipose tissue browning pathway, is predicted to be upregulated (Than et al., 2015). It is possible that the DE loci are not directly involved in the apelin induced pathways and both are altering adipose tissue metabolism, with an unknown final effect on adipose tissue.

Inflammation and stress response

The possible interaction of RAC and HS on animal well-being has been a concern due to an incidence where β-AA supplementation was speculated to have contributed to lameness in cattle during stressful conditions (Grandin, 2018). Our data do show an interaction of RAC and HS on loci and pathways involved in inflammation and oxidative stress. Both proinflammatory (*LTC4S, NR4A1, FOS*) and anti-inflammatory loci (*C1QTNF3*) were upregulated (Samuelsson, 1983; Levi-Schaffer and Piliponsky, 2003; Wagner and Eferl, 2005; Pei et al., 2006). *JUNB* is expressed under stress conditions to mediate oxidative stress and apoptosis (Gurzov et al., 2008; Son et al., 2010). The upregulation of these loci is evidence that the interaction of chronic treatment of RAC and HS provoked inflammatory and immune responses in adipose tissue. The predicted changes in inflammation and immune response pathways, however, vary. While five

interleukin pathways are predicted to be downregulated, one is upregulated, and the involvement of two others is unclear. Interleukins are produced by immune and inflammatory cells, with each type having specific functions (Akdis et al., 2016). Despite chronic HS and RAC, the acute phase response pathway was predicted to be upregulated, even after 30 d of treatment possibly in response to the upregulated interleukins. The acute phase response in animals is induced by stress and inflammation, and its purpose is to resolve such issues by activating acute phase proteins (Cray et al., 2009). Production of nitric oxide and reactive oxygen species in macrophages pathways were predicted to be downregulated, which correlates with loci such as *JUNB* that mediate oxidative stress. These pathway changes imply that RAC may mediate oxidative stress due to HS, but also promote inflammatory and immune responses with interaction of HS. However, the physiological data did not find the interaction negatively affected wether health or well-being based on the parameters measured (Swanson et al., 2020); thus, the predicted changes based on DE loci are not necessarily indicative of physiological changes.

As inflammation is expected due to heat stress (Min et al., 2016), and is a contributing factor to decreased animal efficiency (Mitlöhner et al., 2001), DE loci due to HS annotated to be involved in the inflammatory process were of interest. The genes, *GSTM1* and *GSTM1-like* (glutathione S-transferase Mu 1), reduce oxidative stress and reduce blood pressure in hypertensive rats (Olson et al., 2019); in the lambs their upregulation serves as a line of evidence that oxidative stress was present. This result also agrees with the predicted downregulation of nitric oxide and reactive oxygen species in macrophages pathway, and upregulation of NRF2-mediated oxidative stress response.

Inflammation pathways such as IL-23 and acute phase response were predicted to be downregulated, supporting that the wethers may had adapted to the effects of HS by 30 d.

Ractopamine supplementation altered inflammation and oxidative stress in the adipose tissue. Inflammatory genes such as *LTC4S, NR4A1, FOS,* and *C1QTNF3,* were upregulated due to the interaction of RAC and HS but downregulated due to RAC alone. *IL-34* (interleukin-34), associated with rheumatoid arthritis (Akdis et al., 2016), and *CFP*, a positive regulator of the complement system, were also downregulated by RAC (Michels et al., 2019). *LTB* (lymphotoxin-beta), another downregulated gene, is highly expressed in the inflamed tissues of rheumatoid arthritis patients and overexpression induces chronic inflammation in mice (O'Rourke et al., 2008; Seleznik et al., 2012). While the transcriptomic analysis shows RAC acts to suppress both pro and anti-inflammatory related activity, this conflicts with the pathway analysis. Pro-inflammatory pathways, such as ones involving interleukins, were predicted to be upregulated while anti-inflammatory pathways were predicted to be downregulated. β-AA have some documented anti-inflammatory effects including suppressing production of interleukin-8 (IL-8), one of the pathways upregulated by RAC in our trial (Farmer and Pugin, 2000; Anderson et al., 2014). Although there is transcriptomic evidence that RAC induced inflammation, the physiological data does not support this, as they found no evidence of RAC negatively affect animal well-being (Swanson et al., 2020). However, there is evidence that RAC reduces oxidative stress. The upregulation of *GSTM1* and *GSTM1-like* and predicted upregulation of NRF2-mediated oxidative stress response indicate that RAC moderated oxidative stress. Though whether this was oxidative stress normally

produced from oxidative metabolism or a response to RAC-induced oxidative stress is unclear and cannot be determined from these data.

Expression of the adipose tissue transcriptome was altered due to the combined impact of heat stress and β-AA supplementation in wether lambs. Due to anecdotal concerns of HS and β-AA interacting to negatively impact well-being of cattle, the results of this study in lambs that identified the differential expression of loci and predicted upregulation of pathways associated with inflammation warrants additional investigation to fully elucidate their role in adipose tissue. Despite this, no interacting effects of HS and β-AA supplementation on animal well-being were apparent in the physiological data. Building a better understanding of the mechanisms by which animals respond to HS and β-AA supplementation will aid in generating improved management practices to improve sustainability of livestock production.

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Table 2.1. Loci differentially expressed due to an interaction of heat stress and ractopamine supplementation in subcutaneous adipose tissue of wether lambs

Loci ID	Log_2 fold change	Adjusted *P*-value
LTC4S	3.758	<0.001
LOC114113045	-24.187	<0.001
JUNB	3.6	0.0015
GPRC5A	6.198	0.0015
SERPINF2	5.356	0.0017
DACT2	3.351	0.0017
SPON2	3.666	0.0017
S100A4	2.539	0.0017
TNXB	1.949	0.0021
MXRA5	2.693	0.0021
FOS	6.174	0.0041
ITGA11	2.956	0.0046
NR4A1	3.040	0.0054
SFRP4	4.545	0.0075
HSD3B7	1.941	0.0075
PPL	1.951	0.0075
DNM1	1.539	0.0093
EHBP1L1	1.095	0.0093
TSKU	1.533	0.0093
THY1	2.166	0.0123
PAMR1	2.825	0.016
C1QTNF3	4.459	0.0175
PLAC9	2.914	0.0175
CD248	2.118	0.0176
FMOD	5.277	0.0176
HTRA3	1.871	0.0190
CLEC4G	3.397	0.0214
MBP	2.877	0.0214
FIBIN	3.811	0.0266
NOVA1	1.28	0.0275
PRIMA1	3.051	0.0286
ANXA8	2.926	0.0286
PTGIS	2.362	0.0286
PFKP	1.357	0.0286
SFXN3	1.491	0.0354
NGEF	4.532	0.0419

Table 2.2. Loci differentially expressed in wether lamb subcutaneous adipose tissue due to ractopamine supplementation

Loci ID	Log_2 fold change	Adjusted *P*-value	Loci ID	Log_2 fold change	Adjusted *P*-value
SERPINF2	-4.230	<0.001	*LRRC17*	-1.502	0.0181
TSKU	-1.317	<0.001	*MRGPRF*	-1.353	0.0181
LOC105604171	-4.319	<0.001	*PRELP*	-1.667	0.0181
MXRA5	-2.015	<0.001	*SEMA3B*	-0.784	0.0185
S100A4	-1.865	<0.001	*FMOD*	-3.235	0.0186
SPON2	-2.712	<0.001	*GPRC5A*	-3.125	0.0209
DACT2	-2.392	<0.001	*LOC105606726*	3.530	0.0209
GSTM1-like	1.145	<0.001	*NSUN3*	0.956	0.0230
CD248	-1.717	<0.001	*SCARF2*	-1.368	0.0230
DNM1	-1.179	<0.001	*FGF18*	-1.885	0.0252
PPM1L	0.895	<0.001	*VASN*	-1.230	0.0252
TNXB	-1.326	<0.001	*COL8A2*	-1.318	0.0285
PLAC9	-2.261	<0.001	*PRR5*	-1.373	0.0285
EHBP1L1	-0.806	<0.001	*PTGIS*	-1.461	0.0285
LTC4S	-2.241	<0.001	*CERCAM*	-0.923	0.0286
IL34	-1.848	<0.001	*BRAT1*	-1.068	0.0316
FKBP10	-1.296	0.0013	*PPP1R18*	-0.799	0.0318
SFXN3	-1.164	0.0018	*LOC105606262*	0.755	0.0325
THY1	-1.536	0.0020	*PAMR1*	-1.628	0.0325
FBLN1	-1.272	0.0023	*CCDC151*	-1.988	0.0328
PPL	-1.320	0.0023	*NUMBL*	-0.899	0.0330
RCN3	-1.222	0.0028	*CILP*	-1.572	0.0333
STAB1	-1.247	0.0029	*GPLD1*	1.578	0.0333
AEBP1	-1.133	0.0030	*HMCN2*	-3.379	0.0333
SFRP4	-3.002	0.0032	*OLFML3*	-0.904	0.0333
C1QTNF1	-1.191	0.0033	*FAM43B*	-3.200	0.0335
C1QTNF3	-3.114	0.0033	*RFX1*	-0.682	0.0335
VCAN	-2.389	0.0033	*DKK3*	-2.170	0.0340
PCDH9	1.761	0.0047	*CD83*	-1.397	0.0344
GPD2	1.425	0.0072	*ALDH3B1*	-0.869	0.0362
HSD3B7	-1.212	0.0072	*COL14A1*	-1.306	0.0362
KCNA6	-1.848	0.0072	*PBLD*	0.832	0.0362
LOC101117184	-4.145	0.0072	*TESK2*	0.818	0.0365
LOC101117955	-2.008	0.0072	*CD55*	-1.657	0.0367
SAA	-4.358	0.0072	*ELN*	-1.541	0.0367
LOC105603423	-6.421	0.0072	*ACAP3*	-0.748	0.0374
LOC105606646	-1.803	0.0072	*F13A1*	-1.574	0.0374
LOC114118399	-0.815	0.0072	*LOC101102402*	0.959	0.0374

CCN4	-3.018	0.0075	*LYL1*	-0.843	0.0374
EFS	-1.126	0.0075	*LOC101103461*	-1.439	0.0378
LTB	-1.523	0.0075	*LOC101110467*	-1.046	0.0388
TMEM119	-1.290	0.0075	*NFATC2*	-1.942	0.0397
IGFBP6	-1.494	0.0086	*PMP22*	-0.948	0.0397
EFEMP2	-1.266	0.0089	*ACBD5*	0.923	0.0399
LOC101123627	-2.285	0.0089	*PRKAR1B*	-0.669	0.0401
LOC114109570	3.267	0.0089	*SGSM2*	-0.855	0.0401
LTBP3	-1.208	0.0101	*SLC46A3*	0.833	0.0407
LOC101104530	-0.997	0.0110	*IARS2*	0.509	0.0431
NAV1	-0.917	0.0110	*KAZALD1*	-1.286	0.0431
PFKP	-0.910	0.0110	*NR4A1*	-1.549	0.0431
LOC105610613	1.675	0.0144	*PDZK1*	3.027	0.0431
B4GALNT1	-2.234	0.0148	*PIEZO2*	-1.646	0.0431
GSTM1	1.493	0.0152	*TIMP1*	-1.140	0.0431
LTB4R	-1.646	0.0161	*VAV3*	1.197	0.0431
FOS	-3.434	0.0172	*SLCO1C1*	1.812	0.0433
LAG3	-0.985	0.0172	*LOC101121916*	-8.701	0.0440
DPYSL3	-1.367	0.0174	*VPS37A*	0.673	0.0467
MRC2	-1.147	0.0177	*HOXD3*	-0.805	0.0467
CFP	-1.562	0.0181	*DOP1A*	0.943	0.0494
LOC101112990	-1.303	0.0181			

Table 2.3. Pathways predicted to be altered in subcutaneous adipose tissue of wether lambs heat-stressed for 30 d

Pathway	Direction	Pathway	Direction
14-3-3-mediated Signaling	Down	LPS-Stimulated MAPK Signaling	Down
3-Phosphoinositide Biosynthesis	Down	Macropinocytosis Signaling	Down
Acute Phase Response Signaling	Down	Neuropathic Pain Signaling in Dorsal Horn Neurons	Down
Apelin Cardiomyocyte Signaling Pathway	Down	nNOS Signaling in Skeletal Muscle Cells	N/A
cAMP-Mediated Signaling	0	NRF2-mediated Oxidative Stress Response	Up
Cardiac Hypertrophy Signaling	Down	Opioid Signaling Pathway	Down
Cardiac Hypertrophy Signaling (Enhanced)	Down	P2Y Purigenic Receptor Signaling Pathway	Down
Coagulation System	0	PCP Pathway	Down
Colorectal Cancer Metastasis Signaling	Down	Phagosome Formation	N/A
Endocannabinoid Neuronal Synapse Pathway	Down	PKCθ Signaling in T Lymphocytes	Down
Endothelin-1 Signaling	Down	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Down
ErbB Signaling	Down	RAR Activation	N/A
Factors Promoting Cardiogenesis in Vertebrates	Down	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	N/A
Gap Junction Signaling	N/A	Role Of NFAT in Cardiac Hypertrophy	Down
GNRH Signaling	Down	Role Of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	N/A
GP6 Signaling Pathway	Down	Role of Tissue Factor in Cancer	N/A
G-Protein Coupled Receptor Signaling	N/A	Superpathway Of Inositol Phosphate Compounds	Down
Growth Hormone Signaling	Down	Triacylglycerol Biosynthesis	0
Hepatic Fibrosis/ Hepatic Stellate Cell Activation	N/A	UVA-Induced MAPK Signaling	Down
HGF Signaling	Down	UVB-Induced MAPK Signaling	Down
IL-12 Signaling and Production in Macrophages	N/A	Wnt/β-catenin Signaling	Down
IL-23 Signaling Pathway	Down	Xenobiotic Metabolism General Signaling Pathway	Down
Leptin Signaling in Obesity	Down		

Table 2.4. Pathways predicted to be altered in subcutaneous adipose tissue of wether lambs supplemented with ractopamine for 30 d

Pathway	Direction	Pathway	Direction
2-ketoglutarate Dehydrogenase Complex	0	Insulin Secretion Signaling Pathway	Up
2-oxobutanoate Degradation I	0	Isoleucine Degradation I	Up
3-Phosphoinositide Biosynthesis	Down	Ketolysis	0
3-Phosphoinositide Degradation	Down	Leucine Degradation I	0
4-1BB Signaling in T Lymphocytes	Up	Leukocyte Extravasation Signaling	Up
4-aminobutyrate Degradation I	0	LPS-stimulated MAPK Signaling	Up
Actin Cytoskeleton Signaling	0	Macropinocytosis Signaling	Down
Acute Myeloid Leukemia Signaling	Up	Mechanisms of Viral Exit from Host Cells	N/A
Acute Phase Response Signaling	Down	Melanocytes Development and Pigmentation Signaling	Up
Adenosine Nucleotides Degradation II	0	Melatonin Signaling	0
Adipogenesis	N/A	Methylmalonyl Pathway	0
AMPK Signaling	Down	Mitochondrial Dysfunction	N/A
Amyloid Processing	Up	Molecular Mechanism of Cancer	N/A
Androgen Signaling	0	Mouse Embryonic Stem Cell Pluripotency	0
Antiproliferative Role of TOB in T Cell Signaling	Down	Natural Killer Cell Signaling	Down
Apelin Adipocyte Signaling Pathway	Up	Netrin Signaling	Down
Apelin Liver Signaling Pathway	Down	Neuregulin Signaling	Up
Apelin Pancreas Signaling Pathway	Up	Neuroinflammation Signaling Pathway	Down
Apoptosis Signaling	Up	Neurotrophin/TRK Signaling	Up
April Mediated Signaling	Down	NGF Signaling	up
Aryl Hydrocarbon Receptor Signaling	Up	NRF2-mediated Oxidative Stress Response	Up
Atherosclerosis Signaling	N/A	Nur77 Signaling in T Lymphocytes	Down
ATM Signaling	Up	Opioid Signaling Pathway	Up
Axonal Guidance Signaling	N/A	Osteoarthritis Pathway	Down
B Cell Activating Factor Signaling	Down	Ovarian Cancer Signaling	Up
B Cell Receptor Signaling	Up	p53 Signaling	Down
Biotin-carboxyl Carrier Protein	0	p70S6K Signaling	Up

assembly			
Breast Cancer Regulation by Stathmin1	N/A	Parkinson's Signaling	N/A
Calcium Signaling	Down	PCP pathway	0
cAMP-mediated signaling	Up	PFKFB4 Signaling Pathway	Up
Cardiac Hypertrophy Signaling (Enhanced)	Down	Phagosome Formation	N/A
Cardiac β-adrenergic Signaling	Up	Phospholipase C Signaling	Down
CCR5 Signaling in Macrophages	0	PKCθ Signaling in T Lymphocytes	Down
CD27 Signaling in Lymphocytes	0	PPARa/RXRa Activation	Down
CD28 Signaling in T Helper Cells	Down	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Down
Cholecystokinin/Gastrin-mediated Signaling	0	Prolactin Signaling	Up
Colorectal Cancer Metastasis Signaling	Down	Protein Kinase A Signaling	Down
Corticotropin Releasing Hormone Signaling	Down	Purine Nucleotides Degradation II (Aerobic)	0
Death Receptor Signaling	0	RANK Signaling in Osteoclasts	Down
Dendritic Cell Maturation	Down	RAR Activation	N/A
Dipthamide Biosynthesis	0	Reelin Signaling In Neurons	Up
D-myo-inosital (1,4,5,6)-Tetrakisphosphate Biosynthesis	Down	Regulation of IL-12 Expression in Activated and Anergic T Lymphocytes	N/A
D-myo-inosital (3,4,5,6)-Tetrakisphosphate Biosynthesis	Down	Regulation Of the Epithelial Mesenchymal Transition By Growth Factors Pathway	0
D-myo-inositol-5-phosphate Metabolism	Down	Regulation of the Epithelial Mesenchymal Transition In Development Pathway	Down
dTMP De Novo Biosynthesis	0	Regulation of the Epithelial-Mesenchymal Transition Pathway	N/A
EGF Signaling	Up	Reintol Biosynthesis	Up
Eicosanoid Signaling	0	Renin-Angiotensin Signaling	Up
ERK/MAPK Signaling	Down	Role NFAT in Regulation of the Immune Response	Down
Estrogen Receptor Signaling	Down	Role of JAK family kinases in IL-6-type Cytokine Signaling	N/A
Factors Promoting Cardiogenesis in Vertebrates	Down	Role of JAK2 in Hormone-like Cytokine Signaling	N/A
FAT10 Cancer Signaling Pathway	Down	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	N/A
Fatty Acid β-oxidation I	Up	Role of NFAT in Cardiac	Down

		Hypertrophy	
Fc Epsilon RI Signaling	Up	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	NA
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	Down	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Up
FcyRIIB Signaling in B Lymphocytes	Up	Role of PKR in Interferon Induction and Antiviral Response	Down
FGF Signaling	Up	SAPK/JNK Signaling	Up
FLT3 Signaling in Hematopoietic Progenitor Cells	Up	Senescence Pathway	Up
Ga12/13 Signaling	Up	Sertoli Cell-Sertoli Cell Junction Signaling	N/A
Gaq Signaling	Down	Signaling by Rho Family GTPases	Down
GDNF Family Ligand-Receptor Interactions	0	Sperm Motility	Up
Glioblastoma Multiforme Signaling	Down	STAT3 Pathway	Up
Glucocorticoid Receptor Signaling	N/A	Stearate Biosynthesis I (Animals)	0
Glutamate Degradation III (via 4-aminobutyrate)	0	Sumoylation Pathway	Up
Glutaryl-CoA Degradation	Up	Superoxide Radicals Degradation	0
GM-CSF Signaling	Up	Superpathway of Inositol Phosphate Compounds	Down
GNRH Signaling	Down	Synaptogenesis Signaling Pathway	Down
GP6 Signaling Pathway	Down	Systemic Lupus Erythematosus In B Cell Signaling Pathway	Down
G-Protein Coupled Receptor Signaling	N/A	T Cell Exhaustion Signaling Pathway	Down
Growth Hormone Signaling	Up	T Cell Receptor Signaling	N/A
Hepatic Fibrosis / Hepatic Stellate Cell Activation	N/A	Tec Kinase Signaling	Down
Hepatic Fibrosis Signaling Pathway	Down	TGF-β Signaling	Up
HER-2 Signaling in Breast Cancer	N/A	Tight Junction Signaling	N/A
HGF signaling	Up	TR/RXR Activation	N/A
HIPPO signaling	Down	Triacylglycerol Biosynthesis	Up
HMGB1 Signaling	Down	Type II Diabetes Mellitus Signaling	0
Human Embryonic Stem Cell Pluripotency	N/A	UVA-Induced MAPK Signaling	Up

Huntington's Disease Signaling	0	UVB-Induced MAPK Signaling	Up
IGF-1 Signaling	Up	Valine Degradation	Up
IL-10 Signaling	N/A	VDR/RXR Activation	0
IL-12 Signaling and Production in Macrophages	N/A	White Adipose Tissue Browning Pathway	Down
IL-15 Production	Up	Wnt/Ca+ pathway	Down
IL-22 Signaling	Up	Wnt/β-catenin Signaling	Up
IL-6 Signaling	Up	Xenobiotic Metabolism AHR Signaling Pathway	Up
IL-8 Signaling	Up	Xenobiotic Metabolism Signaling	N/A
ILK Signaling	Down	β-alanine Degradation I	0

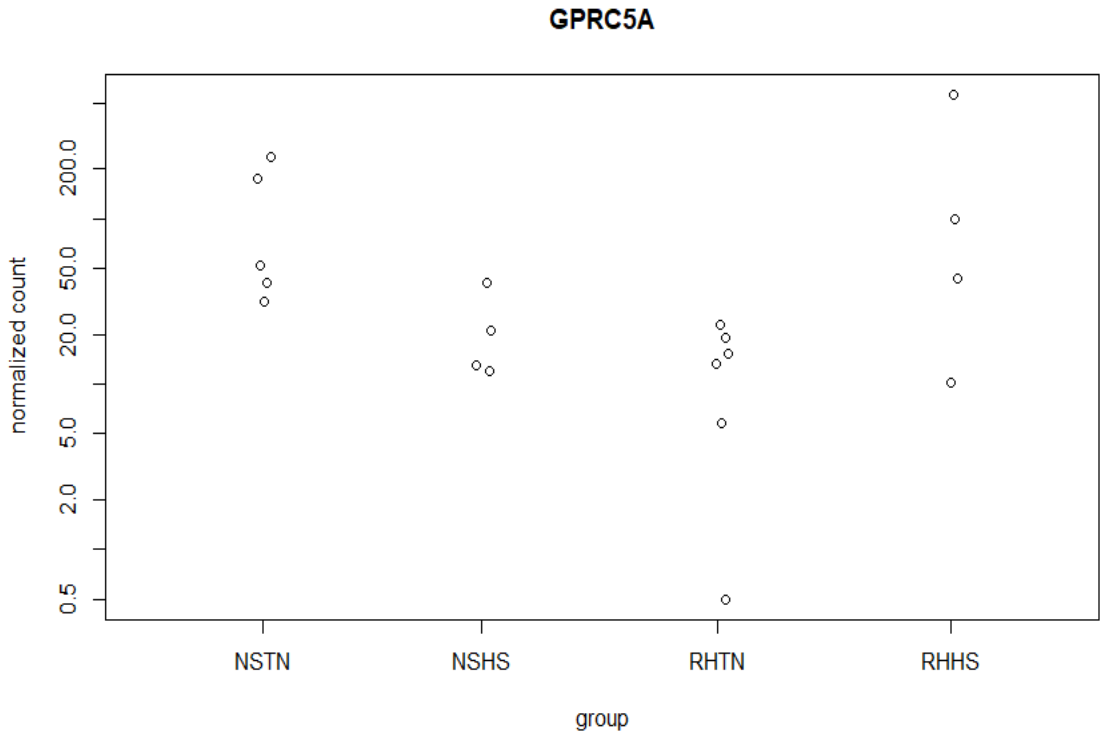
Table 2.5. Pathways predicted to be altered in subcutaneous adipose tissue of wether lambs supplemented with ractopamine and heat stressed for 30 d

Pathway	Direction	Pathway	Direction
Acetyl-CoA Biosynthesis II (From Citrate)	0	IL-22 Signaling	Down
Actin Cytoskeleton Signaling	Up	IL-6 Signaling	Down
Acute Myeloid Leukemia Signaling	Down	IL-7 Signaling Pathway	Down
Acute Phase Response Signaling	Up	IL-8 Signaling	Down
Adrenomedullin signaling pathway	Down	IL-9 Signaling	Down
Agranulyte Adhesion and Diapedesis	N/A	ILK Signaling	Up
AMPK Signaling	Down	Inhibition of ARE-Mediated mRNA Degradation Pathway	Down
Antioxidant Action of Vitamin C	Up	iNOS signaling	0
Apelin Adipocytes Signaling Pathway	Down	Leptin Signaling in Obesity	0
Apelin Cardiomyocyte Signaling Pathway	Down	Leukocyte Extravasation Signaling	Down
Apelin Endothelial Signaling Pathway	Down	Melatonin Signaling	Down
Apelin Liver Signaling Pathway	0	Molecular Mechanisms of Cancer	N/A
Apelin Pancreas Signaling Pathway	0	Neuroinflammation Signaling Pathway	Up
April Mediated Signaling	Up	Neuropathic Pain Signaling in Dorsal horn Neurons	Down
ATM Signaling	Down	Neuroprotective Role of THOP1 in Alzheimer's Disease	Up
Axonal Guidance Signaling	N/A	NGF Signaling	Up
B Cell Activating Factor Signaling	0	nNOS Signaling in Neurons	0
B Cell Receptor Signaling	Up	Notch Signaling	0
Basal cell Carcinoma Signaling	Up	Opioid Signaling Pathway	Down
BEX2 Signaling Pathway	Up	Ovarian Cancer Signaling	0
BMP Signaling pathway	Up	P2Y Purigenic Receptor Signaling Pathway	0
Calcium Signaling	Up	p70S6K Signaling	Down
Cardiac Hypertrophy Signaling	Up	Pancreatic Adrenocarcinoma Signaling	Down
Cardiac Hypertrophy Signaling (Enhanced)	Up	PCP pathway	Up
CCR5 Signaling in Macrophages	Down	Phagosome Formation	N/A

CD28 Signaling in T helper Cells	Down	Phospholipase C Signaling	0
CDK5 Signaling	Up	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Down
Cell Cycle Regulation by BTG Family Proteins	0	Protein Kinase A Signaling	Up
Cellular Effects of Sildenafil (Viagra)	N/A	RANK Signaling in Osteoclasts	Down
Ceramide Signaling	Up	RAR Activation	N/A
Circadian Rhythm Signaling	N/A	Reelin Signaling in Neurons	Down
Colorectal Cancer Metastasis Signaling	Up	Regulation of the Epithelial Mesenchymal transition in Development Pathway	Up
Complement System	Up	Regulation of the Epithelial Mesenchymal Transition by Growth Factors Pathway	Up
Corticotropin Releasing Hormone Signaling	Up	Regulation of IL-2 Expression in Activated and Anergic T lymphocytes	N/A
CTLA4 Signaling in Cytotoxic T Lymphocytes	N/A	Renin-Angiotensin Signaling	Down
CXCR4 Signaling	Down	RhoA Signaling	Up
Dendritic Cell Maturation	Up	Role of JAK family kinases in IL-6-type Cytokine Signaling	N/A
Dopamine Receptor Signaling	0	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	N/A
Dopamine-DARPP32 Feedback in cAMP Signaling	0	Role of NFAT in Cardiac Hypertrophy	Down
EGF Signaling	Down	Role of NFAT in Regulation of the Immune Response	Up
Endocannabinoid Cancer Inhibition Pathway	0	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	N/A
Endocannabinoid Developing Neuron Pathway	Down	Role of Pattern Recognition in Recognition of Bacteria and Viruses	Down
Endocannabinoid Neuronal Synapse Pathway	0	Semaphorin Neuronal Repulsive Signaling Pathway	Up
ERK/MAPK Signaling	0	Senescence Pathway	Up
Estrogen Receptor Signaling	Up	Sertoli cell-Sertoli Cell Junction Signaling	N/A
Factors Promoting Cardiogenesis in Vertebrates	Up	Sperm Motility	Down
Gap Junction Signaling	N/A	Sphingomyelin Metabolism	0
Gaq Signaling	Down	Sphingosine-1-phosphate Signaling	Up

GDNF Family Ligand-Receptor Interactions	Up	Synaptic Long Term Potentiation	Up
Glioblastoma Multiforme Signaling	Up	Synaptogenesis Signaling Pathway	Up
Glioma Invasiveness Signaling	Up	Systemic Lupus Erythematosus in B Cell Signaling Pathway	Up
GNRH Signaling	Up	T Cell Exhaustion Signaling Pathway	Up
Gp6 Signaling Pathway	Up	T Cell Receptor Signaling	N/A
Granulocyte Adhesion and Diapedesis	N/A	Tec Kinase Signaling	Down
Hepatic Fibrosis / Hepatic Stellate Cell Activation	N/A	TGF-β Signaling	Up
Hepatic Fibrosis Signaling Pathway	Up	Tight Junction Signaling	N/A
HGF Signaling	Down	TR/RXR Activation	N/A
Histamine Degradation	0	TREM1 Signaling	Up
HMGB1 Signaling	Up	Type II Diabetes Mellitus Signaling	Down
Human Embryonic Stem Cell Pluripotency	N/A	UVA-Induced MAPK Signaling	Down
IL-1 Signaling	0	UVB-Induced MAPK Signaling	Down
IL-10 Signaling	N/A	UVC-Induced MAPK Signaling	0
IL-12 Signaling and Production in Macrophages	N/A	White Adipose Tissue Browning Pathway	Up
IL-15 Production	Up	Wnt/Ca+ pathway	Up
IL-17A Signaling in Gastric Cells	0	Wnt/β-catenin Signaling	Down

Figure 2.1. Normalized expression count of *GPRC5A*



NS=not supplemented, TN=thermoneutral, HS=heat stress, RH=ractopamine

CHAPTER 3: EFFECT OF ZILPATEROL AND HEAT STRESS ON WHITE ADIPOSE TISSUE TRANSCRIPTOME OF BEEF CATTLE

Introduction

Heat stress (HS) initiates negative physiological outcomes that result in substantial financial loss for the livestock industry. For beef cattle production in the USA, HS costs producers an average of $369 million annually (St-Pierre et al., 2003). Heat stress causes a decrease in average daily gain and dry matter intake in cattle (Mitlöhner et al., 2001); this response is partially due to the physiological response to reduce heat production during digestion (Basarab et al., 2003). When the effects of feed intake are eliminated with pair-feeding, heat-stressed animals still exhibit a reduced rate of gain and muscle growth compared to thermoneutral controls, indicating the heat event itself alters the animals' physiological state (Swanson et al., 2020). Chronic HS is also a detriment to the immune system as it increases plasma concentrations of the inflammatory cytokines, TNF-α and IL-6 (Min et al., 2016).

Epinephrine and norepinephrine regulate the animals' physiological response to HS in part by binding to β-adrenergic receptors (β-AR) to activate downstream signaling pathways (Gonzalez-Rivas et al., 2020). In response to acute stress, signaling pathways for lipolysis of circulating and stored triglycerides are activated, and the responsiveness of adipocytes to lipolytic signals increases. Through these mechanisms, chronic stress increases lipogenesis and adipogenesis (Campbell et al., 2009; Peckett et al., 2011; Faylon et al., 2015). In general, the impact of HS on adipose tissue manifests as

decreased carcass fat (Mitlöhner et al., 2001). Considering the impact of HS on fat and health, HS is unfavorable for both the end product and animal well-being.

Beta-adrenergic agonists (β-AA) are feed supplements commonly used in the livestock industry. The β_1-AA ractopamine HCl (RAC) and β_2-AA zilpaterol HCl (ZH) improve cattle growth performance, carcass weight, and longissimus muscle area during the finishing phase (Elam et al., 2009; Lean et al., 2014). β-AA also change carcass composition by altering both intramuscular and intermuscular adipose deposition, thus decreasing 12^{th} rib fat and marbling score (Lean et al., 2014). These supplements induce cellular signaling by binding to G-protein coupled β-AR which activates cAMP (cyclic adenosine monophosphate) and Protein Kinase A signaling pathways. This results in CREB (cAMP response element-binding protein) regulation of gene transcription, similar to the mechanism by which endogenous epinephrine signals (Mersmann, 1998). Beta-adrenergic agonist supplementation also decreases adipose deposition due to increased lipolysis and decreased fatty acid biosynthesis (Johnson et al., 2014). Together, these physiological mechanisms underlie the improved production outcomes.

The adrenergic system is associated with the stress response, contributing to the anecdotal implication that ZH is detrimental to animal well-being (Grandin, 2018). Circulating epinephrine that is elevated by HS and β-AA both activate β-AR, leading to concerns about the use of β-AA in stressed animals (Mersmann, 1998). Several studies have evaluated whether β-AA alter physiological indicators of stress. Peterson et al. (2015) observed that RAC-supplemented pigs had higher incidences of mobility impairment during handling and transport. Hagenmaier et al. (2017) found a diet by

handling intensity interaction where RAC-supplemented cattle with high-stress-handling had the greatest increase of plasma cortisol and epinephrine levels after handling and transport; RAC-supplemented cattle did not have decreased mobility. Cattle supplemented with ZH and subjected to a stress challenge had decreased body temperatures relative to controls (Boyd et al., 2015; Buntyn et al., 2016). β-AA act as vasodilators (Dawes Matthew et al., 1997). Therefore, the observed decreased body temperature in ZH supplemented cattle could be attributed to an increased ability to dissipate heat (Buntyn et al., 2016). Furthermore, in response to an endocrine challenge, ZH-supplemented heifers had lower plasma cortisol and epinephrine levels relative to unsupplemented controls indicating a downregulation of the hypothalamic-pituitary-adrenal axis (Buntyn et al., 2016). The whole blood transcriptome of cattle supplemented RAC had little change relative to controls, including minimal signs of systemic inflammation; individual genes involved in inflammatory pathways that were upregulated included *IFI35*, *TYROBP*, and *TP53INP1* (Burrack et al., 2020). A study of RAC supplementation in pigs also reported a transcriptional stress response as well as changes in metabolic pathways, such as upregulation of amino acid biosynthesis (Brown et al., 2018).

Therefore, the purpose of this study was to determine whether supplementation of the β_2-AA ZH confounded the effects of HS on gene expression and physiological pathways. We also sought to identify how HS and ZH independently altered the subcutaneous adipose tissue transcriptome. These data will help inform livestock production practices during environmental stress and contribute to the understanding of the mechanistic function of β-AA during heat stress.

Materials and Methods

This project was approved by the University of Arizona Institute of Animal Care and Use Committee (IACUC; Protocol 12-396) and the University of Nebraska-Lincoln IACUC (Project 1826). Commercial Red Angus-based (Red Angus sire x Red Angus crossbred dams) steers (260 ± 25 kg) were acclimated to individual tie stalls or pens for 6 d prior to initiation of the trial. Steers were housed in either the control (thermoneutral, TN; THI=68; n=11) or HS (THI=73-85; n=12) conditions for 21 d. The temperature humidity index (THI) of the HS environments cycled daily so it was 85 by midday and then dropped to 73 during the night to simulate a day-night temperature cycle and allow the cattle to recover. Steers were supplemented with ZH (8.38 mg/kg/d; n=12) mixed at 1% in soybean meal as a carrier or only soybean meal (no supplement; NS; n=11) during the 21 d. Heat-stressed steers were fed, *ad libitum*, a diet consisting of 73.2% cracked corn, 13.7% chopped alfalfa, 6.3% molasses, 3.8% soybean meal, 2.1% mineral mix, and 0.9% urea. Thermoneutral steers were pair-fed based on the daily feed intake (percentage of body weight) of HS steers.

Subcutaneous adipose tissue samples were collected via biopsy at d 3 and 10. At the time of biopsy, 2-3 ml of local anesthetic (2% lidocaine HCl) was injected prior to collection of samples through a 5 cm incision approximately 20 cm cranial of the pelvic bone. The samples were flash-frozen in liquid nitrogen. The second biopsy (10 d) was collected from the contralateral loin muscle. The cattle were harvested on d 21 at the University of Arizona Food Products and Safety Laboratory. A third sample of adipose tissue was collected and flash frozen following evisceration and skinning.

RNA from subcutaneous adipose tissue was extracted using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), following the protocol from an Rneasy® Plus Mini Kit. RNA was washed and eluted on columns with DNase I treatment (Direct-zol™ RNA MiniPrep Plus, Zymo, Irvine, CA) according to the manufacturer's instructions. RNA integrity (RIN) and concentration were determined using the Nano chip on the Agilent Bioanalyzer 2100 (Santa Clara, CA, USA). Adipose tissue samples with RIN ≥ 5.8 (N=70) were sequenced using 100 bp single-end, 3' Tag-Seq reads at the Genomic Sequencing and Analysis Facility at the University of Texas at Austin to a targeted minimum depth of 5 million reads/sample. Reads were trimmed using TrimGalore (Martin, 2011) and HTStream (HTStream); minimum read quality was set to 20, the adapter removed, and minimum output read length set to 35 bp. Transcripts were quantified with STAR as annotated in GCF_002263795.1_ARS-UCD1.2 (Dobin et al., 2013).

Statistical analysis of transcript counts was performed with DESeq2 (Love et al., 2014). Loci with 20 or more counts observed across all samples were kept for analysis. Each sample collection time point was individually analyzed with a model design that included supplement, environment, and their interaction; time was expected to impact gene expression as these were growing animals. To identify outlier samples, the normalized count matrix was transformed with regularized log transformation and input into the rrcov R package PcaGrid (Todorov and Filzmoser, 2009). Outliers were detected using a principal component analysis as previously described (Chen et al., 2020). Samples with orthogonal distance or score distance outside of the cutoff values

determined by default parameters were considered outliers. Individual loci with an adjusted *P*-value< 0.05 were considered differentially expressed (DE). Based on our stringent false discovery rate cutoff and analysis there were few loci that were individually differentially expressed. Pathway analysis (Qiagen, Germantown, MD) was conducted using all loci with a raw $P<0.05$ from the analyses of the main effects and interaction to predict changes in molecular pathways. Raw *P*-value was used since the adjusted *P*-value accounts for all the significant loci in a single analysis, but there are various possible loci in a pathway and a single locus could be involved in multiple pathways. Thus, the use of a raw *P*-value allows for the loci to be considered alone until used for analysis with other loci in a pathway.

Results

The mean RIN for sequenced samples was 7.7 ± 0.71. On average, 3,608,311 ± 579,403 reads per sample were obtained with 79.2% ± 6.67% uniquely mapping to the genome. Of 25,019 transcripts with at least one read, 17,432 were included in analyses. After removing five outliers from the adipose tissue data set, there was a minimum of 4 and an average of 5.42 animals per treatment combination group for each timepoint.

There were four DE loci at d 3 (*MISP3, APOL6, SLC25A4, S100A12*), and all were upregulated due to ZH (Table 3.1). On d 10, two loci were DE (*RRAD, ALB*), and were both downregulated due to HS by ZH interaction. No loci were DE among experimental groups at d 21.

There were 509 molecular pathways predicted to be altered ($P < 0.01$) across all timepoint/experimental group combinations: 202 due to environment (Table 3.2), 126 due to supplement (Table 3.3), and 181 due to the environment by supplement interaction (Table 3.4). HS was predicted to downregulate metabolic pathways at d 3 ($P < 0.01$) including those for calcium signaling, glycolysis, and gluconeogenesis. Immune and stress pathways, including those for sirtuin signaling, were predicted to be downregulated, and interferon signaling, acute phase responses, and IL-6 signaling upregulated were predicted to be upregulated by HS at one or more time points. Ten different interleukin (IL) signaling pathways were either upregulated or dysregulated due to HS at d 10 including those for IL-1, 2, 3, 6, 8, 10, 12, 15, 17, and 17A. At d 21, stress pathways including those for iNOS signaling and NRF2-mediated stress response were predicted to be upregulated. Metabolic pathways including glycolysis and cholesterol biosynthesis were also identified as upregulated or dysregulated at d 21 due to HS.

Supplement was predicted to alter metabolic pathways in the adipose tissue at d 3 including the upregulation of fatty acid β-oxidation and oxidative phosphorylation, downregulation of glycolysis, and dysregulation of mitochondrial function. Few metabolic pathways were predicted to be altered by ZH at d 10 and 21: mTOR signaling, protein kinase A signaling, and retinoate biosynthesis. Stress and immune pathways including sirtuin and IL-7 signaling were downregulated at d 3 due to ZH. Acute phase responses and IL-12 signaling pathways were upregulated and interferon signaling was downregulated due to ZH at d 21.

The interaction of HS and ZH was predicted to downregulate oxidative phosphorylation and fatty acid β-oxidation at d 3 and to upregulate glycolysis and sirtuin signaling. At d 10, immune pathways including those for acute phase responses, IL-6, and IL-8 were predicted to be downregulated due to the interaction of HS and ZH. Immune pathways including those for acute phase responses and apoptosis were predicted to be dysregulated at d 21 due to HS by ZH interaction.

Discussion

Although ZH was not predicted to impact stress pathways, HS caused the adipose tissue transcriptome to show evidence of oxidative stress and an inflammatory response attributed to HS. This study provides some of the first transcriptomic evidence that HS and ZH supplementation in beef cattle do not additively affect stress pathways. Instead, it demonstrates that ZH may moderate the unwanted responses to stress by subcutaneous adipose tissue. Expectedly, the responses to environmental conditions and supplement changed with time, likely due to adaptation to HS and desensitization of β-AR after chronic stimulation (Lohse et al., 1990). These data contribute to the growing literature demonstrating that ZH supplementation in livestock acts to moderate physiological and transcriptomic responses to HS.

Transcriptome data provide evidence of a strong inflammatory response to HS in adipose tissue. Our previous work in sheep has demonstrated that chronic HS induces inflammation, as indicated by increased plasma TNFα levels, greater circulating lymphocyte and granulocyte concentrations, and increased plasma insulin relative to

glucose concentrations (Barnes et al., 2019; Swanson et al., 2020). This coincides with physiological responses such as hyperventilation and hyperthermia (Swanson et al., 2020). The predicted upregulation of interferon signaling at d 3 and the dysregulation of acute phase responses and interleukin pathways at d 10 are evidence of inflammation due to HS in adipose. Increases in circulating interleukins are typically stimulated during an immune response and regulate acute phase responses and inflammation (Akdis et al., 2016). Stress, infections, and inflammation activate acute phase responses which initiate reactions that heal damaged tissues and aid the return to homeostasis (Cray et al., 2009).

Circulating catecholamines induced by HS and ZH-supplemented through the diet both stimulate the β adrenergic system, and thus, there has been concern over possible compounding effects on animal health and well-being (Grandin, 2018). In this study, 21 d ZH supplementation simultaneous with HS exposure did alter the HS-induced changes in stress and inflammation gene expression in adipose tissues. The acute phase response and six of the ten interleukin pathways that were predicted to be upregulated or dysregulated at d 10 in unsupplemented animals exposed to HS were instead downregulated or not affected in animals exposed to HS and supplemented with ZH. In other studies, the β_2-AA, isoproterenol, inhibited IL-8 production in human monocytes (Farmer and Pugin, 2000) and suppressed IL-2 receptors in human lymphocytes (Feldman et al., 1987). Salbutamol, another β_2-AA, inhibited the production of IL-12 in human monocytes (Panina-Bordignon et al., 1997). Based on our results and the previous literature, we postulate that ZH had an inhibitory effect on interleukin production induced by HS. ZH appeared to have a beneficial role in animal well-being, which did not support the

previously-suggested additive impact of β-AA and environmental stress on inflammatory and stress responses of cattle.

We observed only minimal evidence that ZH induced inflammation in adipose tissues. After 21 d of daily supplementation, ZH upregulated pathways associated with the acute phase responses and the complement system but also downregulated interferon signaling. Moreover, ZH supplementation in cattle exposed to HS reversed the enhancement of inflammatory pathways observed in unsupplemented cattle exposed to HS at day 21. This, together with downregulation of the acute phase responses due to HS by ZH interaction, indicate ZH supplementation suppressed HS-induced inflammation. In humans, β_2-AA were developed as bronchodilators due to their anti-inflammatory effects such as suppressing pro-inflammatory activities of neutrophils (Anderson et al., 2014). This is further supported by biological measures taken during the present study, where ZH supplementation in steers exposed to HS had lower rectal temperatures than unsupplemented steers exposed to HS (Grijalva, 2020). A less severe increase in rectal temperature was also observed in heat-stressed wethers when supplemented with RAC compared to those that were unsupplemented (Swanson et al., 2020). However, in another study, it was reported that HS lambs supplemented with ZH had higher rectal temperatures than unsupplemented heat stress lambs (Barnes et al., 2019). These differing outcomes may be due to differences in RAC and ZH.

HS resulted in a shift in genes associated with in cellular metabolism. At d 3, changes in gene expression predict the downregulation of glycolysis and gluconeogenesis in adipose tissues, perhaps consistent with switch from glucose to fatty acids as an energy

source. This result was consistent with our previous observation of increased circulating lipids in heat-stressed lambs (Swanson et al., 2020); both studies used pair-feeding to negate differences due to intake, and thus these metabolic changes can be attributed to direct effects of HS on physiological function. However, after 21 d, glycolysis in the steers undergoing HS was predicted to be upregulated in adipose tissue, indicating another switch in metabolism. In addition, adipocytes from these heat-stressed cattle had reduced rates of epinephrine-stimulated fatty acid mobilization compared to animals in the thermoneutral environment consistent with reduced utilization of fat for energy (Reith et al., 2020). Although this could occur from the depletion of fatty acid reserves, there was no difference in 12th rib fat thickness or marbling score due to HS at harvest (Grijalva, 2020).

Acute ZH supplementation also altered adipose tissue metabolism, as both oxidative phosphorylation and fatty acid β-oxidation were predicted to be upregulated at d 3. The upregulation of oxidative phosphorylation is likely due to the improved mitochondrial capacity from ZH supplementation (Sieck et al., 2020). Furthermore, *SLC25A4* was upregulated by ZH at d 3. *SLC25A4,* Solute Carrier Family 25 Member 4, encodes adenine nucleotide translocator 1 (ANT1), a mitochondrial protein that exchanges ADP for ATP across the mitochondrial inner membrane (Klingenberg, 2008). ANT1 regulates oxidative flux when there are low levels of ADP, which is associated with a low-demand for ATP (Willis et al., 2018). Upregulation of *SLC25A4* due to ZH in adipose tissue suggests increased ADP/ATP exchange and oxidative metabolism in the mitochondria, contributing to upregulated oxidative phosphorylation. The predicted

increase of fatty acid β-oxidation due to ZH is supported by the upregulation of *APOL6* at d 3. *APOL6,* Apolipoprotein L6, is part of a family of apolipoproteins that bind and transport lipids (Liu et al., 2005). As fatty acid β-oxidation increases, an increase in APOL6 transporting the fatty acids within the cell would become necessary. Although ZH had acute effects on adipose tissue metabolism, some of these effects appeared to be suppressed by concurrent HS. Even though ZH should improve mitochondria efficiency during stress, transcriptome data predict the interaction of HS and ZH downregulates oxidative phosphorylation in adipose tissue at 3 d. Thus, acute ZH supplementation likely increased metabolic activity in adipose tissue in the absence but not in the presence of HS.

In our study we found that ZH supplementation did not exacerbate the adipose tissue transcriptome response to HS. Instead, genes with altered expression due to ZH supplementation were predicted to mitigate the inflammatory response to HS in adipose tissue. Optimizing cattle efficiency and upholding high standards of animal well-being are priorities of the beef industry, and these data indicate that the use of β-AA may benefit well-being in heat-stressed animals. Furthermore, increasing our understanding of the molecular mechanisms of HS can lead to the development of novel mitigation strategies.

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Table 3.1. Loci that were differently expressed in the adipose tissues of heat-stressed Red Angus steers supplemented with zilpaterol for 21 d.

Time (d)	Effect	Loci ID	Log_2 Fold Change	P_{adj}
3	Supplement	*MISP3*	1.448	0.005
		S100A12	1.966	0.047
		SLC25A4	5.396	0.047
		APOL6	1.448	0.047
10	Interaction	*RRAD*	-3.138	0.007
		ALB	-9.665	0.046

Table 3.2. Pathways predicted to be altered in adipose tissues of heat-stressed Red Angus steers for 21 d.

Time	Pathway	Z-Score	Pathway	Z-Score
3	3-phosphoinositide Biosynthesis	-1.667	Glycolysis I	-2.646
	3-phosphoinositide Degradation	-1.667	Hepatic Fibrosis Signaling Pathway	-0.853
	Actin Cytoskeleton Signaling	-3.207	ILK Signaling	-3
	Activation of IRF by Cytosolic Pattern Recognition Receptors	1.134	iNOS Signaling	1
	Agranulocyte Adhesion and Diapedesis	N/A	Interferon Signaling	2.236
	Apelin Cardiomyocyte Signaling Pathway	-2.828	Mevalonate Pathway I	N/A
	Calcium Signaling	-3	Necroptosis Signaling Pathway	0.632
	cAMP-mediated signaling	-0.832	nNOS Signaling in Neurons	N/A
	Cardiac Hypertrophy Signaling	-3.207	nNOS Signaling in Skeletal Muscle Cells	N/A
	Cardiac Hypertrophy Signaling (Enhanced)	-1.342	Opioid Signaling Pathway	-2.673
	Cardiac β-adrenergic Signaling	-0.905	Osteoarthritis Pathway	0.632
	Cell Cycle Control of Chromosomal Replication	-1.633	PFKFB4 Signaling Pathway	-1.342
	Cellular Effects of Sildenafil (Viagra)	N/A	Phospholipase C Signaling	-3.051
	Clathrin-mediated Endocytosis Signaling	N/A	Protein Kinase A Signaling	-0.943
	Corticotropin Releasing Hormone Signaling	0.707	Regulation of Actin-based Motility by Rho	-3
	Creatine-phosphate	N/A	RhoA Signaling	-1.897

3	Biosynthesis			
	CXCR4 Signaling	-1.265	RhoGDI Signaling	2.887
	Death Receptor Signaling	1.89	Role of CHK Proteins in Cell Cycle Checkpoint Control	0
	D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	-1.667	Semaphorin Neuronal Repulsive Signaling Pathway	-2.333
	D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	-1.667	Senescence Pathway	-0.535
	D-myo-inositol-5-phosphate Metabolism	-1.667	Sertoli Cell-Sertoli Cell Junction Signaling	N/A
	Dopamine-DARPP32 Feedback in cAMP Signaling	-2.333	Signaling by Rho Family GTPases	-3.051
	Endocannabinoid Cancer Inhibition Pathway	0.302	Superpathway of GGPP Biosynthesis I (via Mevalonate)	N/A
	Epithelial Adherens Junction Signaling	N/A	Synaptic Long Term Potentiation	-1.667
	Estrogen Receptor Signaling	-2	Thrombin Signaling	-0.378
	Gluconeogenesis I	-2.236	Tight Junction Signaling	N/A
10	14-3-3-mediated Signaling	0.302	Inhibition of Angiogenesis by TSP1	2.236
	Acute Phase Response Signaling	1.342	Inhibition of Matrix Metalloproteases	0
	Adipogenesis pathway	N/A	JAK/Stat Signaling	1.414
	Adrenomedullin signaling pathway	1	Leukocyte Extravasation Signaling	-1.387
	Agranulocyte Adhesion and Diapedesis	N/A	LXR/RXR Activation	3.153
	Apelin Endothelial Signaling Pathway	0.905	Molecular Mechanisms of Cancer	N/A
	Apoptosis Signaling	0.707	Mouse Embryonic Stem Cell Pluripotency	1.265
	Aryl Hydrocarbon Receptor Signaling	0.333	MSP-RON Signaling In Macrophages Pathway	0
	Axonal Guidance Signaling	N/A	mTOR Signaling	1.155
	B Cell Receptor Signaling	0.535	Natural Killer Cell Signaling	-1.604
	BER pathway	N/A	NF-κB Signaling	-1
	BMP signaling pathway	0.707	NRF2-mediated Oxidative Stress Response	0.632
	Cardiac Hypertrophy Signaling	1.069	Opioid Signaling Pathway	2.236
	Cardiac Hypertrophy Signaling (Enhanced)	0.333	Ovarian Cancer Signaling	1.414
	CD27 Signaling in Lymphocytes	0.816	P2Y Purigenic Receptor Signaling Pathway	0.905
	CD40 Signaling	0.816	p38 MAPK Signaling	-0.333

10	Cell Cycle: G1/S Checkpoint Regulation	0	p53 Signaling	-1
	Chemokine Signaling	1.667	Pancreatic Adenocarcinoma Signaling	3
	Cholecystokinin/Gastrin-mediated Signaling	0.258	Phospholipase C Signaling	-0.832
	Chronic Myeloid Leukemia Signaling	N/A	PPAR Signaling	-0.905
	Clathrin-mediated Endocytosis Signaling	N/A	PPARα/RXRα Activation	0
	Coagulation System	0.447	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.213
	Colorectal Cancer Metastasis Signaling	2	Prolactin Signaling	1
	CXCR4 Signaling	0.577	Protein Kinase A Signaling	-1.8
	EIF2 Signaling	-2	PTEN Signaling	-1.604
	Endocannabinoid Cancer Inhibition Pathway	-1.069	PXR/RXR Activation	N/A
	Endocannabinoid Developing Neuron Pathway	1.667	RAR Activation	N/A
	Endothelin-1 Signaling	1.807	Regulation Of The Epithelial Mesenchymal Transition By Growth Factors Pathway	2.324
	Ephrin B Signaling	1.134	Regulation of the Epithelial-Mesenchymal Transition Pathway	N/A
	Ephrin Receptor Signaling	1.604	Relaxin Signaling	2.236
	Epithelial Adherens Junction Signaling	N/A	RhoGDI Signaling	0
	ErbB2-ErbB3 Signaling	0.816	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	N/A
	ERK/MAPK Signaling	-0.5	Role of MAPK Signaling in the Pathogenesis of Influenza	N/A
	ERK5 Signaling	1.414	Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	1.342
	Erythropoietin Signaling Pathway	1.069	Role of NFAT in Cardiac Hypertrophy	1.155
	Estrogen Receptor Signaling	1.964	Role of NFAT in Regulation of the Immune Response	0.832
	Factors Promoting Cardiogenesis in Vertebrates	-0.905	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	N/A
	FAK Signaling	N/A	Role of Tissue Factor in Cancer	N/A
	FXR/RXR Activation	N/A	SAPK/JNK Signaling	0.333

10	G Beta Gamma Signaling	1	Sertoli Cell-Sertoli Cell Junction Signaling	N/A
	GADD45 Signaling	N/A	Signaling by Rho Family GTPases	1.387
	Gap Junction Signaling	N/A	Sirtuin Signaling Pathway	-0.229
	Germ Cell-Sertoli Cell Junction Signaling	N/A	SPINK1 General Cancer Pathway	-0.378
	Glioma Invasiveness Signaling	0.707	STAT3 Pathway	-0.302
	GNRH Signaling	1.213	Sumoylation Pathway	0.333
	Gα12/13 Signaling	0.535	Synaptic Long Term Potentiation	0.577
	Hepatic Fibrosis Signaling Pathway	1	Synaptogenesis Signaling Pathway	3.657
	HER-2 Signaling in Breast Cancer	1	Systemic Lupus Erythematosus In B Cell Signaling Pathway	0.943
	Hereditary Breast Cancer Signaling	N/A	T Cell Exhaustion Signaling Pathway	0
	HGF Signaling	0.302	Tec Kinase Signaling	-0.816
	HIF1α Signaling	1.807	Telomerase Signaling	0.378
	Huntington's Disease Signaling	0.707	TGF-β Signaling	1.134
	IGF-1 Signaling	0.632	Th1 Pathway	-1.89
	IL-1 Signaling	0.816	Thrombopoietin Signaling	1.134
	IL-10 Signaling	N/A	Thyroid Cancer Signaling	1
	IL-12 Signaling and Production in Macrophages	N/A	Tight Junction Signaling	N/A
	IL-15 Signaling	2.121	TNFR2 Signaling	N/A
	IL-17 Signaling	2	Toll-like Receptor Signaling	-1.342
	IL-17A Signaling in Gastric Cells	2	TR/RXR Activation	N/A
	IL-2 Signaling	0.816	Tumor Microenvironment Pathway	2.183
	IL-3 Signaling	0.707	UVC-Induced MAPK Signaling	0.816
	IL-6 Signaling	1.291	VEGF Family Ligand-Receptor Interactions	1.414
	IL-8 Signaling	2.558	VEGF Signaling	0.632
	ILK Signaling	0.258	Virus Entry via Endocytic Pathways	N/A
21	Activation of IRF by Cytosolic Pattern Recognition Receptors	-0.816	NF-κB Signaling	-0.905
	Cholesterol Biosynthesis I	N/A	NRF2-mediated Oxidative Stress Response	0.447
	Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	N/A	PDGF Signaling	0
	Cholesterol Biosynthesis III	N/A	Phenylalanine Degradation IV	N/A

21	(via Desmosterol)		(Mammalian, via Side Chain)	
	Coronavirus Pathogenesis Pathway	1.941	Protein Ubiquitination Pathway	N/A
	EIF2 Signaling	-3	PTEN Signaling	1.667
	Glutathione-mediated Detoxification	0	Role of JAK1, JAK2 and TYK2 in Interferon Signaling	N/A
	Glycolysis I	1	Sirtuin Signaling Pathway	-1
	HER-2 Signaling in Breast Cancer	-2.887	Superpathway of Cholesterol Biosynthesis	2
	iNOS Signaling	-1.342	Zymosterol Biosynthesis	N/A
	mTOR Signaling	N/A		

Table 3.3. Pathways predicted to be altered in adipose tissues of zilpaterol-supplemented Red Angus steers for 21 d.

Time	Pathway	Z-Score	Pathway	Z-Score
3	14-3-3-mediated Signaling	-1	Melanoma Signaling	-1
	Amyotrophic Lateral Sclerosis Signaling	-1.342	Melatonin Degradation II	N/A
	Cancer Drug Resistance By Drug Efflux	N/A	Mevalonate Pathway I	N/A
	Cell Cycle Control of Chromosomal Replication	-2.828	Mitochondrial Dysfunction	N/A
	Coronavirus Pathogenesis Pathway	-1.069	Mitotic Roles of Polo-Like Kinase	-0.447
	EIF2 Signaling	-0.378	mTOR Signaling	-0.816
	Endometrial Cancer Signaling	-1.342	NF-κB Activation by Viruses	-0.816
	ErbB2-ErbB3 Signaling	-1.89	nNOS Signaling in Neurons	N/A
	Estrogen Receptor Signaling	-0.655	nNOS Signaling in Skeletal Muscle Cells	N/A
	Fatty Acid β-oxidation I	2.121	Oxidative Phosphorylation	4.899
	FcγRIIB Signaling in B Lymphocytes	0	PDGF Signaling	-1.134
	Gap Junction Signaling	N/A	Prostate Cancer Signaling	N/A
	Glutaryl-CoA Degradation	2	Regulation of Cellular Mechanics by Calpain Protease	-0.447
	Glycine Betaine Degradation	N/A	Regulation of eIF4 and p70S6K Signaling	0
	Glycolysis I	-1.342	Role of NFAT in Cardiac Hypertrophy	-0.577
	GM-CSF Signaling	-1.134	Sirtuin Signaling Pathway	-0.655
	Hepatic Fibrosis Signaling Pathway	-1.698	Superpathway of Cholesterol Biosynthesis	N/A
	HER-2 Signaling in Breast Cancer	-1.941	T Cell Receptor Signaling	N/A
	IL-7 Signaling Pathway	-1.342	Tetrapyrrole Biosynthesis II	N/A
	Isoleucine Degradation I	1.633	Thyroid Cancer Signaling	-1.414
	Ketogenesis	N/A	Tryptophan Degradation III (Eukaryotic)	2
	Ketolysis	N/A	Tumor Microenvironment Pathway	-0.577
	Kinetochore Metaphase Signaling Pathway	-1.414	Type II Diabetes Mellitus Signaling	0
	Maturity Onset Diabetes of Young (MODY) Signaling	N/A	Valine Degradation I	1.342
10	Actin Cytoskeleton Signaling	-0.333	Melanoma Signaling	-1
	Actin Nucleation by ARP-WASP Complex	-1	Molecular Mechanisms of Cancer	N/A

10	Agranulocyte Adhesion and Diapedesis	N/A	mTOR Signaling	0.905
	Axonal Guidance Signaling	N/A	Neuregulin Signaling	0.447
	Cell Cycle Control of Chromosomal Replication	0.447	p53 Signaling	-1
	Choline Biosynthesis III	N/A	Pancreatic Adenocarcinoma Signaling	2.449
	Chronic Myeloid Leukemia Signaling	N/A	Phospholipase C Signaling	-0.905
	Clathrin-mediated Endocytosis Signaling	N/A	Protein Kinase A Signaling	0.832
	Colorectal Cancer Metastasis Signaling	-1.414	PTEN Signaling	-0.333
	CXCR4 Signaling	-0.302	Regulation of Actin-based Motility by Rho	-0.447
	EIF2 Signaling	-2.714	RhoGDI Signaling	0
	Endothelin-1 Signaling	0	Role of Tissue Factor in Cancer	N/A
	Ephrin B Signaling	0.447	Sertoli Cell-Sertoli Cell Junction Signaling	N/A
	Ephrin Receptor Signaling	0.632	Signaling by Rho Family GTPases	0
	G Beta Gamma Signaling	-0.378	Sphingosine-1-phosphate Signaling	0
	Glioblastoma Multiforme Signaling	-1.134	SPINK1 General Cancer Pathway	-1.633
	Hepatic Fibrosis Signaling Pathway	0	Spliceosomal Cycle	2.449
	HIF1α Signaling	0.258	Tec Kinase Signaling	0
	IL-15 Signaling	0.816	Thrombin Signaling	-0.707
	IL-8 Signaling	0	Trehalose Degradation II (Trehalase)	N/A
	Leukocyte Extravasation Signaling	-1.508	Virus Entry via Endocytic Pathways	N/A
	Macropinocytosis Signaling	0.447		
21	Activation of IRF by Cytosolic Pattern Recognition Receptors	-0.333	LPS/IL-1 Mediated Inhibition of RXR Function	-2.449
	Acute Phase Response Signaling	1.789	LXR/RXR Activation	4.899
	Androgen Biosynthesis	N/A	Maturity Onset Diabetes of Young (MODY) Signaling	N/A
	Arginine Degradation VI (Arginase 2 Pathway)	N/A	Methylglyoxal Degradation III	N/A
	Atherosclerosis Signaling	N/A	mTOR Signaling	N/A
	BEX2 Signaling Pathway	-1.633	Pathogenesis of Multiple Sclerosis	N/A
	Clathrin-mediated Endocytosis	N/A	Production of Nitric Oxide and	0.775

21	Signaling		Reactive Oxygen Species in Macrophages	
	Coagulation System	0	PXR/RXR Activation	N/A
	Complement System	1.89	Retinoate Biosynthesis I	2.236
	Coronavirus Pathogenesis Pathway	2.333	Role of Hypercytokinemia/ hyperchemokinemia in the Pathogenesis of Influenza	-3
	Extrinsic Prothrombin Activation Pathway	N/A	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	0.378
	FXR/RXR Activation	N/A	Role of Tissue Factor in Cancer	N/A
	Glutathione-mediated Detoxification	N/A	Stearate Biosynthesis I (Animals)	1.342
	GP6 Signaling Pathway	0.302	Tyrosine Degradation I	N/A
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	N/A	Urea Cycle	N/A
	IL-12 Signaling and Production in Macrophages	N/A	Xenobiotic Metabolism AHR Signaling Pathway	2.646
	Interferon Signaling	-2.236	Xenobiotic Metabolism CAR Signaling Pathway	2.887
	Intrinsic Prothrombin Activation Pathway	1.667		

Table 3.4. Pathways predicted to be altered in adipose tissues of heat-stressed, zilpaterol-supplemented Red Angus steers for 21 d.

Time	Pathway	Z-Score	Pathway	Z-Score
3	14-3-3-mediated Signaling	N/A	Mitochondrial Dysfunction	N/A
	Axonal Guidance Signaling	N/A	Molecular Mechanisms of Cancer	N/A
	B Cell Receptor Signaling	0.577	Necroptosis Signaling Pathway	0.333
	Breast Cancer Regulation by Stathmin1	2.041	Netrin Signaling	2.236
	Cardiac Hypertrophy Signaling	2.309	nNOS Signaling in Skeletal Muscle Cells	N/A
	Cardiac Hypertrophy Signaling (Enhanced)	1.886	Opioid Signaling Pathway	2.53
	Cellular Effects of Sildenafil (Viagra)	N/A	Oxidative Phosphorylation	-3
	Coronavirus Replication Pathway	1	P2Y Purigenic Receptor Signaling Pathway	2.646
	Dopamine-DARPP32 Feedback in cAMP Signaling	2.828	PDGF Signaling	0.816
	Endocannabinoid Neuronal Synapse Pathway	1.134	Phospholipase C Signaling	1.897
	Epithelial Adherens Junction Signaling	N/A	PI3K Signaling in B Lymphocytes	2.333
	ERK5 Signaling	0.816	PKCθ Signaling in T Lymphocytes	1.134
	Estrogen Receptor Signaling	2.324	Remodeling of Epithelial Adherens Junctions	N/A
	Estrogen-Dependent Breast Cancer Signaling	2	RhoA Signaling	1.134
	Fatty Acid β-oxidation I	-2	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	N/A
	GADD45 Signaling	N/A	Role of NFAT in Cardiac Hypertrophy	1.508
	Gap Junction Signaling	N/A	Serine Biosynthesis	N/A
	Germ Cell-Sertoli Cell Junction Signaling	N/A	Sertoli Cell-Sertoli Cell Junction Signaling	N/A
	Glycolysis I	1	Sirtuin Signaling Pathway	0.905
	GM-CSF Signaling	1.342	Superpathway of Serine and Glycine Biosynthesis I	N/A
	GNRH Signaling	1.667	Systemic Lupus Erythematosus In B Cell Signaling Pathway	0.258
	Gustation Pathway	N/A	Thrombin Signaling	1
	Hepatic Fibrosis Signaling Pathway	2.673	Tumor Microenvironment Pathway	1.667
	HGF Signaling	1.134	Valine Degradation I	N/A

3	Isoleucine Degradation I	N/A		
10	14-3-3-mediated Signaling	0.333	IL-3 Signaling	0
	3-phosphoinositide Biosynthesis	-0.832	IL-6 Signaling	-1.604
	3-phosphoinositide Degradation	-0.277	IL-8 Signaling	-1.147
	Actin Cytoskeleton Signaling	-1.069	ILK Signaling	-1.604
	Actin Nucleation by ARP-WASP Complex	0.378	Inhibition of Angiogenesis by TSP1	-2
	Acute Myeloid Leukemia Signaling	0	Integrin Signaling	-1
	Acute Phase Response Signaling	-1.886	Intrinsic Prothrombin Activation Pathway	-0.816
	Aldosterone Signaling in Epithelial Cells	-1	JAK/Stat Signaling	0.302
	Apelin Cardiac Fibroblast Signaling Pathway	0.816	Leukocyte Extravasation Signaling	0.775
	Aryl Hydrocarbon Receptor Signaling	0.905	LXR/RXR Activation	-3.441
	Atherosclerosis Signaling	N/A	Maturity Onset Diabetes of Young (MODY) Signaling	N/A
	Axonal Guidance Signaling	N/A	Melanoma Signaling	1.134
	B Cell Receptor Signaling	0.258	Molecular Mechanisms of Cancer	N/A
	Cancer Drug Resistance by Drug Efflux	N/A	MSP-RON Signaling In Cancer Cells Pathway	-1.291
	Cardiac Hypertrophy Signaling	-1	MSP-RON Signaling In Macrophages Pathway	0
	Cardiac Hypertrophy Signaling (Enhanced)	-0.365	mTOR Signaling	-0.277
	Caveolar-mediated Endocytosis Signaling	N/A	Neuregulin Signaling	0.378
	Cell Cycle: G1/S Checkpoint Regulation	0	Non-Small Cell Lung Cancer Signaling	0.447
	Chemokine Signaling	-0.707	NRF2-mediated Oxidative Stress Response	-0.632
	Cholecystokinin/ Gastrin-mediated Signaling	-0.905	Nur77 Signaling in T Lymphocytes	0
	Chronic Myeloid Leukemia Signaling	N/A	Opioid Signaling Pathway	-1.043
	Clathrin-mediated Endocytosis Signaling	N/A	Ovarian Cancer Signaling	-0.378
	Coagulation System	-0.378	P2Y Purigenic Receptor Signaling Pathway	-0.302
	Colorectal Cancer Metastasis Signaling	-0.688	Pancreatic Adenocarcinoma Signaling	-2.121
	CXCR4 Signaling	-0.832	PDGF Signaling	0

10	D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	-0.577	Phospholipase C Signaling	0.243
	D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	-0.577	PI3K/AKT Signaling	0.632
	D-myo-inositol-5-phosphate Metabolism	-0.535	PPAR Signaling	0
	EIF2 Signaling	2.138	PPARα/RXRα Activation	-1.069
	Endocannabinoid Cancer Inhibition Pathway	0.277	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-2
	Endocannabinoid Developing Neuron Pathway	-0.632	Protein Kinase A Signaling	-0.408
	Endometrial Cancer Signaling	-0.378	PTEN Signaling	0
	Ephrin Receptor Signaling	-1.604	Regulation of eIF4 and p70S6K Signaling	0
	ERK/MAPK Signaling	0	Renal Cell Carcinoma Signaling	-0.447
	Extrinsic Prothrombin Activation Pathway	N/A	RhoGDI Signaling	1.265
	FXR/RXR Activation	N/A	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	N/A
	GADD45 Signaling	N/A	Role of NFAT in Cardiac Hypertrophy	-0.535
	Gap Junction Signaling	N/A	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	N/A
	Germ Cell-Sertoli Cell Junction Signaling	N/A	Role of Tissue Factor in Cancer	N/A
	Glioblastoma Multiforme Signaling	0	Senescence Pathway	-1.414
	Glioma Invasiveness Signaling	0.333	Sertoli Cell-Sertoli Cell Junction Signaling	N/A
	Glioma Signaling	0.707	Signaling by Rho Family GTPases	-2.673
	Glucocorticoid Receptor Signaling	N/A	S-methyl-5-thio-α-D-ribose 1-phosphate Degradation	N/A
	Gαq Signaling	0	SPINK1 General Cancer Pathway	0.707
	Hepatic Fibrosis Signaling Pathway	-0.186	STAT3 Pathway	0.302
	HER-2 Signaling in Breast Cancer	-0.688	Sumoylation Pathway	1.414
	Hereditary Breast Cancer Signaling	N/A	Superpathway of Inositol Phosphate Compounds	-0.775
	HGF Signaling	-0.632	T Cell Receptor Signaling	N/A
	HIF1α Signaling	-1.789	Tec Kinase Signaling	0

10	HMGB1 Signaling	-1.508	Telomerase Signaling	-0.302
	HOTAIR Regulatory Pathway	0	Thrombin Signaling	-1.387
	IGF-1 Signaling	0.632	Tumor Microenvironment Pathway	-1.698
	IL-12 Signaling and Production in Macrophages	N/A	Virus Entry via Endocytic Pathways	N/A
	IL-15 Signaling	-1.414	Wnt/β-catenin Signaling	2.324
	IL-2 Signaling	0	α-Adrenergic Signaling	0
21	Acute Phase Response Signaling	-0.632	LPS/IL-1 Mediated Inhibition of RXR Function	N/A
	Apoptosis Signaling	0.447	LXR/RXR Activation	-3.317
	Caveolar-mediated Endocytosis Signaling	N/A	mTOR Signaling	N/A
	Coagulation System	-2.236	NRF2-mediated Oxidative Stress Response	0
	Complement System	N/A	PPARα/RXRα Activation	-1.633
	EIF2 Signaling	2	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-0.707
	Extrinsic Prothrombin Activation Pathway	N/A	PTEN Signaling	-0.378
	FXR/RXR Activation	N/A	PXR/RXR Activation	N/A
	GP6 Signaling Pathway	-1.508	Role of Tissue Factor in Cancer	N/A
	Histidine Degradation III	N/A	UVA-Induced MAPK Signaling	N/A
	Intrinsic Prothrombin Activation Pathway	-2.449		

CHAPTER 4: CHANGES IN FATTY ACID MOBILIZATION IN VISCERAL ADIPOSE TISSUE DUE TO ZILPATEROL AND HEAT STRESS IN CATTLE

Introduction

Growth and feed efficiency of cattle is improved by supplementation with the beta-adrenergic agonists (β-AA) ractopamine hydrochloride (RAC; β_1-AA) or zilpaterol hydrochloride (ZH; β_2-AA) (Elam et al., 2009). Supplementation can decrease carcass fatness and marbling, but the effects are not consistent, possibly due to type of β-AA, time on supplement, or cattle breed (Elam et al., 2009; Brown et al., 2014; Boyd et al., 2015). β-AA supplementation alters adipose deposition by inhibiting fatty acid biosynthesis and promoting lipolysis of stored triacylglycerols into free fatty acids (Kim et al., 2010; Johnson et al., 2014). However, beta-adrenoceptors (β-AR) desensitize with chronic activation (Mills et al., 1990; Re et al., 1997; Avendaño-Reyes et al., 2006), thus supplementation is limited to the last 20-40 d of feeding.

The annual economic impact of heat stress was estimated to cause a loss of $369 million to the US beef industry (St-Pierre et al., 2003). Heat-stressed livestock have reduced growth rates, dry matter intake, and average daily gain (Mitlöhner et al., 2001; St-Pierre et al., 2003). Fat thickness tends to decrease due to heat stress, though in some studies, the only noted effect of heat stress on adipose is increased dressing percentage (Mitlöhner et al., 2001; Gaughan et al., 2010). Epinephrine, released in response to stress (Tai et al., 2007), induces lipolysis in adipose (Samra et al., 1996). In response to acute

stress, signaling pathways for lipolysis of circulating and stored triglycerides are activated, while chronic stress increases lipogenesis and adipogenesis (Campbell et al., 2009; Peckett et al., 2011). In cattle, heat stress also increases the responsiveness of adipocytes to lipolytic signals, increasing lipolysis (Faylon et al., 2015).

The objective of this study was to understand how heat stress and β-AA independently and interactively affect adipose tissue metabolism. Prior work identified minimal impact of RAC on skeletal muscle metabolic properties and transcriptome, while ZH altered both (Kubik et al., 2018; Barnes et al., 2019). Based on the known effect of ZH on marbling and carcass fatness, we therefore hypothesized that ZH may also be affecting adipose metabolism; specifically, that lipolytic activity is increased due to heat and β-AA in an additive fashion. We tested this hypothesis in ZH-supplemented cattle exposed to heat stress for 21 d, using epinephrine to induce fatty acid mobilization.

Materials and Methods

This project was approved by the University of Arizona Institute of Animal Care and Use Committee (IACUC; Protocol 12-396) and the University of Nebraska-Lincoln IACUC (Project 1826). Commercial Red Angus-based (Red Angus sire x Red Angus crossbred dams) steers (260 ± 25 kg) were acclimated to individual tie stalls or pens for 6 d prior to initiation of the trial. Steers were housed in either the control (thermoneutral, TN; THI=68; n=11) or HS (THI=73-85; n=12) conditions for 21 d. The temperature humidity index (THI) of the HS environments cycled daily so it was 85 by midday and

then dropped to 73 during the night to simulate a day-night temperature cycle and allow the cattle to recover. Steers were supplemented with ZH (8.38 mg/kg/d; n=12) mixed at 1% in soybean meal as a carrier or only soybean meal (no supplement; NS; n=11) during the 21 d. Heat-stressed steers were fed, *ad libitum*, a diet consisting of 73.2% cracked corn, 13.7% chopped alfalfa, 6.3% molasses, 3.8% soybean meal, 2.1% mineral mix, and 0.9% urea. Thermoneutral steers were pair-fed based on the daily feed intake (percentage of body weight) of HS steers.

At harvest (d 21), visceral adipose tissue was collected to determine fatty acid mobilization in a method adapted from Raclot and Groscolas (1993). Briefly, modified Krebs Ringer buffer (MKRB) was made (9 g of KRB (Sigma), 900 ml ddH_2O, 15 mM $NaHCO_3$, 2.5 mM $CaCl_2$ dihydrate, and 4% fatty acid free BSA, pH adjusted to 7.4 and sterilized). Visceral adipose tissue was minced, strained (200 µM), washed (37°C MKRB) and minced a second time. Adipose tissue (400±10 mg) was added to 5ml MKRB containing 0 or 1 µM epinephrine. Samples were incubated in a shaking water bath (2h, 37°C), and the media filtered (2.4cm glass microfiber filter) and stored at -80°C. Free fatty acids (FFA) were quantified by colorimetric detection (Sigma Aldrich Free Fatty Acid Quantification Kit), read at 570 nm (BioTek EPOCH). Free fatty acid concentrations were calculated using a 0 to 4nmol/µL standard curve for palmitic acid and analyzed using the PROC MIXED procedure (SAS Institute Inc., Cary, NC, USA). The model design had fatty acid concentration as the response variable with supplement, environment, and the interaction thereof as the independent variables . *P*-values <0.05 were considered significant.

Results

There was no interaction between environment and supplement for *ex vivo* FFA mobilization from steer adipose. FFA concentration did not differ among groups at 0μM (Figure 4.1). At 1μM epinephrine, FFA was greater ($P<0.05$) in TN than HS. All treatment groups responded to epinephrine, with FFA concentration greatest in TN/ZH (Figure 4.1).

Discussion

These analyses, conducted on visceral adipose after 21 d of heat stress and supplementation with ZH failed to find evidence of an interaction between the treatments on fatty acid mobilization. ZH supplementation alone, however, increased the FFA-mobilization response to epinephrine even after 21 d, compared to unsupplemented animals. ZH preferentially binds β_2-AR (Verhoeckx et al., 2005; Baxa et al., 2010), while epinephrine is non-selective, binding to β_1-AR and β_2-AR without preference (Lodish et al., 2000). The heightened effect in the presences of both ZH and epinephrine could be due to the latter acting on β_1-AR with greater frequency than in the absence of ZH. The chronic effect of β-AA on adipose tissue metabolism can be difficult to observe from a single time-point. β-AA enhance lipid mobilization, as supported by our data, but also increase fatty acid synthesis, which would increase the amount of fatty acid available for mobilization (Yang and McElligott, 1989; Moibi et al., 2000). Since approximately the same amount of visceral adipose tissue used for fatty acid mobilization of each animal,

and the basal concentrations of fatty acid were not different between experimental groups, there was an equal amount of fatty acid to be utilized for mobilization. Thus, increased fatty acid mobilization in adipose tissue can be directly attributed to ZH treatment. These data support the continued activity of β-AR despite 21 d of supplementation.

Decreased FFA mobilization due to heat stress was possibly an effect of chronic exposure to heat stress-induced stimulation of the adrenergic system via epinephrine over 21 d of treatment. It is possible that epinephrine bound both β-AR isoforms of the adipose tissue during the trial, chronically activating them to the point of desensitization therefore resulting in a lesser response to stimulation. This result is supported by a prior heat stress study of dairy cows where fatty acid mobilization was decreased in response to epinephrine stimulation after 9 d of heat stress compared to that of thermoneutral cows (Baumgard et al., 2011). In fact, in our previous study with lambs, circulating epinephrine was increased due to heat stress (Swanson et al., 2020). If the steers responded likewise, it is reasonable to speculate that both β_1-AR and β_2-AR became desensitized and less responsive to epinephrine over 21 d of treatment compared to thermoneutral animals.

This study provides evidence that increased lipolysis is a mechanism by which ZH can reduce adipose tissue deposition. Conversely, HS impaired fatty acid mobilization at 21 d, presumably via β-AR desensitization due to chronic stimulation. Finally, no interacting effects of HS and β-AA supplementation on mechanisms that would impact animal well-being were apparent. Building a better understanding of the mechanisms by which animals respond to HS and β-AA supplementation will aid in

generating improved management practices to improve sustainability of livestock production.

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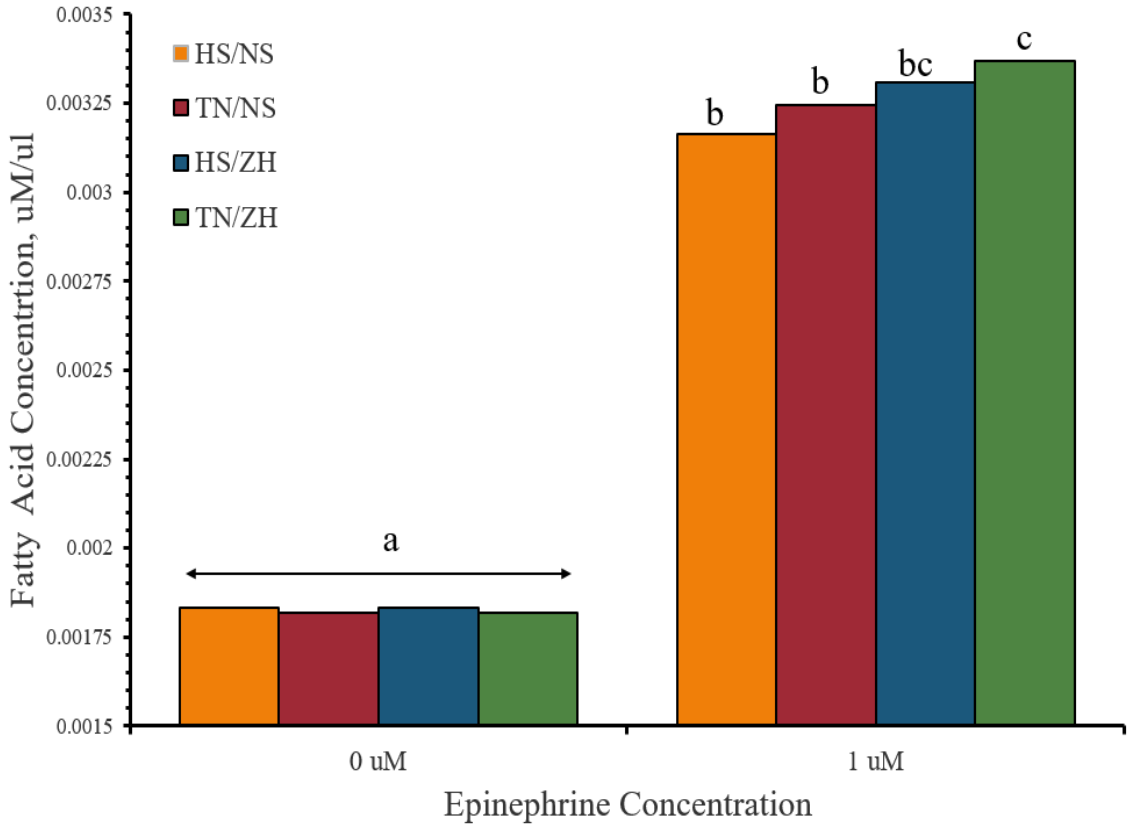
Figure 4.1. Fatty acid concentration of adipose stimulated by 1 µM of epinephrine after 21 d of ZH supplementation and HS exposure



TN=thermoneutral, HS=heat stress, NS=no supplement, ZH=zilpaterol