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Novel mechanisms involved with lipid metabolism in adipose tissue of dairy cows

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ABSTRACT

Proper lipid metabolism is essential to the maintenance of milk production and fitness in the modern dairy cow. Little is known about the regulation of lipid metabolism in adipose tissue in dairy cattle, including the roles of adipokines and lipid droplet associated proteins. Therefore, the objectives of this study were to characterized changes in transcript abundances of adipokines during three different energy altering models, and lipid droplet associated proteins throughout the transition period and with leptin induced lipolysis. Transcript abundances of leptin decreased during feed restriction, while angiopoietin like protein 4 (ANGPTL4) increased during early lactation, feed restriction, and following growth hormone administration. Therefore, reduced lipid uptake by adipose tissue during declining energy balance may be due to ANGPTL4 inhibiting lipoprotein lipase. Phosphorylation of hormone sensitive lipase (HSL) and perilipin increased during early lactation, but protein abundance of adipose triglyceride lipase (ATGL), the rate limiting step in lipolysis, decreased. Unlike lipid catabolism during early lactation, leptin induced lipolysis was mild, and protein abundance of ATGL and phosphorylation of STAT3 increased. These responses were attenuated with the addition of a STAT3 inhibitor. Therefore, leptin increased ATGL protein abundance through STAT3. This novel regulation of ATGL by STAT3 demonstrates a potential mechanism that allows for the differential regulation of HSL and ATGL. In conclusion, this research identified two novel mechanisms of lipid metabolism in dairy cattle which may allow for the development of strategies to optimize lipid metabolism.
CHAPTER 1. GENERAL INTRODUCTION

1.1 THESIS ORGANIZATION

This dissertation presents the author’s research in the alternative thesis format. The first chapter contains a general introduction of the dairy industry and emerging roles of adipose tissue, objectives, and a review of relevant literature. Since the research presented in this dissertation pertains to adipokines and lipid droplet associated proteins, the review of relevant literature is split into these two sections with these sections divided by adipokine or protein. Review of relevant literature for each adipokine or protein identifies additional nomenclature, describes the known function of the adipokine or protein, and discusses current knowledge in dairy cattle.

Chapters 2 and 3 are manuscripts published in Domestic Animal Endocrinology and Journal of Dairy Science, respectively. Chapter 2 is a manuscript that characterizes changes in leptin, adiponectin, adiponectin receptor 2 and angiopoietin like protein 4 transcript abundances in three energy altering models. Chapter 3 is a manuscript that characterizes the changes in protein abundance of perilipin, phosphorylated perilipin, hormone sensitive lipase, phosphorylated hormone sensitive lipase, adipose triglyceride lipase and comparative gene identity-58 during the transition period in dairy cattle. Chapter 4 is a manuscript in preparation for publication in American Physiology Journal’s Endocrinology and Metabolism. This chapter is a manuscript that characterizes leptin induced lipolysis in bovine adipocytes and identifies potential mechanisms utilized by leptin to induce
lipolysis. Two additional methods were utilized to better characterize this mechanism. They are included as appendix A and B. Appendix A characterizes the translocation of hormone sensitive lipase and adipose triglyceride lipid to the lipid droplet. This appendix may be combined with chapter 4 in the final publication. Appendix B identifies a potential promoter region of adipose triglyceride lipase that is bound by STAT3 during leptin treatment. This chapter contains preliminary data and additional replication is needed prior to publication. Following Chapter 4 is a chapter that summarizes the major finding from all work in this dissertation. Additionally, hypotheses and proposals of additional research are included for each major finding that pertain to the basic understanding of lipid metabolism and implications for the dairy industry.

1.2 INTRODUCTION

1.2.1 Dairy industry background

Demands for improved efficiency of agricultural systems are a result of declining farmland and increasing world population. For the dairy industry, efficient cows are classified as those that produce similar or greater quantities of milk while consuming less than expected quantities of feed. Nutritional, genetic, and management approaches can be used to optimize efficiency within dairy herds. Differences between individual efficiency can be dependent on appetite, nutrient digestion and absorption, maintenance requirements, nutrient partitioning, and utilization energy for production (Korver, 1988). The differences in metabolic efficiencies between tissues may play a critical role in the overall efficiency of cattle.
However, the mechanisms that contribute to the efficiency of individual tissues are not well understood.

Energy requirements fluctuate over the course of a single lactation. Following parturition, energy intake is often not sufficient to meet the elevated energy requirements for milk synthesis. Body energy reserves, in particular adipose tissue, are mobilized to meet this energy deficit. This state is often referred to as negative energy balance. As the lactation cycle progresses, milk synthesis stabilizes, and eventually declines. This period is accompanied by increasing feeding intake. Typically between 45 and 83 days in milk (DIM) energy intake exceeds energy requirements and allows for body energy reserves to be recuperated in preparation for the next lactation (Banos and Coffey, 2010; Butchereit et al., 2011; Hüttmann et al., 2009; Spurlock et al., 2012).

Although cows that utilized body energy reserves instead of consuming feed to meet energy requirements may appear to be more feed efficient, times of severe or prolonged negative energy balance can reduce overall fitness. Declining reproductive fitness has been a leading reason for culling decisions in the United States (Stevenson, 2009). Various measurements of reproduction, including days to first ovulation and days open, have been negatively associated with severe or prolonged negative energy balance during early lactation (Patton et al., 2007; Veerkamp et al., 2000). Additionally, the incidences of metabolic diseases, displaced abomasum and reproductive disorders increase during early lactation. Since pre-partum health can influence these diseases, they are often referred to as transitional diseases, which are usually diagnosed during the transition period, or
three weeks prior to and after parturition (LeBlanc, 2010). Metabolic diseases and
displaced abomasum are often associated with negative energy balance due to the
etiology of the disease. For instance, ketosis is diagnosed by observing elevated
circulating ketone bodies which are a by-product of incomplete gluconeogenesis due
to high lipid mobilization. Reproductive disorders, such as retained placenta and
metritis, can occur in cows regardless of energy status. However, reproductive
disorders are more likely to occur in cows in negative energy balance due to
decreased peripheral blood neutrophil function (Galvão et al., 2010; Hammon et al.,
2006). Therefore, understanding mechanisms involved that enable cows to best
manage negative energy balance and the transition period may prevent reduced
fitness.

1.2.2 Emerging roles of adipose tissue

Traditionally, adipose tissue has been viewed as a reservoir of densely stored
excess energy which can be mobilized for use during times of energy shortage, or
provide heat during times of cold exposure. Since the discovery of leptin in 1994
(Zhang et al., 1994) research has redefined adipose tissue as a dynamic endocrine
organ which is capable of regulating satiety, reproduction, immune functions,
angiogenesis, lipid metabolism in adipose and skeletal muscle, and glucose
metabolism. These novels roles, along with the increased prevalence of obesity,
have spawned an increased interest in adipose tissue (Figure 1). However, many of
the important factors that contribute to these functions lack characterization or are
poorly understood in dairy cattle.
1.3 OBJECTIVES

The overall objective of this dissertation was to characterize novel proteins involved in lipid metabolism of adipose tissue in Holstein cows to expand our understanding of potential mechanisms that contribute to the regulation of energy balance in dairy cattle. To accomplish this overall objective, three studies were conducted to 1) determine changes in the transcript abundance of adipokines in adipose tissue of cows with altered energy balance 2) define the regulation of novel lipolytic proteins during the transition from late pregnancy through early lactation and 3) determine mechanisms involved in leptin induced lipolysis. Results from this dissertation contribute to the understanding of lipid metabolism in dairy cattle.

1.4 REVIEW OF RELEVANT LITERATURE

1.4.1 Adipokines

Adipose tissue is a dynamic tissue capable of communicating with other tissues through the secretion of hormones. The first adipose derived hormones to be investigated had the ability to bind cytokine-like receptors and were called adipocytokines. As the number of secreted factors from adipose tissue increased and their known functions expanded, adipocytokine, or simply adipokine, is now used to identify any factor secreted by adipose tissue. To date, over 260 adipokines have been identified (Lehr et al., 2012). They contribute not only to the regulation of energy homeostasis, but to a wide range of functions in the body. For the sake of brevity, this review will focus on three adipokines that have been identified as possibly regulating energy homeostasis in dairy cattle.
1.4.1.1 Leptin

Leptin was discovered in a population of mice (ob/ob) that were obese due to a spontaneous mutation in the obese gene that prevented the synthesis of functional leptin (Zhang et al., 1994). These mice were not only morbidly obese, but infertile. Restoration of proper circulating leptin concentrations through the injection of recombinant leptin protein decreased adiposity (Pelleymounter et al., 1995). Leptin has since been observed to reduce obesity in humans with leptin deficiencies (Montague et al., 1997). Additionally, leptin can significantly reduce feed intake in mice (Halaas et al., 1995), pigs (Barb et al., 2006) and sheep (Henry et al., 1999) with normal leptin expression, indicating that leptin could be a potential tool to increase weight loss in obese humans. However, obesity is a complex disease that is often accompanied by leptin resistance and increasing circulating leptin concentration does not induce weight loss (Coppari and Bjørbaek, 2012).

Leptin regulates body weight by inhibiting food consumption. As observed with the ob/ob mice and mice deficient in the leptin receptor (db/db), the disruption of leptin signaling prevents satiety and allows for over consumption of food and subsequently obesity. Normally, adiposity increases circulating leptin concentrations to decrease food consumption. However, leptin signaling can be blunted with excess circulating leptin due to obesity, creating a state of leptin resistance. Inversely, circulating leptin concentrations decline during times of energy deficiency. This is expected to increase appetite, driving food consumption. Since circulating leptin concentrations are correlated to adipose tissue mass, decreased leptin concentrations could be confounded with adipose tissue loss. However, decreases
in leptin occurs prior to changes in weight (Maffei et al., 1995). Additionally, changes in physiological status, such as parturition, are associated with changes in leptin concentrations. For example, leptin concentrations decline following parturition in humans (Saucedo et al., 2011). However, this decline in circulating leptin concentrations coincided with weight loss, and is difficult to conclude if the changes in leptin concentrations are a result of physiological status or simply the loss of fetus weight. Regardless, leptin has been shown to be an important adipokine in regulating energy homeostasis.

Leptin has been associated with reducing appetite through signaling in the hypothalamus. Five isoforms of the leptin receptor (a-e) have been identified, but leptin signaling occurs primarily through the long form of the leptin receptor, often referred to as leptin receptor b. Isoforms a, c, d, and e are shortened forms of the leptin receptor and cannot completely activate the leptin signaling cascade. Binding of the long form of the leptin receptor by leptin in the hypothalamus activates the Janus kinase (JAK) /Signal Transducer and activator of Transcription (STAT) pathway, inhibiting neuropeptide Y (NPY) and agouti-related protein (AgRP) while activating α-melanocyte-stimulating hormone (αMSH). Increases in αMSH signals satiety, while decreases in NPY and AgRP reduce appetite signals.

Leptin also induces signaling in other cell types including cardiomyocytes via ERK1/2, and p38 (Rajapurohitam et al., 2012), oocytes via JAK2/STAT3, ERK1/2, and p38 (Arias-Alvarez et al., 2010; Lin et al., 2009), and adipocytes via Jak2/STAT3. Other than to acknowledge that leptin can signal through multiple cell types, and utilize different secondary messenger to initiate downstream signaling,
this review will not go into detail about leptin signal transduction in all cell types and will focus on adipocytes.

Leptin induces lipolysis in rodent (Bendinelli et al., 2000; Frühbeck et al., 2001; Rodríguez et al., 2003; Wang et al., 1999), human (Singh et al., 2012) and pig (Ajuwon et al., 2003; Li et al., 2010; Ramsay, 2001) adipocytes through activation of Jak2/STAT3. Observed increases in lipolysis in both adipocytes and adipose tissue explants are milder than when stimulated by isoproterenol, and also result in an increase in only glycerol concentrations (Ajuwon et al., 2003; Li et al., 2010; Wang et al., 1999). However, exposure of ovine adipose tissue explants to ovine leptin did not increase lipolysis or activate STAT3 or STAT5 (Newby et al., 2001). These data suggest leptin that may be able to regulate feed intake in all species, but only alter lipid metabolism in monogastric species. Additionally, the contribution of lipolytic activity by HSL and ATGL remain unknown at this time. In porcine adipocytes stimulated with leptin, ATGL protein abundance was decreased and transcript abundances increased. Although Li et al. went on to determine that ATGL transcript and protein abundances were affected by activation of JAK, mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor (PPAR)γ, the contribution of these pathways to leptin induced lipolysis remains unknown (Li et al., 2010).

Leptin is one of the most studied adipose tissue related proteins in dairy cattle due to its potential effects in regulating energy intake and fertility. Similar to rodents and humans, circulating leptin concentrations are positively correlated with body condition scores (BCS), nutritional status, and stage of lactation (Block et al., 2001,
Declining circulating leptin or transcript abundances during feed restriction or early lactation is expected to stimulate feed intake to improve energy status in cattle (reviewed by Ingvartsen and Boisclair, 2001). The presence of leptin is required for fertility in mice and may be required to allow ovulation (reviewed by Wathes, 2012). Additionally, single nucleotide polymorphisms (SNP) are associated with milk production, energy intake, and fertility. However, these SNPs are not consistently validated across multiple populations. For example, the promoter SNP, A1457G was associated with growth and fertility in a study using 509 Holstein Friesian cows (Clempson et al., 2011), but were not associated in a different population with 43,117 lactation records on each of 848 sires (Giblin et al., 2010). Although much is understood about leptin in dairy cattle, questions still remain about the effects of leptin at the tissue level.

### 1.4.1.2 Adiponectin

Adiponectin was discovered by four groups, and has since been identified to regulated insulin sensitivity and atherosclerosis (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995). Since adiponectin was identified independently, it is also known as Acrp30, AdipoQ, and gelatin binding protein (GBP28). In this manuscript, it will simply be referred to as adiponectin. Adiponectin is an adipokine that improves insulin sensitivity regardless of adiposity. Expression of adiponectin is lower in obese compared lean individuals and is increased with weight loss (Basu et al., 2007; Bobbert et al., 2005; Bodles et al., 2006) which can improve insulin sensitivity (Yamauchi et al., 2001). Insulin sensitivity can be improved in obese ob/ob mice by overexpression of adiponectin (Kim et al., 2007).
Thus adiponectin may be an adipokine that is essential for insulin sensitivity, and has been considered an independent risk factor for the development of metabolic syndrome and type II diabetes.

Adiponectin signals through two receptors to increase β-oxidation and subsequently improve insulin sensitivity. Both receptors are expressed in muscle, adipose tissue, and liver. However, abundances and signaling affinity are tissue specific. Activation of the either receptor is expected to increase activation of AMPK and subsequently β-oxidation in liver, or glucose uptake and β-oxidation in skeletal muscle (Figure 4, reviewed by Kadowaki and Yamauchi, 2005). Additionally, adiponectin may utilize a similar pathway to induce lipolysis in adipocyte, but this pathway has yet to be confirmed. In adipocytes, adiponectin receptor 2 is more responsive to exterior stimuli suggesting it maybe the primary signaling receptor in adipocytes (Fasshauer et al., 2004; Fu et al., 2007; Kudoh et al., 2011; Yamauchi et al., 2003).

Adiponectin circulates at low, medium, and high molecular weights in 3, 6, or 12-18 multimers complexes, respectively (Suzuki et al., 2007). Activities of these multimers may be tissue dependent. For example, high molecular weight adiponectin has been reported to activate nuclear factor kappa light chain enhancer of activated B cells (NFκB) in myocytes, but activates AMPK in hepatocytes (Lago et al., 2007). High molecular weight adiponectin has been associated with insulin sensitivity during times of insulin resistance following weight loss or treatment with PPAR γ agonist (Basu et al., 2007; Bobbert et al., 2005; Bodles et al., 2006). Additionally, a truncated or globular form of adiponectin has been observed in
circulation, and may be cleaved by active monocytes and/or neutrophils through the secretion of leukocyte elastase (Waki et al., 2005). The globular portion of adiponectin has been observed to increase lipid clearance, LPL activity, and insulin sensitivity (Combs et al., 2001) while reducing atherosclerotic lesions (Yamauchi et al., 2003).

Due to this complexity, characterization of adiponectin has been limited in dairy cattle. Adiponectin transcript abundances in adipose tissue do not change during the last part of gestation through the first 2 months of lactation (Giesy et al., 2012; Khan et al., 2013; Lemor et al., 2009). Adiponectin receptors 1 and 2 decreased in adipose tissue from 7 day pre-partum to 22 days postpartum (Lemor et al., 2009). In contrast, others have not observed a change in adiponectin receptor 1 or 2 abundance between late gestation and early lactation in adipose tissue of dairy cows (Giesy et al., 2012; Sadri et al., 2011). Recently, total plasma adiponectin, and molecular weight isoforms of adiponectin were measured using a bovine specific antibody. Separation of adiponectin by molecular weight demonstrated that the majority of adiponectin was high molecular weight. Both circulating high molecular weight adiponectin and total adiponectin declined 1 week prior to parturition and remained low for the first 7 weeks of lactation for the 4 cows used in this study (Giesy et al., 2012).

1.4.1.3 Angiopoietin-like protein 4

The generation of the PPAR α null mouse to search for unknown mechanisms involved in the regulation of fatty acid oxidation in the liver during times of fasting led to the discovery of angiopoietin-like protein 4 (ANGPTL4) (Kersten et al., 2000).
Through subtraction hybridization of liver between wild type and PPAR α null mice, Kersten et al. identified a novel sequence that was highly expressed in adipose tissue and elevated during times of fasting (2000). Subsequently, this factor was called fasting induced adipocyte factor (FIAF). Due to its angiopoietin-like structure, FIAF is also known as ANGPTL4. For consistency, ANGPTL4 will be used throughout. Additional angiopoietin like proteins have been identified, and have roles in angiogenesis (ANGPTL 1, 2, 3, 4 and 6), hematopoietic stem cell activity (ANGPTL 5 and 7; Zhang et al., 2006), or energy metabolism (ANGPTL 3, 4 and 6; as reviewed by Hato et al., 2008). This review will focus on ANGPTL4 due to its potent role in regulating lipid uptake and lipolysis.

Dysregulation of ANGPTL4 resulted in altered circulating lipid profiles. Mild over expression of ANGPTL4 in mice resulted in a 50% reduction in adipose tissue mass and increased circulating triacylglycerides, free fatty acids, glycerol, cholesterol and high density lipoproteins (HDL). Functional assays confirmed decreased lipid uptake suggesting ANGPTL4 can inhibit lipid uptake by adipose tissue (Mandard et al., 2006). These results were when ANGPTL4 deficiency mice had a 48% decrease in circulating triacylglyceride levels compared to wild type mice under fasted conditions. This decrease was due to decreased abundance of very low density lipoproteins (VLDL), suggesting increased uptake of dietary lipids. Additionally, circulating triacylglycerides were decreased 77% compared to wild type mice when both groups of mice were fed a high fat diet prior to fasting (Mandard et al., 2006). These data suggest ANGPTL4 has a critical role in regulating lipid uptake during times of fasting.
Lipids circulate as lipoproteins for transport to various tissues in the body. Chylomicrons are formed from dietary lipids in enterocytes and released into the lymphatic system, while other lipoproteins are synthesized in the liver. Lipoproteins have proteins embedded in the phospholipids that interact with lipoprotein receptors along the vasculature. Lipid uptake by tissue is mediated through the binding of chylomicrons or lipoproteins to the lipoprotein receptors which allow active lipoprotein lipase (LPL) to hydrolyze lipid to free fatty acids and glycerol. The decrease in available VLDL in fasted ANGPTL4 null mice would suggest that dietary lipid are hydrolyzed by LPL and taken up by surrounding tissue.

Mice lacking ANGPTL4 have increased LPL activity suggesting ANGPTL 4 inhibits lipid uptake by interacting with LPL. Through the use of enzyme linked immune sorbent assays (ELISA). Lee et al. were able to demonstrate that ANGPTL4 binds LPL. Additionally, they were able to generate an antibody which recognizes amino acid residues 29-53. This antibody prevented ANGPTL4 from binding LPL, and when given to mice decreased circulating triacylglyceride to level comparable to ANGPTL4 null mice. Additionally, the introduction of a point mutation that change amino acid residue 40 from glutamate to lysine resulted in similar phenotypes as the ANGPTL4 null mice (Lee et al., 2009). These data demonstrate that ANGPTL4 inhibits LPL by directly binding LPL, preventing the dimerization, and activation of LPL (Kersten, 2009). However, fatty acids can reduce this interaction (Robal et al., 2012).

To determine the importance of individual tissue expression of ANGPTL4 the ability of ANGPTL4 to inhibit LPL peripherally or locally needs to be considered.
Expression of LPL is tissue specific and dependent upon physiological status. For example, under fasted conditions, adipose tissue is releasing, and not storing lipids. During these conditions, adipose tissue LPL decreases. However, muscle which is in need of energy substrates increases muscle LPL activity. Therefore, it becomes important to determine if ANGPTL4 secreted by adipose tissue inhibits muscle LPL. Increased expression of intestinal \textit{ANGPTL4} but not adipose or liver has been linked to decreased adiposity in germ-free mice (Bäckhed et al., 2004, 2007). These mice were also resistant to obesity, however; germ free mice lacking ANGPTL4 were not (Bäckhed et al., 2004). Therefore, ANGPTL4 can act peripherally to inhibit LPL and prevent obesity. Interestingly, germ free rats had similar adiposity as conventionally raise rats despite increased intestinal \textit{ANGPTL4} expression. However, \textit{ANGPTL4} expression in adipose tissue was decreases suggesting a tissue specific regulation of LPL activity (Swartz et al., 2013). These conflicting results indicated conclusions about individual tissue expression of \textit{ANGPTL4} should be drawn very carefully.

Angiopoietin-like protein 4 abundance increases with fasting and enhances lipolysis in adipocytes. Gray et al. observed a reduction in glycerol released from adipose tissue explants from \textit{ad libitum} fed and fasted ANGPTL4 null mice compared to adipose tissue explants from wild type mice (Gray et al., 2012). This reduction was accompanied by a decrease in phosphorylation of HSL at serine 660, phosphorylated perilipin at 492 and ATGL protein abundance suggesting reduced activation of the PKA pathway with the absence of ANGPTL4. These results were confirmed by experiments utilizing chemicals that stimulated lipolysis at various points along the pathway. Maximal lipolysis was not achieved with the administration
of isoproterenol, which activates β-adrenergic receptors, in ANGPTL4 null adipocytes. While lipolysis induced with forskolin, which activates adenylate cyclase, 3-isobutyl-1-methylxanthine (IBMX), which prevents hydrolysis of cAMP, and 8-Br-cAMP, which is cAMP that is resistant to hydrolysis, were not different in ANGPTL4 null mice compared to wild type adipocytes (Gray et al., 2012). These data suggests that ANGPTL4 is required for maximal lipolysis and acts up stream of adenylate cyclase.

Angiopoietin-like protein 4 has been the only angiopoietin like protein described in cattle, and was originally characterized from tissue of 2 mature Angus based steers (Mamedova et al., 2010). Protein and transcript expression of ANGPTL4 were most abundant in liver and adipose tissue compared to intestinal tissue and pancreas. However, omasum protein also showed high expression (Mamedova et al., 2010). Characterization of differentially expression genes in adipose tissue of first lactation cows pre-partum (30 days) and post-partum (14 DIM), showed a 2 fold increase in ANGPTL4 post-partum (Sumner-Thomson et al., 2011). Given the potency of ANGPTL4 in mice, additional characterization of ANGPTL4 is necessary.

1.4.2 Lipolytic proteins

Catabolism of triacylglycerides is primarily stimulated through the protein kinase A (PKA) pathway. During times of energy deficiency, particular during early lactation, this pathway is critical in supplying energy substrates to peripheral tissues. Activation of the PKA pathway occurs through the binding of catecholamines (e.g. epinephrine, norepinephrine) to beta adrenergic receptors (β-AR). Activation of
adenylate cyclase occurs when the G-coupled proteins are released from β-ARs (Figure 2). Adenylate cyclase elevates intracellular concentration of cyclic adenosine monophosphate (cAMP) by converting adenosine triphosphate (ATP) to cAMP. Activation of PKA occurs when cAMP binds and activates two catalytic subunits of PKA. Cytosolic hormone sensitive lipase (HSL) is phosphorylated by PKA and subsequently translocates to the lipid droplet. Triacylglycerides at lipid droplet surface are catabolized by phosphorylated HSL and monoglyceride lipase resulting in 3 free fatty acids (FFA) and glycerol.

Additional proteins associated with the lipid droplet have been identified as critical regulators of lipolysis in adipocytes. Perilipin, a member of the PAT (Perilipin, Adipophilin, Tail Interacting Protein at kilodalton 47 (TIP47)) protein family, coats the lipid droplet, preventing HSL from hydrolyzing lipids at the lipid droplet surface. When phosphorylated by PKA, perilipin can facilitate the catabolism of triacylglycerides by allowing phosphorylated HSL to interact with the lipid droplet surface (Granneman et al., 2007). Additionally, cytosolic adipose triglyceride lipase (ATGL) translocates to the lipid droplet surface when PKA phosphorylates perilipin and releases comparative gene identity-58 (CGI-58; also known as α/β hydrolase domain containing 5) from the lipid droplet to remove the inhibitory protein G0/G1 switch protein (G0S2) from ATGL. The translocation of ATGL to the lipid droplet allows for the initial hydrolyzes triacylglycerides to diacylglycerides (Zimmermann et al., 2004).

Lipolysis primarily occurs in adipocytes, but can occur in other cell types that store small amounts lipids. The focus of this review will pertain to literature relevant
to the regulation of lipolysis in adipose tissue. However, differences between the regulation of lipolysis in adipose tissue and other tissues will occasionally point out.

1.4.2.1 Hormone sensitive lipase

Hormone sensitive lipase was identified in the early 1960s and until recently was thought to be the rate limiting step in hormone stimulated lipolysis (Vaughan et al., 1964). Activation of HSL can be through PKA, adenosine monophosphate activated kinase (AMPK) (Garton and Yeaman, 1990; Garton et al., 1989), extracellular signal-regulated kinase (ERK) (Greenberg et al., 2001), glycogen synthase kinase-4 (Garton and Yeaman, 1990; Olsson et al., 1986), and protein kinase C (Carmen and Víctor, 2006; Fricke et al., 2004), but is primarily regulated by the PKA and AMPK pathways. Increases in intracellular concentration of cAMP activate PKA which phosphorylates HSL and allows for the hydrolysis of tri- and diacylglycerides (Figure 2). Increased intracellular concentration of adenosine monophosphate (AMP) leads to the activation of AMPK, and prevents PKA phosphorylation of HSL (Garton and Yeaman, 1990). However, it is not well understood if activation of AMPK stimulates (Gaidhu et al., 2009), inhibits (Daval et al., 2005), or does not affect (Chakrabarti et al., 2011) lipolysis in adipocytes.

Activation of HSL can be through phosphorylation at 5 different serine residues (Anthonsen et al., 1998; Garton and Yeaman, 1990). Three of the five serine residues (563, 659, 660) are phosphorylated by PKA. Serine residue 563 was the first residue to be described (Egan et al., 1992). Serine residues 659 and 660 are also phosphorylated by PKA and regulate activity of HSL more than 563 (Anthonsen et al., 1998). The two remaining phosphorylated residues undergo PKA
independent phosphorylation. Serine residue 565 has been showed to be phosphorylated by AMPK (Garton et al., 1988). Due to its proximity to serine residue 563, phosphorylation of serine residue 565 may inhibit PKA phosphorylation of serine 563 (Carmen and Víctor, 2006; Daval et al., 2005). Phosphorylation of HSL serine residue 600 was identified to be phosphorylated by ERK, and may increase lipolysis (Greenberg et al., 2001). Phosphorylation of HSL by PKA increases the hydrophobicity of HSL (Krintel et al., 2009) that induces the translocation of HSL (Egan et al., 1992) to the lipid droplet within 5 minutes of stimulation (Brasaemle et al., 2000a). Once at the lipid droplet surface, PKA phosphorylated HSL interacts with phosphorylated perilipin to hydrolyze triacylglycerides (Carmen and Víctor, 2006; Fricke et al., 2004; Miyoshi et al., 2006; Moore et al., 2005).

Phosphorylation sites were identified and labeled according to their position in the rat HSL amino acid sequence. Although sites are not located in the exact same locations in other species, they continued to be referred to by their originally identified location in rat. Additionally, these sites were identified prior to the release of the rat genome in 2004 (Gibbs et al., 2004). It is therefore not surprising that these sites are not located at the same amino acid as originally identified (Figure 3). Comparison of the rat and the bovine amino acid sequence revealed identical amino acid residues that correspond to serine 563, 565, and 660 (Figure 3, Flicek et al., 2012). This conservation between species suggests that these sites may have similar activities between rodents and cattle.

In dairy cattle, the conserved serine residues of HSL are more critical in regulating lipolysis than transcript or protein abundances in adipose tissue.
Transcript abundances of *HSL* have been reported to increase with feed restriction in sheep and cattle (Bonnet et al., 1998), and in the first 90 of lactation compared to pre-partum samples (Sumner and McNamara, 2007). However, others have reported no change in *HSL* mRNA abundance throughout lactation (Elkins and Spurlock, 2009; Khan et al., 2013; Sadri et al., 2011; Sumner-Thomson et al., 2011). Total protein abundance has been reported to increase (Locher et al., 2011) or remain constant (Elkins and Spurlock, 2009) during early lactation compared to pre-partum. However, both studies showed an increase in phosphorylation of HSL at serine 563 (Elkins and Spurlock, 2009; Locher et al., 2011). Additionally, Locher et al. observed an increase in phosphorylation of HSL at serine 660 (Locher et al., 2011), which was not examined by Elkins and Spurlock (2009).

1.4.2.2 PAT proteins

The PAT family is named after the original three member of this family: Perilipin, Adipophilin, and TIP-47 (Londos et al., 1999). The remaining two members are S3-12 and OXPAT (Ducharme and Bickel, 2008). Due to a conserved perilipin domain in these proteins, they are also known as: Perilipin 1 (perilipin), perilipin 2 (adipophilin), perilipin 3 (TIP47), perilipin 4 (S3-12), and perilipin 5 (OXPAT). For continuity, this review will refer to this family by their PAT family names.

As with perilipin, members of the PAT family coat the lipid droplet to allow for storage of lipid. During the maturation of white adipocytes, multiple lipid droplets form from the endoplasmic reticulum (ER) and eventually unite into a single large lipid droplet. Newly formed lipid droplets are coated with S3-12 and TIP-47. As lipid droplets enlarge through fusion with other lipid droplets and lipid uptake, adipophilin
begins to coat the lipid droplet. Once the lipid droplet becomes large and centrally located, perilipin begins to replace S3-12, TIP47, and adipophilin (reviewed by Ducharme and Bickel, 2008).

Although the functions of PAT proteins can be redundant, only perilipin is critical for lipolysis in white adipose tissue. Tail interacting protein-47 can compensate for decreased adipophilin, because mice lacking adipophilin had increased expression of TIP-47. However, the loss of both TIP-47 and adipophilin increased lipolytic activity as observed by the reduced amount of lipid in the lipid droplet and the number of lipid droplets (Sztalryd et al., 2006). Adipophilin can protect the lipid droplet from lipolysis, but it is less efficient than perilipin because basal, or maintenance lipolysis was elevated in perilipin null mice. Additionally, stimulated lipolysis was decreased in perilipin null mice suggesting adipophilin does have the capabilities to facilitate stimulated lipolysis, but cannot completely rescue perilipin null adipocytes (Tansey et al., 2001). Although OXPAT may regulate lipolysis via regulation of ATGL, it has been observed to be active only in highly oxidative tissues such as skeletal and cardiac muscle, where perilipin expression is low (Blanchette-Mackie et al., 1995; Greenberg et al., 1993; Servetnick et al., 1995).

Since the focus of this review is lipolysis in adipose tissue, the regulation of perilipin in lipolysis will only be reviewed.

Phosphorylation of perilipin occurs at six different serine residues, and phosphorylation of some but not all are required for maximal lipolysis. Phosphorylation of perilipin occurs at serine residues 81, 222, 276, 433, 492, and 517 (Miyoshi et al., 2006). Adipocytes from perilipin null mice transfected with
perilipin that contained serine to alanine mutations at all six phosphorylation
residues (Tansey et al., 2003) or the 3 amino terminal (serine 433, 492, and 517)
residues (Miyoshi et al., 2006) had attenuated lipolysis. Confirmation of these
findings in adipocytes showed that the amino-terminal serine, 517, was responsible
for the majority of lipolytic response (Miyoshi et al., 2007), while serine 492
contributes to lipolytic activity and lipid droplet fragmentation (Marcinkiewicz et al.,
2006; Miyoshi et al., 2007). Additionally, lipolysis in Chinese Hamster Ovary (CHO)
was attenuated when perilipin with serine to alanine mutations of the 3 carboxyl
serine residues (serine 81, 222, 276), but did not observe a reduction in lipolysis
when the three amino terminal serine residues were mutated (Miyoshi et al., 2006).
Chinese hamster ovary cells do not naturally contain lipolytic proteins and the lack of
these proteins may contribute to the contrasting results.

Since perilipin acts as a barrier to the lipid droplet, it was originally
hypothesized that phosphorylation of perilipin resulted in the translocation of perilipin
away from the lipid droplet (Daval et al., 2005; Tansey et al., 2001). However,
fluorescence resonance energy transfer (FRET) and the use of immunofluorescence
revealed that perilipin remained at the lipid droplet surface during stimulated and
basal lipolysis (Granneman et al., 2007; Moore et al., 2005). Perilipin regulates
lipolysis through its interactions with HSL and CGI-58. Translocation of HSL was
decreased in adipocytes lacking perilipin following hormone stimulation (Sztalryd et
al., 2003). In contrast, adipocytes containing full length perilipin with mutations of
the phosphorylation sites had normal translocation of HSL to the lipid droplet
surface, but attenuated lipolysis (Granneman et al., 2007; Miyoshi et al., 2006).
These results suggest that perilipin, but not phosphorylation of perilipin, is required for translocation of HSL to the lipid droplet. Hormone sensitive lipase binds perilipin at two distinct regions, amino acids between residues 141-200 and 406-480 (Shen et al., 2009). However, it is unknown if these interacting are critical for HSL activity. Regardless, perilipin is required to translocate to the lipid droplet and phosphorylation of perilipin is required to allow HSL access to the lipid droplet.

Perilipin sequesters CGI-58 at the lipid droplet, but is released once perilipin is phosphorylated by PKA. Phosphorylation of perilipin at serine residue 517 is required for the translocation of CGI-58 from the lipid droplet (Miyoshi et al., 2007). Additionally, phosphorylation of perilipin at serine residue 492 and serine residue 517 was critical for CGI-58 translocation to the cytosol to activate ATGL in COS-1 cells (Granneman et al., 2009). The ability of perilipin to regulate the two major lipases during stimulated lipolysis suggests that perilipin is a master regulator of lipolysis.

Similar to in vitro cell culture models, phosphorylation of perilipin was associated with lipolytic activity in dairy cattle. Transcript abundances of perilipin either increase (Sumner and McNamara, 2007) at 90 DIM or remain constant (Elkins and Spurlock, 2009; Sadri et al., 2011; Sumner-Thomson et al., 2011) throughout lactation when compared to pre-partum abundances. Total protein abundances of perilipin and mRNA transcript abundances were similar between adipose tissue from early (5-14 DIM) and mid lactation (176-206 DIM) cows. However, phosphorylation of perilipin abundance increased in with cows in early lactation, when adipose tissue mobilization was increase as determined by indicators of lipolysis. Additionally,
glycerol and non-esterified fatty acids (NEFA) were positively correlated with phosphorylation of perilipin (Elkins and Spurlock, 2009). These data suggest that phosphorylation of perilipin may be a critical regulator of lipolysis in the dairy cow during times of negative energy balance.

1.4.2.3 Adipose triglyceride lipase and its co-activators

Adipose triglyceride lipase was discovered in 2004 by 3 different groups, (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004). As a result, ATGL is also known as desnutrin, calcium-independent phospholipase A2, and patatin-like phospholipase domain containing 2 (PNPLA2), and will be referred to as ATGL throughout this review. Adipose triglyceride lipase only has the ability to hydrolyze triacylglycerides to diacylglycerides (Zimmermann et al., 2004). The absence of ATGL reduces stimulated lipolysis in adipocytes (Vaughan et al., 1964). Since HSL can hydrolysis both tri- and diacylglycerides, ATGL is considered the rate limiting step in lipolysis (Haemmerle et al., 2006). In addition to the involvement of ATGL in PKA dependent lipolysis, ATGL can regulates lipid droplet size through basal or maintenance lipolysis (Miyoshi et al., 2008).

The absence of functional ATGL and its cofactor, CGI-58, cause Neutral Lipid Storage Disease (NLSD), which is a slight to severe accumulation of neutral lipids in non adipose tissue depots (Akiyama et al., 2007; Campagna et al., 2008). Mutations of the carboxyl-terminal regions of human ATGL are associated with NSLD (Akiyama et al., 2007; Campagna et al., 2008). Initially, these mutations were considered to prevent ATGL activity, but in vitro work demonstrated that ATGL lacking the full carboxyl-terminal end had increased triglyceride hydrolase activity
relative to wild-type ATGL. However, these mutated forms of ATGL were not able to translocate to the lipid droplet which prevented lipolysis (Schweiger et al., 2008). Therefore, mutation in the C-terminal region of ATGL associated with NSLD may decrease translocation of ATGL and not activity of ATGL.

Mutations of CGI-58 have been associated with a more severe form of NLSD. Humans with this form of the disease not only have accumulation of neutral lipids in non-adipose tissue, but have congenital ichthyosiform erythroderma. Congenital ichthyosiform erythroderma is also known as nonbullous congenital ichthyosiform erythroderma which is characterized by increased hyperkeratosis, eclabion, ectropion and alopecia. Mice deficient in CGI-58 have systemic lipid accumulation, hepatic steatosis, and severe skin permeability barrier defect. These mice typically die within hours of birth, suggesting CGI-58 is critical to lipid metabolism and suggesting roles outside of those regulating ATGL (Radner et al., 2010). Mutations in CGI-58 have been reported in NSLD with ichthyosiform erythroderma, but not all are associated directly with decreased activation of ATGL (reviewed by Yamaguchi and Osumi, 2009). Therefore, inhibition of ATGL activity must be confirmed when determining the role of particular mutations in CGI-58.

Regulation of ATGL may occur at three different points: protein-protein interaction, phosphorylation, and protein abundance. Adipose triglyceride lipase interacts with CGI-58 and G0S2 to regulate its translocation to the lipid droplet (Figure 4). G0/G1switch protein 2 binds to cytosolic ATGL between the first 254 amino acids residues (Cornaciu et al., 2011), and may specifically bind within the patatin domain (amino acid residues 10-178, Figure 4) to prevent the translocation of
ATGL to the lipid droplet (Lu et al., 2010b; Schweiger et al., 2012). Additionally, G0S2 binds to the truncated form of ATGL associated with NLSD (stop codons introduced at amino acid 289 and 319 of 486 amino acids). Instead of inhibiting translocation, the truncated ATGL/G0S2 complex translocates to the lipid droplet (Schweiger et al., 2012). These data suggest that the N-terminal region of ATGL is critical for inhibition of activity via G0S2. Comparative gene identity is required for the translocation of ATGL to the lipid droplet surface, by removing G0S2. Mutations of CGI-58 associated with NLSD prevent CGI-58 from activating ATGL. Amino acid residue 130 and residue 260 of CGI-58 increase ATGL activity during stimulated lipolysis in COS-7 cells (Gruber et al., 2010; Lass et al., 2006). Additionally, the carboxyl-terminal region of ATGL is critical for CGI-58 binding, in particular truncations before amino acids 255 and 254 significantly reduced triglyceride hydrolase activity (Cornaciu et al., 2011). Residues 130-260 of CGI-58 potentially interact with the carboxyl-terminal end of ATGL. However, the direct interaction between these residues has yet to be determined.

Phosphorylation sites have been identified for ATGL, but their functions remain unclear. A phosphorylation site was discovered within ATGL shortly after its identification. However, it was not shown to be phosphorylated by PKA, and subsequently assumed not to be involved in simulated lipolysis (Zimmermann et al., 2004). Two phosphorylation sites were identified by liquid chromatograph-tandem mass spectrophotomertry from lipid droplets in HeLa cells. One of these sites (serine residue 404) was predicted to have an ERK consensus site (Bartz et al., 2007). Additionally, five putative murine PKA and AMPK phosphorylation residues were
identified in Cos-7 cells including included both serine residues identified by Bartz et al. (2007). Mutation of the murine serine residue 406 to alanine, which corresponds with human serine residue 404, was associated with decreased *in vitro* triglyceride hydrolase activity, and radiolabelled oleate release in Cos-1 cells (Pagnon et al., 2012). Additionally, phosphorylation of this site can occur via AMPK during times of increased lipolysis (Ahmadian et al., 2011). However, research is needed to fully understand the relationship of this phosphorylation site and its role in *in vivo* lipolysis.

Given the difficulty of measuring protein-protein interactions *in vivo*, researcher have been determining if ATGL transcript or protein abundance differ between various lipolytic inducing treatments. Human studies observed increased ATGL protein abundance during fasting, but not following an hour of exercise in men. The increase in ATGL protein abundance coincided with a decrease in G0S2 protein and mRNA. However, HSL and CGI-58 remained constant suggesting increased ATGL abundance maybe associated with lipolysis (Nielsen et al., 2011). It should be noted this study did not explore protein abundance of phosphorylated HSL. In swine, ATGL transcript and protein abundance increased in restricted feed studies, but porcine ATGL decreased with β-agonist (Deiuliis et al., 2008). In broiler chickens and quail, ATGL mRNA and protein abundance increase after 24 hours of fasting this coincided with elevated NEFA concentrations. Protein and transcript abundances of ATGL returned to with 8 hours of refeeding (Serr et al., 2009). However, others have reported decreased ATGL abundance with lipolytic stimuli. For example, ATGL abundance decreased in 3T3-L1 cells when treated with
isoproterenol and tumor necrosis factor α (TNFα; (Kralisch et al., 2005). Differential expression of ATGL mRNA and protein was observed with porcine adipocytes derived from stromal vascular cells (Li et al., 2010). A summary of this information is in table 1. Although the majority of researchers have reported increased transcript and protein abundances with elevated lipolytic activity, a better understanding is needed to study in vivo activity of ATGL.

In dairy cattle, research is limited to a single publication, aside from material in this manuscript. The authors reported increased ATGL transcript abundances in cows from low genetic merit sires at 28 and 56 DIM compared to cows from high genetic merit sires. However, no differences were observed between samples taken 21 days pre-partum and 7, 28 and 56 days post partum. It should be noted that this study used 6 cows per group, and set the significance at P < 0.1 (Khan et al., 2013).

1.4.3 Conclusion

Since the discovery of leptin in 1994, our understanding of adipose tissue is more complex due to recently identified mechanisms. Many of these mechanisms can regulate energy homoeostasis, but have not been characterized in dairy cattle. Therefore the exploration of these recently identified mechanisms can expand our understanding of lipid metabolism in dairy cattle. This is particularly important since lipid mobilization is critical to meeting energy requirements during early lactation, and severe or prolonged mobilization of adipose tissue is associated with metabolic diseases and may contribute to reduced fitness.
### 1.5 TABLES

**Table 1.** Summary of ATGL abundance during times of lipolysis.

<table>
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<tr>
<th>Condition</th>
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<th>ATGL</th>
<th>Authors</th>
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<td>Protein</td>
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<td>2011</td>
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<td>--</td>
<td>--</td>
<td>Neilson et al.</td>
<td>2011</td>
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Abbreviations: CGI-58, comparative gene identity- 58; G0S2, G0/G1 switch protein 2; ATGL, adipose triglyceride lipase; mRNA, messenger RNA; hr, hour

<sup>a</sup> Fasting refers to the complete withdraw of feed, while feed restriction refers to a reduction in available feed.

<sup>b</sup> Poultry refers to broiler chickens and quail.
1.6 FIGURES

Figure 1. Number of manuscripts by year. The bars indicated the number of searchable manuscripts on Google scholar identified with the keywords adipose tissue and obesity. The year leptin was discovered is indicated with an arrow.

Figure 2. Hormone stimulated lipolysis. 1) Activation of β-adrenergic receptors by isoproterenol increases intracellular concentrations of cAMP via activation of adenylate cyclase through the activation of G-proteins. 2) Protein kinase A (PKA) is activated by cAMP and subsequently phosphorylates perilipin at 6 serine residues and hormone sensitive lipase (HSL) at 3 serine residues. 3) Phosphorylation of perilipin allows for the activation of adipose triglyceride lipase (ATGL) through the release comparative gene identity-58 (CGI-58) from the lipid droplet and 4) phosphorylated HSL translocates to the lipid droplet and interacts with phosphorylated perilipin to access lipids. Hydrolysis of triacylglyceride by ATGL results in diacylglyceride and a free fatty acid. Active HSL hydrolyzes triacylglycerides and diacylglycerides producing 2 or 1 fatty acids, respectively.

Figure 3. Comparison of HSL phosphorylation sites in rat and bovine hormone sensitive lipase. The alignment of the amino acid sequence of rat and bovine hormone sensitive lipase (HSL) was done using the CLUSTALW feature built into the ENSEMBL website (www.ensembl.org). Highlighted amino acid sequence represents published rat amino acid sequences used to characterize the phosphorylation sites. The specific serine residues phosphorylated are larger in size.
and bolded. Serine residue 600 (rat amino acid 900 and bovine amino acid 569) is in larger red text. “**” indicates residues within the column that are identical. “.:” indicates residues within the column that are conserved substitutions. “.:” indicates residues within the column that are semi-conserved substitutions.

**Figure 4. Protein structure of adipose triglyceride lipase.** The alignment of the amino acid sequence of mouse and bovine adipose triglyceride lipase (ATGL) was done using the CLUSTAL W feature built into the ENSEMBL website (www.ensembl.org). Amino acid sequence that is highlighted in gray represents the published patatin domain in the murine amino acid sequence. Within the patatin domain, it is expected that G0/S1 switch protein 2 (G0S2) binds to and inhibits ATGL activity. The hydrophobic domain is highlighted in blue. The specific serine residues that potentially interact with comparative gene identity-58 (CGI-58) are bolded within the sequence. “**” indicates residues within the column that are identical. “.:” indicates residues within the column that are conserved substitutions. “.:” indicates residues within the column that are semi-conserved substitutions.

**Figure 5. Adenosine monophosphate kinase activated lipolysis.** Activation of adenosine monophosphate kinase (AMPK) occurs through 1) Increases in intracellular adenosine monophosphate (AMP) concentrations allow for the phosphorylation of AMPK or 2) external stimuli. Phosphorylated AMPK can 3) phosphorylate hormone sensitive lipase (HSL), potentially increasing lipolysis. Additionally, adipose triglyceride lipase (ATGL) may be phosphorylated by AMPK.
allowing for it to hydrolyze triacylglycerides. However, perilipin and comparative gene-identity 58 (CGI-58) are not activated. Activation of AMPK increases β-oxidation by phosphorylating and subsequently inhibiting Acetyl Co-A Carboxylase (ACC). This relieves the inhibition on carnitine palmitoyltransferase I b (CPT-1b), allowing fatty acids to be transported and oxidized in the mitochondria.
Figure 3
Figure 4

**Patatin domain**

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***Hydrophobic domain***
1.7 REFERENCES


CHAPTER 2. ADIPOSE TISSUE ANGIPOIETIN-LIKE PROTEIN 4
MESSENGER RNA CHANGES WITH ALTERED ENERGY BALANCE
IN LACTATING HOLSTEIN COWS

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2.1 ABSTRACT

Negative energy balance at the onset of lactation is unfavorably associated with fitness traits in high producing dairy cows. Angiopoietin-like protein 4 (ANGPTL4) is an adipokine that has been associated with the regulation of lipid metabolism through the inhibition of lipoprotein lipase activity and regulation of lipolysis. Expression of ANGPTL4 messenger RNA (mRNA) increases during early lactation, but its regulation with changing energy status is currently unknown. Accordingly, the objective of this study was to determine if ANGPTL4 mRNA abundance is responsive to declining energy balance induced by the transition from pregnancy to lactation, feed restriction, and GH administration in lactating dairy cows. The mRNA abundance of leptin, adiponectin, and adiponectin receptor 2 were also measured to compare adipokine mRNA profiles during changes in energy metabolism. Repeated adipose tissue biopsies were taken from different cows during transition from late pregnancy to lactation (n=26), feed restriction (n=19), and
GH administration (n=20). As expected, milk yield increased with the onset of lactation and GH administration (P < 0.01), but declined during feed restriction. Energy balance declined in each experiment, resulting in negative energy balance at the onset of lactation and following feed restriction. ANGPTL4 mRNA abundance increased two- to six-fold with declining energy balance in each experiment. Leptin mRNA declined with feed restriction, and adiponectin mRNA decreased with the onset of lactation. The consistency and magnitude of the increase in ANGPTL4 mRNA across multiple models of altered energy balance identifies it as an adipokine that is uniquely responsive to changes in energy balance in the lactating dairy cow.

**Keywords:** Leptin, Adiponectin, Growth Hormone, Transition, Feed Restriction

### 2.2 INTRODUCTION

The onset of lactation in high yielding dairy cows typically leads to a period of negative energy balance. Energy stored in body tissues, particularly adipose tissue, is then mobilized to compensate for the energy deficit. A greater understanding of metabolic changes that occur in response to altered energy status in dairy cows is needed, because severe or prolonged negative energy balance is unfavorably associated with reproduction, health and fitness traits [1]. Adipose tissue is recognized as an important endocrine tissue that participates in the regulation of energy homeostasis through the secretion of signaling proteins, or adipokines. Some adipokines, including leptin and adiponectin, are recognized as important modulators of energy metabolism [2], whereas the role of other adipokines is less defined. Angiopoietin-like protein 4 (ANGPTL4) was initially identified as a novel peroxisome
proliferator-activated receptor target gene that was rapidly up-regulated in response to fasting in rodents [3]. This secreted protein is associated with lipid metabolism via inhibition of lipoprotein lipase (LPL) and enhanced lipolysis [4–7], and has been studied in the context of immune response [8] and angiogenesis [9]. The messenger RNA (mRNA) abundance of adipose ANGPTL4 increases with pregnancy and lactation in rodents [10], and the transition from pregnancy to lactation in cattle [11]. Thus, the underlying hypothesis of the current research is that ANGPTL4 is an adipokine whose expression is altered in response to changes in energy status in lactating dairy cows, and that altered expression of ANGPTL4 facilitates the regulation of lipid metabolism.

Three models of altered energy balance in lactating dairy cows are utilized in this research, including the transition from late pregnancy to lactation, feed restriction, and administration of bovine GH. During the transition from pregnancy to lactation, negative energy balance occurs as a result of the rapid increase in energy utilization for milk synthesis that is not offset by increased energy intake [12]. Negative energy balance can also be induced by feed restriction, which is a consequence of limited energy intake. In this model, milk synthesis decreases, but this adaptation does not fully alleviate the energy imbalance. Finally, administration of GH results in a shift in energy partitioning to favor milk synthesis. Although energy intake eventually increases, the delay in response relative to the increase in milk production results in a transient period of reduced energy balance [13].

Leptin mRNA expression and secretion have been investigated using similar models of altered energy balance in dairy cows. In general, leptin mRNA abundance
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and secretion [14–17] decrease with the transition from pregnancy to lactation and during feed restriction, but changes in leptin abundance in response to GH administration are not evident [16,18]. Leptin mRNA abundance was measured in the current experiment for comparison to ANGPTL4 across experimental models. In addition, adiponectin is a significant determinant of insulin sensitivity, intertissue communication, and immune function in nonruminant species [19–21]. Signaling by adiponectin occurs through adiponectin receptor 1 or adiponectin receptor 2, with adiponectin receptor 2 recognized as the predominant functional receptor in adipose tissue [22,23]. Currently, the regulation of adiponectin and adiponectin receptor 2 in response to changing energy status is not well characterized in cattle. Therefore, specific objectives of this research were to quantify changes in mRNA abundance of ANGPTL4, leptin, adiponectin and adiponectin receptor 2 in 3 models of altered energy status in lactating dairy cows. These objectives contribute to efforts to understand the role of adipokines during changes in energy metabolism in Holstein cows.

2.3 MATERIALS AND METHODS

All procedures involving the use of animals were approved by the Iowa State University Institutional Animal Care and Use Committee.

2.3.1 Animals

A total of 65 cows were allocated across 3 experiments and fed ad libitum a consistent total mixed ration (TMR) formulated to meet or exceed all nutritional requirements (1.41 or 1.65 MCal of NEI per kg of dry matter for pre and postpartum diets, respectively) [24], unless noted. Daily individual feed intake was measured for
the duration of all experiments using the Calan Broadbent Feeding system (American Calan, Northwood NH, USA). Cows were trained and acclimated to the feeding system 4 to 6 d prior to initiation of each experiment. Daily feed intake was calculated as the difference between the quantity of TMR provided daily, and orts remaining daily, expressed on a dry matter basis. Dry matter and NE\textsubscript{i} content were determined from weekly TMR samples (Dairyland Laboratory, Arcadia, WI) that represented pooled samples from $\geq 3$ individual feedings. Samples of orts were also analyzed for dry matter and energy content, and these values were used in final calculation of dry matter and energy intake. Milk yield was recorded at each milking, and milk samples were collected weekly during a morning milking for determination of milk components (Dairy Lab Services, Dubuque, IA). Body weights were measured weekly following a morning milking (lactating cows) or at a consistent time of day (non-lactating cows). Energy balance was calculated as previously described [25], according to equations published by the National Research Council [24].

Subcutaneous adipose tissue samples were biopsied from the tailhead region under local anesthesia (2% lidocaine) using a minimally invasive procedure, as previously described [25,26]. Four 1-g adipose tissue samples were extracted at each biopsy, immediately frozen in liquid nitrogen, and stored at -80° C until analysis. Biopsies were taken from alternating sides of cows.

2.3.1.1 Transition experiment

Twenty-six multiparous Holstein cows were moved to pens equipped with the Calan Broadbent Feeding system (American Calan, Northwood, NH, USA) 4 wk prior to expected parturition. Adipose tissue samples were collected to target 21 d
antepartum (AP), and 5, 21, and 150 d postpartum (PP). Samples and data collected on these days were designated as 21 d AP, 5 d PP, 21 d PP, and 150 d PP, respectively. Five cows were removed from the study after 21 d PP due to prolonged illness or injury between 21 and 150 d PP. Blood samples were collected weekly via jugular venipuncture starting ~ 3 wk AP and continuing through 4 wk PP, and again following the adipose tissue biopsy at 150 d PP. Serum was analyzed for NEFA concentration using a colorimetric NEFA assay (Randox Laboratories, Co. Antrium, United Kingdom) according to the manufacturer’s recommended protocol.

2.3.1.2. Feed restriction experiment

Nineteen multiparous Holstein cows were evaluated in 2 replicates (n=8, n=11) separated in time by 1 mo. Cows in the first and second replicate averaged 183 ± 5 and 178 ± 4 d in lactation, respectively, when adipose tissue was first sampled. Baseline feed intake was measured for 5 d prior to initiation of feed restriction. Feed was restricted to achieve a targeted energy balance of -10 Mcal/d, similar to energy balance during the transition period, with a maximum restriction of 50% of baseline feed intake. The quantity of feed provided to each cow was adjusted on day 3 of the experiment to account for changes in milk production. Feed was provided to cows 4 times daily during the feed restriction period. Adipose tissue was sampled on experimental day -6, 1, and 4 relative to initiation of feed restriction on day 0. Blood samples were collected immediately after each biopsy for analysis of NEFA, as previously described in section 2.3.1.1. One cow from replicate 1 lacked sufficient adipose tissue for two biopsies on the same side. Blood and adipose tissue were not sampled on day 1 for this cow.
2.3.1.3. Growth hormone experiment

Twenty multiparous Holstein cows were evaluated in 2 replicates separated in time by 1 d. Cows in the first and second replicate averaged 186 ± 6 and 180 ± 6 d in lactation, respectively, when adipose tissue was first sampled. Baseline feed intake was measured for 5 d prior to administration of GH. Bovine GH was administered as a single dose of Posilac (500mg sometribove zinc; Elanco, Greenfield, IN), given via a subcutaneous injection in the neck. Adipose tissue was sampled on experimental days -4, 3, and 7 relative to GH administration on day 0. Blood samples were collected immediately following each biopsy for analysis of NEFA, as previously described in section 2.3.1.1. For the feed restriction and GH experiments, samples collected on days -6 and -4, respectively, were used as a baseline control. Although we considered utilizing a portion of the cows as an untreated control group to identify potential environmental effects associated with day of biopsy, assignment of these cows to a second replicate for increased experimental power was deemed more appropriate.

2.3.4. Quantitative real-time PCR

Approximately 1 g of subcutaneous adipose tissue was used for RNA extraction following a guanidine thiocyanate-chloroform-phenol protocol [27]. Genomic DNA contamination was removed using DNA-free according to the manufacturer’s protocol (AM1906; Ambion, Life Technologies, Grand Island, NY). Quality and quantity of RNA was assessed by absorbance measurement using a Nanodrop (Thermo Scientific, Waltham, MA). Complementary DNA was generated from 2.5 µg of RNA using Moloney Murine Leukemia Virus Reverse Transcriptase
with all other conditions according to manufacturer’s protocol (Invitrogen, 28025-013). The absence of genomic DNA was confirmed by PCR amplification of reference genes, β-actin or 60S ribosomal protein L32 (RPL32). Primers targeting these genes (Table 1) spanned 131 and 588-bp introns, respectively. All primer sequences and amplicon sizes for PCR are provided in Table 1. Quantitative PCR was carried out under the following conditions for all primers: 95° C for 2 min followed by 40 repeated cycles of 95° C for 30 s, 50° C for 30 s, and 72° C for 45 s. The PCR products were then held at 95° C for 1 min, followed by 56° C for 1 min. Finally, a melt curve was run to confirm each PCR resulted in a single product. The melt curve increased from 56° C to 96° C, with an increase of 0.5° C every 10 s. Quantitative PCR was run in a 96-well format on a MyiQ thermocycler (Bio Rad, Hercules, CA) using SYBR Green chemistry (Bio-Rad, Hercules, CA) according to manufacturer’s protocol, except all reactions were run at half volume. A serially diluted standard curve (~ 2.0 x 10^2 to 2.0 x 10^8 copies of a plasmid containing the PCR product of interest) was included with each assay as a positive control and for evaluation of PCR efficiency. Negative controls were included in each PCR plate. All samples and controls were run in duplicate, and the average of duplicate threshold cycle (Ct) values was used in statistical analyses. Leptin and ANGPTL4 in the transition period were normalized to β-actin. Adiponectin and adiponectin receptor 2 in the transition experiment and all genes in the feed restriction and GH experiments were normalized to RPL32.
2.3.5. Statistical analysis

2.3.5.1. Production and metabolic traits

Differences in production traits and NEFA among biopsy days were determined within experiment using a repeated measures model implemented by the PROC MIXED procedure of SAS [28]. The analysis model included biopsy day as a fixed factor, replicate as a random factor (feed restriction and GH experiments), and cow as a random repeated factor sampled across biopsy day. Covariance matrices, including compound symmetry (CS), autoregressive heterogeneity (ARH), and unstructured (US) were evaluated. The CS or ARH matrix was used in analyses of production data, based on Akaike information content. Non-esterified fatty acid concentrations were analyzed following a natural log transformation to ensure a normal distribution of residuals. For the transition experiment, the weekly NEFA sample collected closest to the day of biopsy was used for analysis. When the overall effect of biopsy day was significant (P < 0.05), differences among biopsy days were determined through pairwise comparisons using the PDIFF procedure of SAS [28]. Means and SEMs for production traits and NEFA are presented in Table 2. Because the 21 d AP biopsies were taken over a range of 26 d for the transition experiment, correlations between actual day AP, and energy balance and NEFA were investigated for these samples using the PROC CORR procedure of SAS [28].

2.3.5.2. Quantitative PCR data

The ΔΔCt analysis method was used to evaluate results of quantitative PCR. The average Ct for each sample and gene was normalized by subtracting the average Ct of the reference gene [29]. Each PCR run was tested for PCR efficiency
using the diluted standard curve. The Ct values for leptin and adiponectin in the transition experiment were adjusted for PCR efficiency, as previously described [30], because they had PCR efficiencies outside an acceptable range of 90 to 110% (72% and 168%, respectively). Differences in mRNA abundance were determined within experiment by analysis of the normalized Ct values, as previously described [30]. The mixed model analysis included the same fixed and random factors as described in section 3.3.5.1., except that PCR plate was also included as a random effect. When the overall effect of biopsy day was significant (P ≤ 0.05), differences among biopsy days were determined through pairwise comparisons using the PDIFP procedure of SAS [28]. Results are presented as relative fold change [29] and expressed relative to 21 d AP for the transition period, day -6 for the feed restriction experiment, or day -4 for the GH experiment. Standard errors of the means of the fold change were calculated as previously described [30]. Correlations between normalized Ct values and actual days AP were calculated for the 21 d AP samples of the transition experiment, as described in section 2.3.5.1.

2.4 RESULTS

2.4.1 Transition period

Milk synthesis increased during the transition period from 5 to 21 d PP, and remained elevated from 21 to 150 d PP (Table 2). The increase in energy requirements during the transition period resulted in negative energy balance during the first 21 d PP, as well as increased NEFA concentrations (Table 2). Serum concentrations of NEFA were greater at 5 d PP compared to all other sampling times.
(Table 2). Correlations of energy balance and NEFA with days AP were non-significant.

The mRNA abundance of β-actin and RPL32 mRNA did not change with sampling day or treatment (P = 0.209, and P = 0.257 to 0.385 for β-actin and RPL32, respectively). Additionally, no consistent trend in mRNA abundance across experimental treatments was observed, and variability across all treatments was similar for both genes (Figure 1). Thus, both β-actin and RPL32 are appropriate reference genes for these experiments.

The transition period was accompanied by significant changes in ANGPTL4 and adiponectin mRNA abundance (P < 0.001, P = 0.050, respectively). The mRNA abundance of ANGPTL4 was greater at 21 d AP, 5 d PP, and 21 d PP compared to 150 d PP (P = 0.003, P < 0.0001, P < 0.0001, respectively), and was elevated at 5 d PP and 21 d PP compared to 21 d AP (P = 0.012 and P = 0.006, respectively; Figure 2A). Adiponectin mRNA decreased at 5 and 21 d PP compared to 21 d AP (P = 0.011 and P = 0.038, respectively; Figure 2B). Leptin and adiponectin receptor 2 mRNA abundance did not change with biopsy day (P = 0.083 and P = 0.483, respectively). A correlation between AP sampling day and normalized Ct values for leptin was observed (r = -0.52, P = 0.01). Correlations for AP sampling day with ANGPTL4, adiponectin, and adiponectin receptor 2 were not significant (P > 0.10). Analyses of ANGPTL4, adiponectin receptor 2, leptin, and adiponectin utilized the CS, ARH, US, and CS covariance matrices, respectively.
2.4.2 Feed restriction

Restricting feed intake to mimic transition energy balance during mid lactation resulted in a decrease in milk production within 1 d of feed restriction, and milk yield continued to decrease throughout the 4 d experiment (Table 2). Energy balance decreased after 1 d of feed restriction and remained negative through the 4 d restriction. As designed, feed restriction resulted in a similar degree of negative energy balance as observed during early lactation (Table 2). Plasma NEFA concentrations increased on days 1 and 4 of feed restriction, relative to the day -6 concentration (Table 2). Days in lactation were similar between cows in the two experimental replicates (P = 0.42), and the effect of replicate was non-significant in all analyses (P > 0.20).

The quantity of RNA or quality of cDNA was unacceptable for >1 samples from 3 cows. Therefore, all mRNA data from these cows were excluded from PCR analyses. Changes in leptin and ANGPTL4 mRNA abundance were observed following feed restriction (P = 0.005, P = 0.002, respectively). Leptin mRNA abundance decreased following 4 d of feed restriction (P < 0.001; Figure 2C), while ANGPTL4 mRNA abundance increased with 1 and 4 d of feed restriction (P = 0.002 and P = 0.034, respectively; Figure 2A). The overall effect of biopsy day on adiponectin (P = 0.129; Figure 2B) and adiponectin receptor 2 (P = 0.399; Figures 2D) mRNA abundance was not significant. Analyses of all genes for the feed restriction experiment utilized the CS covariance matrix.
2.4.3 Growth hormone administration

Milk production increased and energy balance decreased over the 7 d after GH administration (Table 2). Average energy balance of GH treated cows remained slightly positive throughout the experiment, but altered lipid metabolism was confirmed by an increase in NEFA concentration (Table 2). Days in lactation were similar between cows in the two experimental replicates ($P = 0.50$), and the effect of replicate was non-significant in all analyses ($P > 0.20$).

Changes in ANGPTL4 mRNA abundance were observed following GH administration ($P < 0.001$), as it increased 3 and 7 d following GH treatment ($P < 0.001$; Figure 2A). The overall effect of biopsy day was not significant for leptin ($P = 0.063$; Figure 2C), adiponectin ($P = 0.075$; Figure 2B) or adiponectin receptor 2 ($P = 0.902$; Figure 2D) mRNA abundance following GH administration. Analyses of ANGPTL4, adiponectin receptor 2 and leptin utilized the CS covariance matrix, and analysis of adiponectin utilized the ARH matrix.

2.5 DISCUSSION

This study utilized 3 different models of declining energy balance to characterize the mRNA profile of ANGPTL4 as part of an overall objective to understand the potential role of ANGPTL4 in energy homeostasis in lactating dairy cows. Although different cows were utilized in each experiment, basal production and metabolic status were similar among cows in each experiment. Each experiment successfully reduced energy balance, with negative energy balance occurring in the transition and feed restriction experiments. However, altered energy status occurred via different mechanisms. In the transition experiment, negative energy balance at 5
and 21 d PP occurred because energy intake did not keep pace with increased energy requirements for milk synthesis at the onset of lactation. The feed restriction experiment also resulted in negative energy balance due to insufficient energy intake, but daily milk production decreased with declining energy balance in this experiment, in contrast to increasing milk production with the onset of lactation. Administration of GH resulted in a significant reduction in energy balance, although the average energy balance across cows remained slightly positive. This decline in energy balance was primarily driven by increasing milk production, although a small decline in feed intake was also observed. Importantly, each model led to increased circulating NEFA concentrations, indicating altered lipid metabolism.

It should be noted that blood sampling for NEFA analysis occurred immediately after the biopsy procedure in the feed restriction and GH experiments, and for the 150 d PP samples of the transition experiment. Stress may alter circulating concentrations of catecholamines, which may in turn impact circulating NEFA concentrations [31]. Thus, the stress of the biopsy procedure (~ 15 min in duration) may have impacted NEFA concentrations in the present study. Basal NEFA concentrations observed in this study (0.14 to 0.18 mM) are slightly greater than those previously reported in similar studies (0.09 to 0.13 mM, [16,32]). However, the magnitude of change in response to GH administration and short term feed restriction are similar. Thus, the potential impact of the biopsy procedure on circulating NEFA concentrations in this study is likely minimal.

The abundance of ANGPTL4 mRNA increased with declining energy balance in lactating dairy cows, regardless of whether the change in energy status occurred
via the onset of lactation, feed restriction, or GH administration. These results support and extend existing data describing increased ANGPTL4 expression with pregnancy and the onset of lactation in mice and cattle [10,11]. It is important to note that ANGPTL4 mRNA abundance changed dramatically at all time points investigated, even with relatively small changes in energy status. Thus, ANGPTL4 may serve as an early autocrine/paracrine or endocrine signal for changing energy status in lactating cows.

Angiopoietin-like protein 4 impacts lipid metabolism through the inhibition of LPL. Specifically, ANGPTL4 converts active LPL dimers to inactive monomers, thereby limiting the release of lipids from circulating chylomicrons [33]. Lipoprotein lipase is expressed and active at multiple tissues including adipose, cardiac and skeletal muscle, and the mammary gland. However, LPL activity is often regulated in a tissue specific manner [34], and this regulation may be due in part to ANGPTL4. For example, cardiac-specific over-expression of ANGPTL4 in mice resulted in a significant decrease in cardiac LPL activity and triglyceride content, but liver, skeletal muscle, and adipose LPL activity were not altered [35]. In cattle, the activity of adipose LPL decreases with the onset of lactation [36], contributing to the partitioning of energy toward the mammary gland to support milk synthesis. The regulation of LPL may be due in part to reduced mRNA expression, as reduced LPL mRNA has been described with the onset of lactation [11] and in response to GH administration [37]. However, changes in LPL activity in the absence of altered mRNA have also been described in non-lactating cows following feed restriction [38], suggesting a mechanism for post-transcriptional regulation of LPL in cattle. Data
from the current study support the hypothesis that \textit{ANGPTL4} mRNA is regulated in response to changing energy status in lactating dairy cows as part of a mechanism to partition energy away from adipose tissue through inhibition of LPL activity.

Lipolytic effects have also been associated with \textit{ANGPTL4}. Transgenic mice that mildly overexpressed \textit{ANGPTL4} in adipose tissue had approximately 50\% less white adipose tissue mass compared to their non-transgenic counterparts, while consuming similar quantities of feed \cite{6}. These mice also had increased circulating triglycerides, likely due to the inhibitory effect of \textit{ANGPTL4} on LPL, and increased circulating NEFA and glycerol concentrations. Increased circulating triglycerides and NEFA were also observed 30 min after injection of recombinant \textit{ANGPTL4} in mice \cite{4}. The increase in circulating NEFA and glycerol with increased \textit{ANGPTL4} suggest a direct lipolytic effect of \textit{ANGPTL4} \cite{4,6}. Additionally, mice lacking \textit{ANGPTL4} had diminished lipolysis following stimulation by catecholamines and glucocorticoids. In the same study, lipolysis was rescued with the addition of \textit{ANGPTL4} to adipocytes isolated from the \textit{ANGPTL4} deficient mice \cite{7}. These results suggest \textit{ANGPTL4} contributes to changes in lipid metabolism that favor mobilization and utilization of energy from lipid reserves, while inhibiting lipid storage.

\textit{Angiopoietin-like protein 4} is expressed by multiple tissues in cattle, with greatest expression in liver and adipose tissue \cite{39}. Increased liver \textit{ANGPTL4} mRNA abundance with feed restriction has been described in cattle \cite{40}, and is consistent with the current results for adipose tissue. Circulating concentrations of \textit{ANGPTL4} were not measured in the current study, and the relative importance of circulating concentration versus local expression of \textit{ANGPTL4} in adipose depots is
currently unknown. However, further investigation of ANGPTL4 as an adipokine closely linked to physiological changes associated with altered energy status, particularly in the context of lactation, is warranted.

Leptin mRNA abundance decreased with feed restriction, consistent with previous results [16]. Although a numerical decline in leptin mRNA was observed with the onset of lactation in the current experiment, this change did not achieve statistical significance. A non-significant decline in leptin mRNA from pregnancy to lactation has been described previously [15,17], while others have reported consistent and statistically significant declines in leptin mRNA and circulating plasma levels at the onset of lactation [14]. Differences in statistical significance among studies may be related to differences in the timing of sampling or the severity of negative energy balance occurring across experiments. For example, the correlation between AP sampling day and leptin mRNA abundance observed in the current study suggests leptin mRNA may increase in the pre-partum period as cows approach parturition. This trend may influence the magnitude of change in leptin mRNA observed between AP and PP samples. Regardless, a consistent decline in leptin expression with the onset of lactation has been described across multiple studies, and this regulation has been specifically associated with negative energy balance [14]. In the current study, there was a trend for a decline in leptin mRNA 7 d following GH administration. Previously, changes in plasma leptin concentrations following 3 d of GH administration were not evident [16,18]. It is unlikely that GH has a direct effect on leptin expression, but results from the current research suggest a potential transient decline in leptin mRNA with altered energy balance
following GH administration. Both autocrine and endocrine actions of leptin have been described, as it can directly stimulate lipolysis in adipose tissue and act through the central nervous system to regulate appetite [41]. It has been proposed that the primary consequence of declining leptin concentration in cattle is appetite regulation [14]. This hypothesis is consistent with the increase in feed intake that occurs in early lactation, and the need for increased energy intake with declining energy balance due to feed restriction or GH administration.

Adiponectin mRNA abundance decreased with the onset of lactation in the current study, in contrast to previous results that described no change in adiponectin mRNA with the onset of lactation [15]. However, changes in adiponectin mRNA were not observed with feed restriction or GH administration in the current experiments. Thus, adiponectin mRNA is not consistently regulated in response to altered energy balance. Adiponectin is recognized as an important regulator of insulin sensitivity [21], and the decline in adiponectin mRNA with the onset of lactation coincides with an expected decrease in insulin sensitivity in lactating cows [42]. However, additional research is needed to fully explore the relationships between adiponectin mRNA and protein expression, its circulating multimeric complexes [43], and insulin signaling in dairy cattle. Adiponectin signals through two receptors, adiponectin receptor 1 and 2. Adiponectin receptor 2 is recognized as the primary functional adiponectin receptor in adipose of rodents, humans, and cattle [22,23,44], and was the receptor of primary interest in the current study. Preliminary analyses confirmed that adiponectin receptor 1 was lowly expressed in the bovine adipose tissue investigated (data not shown), but further optimization of experimental conditions
was not pursued. Significant changes in adiponectin receptor 2 mRNA were not observed in any of the experimental models investigated in this study. Previous investigations of adiponectin receptor 2 mRNA report inconsistent results, as adiponectin receptor 2 mRNA either decreased [15] or did not change following parturition [17]. In the current study, mRNA abundance for adiponectin receptor 2 was highly variable across all experiments.

In summary, this research identifies ANGPTL4 as an adipokine that is markedly responsive to changes in energy balance in the lactating dairy cow. Abundance of ANGPTL4 mRNA consistently increased with declining energy balance across 3 models of altered energy balance. The regulation of ANGPTL4 differs from that of leptin and adiponectin, suggesting a fundamental role for this adipokine in the regulation of energy metabolism in the dairy cow. Additionally, the mRNA abundance of ANGPTL4 increased dramatically at the earliest time point sampled in each model. These observations differ from previous work in rodents that concluded ANGPTL4 is not an indicator of overall nutritional status [45]. Future studies to determine whether ANGPTL4 participates directly in the regulation of lipolysis in the adipocyte will be of particular importance.

2.6 ACKNOWLEDGMENTS

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### 2.7 TABLES

**Table 1.** Sequences of primers used for quantitative PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Accession Number</th>
<th>Exon</th>
<th>Amplicon Size (nucleotides)</th>
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<tr>
<td>ANGPTL4</td>
<td>5'- AAG TGG ATT GTT CCA GAT CC -3'</td>
<td>NM_001046043.2</td>
<td>4</td>
<td>148</td>
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<td>ANGPTL4</td>
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<td>5</td>
<td></td>
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<tr>
<td>AdipQ R2</td>
<td>5'- CAT CTG GAC ACA TCT CTT GG -3'</td>
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<td>269</td>
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<td>AdipQ R2</td>
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<td></td>
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<tr>
<td>Adiponectin</td>
<td>5'- TCC TAC TTC CAC CCT GAC TGA -3'</td>
<td>NM_174742.2</td>
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<tr>
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<td></td>
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<tr>
<td>RPL32</td>
<td>5'- GGA CCA AGA AGT TCA TTA GGC - 3'</td>
<td>NM_001034783.1</td>
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<td>138</td>
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<tr>
<td>RPL32</td>
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<td>β-Actin</td>
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Abbreviations: ANGPTL4, angiopoietin-like protein 4; AdipQ R2, adiponectin receptor 2; RPL32, 60S ribosomal protein L32

Reference genes used for normalization of mRNA abundance.
Table 2. Mean ± SEM of daily milk yield, BW, dry matter intake, energy balance, and circulating concentrations of NEFA in lactating Holstein cows during altered energy balance due to the onset of lactation (transition), feed restriction, or following administration of bovine GH.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>21 d AP</th>
<th>5 d PP</th>
<th>21 d PP</th>
<th>150 d PP</th>
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<tr>
<td>Days in Lactation</td>
<td>18.9±1.0</td>
<td>5.6±0.2</td>
<td>22.3±0.4</td>
<td>154.2±0.5</td>
</tr>
<tr>
<td>Range in Days of Lactation</td>
<td>6 to 32</td>
<td>5 to 9</td>
<td>21 to 31</td>
<td>151 to 161</td>
</tr>
<tr>
<td>Milk Yield (kg/d)</td>
<td>-</td>
<td>36.2±1.1</td>
<td>46.8±1.1</td>
<td>43.6±1.2</td>
</tr>
<tr>
<td>BW (kg)</td>
<td>713a±15</td>
<td>624b±13</td>
<td>598c±11</td>
<td>625b±11</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>11.35a±0.62</td>
<td>16.81b±0.68</td>
<td>21.66c±0.62</td>
<td>26.15d±0.70</td>
</tr>
<tr>
<td>EB (MCal/d)</td>
<td>2.56a±0.49</td>
<td>-7.38b±1.29</td>
<td>-7.22b±1.03</td>
<td>4.91c±1.17</td>
</tr>
<tr>
<td>NEFA (mM)</td>
<td>0.18a±0.02</td>
<td>0.75c±0.09</td>
<td>0.38b±0.04</td>
<td>0.17a±0.02</td>
</tr>
</tbody>
</table>

Feed Restriction

<table>
<thead>
<tr>
<th>Day -6</th>
<th>Day 1</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=19</td>
<td>n=19</td>
<td>n=19</td>
</tr>
<tr>
<td>Days in Lactation</td>
<td>180±5</td>
<td>188±5</td>
</tr>
<tr>
<td>Range in Days of Lactation</td>
<td>157 to 206</td>
<td>165 to 214</td>
</tr>
<tr>
<td>Milk Yield (kg/d)</td>
<td>38.7a±1.0</td>
<td>31.0b±1.1</td>
</tr>
<tr>
<td>BW (kg)</td>
<td>664±12</td>
<td>664±12</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>23.91a±0.53</td>
<td>14.63b±0.39</td>
</tr>
<tr>
<td>EB (MCal/d)</td>
<td>5.00a±1.23</td>
<td>-6.17b±0.69</td>
</tr>
<tr>
<td>NEFA (mM)</td>
<td>0.14a±0.007</td>
<td>0.33b±0.03</td>
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</table>

Growth Hormone

<table>
<thead>
<tr>
<th>Day -4</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=20</td>
<td>n=20</td>
<td>n=20</td>
</tr>
<tr>
<td>Days in Lactation</td>
<td>183±6</td>
<td>190±6</td>
</tr>
<tr>
<td>Range in Days of Lactation</td>
<td>154 to 216</td>
<td>161 to 223</td>
</tr>
<tr>
<td>Milk Yield (kg/d)</td>
<td>40.9a±1.3</td>
<td>44.6b±1.5</td>
</tr>
<tr>
<td>BW (kg)</td>
<td>713±10.6</td>
<td>713±10.6</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>27.41a±0.57</td>
<td>26.50a,b±0.57</td>
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<td>EB (MCal/d)</td>
<td>6.47a±0.91</td>
<td>2.46b±0.82</td>
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<tr>
<td>NEFA (mM)</td>
<td>0.17a±0.01</td>
<td>0.35b±0.04</td>
</tr>
</tbody>
</table>

a,b,c Means within experiment with different superscripts differ, P < 0.05.
For all experiments and traits, the overall effect of experimental time point was significant (P < 0.01).

Means and SEM were back-transformed following statistical analysis.

The average of three BW was used to represent all three experimental time points, as maintenance energy requirements were not expected to change over the short duration of the experiment.

Abbreviations: d AP, days antepartum; d PP, days postpartum; DMI, dry matter intake; EB, energy balance.
2.8 FIGURES

**Figure 1. Quantification of reference genes in three models of altered energy status.** (A) Quantitative PCR cycle thresholds (± SEM) for β-actin and 60S ribosomal protein L32 (RPL32) from adipose tissue representing cows transitioning from late pregnancy to lactation. Sampling days include approximately 21 d AP (-21; n=26), and 5 (n=26), 21 (n=26) or 150 (n=21) d PP. (B) Quantitative PCR cycle thresholds (± SEM) for RPL32 following feed restriction or growth hormone administration. Sample 1, 2 and 3 represent days -6, 1, and 4 relative to initiation of feed restriction on day 0, or days -4, 3, and 7 relative to administration of growth hormone on day 0, respectively.

**Figure 2. Relative adipose tissue mRNA abundance of (A) angiopoietin like protein 4 (ANGPTL4), (B) adiponectin, (C) leptin, and (D) adiponectin receptor 2 in three models of altered energy status.** Samples in the transition experiment represent approximately 21 d AP (-21; n=26), and 5 (n=26), 21 (n=26) or 150 (n=21) d PP. Samples for the feed restriction (n=16) and GH (n=20) experiments are expressed as days relative to the initiation of feed restriction or GH administration on day 0. Data are expressed as mRNA fold-change (± SEM) relative to mRNA abundance in the 21 d AP, day -6, or day -4 sample for the transition, feed restriction, and GH experiments, respectively. In the transition experiment, leptin and ANGPTL4 were normalized to β-actin, and adiponectin and adiponectin receptor 2 were normalized to 60S ribosomal protein L32 (RPL32). All genes were normalized to RPL32 in the feed restriction and
GH experiments. If the overall effect of sampling day was significant (P ≤ 0.05), means with different letters within experiment are different (P < 0.05).
Figure 1
Figure 2

A. Transition  Feed restriction  GH

B. Transition  Feed restriction  GH

C. Transition  Feed restriction  GH

D. Transition  Feed restriction  GH
2.9 REFERENCES


CHAPTER 3. COORDINATION OF LIPID DROPLET-ASSOCIATED PROTEINS DURING THE TRANSITION PERIOD OF HOLSTEIN DAIRY COWS

A paper published in the Journal of Dairy Science

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3.1 ABSTRACT

Dairy cows often experience negative energy balance with the onset of lactation, and severe or prolonged negative energy balance can contribute to declines in overall fitness. Energy stores, in adipose tissue triacylglycerides, are mobilized during times of energy deficit, and recent research has implicated several proteins associated with the lipid droplet as lipolytic regulators. The objective of this study was to determine if these novel proteins associated with lipolytic regulation are altered with the changing metabolic demands of lactation. Weekly blood samples were collected from 26 Holstein cows from 21 d prior to expected parturition through 28 d postpartum, and again at 150 d postpartum. Serum non-esterified fatty acids, glycerol, and beta hydroxybutyrate were measured. Energy balance was calculated from daily feed intake and milk yield, weekly body weight, and monthly milk component measurements. Adipose tissue biopsies were taken 21 d prior to expected parturition (-21), and 5, 21, and 150 d postpartum. Semi-quantitative western blotting was used to measure abundance of hormone sensitive
lipase (HSL), phosphorylated HSL, perilipin, phosphorylated perilipin (PPLIN), adipose triglyceride lipase (ATGL), and comparative gene identity-58 (CGI-58). Abundance of ATGL was less at 5 and 21 DIM compared to -21 and 150 DIM, even though cows were in negative energy balance and experiencing increased rates of lipolysis in early lactation. In contrast, phosphorylated HSL and PPLIN increased with increasing lipolysis immediately after parturition. Additionally, PPLIN was negatively correlated with milk yield at 5, 21, and 150 d postpartum, and negatively correlated with feed intake and energy balance at 21 d postpartum. This result is consistent with the hypothesis that phosphorylation of perilipin is responsive to signals for increased triacylglyceride mobilization. Finally, a consistent negative correlation between abundance of perilipin and CGI-58 proteins was observed throughout the transition period. These results confirm that novel lipolytic proteins in adipose tissue are regulated at the level of protein abundance and phosphorylation during the periparturient period and into mid lactation.

**Keywords:** perilipin, adipose triglyceride lipase, transition period, energy balance

### 3.2 INTRODUCTION

Milk production per cow has doubled over the last 40 yr, resulting in increased energy requirements throughout lactation (AIPL, 2010). It is widely recognized that energy demands due to the initiation of milk synthesis in early lactation are often not met by energy intake, causing cows to experience negative energy balance. Although a period of negative energy balance is a natural part of the lactation cycle, severe and prolonged negative energy balance can contribute to declines in fitness traits, including fertility (Lopez et al., 2005; Veerkamp et al., 2000), udder health (Banos et al., 2006),
locomotive problems (Collard et al., 2000), and disease susceptibility (Hammon et al., 2006; van Knegsel et al., 2007).

During times of negative energy balance, mobilization of energy reserves from adipose tissue is a primary mechanism by which dairy cows compensate for their energy deficit. It is well established that activation of hormone-sensitive lipase (HSL) via protein kinase A (PKA) is a critical pathway to the regulation of lipolysis. More recently, additional proteins have been associated with lipolytic regulation (Figure 1). Perilipin (PLIN) is a phosphoprotein that associates with lipid droplets. In the basal state, PLIN protects the lipid droplet from HSL mediated lipolysis (Brasaemle et al., 2000b) and co-localizes with the protein comparative gene identity-58 (CGI-58) (Subramanian et al., 2004; Yamaguchi et al., 2006). The phosphorylation of PLIN by PKA stimulates a conformational change in PLIN that provides HSL access to the lipid droplet and facilitates the interaction between HSL and its lipid substrate (Miyoshi et al., 2006). Additionally, phosphorylation of PLIN results in the release of CGI-58 from the lipid droplet. When CGI-58 is not associated with PLIN, it acts as an activator of adipose triglyceride lipase (ATGL) in the cytosol. The lipase ATGL can then translocate to the lipid droplet where it hydrolyzes triacylglycerides to diacylglycerides (Granneman et al., 2007; Schweiger et al., 2008) (Figure 1). It has been suggested that the hydrolysis of triacylglycerides to diacylglycerides by ATGL is the rate limiting step in lipolysis (Haemmerle et al., 2006), and that phosphorylation of PLIN is a “master regulator” of lipolysis through its control of both HSL and ATGL, with the latter mediated by CGI-58 (Miyoshi et al., 2007; Moore et al., 2005).
Appropriate regulation of lipolysis is critical for dairy cows to maintain health and productivity in times of negative energy balance. However, the coordinated regulation of HSL, PLIN, ATGL, and CGI-58 has not yet been studied in cattle. Thus, the current research was undertaken to determine if these proteins are altered at the level of protein abundance or phosphorylation with the changing metabolic demands of lactation. First, we quantified changes in protein abundance of HSL, PLIN, ATGL and CGI-58, as well as phosphorylation of HSL and PLIN, over the transition period. Second, we defined the relationship of these lipolytic proteins with traits influencing energy balance. Finally, we identified correlated changes among the lipolytic regulatory proteins.

### 3.3 MATERIALS AND METHODS

All procedures involving the use of animals were approved by the Iowa State University Institutional Animal Care and Use Committee.

#### 3.3.1 Animals

Individual feed intake was measured daily on 26 multiparous (13, 7, and 6 cows in lactation 2, 3, and 4, respectively) Holstein cows using the Calan Broadbent Feeding system®. Feed intake was measured from approximately 30 d prior to parturition through 150 DIM. Pre-calving and lactating cows were fed once or twice daily, respectively, and orts were removed and weighed daily. Cows were provided ad libitum access to a consistent total mixed ration (TMR) formulated to meet or exceed dry or lactating cow nutritional requirements (Clark, B.D., Erdman, R., Goff, J., Grummer, R., Linn, J., Pell, A., Schwab, C., Tomkins, T., Varga, G., Weiss, 2001). Lactating and non-
lactating cows were weighed weekly following their morning milking and in early afternoon, respectively. Body condition scores (BCS) were determined weekly using a 1 to 5 scale (Elanco, 1996).

3.3.2 Milk Production and Milk Components

Cows were milked twice daily with milk yield recorded at each milking. The software program Bestpred (Cole et al., 2009) was used to predict daily milk fat and protein percent based on monthly DHIA test day results. Additional milk samples were taken at the morning milking and analyzed for lactose content by a commercial laboratory (Dairy Lab Services, Dubuque, IA).

3.3.3 Blood and Tissue Sample Collection

Blood was collected weekly via jugular venipuncture beginning at approximately 21 d prior to parturition through 28 DIM, and again at the completion of the study, approximately 150 DIM. Serum was stored at -80°C until future analysis. Serum glycerol concentrations were measured using Free Glycerol Reagent (Sigma Aldrich, F6428, St. Louis, MO) according to manufacturer’s protocol. Non-esterified fatty acids (NEFA) and BHBA concentrations were analyzed from serum using commercially available kits according to the manufacturer’s protocol (NEFA and Ranbut assay kits, respectively, Randox Laboratories, Co. Antrim, United Kingdom).

Subcutaneous adipose tissue was collected from the tailhead region using a minimally invasive procedure. Briefly, the tailhead region was shaved and cleaned prior to administration of local anesthetic (2% Lidocaine). Following standard surgical
preparation, a straight line incision (approximately 3 cm) was made. Tissue was excised using forceps and scalpel blade, and incisions were closed using surgical staples. With multiple sampling from each cow, samples were taken from alternating sides of the cow, and only from areas devoid of scar tissue. Four 1 g samples of adipose tissue were collected at each biopsy. Biopsies were taken from each cow at 8 to 31 d prepartum (-21 DIM; n=26), 5 to 9 DIM (5 DIM; n=26), 21 to 31 DIM (21 DIM; n=26), and 151 to 161 DIM (150 DIM; n=21). Adipose tissue was immediately frozen in liquid nitrogen and stored at -80°C until protein analysis.

3.3.4 Semi-Quantitative Western Blotting

Semi-quantitative western blotting assays were optimized for each protein of interest to validate linearity of detection for the range of protein abundance for each protein of interest. Proteins were extracted from adipose tissue samples and prepared for Western blotting as previously described (Elkins and Spurlock, 2009). Protein concentrations were determined by BCA assay (Pierce Protein Research, 23227, Rockford, IL) and used to standardize the quantity of total protein loaded on each gel. Proteins (10 to 400 µg per lane, depending on protein of interest) were separated overnight through 10% [for detection of phosphorylated PLIN (PPLIN)] or 8 % (for detection of all other proteins) SDS-PAGE gels, and transferred to polyvinylidene fluoride membranes. Membranes were incubated with the following primary antibodies: PLIN (Chemicon, AB10200; 1:1000), phosphorylated PLIN (Chemicon, AB10200; 1:5000), HSL (Cell Signaling, 4107;1:1000), Phospho HSL (PHSL) at Ser 563 (Cell Signaling, 4139;1:500), ATGL (Cell Signaling, 2138 ; 1:500), and CGI-58 (Everest Biotech, EB07657;1:200). Following incubation with secondary antibody [anti-goat IgG
horseradish peroxidase-linked whole antibody from Santa Cruz Biotechnology (Santa Cruz, CA) for CGI-58, or anti-rabbit IgG horseradish peroxidase-linked whole antibody from GE Healthcare (Pittsburgh, PA) for all other proteins], proteins were detected using the ECL Plus Western Detection Kit (Amersham Pittsburgh, PA) and imaged with Alpha Innotech Imager (FluorChem FC2, Cell Biosciences, Santa Clara, CA). Detected protein band areas were quantified using Total Lab software for 1D analysis (TL100, v2009; Total Lab Limited, Newcastle upon Tyne, UK). All samples were run on duplicate gels. Protein abundance was normalized to abundance of the protein of interest quantified from a standard. The standard was an arbitrarily chosen sample that was loaded in lanes flanking samples from each cow on each gel. Normalization was achieved by subtracting the average standard protein abundance (measured in 2 lanes) from each sample. Normalized protein abundance was averaged across duplicate gels, and a constant was added to the normalized value of each sample for ease of statistical analysis and interpretation. For PPLIN, only the phosphorylated band (upper band) was used for data analysis.

3.3.5 Energy Balance Calculations

Net energy balance was predicted using published equations (Clark, B.D., Erdman, R., Goff, J., Grummer, R., Linn, J., Pell, A., Schwab, C., Tomkins, T., Varga, G., Weiss, 2001). Samples of the TMR were collected from a minimum of three feedings per week, pooled, and analyzed by a commercial laboratory (Dairyland Laboratories, Arcadia, WI) using wet chemistry. Net energy for lactation of the TMR was determined using Ohio Agricultural Research and Development Center (OARDC) equations for cows at 3 multiples of maintenance. Net energy of feed intake (NEi) was calculated as
the product of daily feed intake (kg), dry matter percentage of the TMR, and NE_L of the TMR. Daily feed intake was the average intake over three consecutive days within three days of tissue collection of lactating cows, or within 7 days of tissue collection for non-lactating cows. The NE_L was calculated as [(0.0929 x fat% + 0.0563 x crude protein% + 0.0395 x lactose%) x kg milk yield] using the average of three days of actual milk yield (tissue collection day, +/- 1d), fat and protein percent predicted from the Bestpred program (Cole et al., 2009) for the day of tissue collection, and the average of two lactose percent estimates from samples collected within 4 days before and after the day of tissue collection. Net energy for maintenance (NE_M) was estimated as [0.08 * (BW^{0.75} kg)]. Body weight was the average of two measurements, taken within one week before and after the day of tissue collection. For energy balance estimates at 5 DIM, BW represented the first weight measured after the tissue collection day if the previous week’s weight was taken prior to calving. Net energy of pregnancy (NE_P) was estimated as [(0.00318 x d of gestation – 0.0352) x (calf birth weight/45)]/0.218. Energy balance for lactating cows was calculated as NE_I – (NE_M + NE_LAC), and for non-lactating cows as NE_I – (NE_M + NE_P).

3.3.6 Data Analysis

All data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene’s test, respectively (SAS/STAT User’s Guide, 1999). Serum metabolite data were non-normally distributed and lacked homoscedasticity, and were transformed using the natural log transformation (log_e) to achieve normality. Abundance of HSL and PHSL were also non-normally distributed. Multiple transformations were evaluated but none resulted in a normal distribution. The natural log transformation (log_e) transformation
was used for analysis of these proteins, because the mixed model analysis failed to converge when non-transformed data were used. Results of the mixed model analysis were confirmed using the Kruskal-Wallis test ((SAS/STAT User’s Guide, 1999); results not shown). Differences among biopsy days were determined using the mixed model methodology with repeated measures in SAS (SAS/STAT User’s Guide, 1999). Adipose tissue biopsy day, or week of blood sampling were classified as categorical fixed effects, and cow was included in the model as a random factor. Day or week of sampling was the random factor. Alternate covariance structures were tested, and compound symmetry (CS), unstructured (UN), or heterogeneous first order autoregressive structure (ARH) resulted in models with the best fit. Correlations were determined using the PROC CORR procedure in SAS (SAS/STAT User’s Guide, 1999). Non-parametric Spearman correlations were calculated for all correlations involving transformed variables (NEFA, glycerol, HSL, PHSL). For correlations between protein and metabolite data, metabolite data were used if collected within 3 d of the adipose tissue biopsy.

3.4 RESULTS

Milk production based on 305 d mature equivalent for the completed lactation encompassing the experiment averaged 14,025 kg. As expected, cows utilized body energy reserves with the onset of lactation, as indicated by decreased BW ($P < 0.0001$; Figure 2A) and BCS ($P < 0.0001$; Figure 2B). Energy balance differed significantly across biopsy days, with cows experiencing negative energy balance at 5 and 21 DIM (Figure 2C).
Serum concentrations of NEFA, glycerol, and BHBA were evaluated as indicators of lipolysis. Serum NEFA concentrations differed significantly during sampling ($P < 0.0001$; Figure 3A). Increased NEFA concentrations were observed one week prior to calving through 3 weeks postpartum. Similarly, glycerol concentrations differed during the sampling period ($P < 0.0001$; Figure 3B.) Elevated glycerol concentrations were observed at calving and 1 wk postpartum. Serum BHBA concentrations were increased at calving relative to other weeks evaluated (Figure 3C).

3.4.1 Lipid Droplet-Associated Proteins

The protein abundance of ATGL differed significantly over the sampling period ($P < 0.0001$), with decreased abundance observed at 5 and 21 DIM relative to pre-calving and 150 DIM. Abundance of HSL ($P = 0.43$), PLIN ($P = 0.71$), and CGI-58 ($P = 0.13$) did not differ among sampling times, although there was a numerical trend for increasing CGI-58 with stage of lactation. Phosphorylation of HSL ($P = 0.02$) and PLIN ($P = 0.07$) increased with the onset of lactation. Phosphorylation of HSL was similar pre-calving and at 150 DIM, and elevated at 5 and 21 DIM. Phosphorylated PLIN was significantly elevated at 5 DIM relative to 150 DIM ($P = 0.01$), with intermediate levels observed pre-calving and at 21 DIM (Figure 4).

3.4.2 Correlations between Lipolytic Indicators and Regulatory Proteins

Correlations were calculated to evaluate the relationship between lipolytic indicators and proteins of interest. As expected, a significant ($P < 0.05$) correlation was found for NEFA with PPLIN, PHSL, and ATGL ($r = 0.247, 0.372, \text{ and } -0.376$, respectively). Additionally, significant correlations were observed for milk yield on the
day of biopsy with PPLIN and PHSL ($r = -0.310$ and -0.262, respectively), for DMI with PPLIN ($r = -0.292$), and for energy balance with PHSL and ATGL ($r = -0.293$ and 0.359, respectively. Because these correlations may reflect changes across biopsies, correlations were also calculated within biopsy day. Within biopsy day, correlations between NEFA and proteins of interest were not significant. The correlation between milk yield on the day of biopsy with PPLIN was significant at both 5 and 21 DIM, but with PHSL was only significant at 150DIM. Correlations of milk yield with HSL at 150 DIM, and with PPLIN at 150 DIM showed a statistical trend ($P < 0.10$). Dry matter intake was significantly correlated with PPLIN at 21 DIM and PHSL prior to calving. The correlation with ATGL or PHSL with energy balance was non-significant when analyzed within biopsy day. Energy balance was also correlated with total PLIN abundance at 150 DIM (Tables 1 and 2).

3.4.3 Correlations among Lipolytic Proteins

Analysis of all data revealed a positive correlation between PLIN and HSL ($r = 0.259$, $P = 0.010$). Perilipin and HSL were negatively correlated with CGI-58 ($r = -0.448$, $P < 0.001$ and $r = -0.274$, $P = 0.006$, respectively). Phosphorylation of PLIN and HSL are also correlated ($r = 0.420$, $P < 0.001$). Analysis within biopsy revealed a consistent negative correlation between PLIN and CGI-58 at 5, 21, and 150 DIM. Phosphorylated PLIN was positively correlated with phosphorylated HSL prepartum, and with ATGL at 5 DIM. Finally, ATGL was significantly correlated with phosphorylated HSL prepartum (Tables 3 and 4).
3.5 DISCUSSION

In this study, we describe changes in abundance of lipid droplet-associated proteins over the transition period, and the in vivo coordination of these proteins with energy balance and lipolytic indicators. Three important findings provide novel insight regarding the regulation of these proteins throughout the transition period. First, ATGL protein abundance decreased with the onset of lactation. Second, significant associations between PLIN and energy balance were observed, and third, changes in abundance of PLIN and CGI-58 proteins were coordinately regulated.

Adipose triglyceride lipase was initially identified after HSL knock-out mice maintained normal lipolysis and body composition (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004) Since then, ATGL has been described as a rate-limiting step of lipolysis (Haemmerle et al., 2006). Abundance of ATGL increases in response to caloric restriction and glucocorticoids (Deiuliis et al., 2008; Villena et al., 2004), suggesting that protein abundance increases in response to greater need for lipolytic mobilization of energy due to metabolic or pharmacologic stimuli. Thus in the current study, we expected increased abundance of ATGL at 5 and 21 DIM, when rates of lipolysis were greatest. Surprisingly, a clear and consistent decrease in ATGL was observed at these time points. One potential explanation for this unexpected result is the relative importance of ATGL during times of basal versus PKA mediated lipolysis. It is well documented that ATGL is necessary for both basal and stimulated lipolysis in 3T3-L1 adipocytes and rodent models (Granneman et al., 2007; Haemmerle et al., 2006; Miyoshi et al., 2008). However, results from experiments using human adipocytes indicate ATGL is less important than HSL in regulating PKA mediated lipolysis, whereas
both participate in basal lipolysis (Langin et al., 2005; Rydén et al., 2007). Additionally, ATGL but not HSL or PLIN regulates lipid droplet size under basal conditions in adipocytes derived from mouse embryonic fibroblasts (Miyoshi et al., 2008). Our observation that ATGL abundance is significantly less during negative versus positive energy balance potentially reflects a shift from ATGL-dependent basal lipolysis during positive energy balance, to HSL-dependent PKA stimulated lipolysis during negative energy balance. Furthermore, we hypothesize that this down-regulation of ATGL at the onset of lactation may protect the cow from a potentially detrimental depletion of lipid stores if both lipases were coordinately upregulated. This hypothesis is supported by cell culture experiments, in which ATGL mRNA is down-regulated by isoproterenol, forskolin, and cholera toxin (Kralisch et al., 2005). Potential mechanisms of ATGL regulation warrant further investigation in the transition dairy cow.

Because PLIN is a known phosphorylation target of PKA, we expected its phosphorylation to increase at 5 and 21 DIM. This trend was observed, and is consistent with our previous report describing the abundance of PPLIN in cows in early (5-14 DIM) versus late (176-206 DIM) lactation (Elkins and Spurlock, 2009). However, significant correlations between PPLIN and circulating NEFA and glycerol concentrations within stage of lactation described previously (Elkins and Spurlock, 2009) were not observed within biopsy day in the present study. Likewise, the significant correlations of PHSL with NEFA and glycerol were not observed in the present study. One possible explanation for this discrepancy is that the former study measured NEFA and glycerol from serum samples collected at the time of biopsy, whereas the current study utilized metabolite data from samples collected up to 3 d
before or after the biopsy. The lack of correlation in this study may reflect rapidly changing concentrations of NEFA and glycerol, or transient changes in phosphorylation of PLIN, during early lactation. This explanation is supported by the fact that the correlation between PPLIN and glycerol at 150 DIM, when biopsy and serum samples were collected on the same day, tended toward significance ($r=0.42; P = 0.08$, data not shown) and was consistent with the correlation observed in late lactation cows in the previous study ($r=0.65; P = 0.06$). Alternatively, NEFA and phosphorylated proteins may not be correlated in the current data because of differences in lipolytic rates among adipose tissue depots. In growing steers, increased lipolytic activity in subcutaneous adipose tissue compared to visceral adipose tissue depots (Pothoven et al., 1975; Rule et al., 1992). However, a slightly higher lipolytic rate was observed in perireanal compared with subcutaneous adipose tissue of ewes (Etherton et al., 1977). Furthermore, lactation in sheep appears to alter the lipolytic responses of adipose tissue to β-adrenergic stimulation via depot-specific (Vernon et al., 1995). Thus in the current data, variation in circulating NEFA concentrations may reflect differences in visceral lipolysis, whereas the measurement of protein phosphorylation is from subcutaneous adipose tissue.

Despite the lack of a significant relationship between PPLIN and NEFA within biopsy day, significant correlations between PPLIN and milk yield, DMI, and energy balance were observed in early lactation. Although cause and effect cannot be determined from correlative data, these results are consistent with the hypothesis that PPLIN plays an important role in energy utilization \textit{in vivo}. The negative correlation between PPLIN and milk yield is particularly intriguing. A positive relationship would
have supported a hypothesis that increased energy mobilization through phosphorylation of PLIN supported higher milk production. In contrast, it seems PLIN may be phosphorylated in response to a signal for increased energy mobilization when milk yield is limited by energy availability. Initially (5 DIM), only the relationship between milk yield and PPLIN is evident. However by 21 DIM, it appears that increased PPLIN is associated with cows experiencing more severe negative energy balance due to insufficient feed intake that is ultimately limiting the cow’s milk production. Thus, the combination of milk yield and feed intake, rather than either of these alone, may contribute to variation in PPLIN. However, further research is needed to test this hypothesis and define the mechanisms responsible for the observed correlations.

In addition to the correlation with PPLIN in early lactation, total PLIN protein abundance was positively correlated with energy balance at 150 DIM. This correlation may reflect increasing adiposity and lipid droplet formation with increasing energy balance at 150 DIM. Perilipin was not correlated with BCS at this time point, but the subjective visual evaluation of BCS may have lacked the sensitivity to detect early changes in adiposity. Alternatively, variation in PLIN in the single adipose tissue depot sampled may not be reflective of overall body composition. Regardless, the biological relevance of the correlation between PLIN and energy balance in mid lactation warrants further investigation.

Finally, PLIN protein abundance was negatively correlated with CGI-58 at all sampling times. To our knowledge, this is the first report of coordinated regulation of these proteins in any model. Although PLIN expression is known to be responsive to peroxisome proliferator-activated receptor-γ (Shimizu et al., 2006), estrogen receptor
related receptor (Akter et al., 2008), and RAR-related orphan receptor (ROR)-α (Ohoka et al., 2009), little is known about the regulation of CGI-58. However, expression of PLIN mRNA and protein is specific to adipocytes (Greenberg et al., 1991), whereas CGI-58 is expressed in both pre-adipocytes and adipocytes (Yamaguchi et al., 2007). Therefore, the negative relationship between PLIN and CGI-58 may in part reflect variation in abundance of pre- versus mature adipocytes in the adipose tissue sampled.

3.6 CONCLUSIONS

Proteins involved in the regulation of lipolysis are dynamically regulated throughout the transition period of multiparous Holstein cows. Although ATGL is often described as the rate-limiting step of lipolysis in other species, we show that it is dramatically down-regulated at the onset of lactation when the demand for lipolysis is greatest. In contrast, changes in phosphorylation of PLIN and HSL reflect changes in lipolysis across the time points sampled. Additionally, PPLIN is negatively correlated with milk yield, DMI, and energy balance at 21 DIM, potentially reflecting a complex response to multiple signals of energy demands. Finally, the protein abundance of PLIN and CGI-58 appear to be closely coordinated throughout lactation. Together, these results demonstrate that the abundance of ATGL and phosphorylation of HSL and PLIN are altered throughout the transition period, and potentially contribute to the homeorhetic response to the onset of lactation.

3.7 ACKNOWLEDGEMENTS

This project was supported by National Research Initiative Competitive Grant no. 2009-35206-05222 from the USDA Cooperative State Research, Education, and Extension
Service. The authors would also like to acknowledge S. M. Lonergan, Iowa State University, Ames IA, for technical support in protein detection.
### 3.8 TABLES

**Table 1.** Correlation coefficients (r) between milk production traits and lipid droplet-associated protein abundance and phosphorylation.

<table>
<thead>
<tr>
<th></th>
<th>Milk Yield</th>
<th></th>
<th>Energy Corrected Milk</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 DIM $^a$</td>
<td>21 DIM</td>
<td>150 DIM</td>
<td>5 DIM</td>
</tr>
<tr>
<td><strong>PLIN</strong> $^b$</td>
<td>-0.006</td>
<td>-0.255</td>
<td>-0.079</td>
<td>-0.142</td>
</tr>
<tr>
<td><strong>HSL</strong> $^c$</td>
<td>0.186</td>
<td>-0.220</td>
<td>-0.428 $^§$</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>PPLIN</strong></td>
<td>-0.459*</td>
<td>-0.419*</td>
<td>-0.431 $^§$</td>
<td>-0.251</td>
</tr>
<tr>
<td><strong>PHSL</strong> $^c$</td>
<td>-0.312</td>
<td>-0.168</td>
<td>-0.556*</td>
<td>-0.331 $^§$</td>
</tr>
<tr>
<td><strong>ATGL</strong></td>
<td>0.125</td>
<td>0.162</td>
<td>-0.155</td>
<td>0.204</td>
</tr>
<tr>
<td><strong>CGI-58</strong></td>
<td>-0.202</td>
<td>-0.032</td>
<td>0.348</td>
<td>-0.108</td>
</tr>
</tbody>
</table>

$^a$ 5 DIM indicates proteins from adipose tissue biopsy taken 4 to 9 DIM; 21DIM indicates adipose tissue biopsy taken 21 to 31 DIM; and 150 DIM indicates adipose tissue biopsy taken 151 to 161DIM.

$^b$ Abbreviations: PLIN, perilipin; HSL, hormone sensitive lipase; PPLIN, phosphorylated perilipin; PHSL, phosphorylated hormone sensitive lipase; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identity-58

$^c$ Natural log transformed data were analyzed using Spearman correlation.

$^§ P < 0.1$

$^* P < 0.05$
Table 2. Correlation coefficients (r) between energy balance and intake, and lipid droplet-associated protein abundance and phosphorylation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dry Matter Intake</th>
<th>Energy Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-21 DIM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 DIM</td>
</tr>
<tr>
<td>PLIN</td>
<td>-0.198</td>
<td>-0.163</td>
</tr>
<tr>
<td>HSL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.138</td>
<td>0.173</td>
</tr>
<tr>
<td>PPLIN</td>
<td>-0.111</td>
<td>-0.260</td>
</tr>
<tr>
<td>PHSL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.396*</td>
<td>-0.203</td>
</tr>
<tr>
<td>ATGL</td>
<td>-0.097</td>
<td>-0.074</td>
</tr>
<tr>
<td>CGI-58</td>
<td>0.198</td>
<td>0.158</td>
</tr>
</tbody>
</table>

<sup>a</sup> -21 DIM indicates proteins from adipose tissue biopsy taken 31 to 8 d prior to parturition; 5 DIM indicates adipose tissue biopsy taken 4 to 9 DIM; 21 DIM indicates adipose tissue biopsy taken 21 to 31 DIM; and 150 DIM indicates adipose tissue biopsy taken 151 to 161 DIM.

<sup>b</sup> Abbreviations: NEFA, non-esterified fatty acid; PLIN, perilipin; HSL, hormone sensitive lipase; PPLIN, phosphorylated perilipin; PHSL, phosphorylated hormone sensitive lipase; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identity-58

<sup>c</sup> Natural log transformed data were analyzed using Spearman correlation.

<sup>§</sup> $P < 0.1$

<sup>*</sup> $P < 0.05$
Table 3. Correlation coefficients (r) among lipid droplet-associated protein abundance determined by semi-quantitative western blotting from adipose tissue biopsies collected 4 to 9 DIM (above diagonal) and 21 to 31 DIM (below diagonal).

<table>
<thead>
<tr>
<th></th>
<th>PLIN</th>
<th>HSL</th>
<th>PPLIN</th>
<th>PHSL</th>
<th>ATGL</th>
<th>CGI-58</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLIN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSL</td>
<td>0.131</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPLIN</td>
<td>0.081</td>
<td>-0.311</td>
<td>0.385§</td>
<td>0.476*</td>
<td>-0.178</td>
<td></td>
</tr>
<tr>
<td>PHSL</td>
<td>0.101</td>
<td>-0.004</td>
<td>0.350§</td>
<td>0.201</td>
<td>0.253</td>
<td></td>
</tr>
<tr>
<td>ATGL</td>
<td>0.039</td>
<td>0.039</td>
<td>-0.137</td>
<td>0.186</td>
<td></td>
<td>-0.084</td>
</tr>
<tr>
<td>CGI-58</td>
<td>-0.525*</td>
<td>-0.063</td>
<td>-0.006</td>
<td>0.105</td>
<td>0.129</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PLIN, perilipin; HSL, hormone sensitive lipase; PPLIN, phosphorylated perilipin; PHSL, phosphorylated hormone sensitive lipase; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identity-58

Natural log transformed data were analyzed using Spearman correlation.

§ P < 0.1

* P < 0.05
Table 4. Correlation coefficients (r) among lipid droplet-associated protein abundance determined by semi-quantitative western blotting from adipose tissue biopsies collected 31 to 8 d prior to parturition (above diagonal) and 151 to 161 DIM (below diagonal).

<table>
<thead>
<tr>
<th></th>
<th>PLIN</th>
<th>HSL</th>
<th>PPLIN</th>
<th>PHSL</th>
<th>ATGL</th>
<th>CGI-58</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLIN</td>
<td>0.252</td>
<td>0.050</td>
<td>0.182</td>
<td>0.3632</td>
<td>-0.329</td>
<td></td>
</tr>
<tr>
<td>HSL</td>
<td>0.353</td>
<td>0.291</td>
<td>-0.041</td>
<td>0.097</td>
<td>-0.291</td>
<td></td>
</tr>
<tr>
<td>PPLIN</td>
<td>0.117</td>
<td>-0.016</td>
<td>0.443*</td>
<td>0.223</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>PHSL</td>
<td>0.345</td>
<td>0.049</td>
<td>0.346</td>
<td>0.394*</td>
<td>-0.230</td>
<td></td>
</tr>
<tr>
<td>ATGL</td>
<td>-0.168</td>
<td>0.122</td>
<td>-0.016</td>
<td>-0.025</td>
<td>-0.129</td>
<td></td>
</tr>
<tr>
<td>CGI-58</td>
<td>-0.523</td>
<td>-0.316</td>
<td>-0.290</td>
<td>-0.123</td>
<td>0.252</td>
<td></td>
</tr>
</tbody>
</table>

a Abbreviations: PLIN, perilipin; HSL, hormone sensitive lipase; PPLIN, phosphorylated perilipin; PHSL, phosphorylated hormone sensitive lipase; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identity-58

b Natural log transformed data were analyzed using Spearman correlation.

§ P < 0.1

* P < 0.05
3.9 FIGURES

**Figure 1. Working model of lipolysis.** Beta adrenergic receptors (β-AR) are activated by the binding of catecholamines. This stimulation results in activation of adenylyl cyclase which converts ATP to cAMP. Increased intracellular concentration of cAMP activates Protein Kinase A (PKA) which phosphorylates hormone sensitive lipase (HSL) and perilipin. Phosphorylated HSL translocates to the lipid droplet to hydrolyze triacylglycerides to free fatty acids (FFA) and glycerol. Phosphorylation of perilipin 1) facilitates interactions between phosphorylated HSL and its lipid substrate, and 2) releases comparative gene identity-58 (CGI-58) from the lipid droplet. CGI-58 translocates to the cytosol where it interacts with adipose triglyceride lipase (ATGL). The translocation of ATGL to the lipid droplet allows for the hydrolysis of triacylglycerides to diacylglycerides. Lines with + represent a positive action, a circled ‘P’ represents a phosphorylation event, and dark gray filled shapes represents active proteins.

**Figure 2. Body weight, Body Condition Score and Energy Balance at the time of adipose tissue biopsy.** A.) Body weight was measured weekly beginning approximately 30 days prior to calving through 150 DIM. Body weight around the time of biopsy was a single weight + 3d of the adipose tissue biopsy. B.) Body Condition Scores (BCS) was measured weekly beginning approximately 30 days prior to calving through 150 DIM. BCS around the time of biopsy was a single weight ± 3d of the adipose tissue biopsy. C.) Energy Balance was calculated from feed intake calculated
from daily amounts fed and refused, milk production, and milk components. Differences in letters indicate differences among means, \( P < 0.05 \).

**Figure 3.** **Circulating concentrations of Non-esterified Fatty Acids (A), Glycerol (B) and Beta-hydroxybutyrate (C) at the time of adipose tissue biopsy.** All circulating metabolites were measured weekly during the early afternoon. Weekly measurements of metabolites were from a single blood collection. Following collection, serum was separated and stored at -80 °C until analysis. Data were analyzed following natural log-transformation, but are presented on the linear scale for ease of interpretation. Differences in letters indicate differences among means, \( P < 0.05 \).

**Figure 4.** **Protein abundance of total and phosphorylated lipid droplet-associated proteins.** Adipose tissue samples used for western blotting were taken approximately 21d prior to parturition (31 to 8), and 5 (4 to 9), 21 (21 to 31), and 150 (151 to 161) DIM. Protein abundance of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), perilipin, comparative gene identity-58 (CGI-58), phosphorylated perilipin (PPLIN), and Ser-563 phosphorylated HSL (Phosphorylated HSL) was determined by semi-quantitative western blotting and expressed in relative units, normalized to a common standard. Each inset shows a representative western blot, and the arrow indicates the band used for quantification of PPLIN. Biopsy days are represented as follows: -21, solid white; +5 DIM, diagonal stripe; +21 DIM, gray; +150 DIM, solid black. Lowercase and uppercase letters represent overall differences among means, \( P < 0.05 \).
and $P < 0.1$, respectively. Differences in letters indicate differences among means, $P < 0.05$. 
Figure 1
Figure 2

A. Weight, Kg

B. BCS, score 1 to 5

C. Energy Balance, Mcal
Figure 3

A.

B.

C.
Figure 4
3.10 REFERENCES


CHAPTER 4. SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 INCREASES ADIPOSE TRIGLYCERIDE LIPASE PROTEIN ABUNDANCE DURING LEPTIN INDUCED LIPOLYSIS IN BOVINE ADIPOCYTES

Proposed submission to American Physiology Journal’s Endocrinology and Metabolism

Dawn A. Koltes and Diane M. Spurlock

4.1 ABSTRACT

Regulation of lipid metabolism is critical for the maintenance of milk production and fitness in dairy cattle. Despite the knowledge that leptin can induced lipolysis in adipocytes of monogastrics, it remains unclear if bovine adipocytes undergo leptin induced lipolysis. Additionally, the mechanisms involved in leptin mediated lipolysis remain unclear. Therefore, the objectives of this study were to determine 1) if leptin signaling can induce lipolysis in bovine adipocytes and 2) the potential role of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in leptin stimulated lipolysis. Lipolysis was increased after two hours of exposure to 100 ng/mL of bovine leptin. As expected, phosphorylation of signal transducer and activator of transcription (STAT) 3 increased after leptin exposure. The addition of the STAT3 inhibitor, STAT3TIC, attenuated lipolysis and phosphorylation of STAT3. Phosphorylation of perilipin and HSL at serine 563, and serine 565 were not consistently increased with leptin exposure. However, ATGL protein abundance was increased with leptin
exposure. The leptin-induced increase in ATGL protein abundance was attenuated with the addition of STATISTIC. These results identify for the first time that leptin induces lipolysis in bovine adipocytes. Additionally, increased ATGL protein abundance, but not phosphorylation of HSL or perilipin, indicates that leptin regulates lipolysis via a different mechanism than the traditional catecholamine stimulated pathway.

**Keywords:** Hormone sensitive lipase, perilipin, STATISTIC, glycerol, and non-esterified fatty acids

### 4.2 INTRODUCTION

Leptin is a hormone secreted by adipose tissue in proportion to adipose tissue mass and nutritional status (1, 20). One important role of leptin is to suppress feed intake (6, 15, 24, 26), but it can also act locally to increase lipolysis via the JAK/STAT pathway in rodents and pigs (19, 27, 28, 34). However, the mechanism by which leptin utilizes JAK/STAT to induce lipolysis remains unclear.

Hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are major lipases associated with stimulated lipolysis in adipocytes. Classically, the regulation of lipolysis has been attributed to catecholamines that stimulate lipolysis through the activation of protein kinase A (PKA), which phosphorylates and allows for the translocation of HSL to the lipid droplet (11, 22). It is now recognized that additional proteins also participate in the PKA-dependent regulation of lipolysis. Perilipin, a lipid droplet barrier protein, is phosphorylated by PKA. Phosphorylated perilipin allows 1) access of PKA-phosphorylated HSL to the surface of the lipid droplet (23, 33) and 2) the release of the protein comparative gene identity-58 (CGI-58) from the lipid droplet which
facilitates the translocation of ATGL to the lipid droplet (14). Additional evidence suggests ATGL may play a role in basal lipolysis in rodents and dairy cattle (17, 21, 29); however, the mechanisms by which ATGL are activated is currently under investigation (3, 22, 25).

The dairy cow is a unique model for studying lipid metabolism due to the dramatic changes in lipid turnover that occur throughout a lactation cycle. Following parturition, energy requirements greatly increase due to milk synthesis. These energy requirements are typically not met by feed intake, requiring the mobilization of body energy reserves, in particular adipose tissue, to support lactation. As the lactation cycle progresses, milk synthesis declines and feed intake increases to meet and eventually exceed energy requirements. Energy reserves are then replenished prior to the next lactation cycle (7). The depletion of lipid reserves during early lactation is associated with declining circulating leptin concentrations (1, 8, 9), which may contribute to increasing appetite during this time. However, additional roles of leptin in energy homeostasis in dairy cattle remain unknown. Therefore, the objectives of this research were to: 1) determine if leptin can signal to induce lipolysis in bovine adipocytes and 2) determine the potential role of ATGL and HSL in leptin-stimulated lipolysis.

4.3 MATERIALS AND METHODS

All procedures involving the use of animals were approved by the Iowa State University Institutional Animal Care and Use Committee.
4.3.1 Adipocyte Collection

Approximately 40 g of subcutaneous adipose tissue were collected under local anesthetic (2% lidocaine) from the tailhead region of 15 primiparous mid lactation (300.75 ± 15.21 d in milk) Holstein cows. Adipocytes were isolated using a previously described method (4). Briefly, adipose tissue was minced then digested in cocktail media [20 mM Sodium bicarbonate, 20 mM HEPES, 10 mM D-glucose, and 3% bovine serum albumin (w/v), pH 7.4, containing 1 mg/mL (w/v) of collagenase type I (C2674; Sigma; St. Louis, MO)]. Adipose tissue was digested for 40 minutes at 37°C. Following an additional collagenase digestion (1.8mg/mL of collagenase; w/v), adipocytes were collected by filtration, rinsed, and isolated adipocytes were suspended in media [10g/L low glucose DMEM, 3% bovine serum albumin (w/v), 6.6 mM Sodium bicarbonate, 6.25 mM HEPES, pH 7.2]. Adipocytes were placed in plastic scintillation vials, and allowed to acclimate for 30 minutes prior to treatment at 37°C.

4.3.2 Treatment of Adipocytes

Duplicate vials of adipocytes from eight cows were administered 0ng/mL (Control), 10ng/mL, 100ng/mL, or 200 ng/mL of bovine leptin (CYT-502; ProSpec; Ness-Ziona, Isreal). To determine if the lipolytic effect of leptin was mediated by STAT3, duplicate vials of adipocytes from seven different cows were treated with 0, 1, 1.25, 1.5, 1.75 or 2 μM of STATTIC (2798; Tocris; Bristol, UK) for one hour prior to administration of 100 ng/mL of bovine leptin. As a positive control, a subset of adipocytes from each cow was treated with 100 nM D-L isoproterenol for 2 hours (151358; MP Biomedicals; Solon, OH) or 0 or 2 μM of STATTIC for 1 hour prior to administration of 100 nM D-L isoproterenol (Figures 1, 3). Media was aspirated from under the adipocytes after two
hours after the final treatment. See supplemental figure 1 for a depiction of experimental design. Aspirated media was stored at -80°C until further analysis. Adipocytes were homogenized in a protein homogenization buffer (12, 17), immediately frozen and stored at -80°C until further analysis.

4.3.3 Glycerol and Non-esterified Fatty Acid Assays

Glycerol and non-esterified fatty acid (NEFA) from the aspirated media were measured in triplicate using colorimetric assays in a 96-well plate format. Data were captured using a Tecan Spectrafluor Plus (Tecan Group Ltd). Glycerol concentrations were determined using Free Glycerol Reagent (Sigma; F6428; St. Louis, MO), and NEFA concentrations using the NEFA-HR (2) assay (999-34691, 995-34791, 991-34891, 993-35191, 276-76491; Wako; Richmond, VA), both according to manufacturer’s protocol. Glycerol and NEFA were normalized to protein abundance determined by bicinchoninic acid assay (BCA; 23227, Pierce Protein Biology Products, Rockford, IL) from the same vial. Protein samples were subsequently prepared for western blotting.

4.3.4 Semi-quantitative Western Blotting

Semi-quantitative western blotting assays were performed using previously described methods (12, 17). Briefly, a constant quantity of protein was diluted in Laemmli sample buffer and loaded on either an 8% or a 10% SDS-PAGE gel. See table 1 for antibody information. Gels were transferred to polyvinylidene fluoride membranes (Hybond-P, 45000931; GE Healthcare; Pittsburgh, PA), exposed to primary antibodies, rinsed, and exposed to ECL anti-rabbit horseradish peroxidase IgG antibody (NA9340-1ml; GE Healthcare; Pittsburgh, PA). Membranes were rinsed and then exposed to ECL prime western blotting detection reagent (RPN 2232, GE Healthcare, Pittsburgh, PA),
according to manufacturer’s protocol. Membranes were imaged using an Alpha Innotech Imager (FluorChem FC2, Cell Biosciences, Santa Clara, CA) and protein bands were quantified using Totallab software for 1D analysis (TL100, v2009; Totallab Ltd, Newcastle upon Tyne, UK). Phosphorylated perilipin was determined by quantifying the phosphorylated band (upper band; Supplemental Figure 1 and 2) on the perilipin membranes. All samples were run on duplicate gels. Protein abundance values were normalized to β-actin, and means are presented relative to control samples. For 5 of the 60 gels, β-actin protein abundance was not detected either due to low abundance or accidentally excluded from the membrane. The average β-actin protein abundances for all samples from a single trial were used for normalization of these samples. When β-actin protein abundance was not detected for a single sample within a membrane (23 out of 1442 lanes), that sample was excluded from analysis because using the average β-actin protein abundance resulted in the loss of normality.

4.3.5 Statistical Analysis

A randomized complete block design was utilized for statistical analysis of glycerol, NEFA, and protein abundance for leptin and STATTIC treated adipocytes. For statistical analysis, the PROC MIXED procedure in SAS was utilized. Treatments were blocked within cow. Cow was fit as a random effect, and treatment was fit as a fixed effect. A 2x2 factorial design was used to compare the two factors control and isoproterenol treated adipocytes in the presence or absence of STATTIC. The PROC MIXED procedure in SAS was utilized for statistical analyses (30). In this 2x2 factorial design, cow was fit as a random effect and isoproterenol treatment, STATTIC treatment and the interaction were fit as fixed class effects. Since phosphorylated perilipin was
not detectable in the majority of samples (64 out of 80 samples for the leptin experiment and, 156 out of 176 samples for the STATTIC experiment), a chi-squared test was performed in SAS to test if the presence of phosphorylation was higher than expected with a particular treatment (30). All model residuals were tested for normality. Data that significantly deviated from normality included: total protein abundance for perilipin during leptin treatment; glycerol, NEFA, phosphorylation of HSL at serine 563 and 565, perilipin and STAT3 for the comparison of isoproterenol treatment of cows in the leptin experiment; and NEFA concentrations for the STATTIC treatment. These data were log transformed to induce normality in the residuals. Pairwise comparisons were conducted between control and treatment groups using the contrast statement in PROC MIXED (30). Significance was set at P < 0.05, and tendency set at P < 0.01.

4.4 RESULTS

4.4.1 Leptin treatment

Primary bovine adipocytes exposed to 100nM isoproterenol had a 16 fold increase in glycerol and a 31 fold increase in NEFA concentrations in the media compared to control adipocytes (Figure 1 A and B). The increased lipolytic activity was accompanied by an increase in phosphorylation of HSL at Serine 563 and perilipin (Figure 1C). Total protein abundance of HSL, perilipin, ATGL, STAT3, and phosphorylation of STAT3 at Tyrosine 705 and HSL at Serine 565 were similar between isoproterenol-treated and control adipocytes (Figure 1C). P-values are presented in Table 2.

Glycerol concentrations were altered by leptin treatment after 2 hours (Figure 2A), but NEFA concentrations remained constant (Figure 2B). Glycerol concentrations
increased with 100 ng/mL of bovine leptin compared to control adipocytes (P = 0.016). Leptin stimulation altered the relative abundance of phosphorylated STAT3 at Tyrosine 705, and ATGL protein abundance. Phosphorylation of STAT3 at Tyrosine 705 was elevated by treatment with 10 or 100 ng/mL of bovine leptin (P = 0.021 and P = 0.032, respectively), and ATGL was elevated with 10, 100 or 200 ng/mL of bovine leptin (P = 0.025, P = 0.001, and P = 0.003, respectively). Phosphorylated HSL at Serine 563 tended to be altered with leptin treatment, and was increased with 100 and 200 ng/mL of bovine leptin (P = 0.030 and P = 0.041, respectively). Phosphorylation of HSL at Serine 565, perilipin and total protein abundance of perilipin, HSL, and STAT3 remained constant with leptin treatment (Figure 2C and D). Overall p-values are listed in table 2.

4.4.2 STATTIC treatment

To confirm signaling of leptin was mediated by STAT3 in bovine adipocytes, cells were treated with a STAT3 specific inhibitor, STATTIC. The introduction of STATTIC at 2μM did not alter lipolytic indicators or relative protein abundance in either control or isoproterenol stimulated adipocytes (Figure 3). Adipocytes treated with isoproterenol had elevated glycerol (Figure 3A) and NEFA concentrations (Figure 3B) in the media, and increased phosphorylation of HSL at Serine 563 and perilipin. Total protein abundance of ATGL was significantly altered by isoproterenol treatment, but pairwise comparisons between control and isoproterenol in the presence or absence of STATTIC were not significant. Total protein abundance of HSL, perilipin, STAT3, ATGL, phosphorylation of STAT3 at Tyrosine 705, and HSL at Serine 565 were not altered with isoproterenol treatment (Figure 3C). Overall p-values are listed in table 3.
Treatment of bovine adipocytes with STATTIC (0, 1, 1.25, 1.5, 1.75, 2 μM) plus 100ng/mL of leptin altered overall glycerol concentrations (Figure 4A), while NEFA concentrations were similar across treatments (Figure 4B). Glycerol concentrations were elevated in adipocytes treated with 0 or 1 μM of STATTIC plus 100 ng/mL of leptin compared to control adipocytes (P = 0.036 and P = 0.003, respectively). Overall, relative protein abundances were not significantly altered by leptin treatment (Figure 4C). However, ATGL protein abundance tended to be altered with increased protein abundance in adipocytes treated with 0 or 1.75 μM of STATTIC plus 100 ng/mL of leptin (P = 0.026 and P = 0.026, respectively). Although treatment was not significant overall, phosphorylation of STAT3 at Tyrosine 705 increased with 100 ng/mL of leptin (P = 0.032). Phosphorylation of HSL at serine 563 tended to be altered with STATTIC treatment, but only adipocytes treated with 1.75 μM of STATTIC plus 100 ng/mL of leptin had increased phosphorylation compared with control (P = 0.05). Phosphorylated HSL at serine 565, phosphorylation of perilipin, and total protein abundance of HSL, STAT3 and perilipin remained constant throughout the STATTIC treatment. Overall p-values are listed in table 3.

4.5 DISCUSSION

Leptin is a potent adipokine that can regulate energy balance by altering feed intake (6, 15, 24, 26) and lipid metabolism (2, 5, 16, 19, 27, 28, 34). In dairy cattle, circulating leptin concentrations decline following parturition to increase appetite (1, 8). However, the role of leptin later in lactation when energy intake exceeds energy requirements was unknown. By demonstrating that leptin induces lipolysis in bovine adipocytes, this study has elucidated a novel role for leptin in the dairy cow.
Additionally, the detailed mechanisms involved in leptin induced lipolysis had not been well characterized in previous studies. This study indicates 1) that leptin activated the STAT3 signaling pathway to induced lipolysis, and 2) leptin treatment increased ATGL protein abundance via STAT3.

Leptin induced a slight, but significant increase in lipolysis of bovine adipocytes. The approximately 30% increase in glycerol release was considerably lower than the 1,600% increase in glycerol release during isoproterenol treatment. This mild lipolytic response was similar to responses observed in murine and porcine adipocytes (19, 34). In addition to the similar lipolytic response, murine and porcine adipocytes exposed to leptin had elevated glycerol concentrations but not NEFA concentrations (19, 34). Transcription of genes involved in β-oxidation increased in adipocytes treated with leptin (34) suggesting that NEFA undergo β-oxidation instead of being release into the media. Although we did not directly test for increased β-oxidation, we expected NEFA to undergo β-oxidation in bovine adipocytes due to the similarities of our result to other models. The ability of bovine adipocytes to undergo leptin induced lipolysis and β-oxidation suggest that increased circulating leptin concentrations may regulate adipose tissue accumulation and mobilization during later lactation in dairy cattle.

Physiological circulating concentrations of leptin range between 1 and 20 ng/mL in cattle (1, 8–10, 32, 35). However, leptin consistently induced lipolysis at 100 ng/mL, and not 10 ng/mL of bovine leptin. Porcine and rat adipocytes can undergo leptin induced lipolysis at concentrations slightly elevated from physiological ranges (19, 27, 34). However, maximal lipolytic response occurs with 100 ng/mL of leptin (27, 31). The ability of leptin to induce lipolysis in primary adipocytes at approximately 10 times
circulating concentrations may indicate that either throughout the isolation process the adipocytes become less sensitive to leptin or that intratissue concentrations of leptin are higher than circulating concentrations as suggested by (2).

Phosphorylation of STAT3 was increased at concentrations of leptin similar to circulating concentrations in cattle, as well as concentrations of leptin that induced lipolysis. The ability of leptin to increase phosphorylation of STAT3 at in vivo circulating concentrations indicated that circulating concentrations of leptin can induce signaling in bovine adipocytes. Additionally, phosphorylation of STAT3 increased with leptin concentrations that induced lipolysis in bovine adipocytes. The attenuation of phosphorylation of STAT3 coincided with the attenuation of lipolysis in adipocytes treated with STATTIC. This attenuation confirms the importance of STAT3 to leptin induced lipolysis.

The roles of ATGL and HSL are primarily associated with their participation in catecholamine stimulated lipolysis. Following activation of PKA, phosphorylation of perilipin releases CGI-58 from the lipid droplet surface where it allows ATGL to translocate to the lipid droplet surface. This initial step results in the hydrolysis of triacylglycerides to diacylglycerides. Activated PKA phosphorylates HSL at serine 563, serine 659, and serine 660. The activation of HSL by PKA allows for the translocation of HSL to the lipid droplet (18). However, HSL can be phosphorylated at serine 565 by AMPK, and is expected to prevent phosphorylation at serine 563 and may prevent lipolysis (13).

Leptin treatment increased protein abundance of ATGL, but not phosphorylation of HSL or perilipin. As previously mentioned, isoproterenol was included as a positive
control, and increased phosphorylation of HSL at serine 563 and perilipin. The lack of phosphorylation of HSL and perilipin with leptin treatment indicates these proteins may not be activated by leptin to induce lipolysis. However, leptin increased ATGL protein abundance. This increase in ATGL protein abundance was attenuated with the addition of STATTIC, and coincided with the decrease in lipolysis. This indicated that ATGL may be active during leptin induced lipolysis. Contradicting our results, porcine SVC-derived adipocytes had decreased ATGL protein abundance after 3 hours of leptin treatment at sufficient concentrations to induce lipolysis (19). These differences may be due to differences in cell type, length of exposure or differences between species. However, protein abundance in SVC-derived porcine adipocytes were inversely affected by the JAK inhibitor, AG490 (19). This suggests that the JAK/STAT pathway regulates ATGL in both bovine and porcine adipocytes. However, Li et al. did not determine the effect of the JAK inhibitor on lipolysis. Therefore, it is unclear how the altered ATGL protein abundance may influence leptin induced lipolysis in porcine adipocytes. Since, protein abundance of ATGL does not directly indicate activity of ATGL, these differences in ATGL protein abundance may not reflect differences in ATGL activity, and only differences in the regulation of ATGL protein abundance.

In conclusion, these results indicate that leptin can regulate lipolysis in bovine adipocytes. This provides novel insight into lipid metabolism in the lactating dairy cow since leptin concentrations are known to be altered throughout a lactation cycle (1). Additionally, these results indicate that leptin regulates lipolysis via different mechanisms than isoproterenol. In contrast to increased phosphorylation of HSL and perilipin by PKA, these results demonstrate that STAT3 increased ATGL protein
abundance which may lead to increased lipolysis. This differential regulation of HSL and ATGL during leptin induced lipolysis may provide a mechanism for regulating basal lipolysis.

4.6 ACKNOWLEDGMENTS

This project was supported by National Research Initiative Competitive Grant no. 2009-35206-05222 from the USDA Cooperative State Research, Education, and Extension Service, and J.M. Reecy at Iowa State University for helpful discussions on experimental design.
Table 1. Antibodies used for semi-quantitative western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalog number</th>
<th>Gel percentage</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSTAT3 Try705a</td>
<td>Cell Signaling</td>
<td>9145</td>
<td>8</td>
<td>1:500</td>
</tr>
<tr>
<td>STAT3</td>
<td>Cell Signaling</td>
<td>8719</td>
<td>8</td>
<td>1:500</td>
</tr>
<tr>
<td>ATGL</td>
<td>Cell Signaling</td>
<td>2138</td>
<td>8</td>
<td>1:500</td>
</tr>
<tr>
<td>PHSLSer563</td>
<td>Cell Signaling</td>
<td>4139</td>
<td>8</td>
<td>1:500</td>
</tr>
<tr>
<td>PHSLSer565</td>
<td>Cell Signaling</td>
<td>4137</td>
<td>8</td>
<td>1:500</td>
</tr>
<tr>
<td>HSL</td>
<td>Cell Signaling</td>
<td>4107</td>
<td>8</td>
<td>1:500</td>
</tr>
<tr>
<td>Perilipin</td>
<td>Chemicon</td>
<td>AB10200</td>
<td>10</td>
<td>1:1000</td>
</tr>
<tr>
<td>P-Perilipinb</td>
<td>Chemicon</td>
<td>AB10200</td>
<td>10</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-actin</td>
<td>Cell Signaling</td>
<td>5125</td>
<td>-c</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

a Abbreviations: STAT3, signal transducer and activator of transcription 3; HSL, hormone sensitive lipase; PSTAT3 Try705, phosphorylated STAT 3 at tyrosine 705; ATGL, adipose triglyceride lipase; PHSLSer563, phosphorylated HSL at serine 563; PHSLSer565, phosphorylated HSL at serine 565; P-Perilipin, phosphorylated perilipin

b Phosphorylated perilipin was quantified based on the shift in the protein size between phosphorylated and unphosphorylated protein. The upper band was quantified as phosphorylated perilipin and the lower band as unphosphorylated perilipin.

c β-actin antibody was exposed to all membranes.
Table 2. List of P-values for main effects from the leptin experiment.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Isoproterenol treatment $^a$</th>
<th>Leptin Treatment $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>&lt;0.001</td>
<td>0.017</td>
</tr>
<tr>
<td>NEFA</td>
<td>&lt;0.001</td>
<td>0.985</td>
</tr>
<tr>
<td>STAT3</td>
<td>0.703</td>
<td>0.636</td>
</tr>
<tr>
<td>PSTAT3</td>
<td>0.894</td>
<td>0.039</td>
</tr>
<tr>
<td>Perilipin</td>
<td>0.837</td>
<td>0.789</td>
</tr>
<tr>
<td>PPLIN</td>
<td>0.002$^c$</td>
<td>0.520$^c$</td>
</tr>
<tr>
<td>HSL</td>
<td>0.669</td>
<td>0.236</td>
</tr>
<tr>
<td>PHSL 563</td>
<td>0.021</td>
<td>0.102</td>
</tr>
<tr>
<td>PHSL 565</td>
<td>0.433</td>
<td>0.399</td>
</tr>
<tr>
<td>ATGL</td>
<td>0.082</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Abbreviations: NEFA, non-esterified fatty acid; STAT3, signal transducer and activator of transcription 3; PSTAT3, phosphorylated STAT3 at tyrosine 705; PPLIN, phosphorylation of perilipin; HSL, hormone sensitive lipase; PHSL 563, phosphorylation of HSL at serine 563; PHSL 565, phosphorylation of HSL at serine 565; ATGL, adipose triglyceride lipase

$^a$ P-values for the comparison of control and isoproterenol treated adipocytes.

$^b$ P-values for the main effect of leptin treatment.

$^c$ Phosphorylated perilipin was not detected many of samples from control and leptin treated adipocytes. Therefore, a chi-squared test was used to determine differences in protein abundance.
Table 3. List of P-values for the main effects from the STATATIC experiment.

<table>
<thead>
<tr>
<th>Effect</th>
<th>STATATIC treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Isoproterenol treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Interaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>STATATIC +Leptin&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>0.892</td>
<td>&lt;0.001</td>
<td>0.954</td>
<td>0.048</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.878</td>
<td>&lt;0.001</td>
<td>0.978</td>
<td>0.839</td>
</tr>
<tr>
<td>STAT3</td>
<td>0.109</td>
<td>0.833</td>
<td>0.234</td>
<td>0.748</td>
</tr>
<tr>
<td>PSTAT3</td>
<td>0.818</td>
<td>0.380</td>
<td>0.324</td>
<td>0.143</td>
</tr>
<tr>
<td>Perilipin</td>
<td>0.496</td>
<td>0.464</td>
<td>0.849</td>
<td>0.743</td>
</tr>
<tr>
<td>PPLIN</td>
<td>0.554&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.342&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HSL</td>
<td>0.923</td>
<td>0.746</td>
<td>0.868</td>
<td>0.743</td>
</tr>
<tr>
<td>PHSL 563</td>
<td>0.074</td>
<td>&lt;0.001</td>
<td>0.2524</td>
<td>0.104</td>
</tr>
<tr>
<td>PHSL 565</td>
<td>0.416</td>
<td>0.666</td>
<td>0.776</td>
<td>0.203</td>
</tr>
<tr>
<td>ATGL</td>
<td>0.681</td>
<td>0.038</td>
<td>0.766</td>
<td>0.107</td>
</tr>
</tbody>
</table>

Abbreviations: NEFA, non-esterified fatty acid; STAT3, signal transducer and activator of transcription; PSTAT3, phosphorylated STAT3 at tyrosine 705; PPLIN, phosphorylation of perilipin; HSL, hormone sensitive lipase; PHSL 563, phosphorylation of HSL at serine 563; PHSL 565, phosphorylation of HSL at serine 565; ATGL, adipose triglyceride lipase

<sup>a</sup> P-values for the main effect in analyses of the 2x2.

<sup>b</sup> P-values for the main effect of STATATIC treatment.

<sup>c</sup> Phosphorylated perilipin was not detected in the control and STATATIC/leptin treated adipocytes. Therefore, a chi-squared test was used to determine differences in protein abundance, and prevented calculating the effect of the interaction between STATATIC treatment and Isoproterenol treatment.
4.8 FIGURES

Figure 1. *Comparison of control and isoproterenol treated bovine adipocytes from cows in the leptin experiment.* N = 8 cows per treatment. A) Glycerol concentration normalized to protein concentrations. B) NEFA concentration normalized to protein concentrations. C) Protein abundance normalized to β-actin and expressed relative to control. Representative western blots are shown under the graph for the corresponding antibody. The arrow indicates phosphorylated perilipin. Lane 1: Control Samples, Lane 2: Adipocytes treated with 100nM Isoproterenol. Abbreviations: STAT3, signaling transducer and activator of transcription 3; HSL, hormone sensitive lipase; PSTAT3 Try705, phosphorylated STAT 3 at tyrosine 705; ATGL, adipose triglyceride lipase; PHSL ser563, phosphorylated HSL at serine 563; PHSL ser565, phosphorylated HSL at serine 565. * represents P < 0.05 compared to control.

Figure 2. *Effects of leptin on lipolysis in bovine adipocytes.* N = 8 cows per treatment. A) Glycerol concentration normalized to protein concentrations. B) NEFA concentration normalized to protein concentrations. C) and D) Protein abundance normalized to β-actin expressed relative to control abundance. Representative western blots are shown under the graph for the corresponding antibody. The arrow indicates phosphorylated perilipin band. Lane 1: 0 ng/mL of leptin (Control), Lane 2: 10 ng/mL of leptin, Lane 3: 100 ng/mL of leptin, Lane 4: 200 ng/mL of leptin. Abbreviations: STAT3, signal transducer and activator of transcription 3; HSL, hormone sensitive lipase; PSTAT3 Try705, phosphorylated STAT 3 at tyrosine 705; ATGL, adipose triglyceride lipase;
lipase; PHSL ser563, phosphorylated HSL at serine 563; PHSL ser565, phosphorylated HSL at serine 565. * represents $P < 0.05$ compared to control where the overall effect of leptin was $P < 0.05$. $\S$ represents $P < 0.05$ compared to control where the overall effect of leptin was $P < 0.11$.

**Figure 3. Comparison of control and isoproterenol treated bovine adipocytes in the absence or presence of 2 μM of STAT3C.** N = 7 cows per treatment. A) Glycerol concentration normalized to protein concentrations. B) NEFA concentration normalized to protein concentrations. C) Protein abundance normalized to β-actin and expressed relative to control adipocytes in the absence of 2 μM of STAT3C. Representative western blots are shown under the graph for the corresponding antibody. The arrow indicates phosphorylated perilipin band. Abbreviations: STAT3, signal transducer and activator of transcription 3; HSL, hormone sensitive lipase; PSTAT3 Try705, phosphorylated STAT 3 at tyrosine 705; ATGL, adipose triglyceride lipase; PHSL ser563, phosphorylated HSL at serine 563; PHSL ser565, phosphorylated HSL at serine 565. Differences in subscript represent $P < 0.05$ for differences in isoproterenol treatment.

**Figure 4. Effects of STAT3C on lipolysis in control and leptin treated bovine adipocytes.** N = 7 cows per treatment. A) Glycerol concentration normalized to protein concentrations. B) NEFA concentration normalized to protein concentrations. C) Protein abundance normalized to β-actin and expressed relative to control abundance.
Representative western blots are shown under the graph for the corresponding antibody. The arrow indicates phosphorylated perilipin band. Lane 1: Control Samples (0 µM of STATTIC and 0 ng/mL of leptin), Lane 2: 0 µM of STATTIC plus 100 ng/mL of leptin, Lane 3: 1 µM of STATTIC plus 100 ng/mL of leptin, Lane 4: 1.25 µM of STATTIC plus 100 ng/mL of leptin, Lane 5: 1.5 µM of STATTIC plus 100 ng/mL of leptin, Lane 6: 1.75 µM of STATTIC plus 100 ng/mL of leptin, Lane 7: 2 µM of STATTIC plus 100 ng/mL of leptin. Abbreviations: STAT3, signal transducer and activator of transcription 3; HSL, hormone sensitive lipase; PSTAT3 Try705, phosphorylated STAT 3 at tyrosine 705; ATGL, adipose triglyceride lipase; PHSL ser563, phosphorylated HSL at serine 563; PHSL ser565, phosphorylated HSL at serine 565. § represents a pairwise comparison of P < 0.05 when compared to control when the overall effect of STATTIC treatment was P< 0.11.

**Supplemental Figure 1. Experimental design.** Timelines for treatment during the leptin and STATTIC experiments. Adipose tissue was collected from 8 and 7 cows for the leptin and STATTIC experiment, respectively. Following digestion of adipose tissue, adipocytes were allowed a 30 minute acclimation period prior to treatment. For the leptin experiment, duplicate vials were treated 0, 0, 10, 100, or 200 ng/ml of leptin at time 0. For the STATTIC experiment, duplicate vials of adipocytes were treated with 0, 1, 1.25, 2.5, 1.75, or 2 µM of STATTIC one hour (-1hr) prior to treatment with 100 ng/mL of leptin (0hr). For both experiments, additional vials were treated with 100nM of isoproterenol at time 0. Media and cells were collected after 2 hours of treatment with leptin.
Figure 2

A.

B.

C.

D.
Figure 3

A. 

![Graph showing Glyceral (µM per mg/mL of protein)]

B. 

![Graph showing NEFA (mmol/L per mg/mL of protein)]

C. 

![Bar graph showing Protein Abundance (Relative Units)]

Protein of interest: 
- PSTAT3
- STAT3
- ATGL
- PHSL
  - Ser563
  - Ser565
- HSL
- Perilipin

β-actin

Isoproterenol
- - + + - - + + - + + - + + - + + - + + - + +

2µM SATTIC
- + + + + + + + + + + + + + + + + + + +
Figure 4

A. Glycolysis (μM per mg/mL of protein) vs STATIC (μM) + 100ng/mL of Leptin

B. NRG1 (ng/mL per mg/mL of protein) vs STATIC (μM) + 100ng/mL of Leptin

C. Protein Abundance (Relative Units) vs Protein of interest: PSTAT3, STAT3, ATGL, Perilipin

D. Protein Abundance (Relative Units) vs Protein of interest: PHSL Ser563, PHSL Ser565, HSL
Supplemental figure 1

Leptin Experiment
- Adipose tissue collected
- Adipose tissue digested
- Acclimation period
-0.5 hr 0 hr 2 hr
- Adipocytes plated
- Treatment applied
- Collect media and cells

STATTIC Experiment
- Adipose tissue collected
- Adipose tissue digested
- Acclimation period
-1.5 hr -1 hr 0 hr 2 hr
- Adipocytes plated
- STATTIC treatment applied
- Leptin treatment applied
- Collect media and cells
4.9 REFERENCES


CHAPTER 5. DISCUSSION

5.1 BACKGROUND

Initiation of milk synthesis during early lactation increases energy requirements, which are often not met by feed intake. Body energy reserves, in particular adipose tissue, are mobilized during this time to meet the energy deficit. Severe or prolonged negative energy balance can reduce overall fitness, in particular reproductive fitness (Banos et al., 2006; Collard et al., 2000; Hammon et al., 2006; van Knegsel et al., 2007; Veerkamp et al., 2000). Since reduced reproductive fitness is a leading reason for culling in the dairy industry (Stevenson, 2009), understanding the regulation of lipid metabolism may be critical for preventing the decline in overall fitness.

Research presented in this dissertation characterized previously unknown or poorly understood mechanisms of lipid metabolism in the dairy cow. These mechanisms were investigated in the dairy cow because they regulate lipid uptake and lipolysis in model systems. Two major findings were identified in studies presented in this dissertation. First, transcription of angiopoietin like protein 4 (ANGPLT4) was dramatically altered with declining energy balance. Second, adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) were differentially regulated in bovine subcutaneous adipose tissue. These findings are important because they contribute to our understanding of lipid metabolism in dairy cattle and may allow for strategies to be developed to optimize lipid metabolism and reduce the risk of declining fitness.
5.2 MAJOR FINDINGS

5.2.1 Transcript abundance of angiopoietin like protein 4 (ANGPTL4) increased with declining energy balance

Angiopoietin like protein 4 is a potent adipokine that regulates lipid uptake by preventing the activation of lipoprotein lipase (LPL). Increased transcript abundance of ANGPTL4 during declining energy balance indicates ANGPTL4 may regulate lipid uptake during altered energy balance in dairy cattle. This study was the first to characterize ANGPTL4 transcript abundances during times of altered energy balance. However, the effects of ANGPTL4 on lipid uptake in adipose tissue of dairy cattle remain unclear.

The specificity of ANGPTL4 to inhibit specific LPL isoforms is unknown. In germ free mice, adipose tissue mass is smaller compared to conventionally raised mice. Also, expression of ANGPTL4 in intestinal tissues was greater in germ free compared to conventionally raised mice. It is expected that the increase in intestinal ANGPTL4 transcription prevents LPL activity in adipose tissue, because adipose tissue ANGPTL4 transcript abundances were similar between germ free and conventionally raised mice (Swartz et al., 2013). These results suggest that ANGPTL4 produced by one tissue can inhibit activity of LPL in other tissues. Contrary to this finding, germ free rats had similar adipose tissue mass compared to conventionally raised rats despite increases in intestinal and hepatic ANGPTL4 transcript abundances (Swartz et al., 2013). Reduced adipose tissue ANGPTL4 transcript abundances were suggested to be the cause of adipose tissue mass accumulation. Together these findings suggest that ANGPTL4 may
inhibit LPL in the tissue it is produced or other tissues, and these differences may be species specific.

Angiopoietin like protein 4 is secreted by multiple tissues in cattle (Mamedova et al., 2010). During negative energy balance, LPL activity decreases in adipose tissue and muscle (Bonnet et al., 2004; Van den Top et al., 2005), but increases in the mammary gland (Shirley et al., 1973). These differences in activity of LPL may be due to differences in tissue specific production of LPL isoforms or the ability of ANGPTL4 to inhibit LPL in a tissue specific manner. In cattle, LPL transcript abundances were not altered by negative energy balance in adipose tissue, but LPL activity decreased (Bonnet et al., 2004). This suggests post-transcriptional modifications of LPL instead of differences in LPL production are regulating LPL activity. Our findings in Chapter 2 suggest that post-transcriptional modifications may be mediated by ANGPTL4. To test this hypothesis that ANGPTL4 inhibits LPL activity during declining energy balance in a tissue/isoform specific manner, in vitro assays would need to be conducted to determine the ability of ANGPTL4 to inhibit the activity of LPL from adipose tissue, muscle and mammary gland.

Additionally, changes in transcript abundances of ANGPTL4 were altered with declining energy balance suggesting ANGPTL4 may serve as a biomarker for energy balance. Unlike circulating and transcript abundances of leptin, ANGPTL4 transcript abundances were altered by modest changes in energy balance. For example, energy balance during late pregnancy was 2.56 Mcal/day and 4.91 Mcal/day during mid lactation. Although this difference in energy balance was slight and positive, ANGPTL4 transcript abundance increased in adipose tissue during late pregnancy. Physiological
state has been shown to alter transcript abundance of ANGPTL4 in mice (Joesphs et al., 2007). However, these changes were observed during declining energy balance following growth hormone administration. This consistency between models suggests that ANGPTL4 may be a biomarker for energy balance. To determine if ANGPTL4 could be a useful on-the-farm biomarker for energy balance, characterization of circulating ANGPTL4 concentrations and energy balance are required. Additionally, the correlation between circulating ANGPTL4 and already easily measured traits such as body condition score would need to be evaluated to determine if this potential biomarker would provide additional information about the energy status of a cow.

5.2.2 Differential regulation of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL)

Hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and perilipin are critical regulators of lipid catabolism. Phosphorylation of both HSL and perilipin were increased during early lactation, and negatively correlated with energy balance at 21 days in milk. This indicates the involvement of PKA dependent mechanisms in lipid mobilization during the transition period. However, ATGL protein abundance decreased during early lactation, but increased with leptin induced lipolysis when PKA phosphorylation was not significantly altered. The ability of signal transducer and activator of transcription (STAT) 3 inhibitor, STAT3IC, to attenuate lipolysis and ATGL protein abundance in bovine adipocytes indicates leptin induced lipolysis may be regulated by a STAT3 mediated increase in ATGL protein abundance. Additionally, ATGL translocated to the lipid droplet with leptin administration in stromal vascular cell
(SVC)-derived adipocytes. These results suggest that HSL and ATGL are differentially regulated during leptin induced lipolysis.

Differential regulation of HSL and ATGL was observed during altered energy balance in mid lactation but both HSL and ATGL translocate to the lipid droplet during isoproterenol treatment. Two additional studies were conducted using the energy altering models from Chapter 2. In these studies, protein abundance of ATGL decreased with declining energy balance, while phosphorylation of HSL at serine 563 was not altered with negative energy balance during feed restriction or declining energy balance following growth hormone administration (personal communication, P.Faylon). This would suggest that declining energy balance may decrease ATGL activity. However, both HSL and ATGL translocated to the lipid droplet following isoproterenol stimulation in SVC-derived adipocytes indicating that both HSL and ATGL are involved in PKA-dependent lipolysis in bovine adipocytes. The increased activity of ATGL with isoproterenol stimulated lipolysis suggests ATGL may be activated during the transition period. The ability of leptin to regulate ATGL protein abundance indicates that decreases in ATGL during the transition period may be due to decreased leptin concentrations, and are not reflective of ATGL activity. To test this hypothesis, additional studies are needed to 1) better characterize leptin induced STAT3 binding of the promoter region of ATGL and 2) determine if ATGL activity is increased during the transition period when protein abundances are decreased.

With this additional knowledge, our understanding of lipid metabolism can be utilized to regulate lipolysis. It might be expected that slowing lipolysis during early lactation might attenuate the adverse effects associated with rapid or prolonged
mobilization of lipids. However, at this time it is unknown if inhibiting lipolysis during early lactation would reduce milk production and alleviate negative energy balance or result in the mobilization of energy from muscle to overcome the energy deficit brought on by lactation.

5.3 GENERAL CONCLUSIONS

In conclusion, this dissertation identified ANGPTL4 as a potentially important adipokine in regulating lipid uptake in adipose tissue and a potential biomarker for energy balance. This initial characterization of ANGPTL4 during altered energy balance has important implications for regulating lipid uptake by dairy cattle, particularly during the dry period when excessive accumulation of adipose tissue can increase the risk for metabolic diseases during early lactation. However, additional research is needed to understand the regulation of LPL by ANGPTL4 and the association of circulating concentrations of ANGPTL4 with energy status. Differential regulation of HSL and ATGL was observed in lipolysis of bovine adipocytes. One potential mechanism by which this differential regulation may occur is through a leptin induced STAT3 mediated increase in ATGL protein abundance. However, additional research is needed to understand the activation of ATGL by STAT3 during the transition period prior to the development of strategies to optimize lipid metabolism. Overall, results from this dissertation provide information about lipid metabolism in dairy cattle which may be used to develop strategies to reduce adverse effects of prolonged or severe negative energy balance.
5.4 REFERENCES


Leptin stimulates an increase in adipose triglyceride lipase (ATGL) protein abundance, but not phosphorylation of hormone sensitive lipase (HSL), via signal transducer and activator of transcription (STAT) 3 in bovine adipocytes. However, the mechanisms that regulate the leptin induced increase in ATGL protein abundance and its subsequent activity are unclear. Therefore, the objectives of this study were to determine 1) if ATGL translocates to the lipid droplet with leptin administration and 2) if STAT3 translocates to the nucleus. Stromal vascular cell (SVC) derived bovine adipocytes were treated with 100 nM of isoproterenol or 100 ng/mL of bovine leptin for two hours or were treated for 0, 5, 10, 20 or 120 minutes with 100ng/mL of leptin. Immunofluorescence was used to detected the translocation of ATGL, HSL, comparative gene identity- 58 (CGI-58), G1/G0 switch protein 2 (G0S2), and phosphorylated STAT3. Lipid droplets and nuclei were detected with Bodipy and DAPI, respectively. As expected, HSL and ATGL translocated to the lipid droplet after two hours of exposure to isoproterenol, suggesting PKA-dependent lipolysis involves both
ATGL and HSL. However, only ATGL translocated to the lipid droplet with leptin exposure. These results were confirmed when ATGL translocated to the lipid droplet at 120 minutes of exposure to leptin in a second experiment. Additionally, STAT3 translocated to the nucleus within 10 minutes of leptin exposure suggesting that STAT3 increases transcription of ATGL. In conclusion, ATGL and HSL work in tandem following lipolytic stimulation by isoproterenol, but are differentially regulated by leptin. This differential regulation may occur through increased transcription of ATGL by STAT3.

### A.2 INTRODUCTION

Adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) are the key lipases involved in PKA-dependent lipolysis. Under catecholamine stimulation, protein kinase A (PKA) phosphorylates HSL and perilipin. Phosphorylated HSL translocates to the lipid droplet where it interacts with phosphorylated perilipin to hydrolyze tri- and diacylglycerides. Phosphorylation of perilipin by PKA allows for the translocation of ATGL from the cytosol to the lipid droplet through the release of CGI-58 from the lipid droplet. Comparative gene identity-58 activates ATGL through the release of G0/G1 Switch protein 2 (G0S2) which inhibits ATGL activity (5, 6, 8, 11, 18).

Mechanisms that regulate PKA-dependent lipolysis are well established, but the contribution of these mechanisms during leptin induced lipolysis is not well characterized. Leptin signaling results in the phosphorylation of signal transducer and activator of transcription (STAT) 3 via janus kinase 2 (JAK) (2). The increased STAT3 signaling contributes to increased protein abundance of ATGL during leptin induced lipolysis suggesting STAT3 translocates to the nucleus to increase transcription of
ATGL. Therefore, the objectives of this study were to determine 1) if ATGL translocates to the lipid droplet with exposure to leptin and 2) if phosphorylated STAT3 translocates to the nucleus to potentially increase transcription.

A.3 MATERIALS AND METHODS

A.3.1 Culture of Stromal Vascular Cell Derived Adipocytes

Stromal vascular cells (SVC) were harvested using a previously described method with slight modifications (Ajuwon et al., 2004). Cells were isolated from 2 multiparous mid lactation Jersey cows (Chapter 4). Following collagenase digestion of adipose tissue, SVCs were pelleted and rinsed two times in warmed cocktail media [20 mM Sodium bicarbonate, 20 mM HEPES, 10 mM D-glucose, and 3% bovine serum albumin (w/v), pH 7.4]. Cells were counted using trypan blue staining, plated at 10,000 cells per cm² in 6-well plates or 60 cm petri dishes, and grown on a glass cover slip in low glucose DMEM (D5523, Sigma Aldrich, St. Louis, MO) media containing 5% fetal bovine serum (FBS, s11150, Atlanta Biologicals, Norcross, GA). Media was changed every 4 days until reached 90% confluency. Subsequently, SVC-cells were cultured in differentiation media [10% FBS, 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX, I5879, Sigma-Aldrich, St. Louis, MO) 1μM of dexamethasone (D4902, Sigma Aldrich, St. Louis, MO), and 10ug/ml of insulin (I6634, Sigma Aldrich, St. Louis, MO)] as previously described (19). The first day of differentiation was denoted as day 0. After 48 hours, differentiation media was change to lipogeneic media [10% FBS, 10 μM of troglitizone (71750, Caymen Chemical, Ann Arbor, MI), 10ug/ml of insulin, 2% v/v of fatty acid supplement (F7175-5 mL, Sigma Aldrich, MO), 1mM of Sodium Acetate (S209-500g,
Fisher Scientific, Waltham, MA) on day 2 day of differentiation, and changed every 2 days until 14 days post differentiation.

**A.3.2 Experimental treatment and immunofluorescent labeling**

On day 14, SVC-derived adipocytes were conditioned with low glucose DMEM for one hour prior to the addition of fresh low glucose DMEM media and treatments. For the first experiment, SVC-derived adipocytes were exposed to media (control), 100 ng/mL of bovine leptin (CYT-502, ProSpec, Ness-Ziona, Isreal) or 100nM of isoproterenol (151358; MP Biomedicals; Solon, OH) for two hours. For the second experiment, SVC-derived adipocytes were treated with 100 ng/mL of bovine leptin for 0, 5, 10, 20, 120 or 240 minutes. Following treatments, SVC-derived adipocytes were rinsed twice with phosphate buffered saline (PBS) then fixed for five minutes using 4 % paraformaldehyde. Cells were rinsed three times using PBS and then permeated for five minutes 0.6% Triton-X 100 in PBS. The SVC-derived adipocytes were blocked for one hour in PBS containing 5 % bovine serum albumin after rinsing in PBS. Coverslips with cells were then removed and exposed to primary antibodies overnight. Primary antibodies and their corresponding secondary antibody are listed in Table 1. Cover slips were rinsed in PBS containing 0.5% Tween-20 and then exposed to fluorescently labeled secondary antibodies and either 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, IW-1404, IHC World, Woodstock, MD) to stain nuclei or Bodipy (D-2184, Molecular Probes, Eugene, Oregon) to the lipid droplet, respectively. After one hour of incubation, cover slips were rinsed with PBS containing 0.5% Tween-20 in PBS and placed in Fluorogel with DABCO mounting media on microscope slides (17985-01, Electron Microscopy Sciences, Hatifield, PA) and sealed with nail polish.
A.3.3 Analysis of Immunofluorescence

Fluorescence was captured from a dual labeled coverslip using a Qimaging QICam (Surrey BC, Canada) accompanied by a Leica DMI3000B microscope (Wetzlar, Germany). Each fluorescent wavelength was captured, saved individually, and then compiled by Qimaging software. Five views containing well-formed and well dispersed lipid droplets or nuclei were captured per coverslip. Views were captured from two coverslips per time point from a single cell line for analysis of time dependent translocation of ATGL. All other treatments had one coverslip per treatment for each of the two cows.

Fluorescence was measured using ImageJ software (17). Currently, software is not available to automatically measure the translocation of HSL and ATGL to the lipid droplet. To measure the translocation of HSL and ATGL, the difference in fluorescence at the lipid droplet and cytosol were measured. To determine this difference, a line was extended from the edge of the lipid droplet into the cytosol (Figure 1A). This line was arbitrarily drawn around the lipid droplet, but areas where the line would overlap with another lipid droplet were avoided. The average fluorescence measured between 0 and 0.02 inches was considered to be fluorescence at the lipid droplet. The average fluorescence between 0.06 and 0.0733 inches was considered to be cytosolic fluorescence. These averages were used to determine the increased fluorescence at the lipid droplet by subtracting the average fluorescence at the lipid droplet from the average fluorescence in the cytosol. To prevent bias from the location of the line, fluorescence was measured along five different lines for each lipid droplet and two to three lipid droplets were measured per view. The difference in fluorescence was
averaged by lipid droplet, and was used for statistical analysis. For simplicity, we will refer to the difference in fluorescence at the lipid droplet and the fluorescence in the cytosol as translocation of the protein of interest. To account for changes that occurred within an image, fluorescence was measured at random points within the image. The difference in background fluorescence was quantified to assess overall variability in fluorescence, and was measured as the difference in the average fluorescence between 0 and 0.02 from the average fluorescence between 0.6 and 0.0733.

Translocation of phosphorylated STAT3 to the nucleus was determined by calculating the colocalization of FITC and DAPI using the Mander’s Coefficient plugin from the ImageJ software (17). Four correlation coefficients were determined for each slide. See table 2 for a list of these correlation coefficients and definitions. Five views were measured per slide with one slide per cow. The correlation coefficients were averaged by slide and used for statistical analysis. Views were dropped from the analysis when the colocalization of FITC and DAPI greatly deviated from nucleus (see Figure 1B). Translocation of phosphorylated STAT3 to the nucleus caused a notable increase in the fluorescence in the nucleus compared to the cytosol. This increase in fluorescence was quantified by counting noticeable areas with increase fluorescence in the phosphorylated STAT3 image that corresponded to a DNA nucleus in the corresponding image. Counting these “visible nuclei” allowed for the confirmation of results observed with the Mander’s plugin. These counts will be identified as visual nuclei counts.
A.3.4 Statistical Analysis

Differences in lipid droplet diameter, translocation, and colocalization were estimated using PROC MIXED of SAS(16). Differences in lipid droplet diameter were estimated using treatment (time or stimulant) as a fixed effect, and view was grouped within slide or cow as a random effect. Differences in translocation of HSL, ATGL, CGI-58, and G0S2 were estimated using treatment (time or stimulant) as a fixed effect, lipid droplet diameter and background fluorescence as covariates, and view was grouped within slide or cow as a random effect. Differences in the colocalization of FITC and DAPI for total colocalization (Rtotal), Manders’ adjusted colocalization (Rcoloc), overlapping of FITC with DAPI or overlapping of DAPI with FITC (tM1 and tM2, respectively), and visual nuclei counts were estimated using as a fixed effect of time and cow as a random effect. Residuals were tested for normality and were found to be normally distributed (16). When overall significance was P < 0.05, pairwise comparisons were conducted using the LSmeans statement in SAS (16).

A.4 RESULTS AND DISCUSSION

A.4.1 Translocation of ATGL and HSL to the lipid droplet under β-agonist and leptin stimulation

Lipid droplet size has been associated with differential translocation of ATGL due to differences in perilipin abundance (7). Therefore, the diameter of lipid droplets was measured and included as a covariate. Lipid droplet diameter did not differ across time for cells immunostained for ATGL (P < 0.21, Figure 2) or among different lipolytic stimuli
treatments for cells immunostained for HSL (P < 0.40). Lipid droplet diameter tended to be different between different lipolytic stimulation for SVC-derived adipocytes immunostained for ATGL (P < 0.08; Figure 2).

Translocation of ATGL and HSL was altered by the addition of lipolytic stimuli (P < 0.03, Figure 3; P < 0.01, respectively; Figure 4). Isoproterenol and leptin increased ATGL translocation to the lipid droplet compared to control SVC-derived adipocytes (P = 0.01 and P = 0.04, respectively). No difference in ATGL translocation to the lipid droplet was observed between isoproterenol and leptin treated SVC-derived adipocytes (P = 0.81). Treatment with isoproterenol increased the translocation of HSL to the lipid droplet compared to control and leptin treatment (P < 0.01, and P < 0.01, respectively). However, translocation of HSL to the lipid droplet was not different between control and leptin treated SVC-derived adipocytes (P = 0.70).

The results indicate that translocation of ATGL to the lipid droplet may be regulated by two different mechanisms. As expected, isoproterenol stimulated the translocation of HSL to the lipid droplet through PKA mediated phosphorylation. Additionally, isoproterenol likely stimulated the translocation of ATGL either through direct phosphorylation (14) or indirectly through the phosphorylation of perilipin (7). Leptin administration increased translocation of ATGL, but not HSL, suggesting that ATGL translocates to the lipid droplet via a PKA-independent mechanism during leptin induced lipolysis. One possible explanation is that leptin increases protein abundance of ATGL quicker than its inhibitor, G0S2. Therefore, the observed increase in ATGL translocation to the lipid droplet due to stimulated lipolysis may be a function of overall increases in ATGL protein abundance.
These two mechanisms regulating ATGL translocation may explain the discrepancy between ATGL protein abundance during early lactation and expected activity. During early lactation, circulating leptin concentrations decrease to increase appetite (1, 3, 15), and coincide with decreased ATGL protein abundance (Chapter 3). Additionally, ATGL protein abundance is decreased during feed restriction (P. Faylon, personal communication) when circulating leptin concentrations decrease (4, 9). Therefore, the decrease in ATGL protein abundance may be due to decreased leptin signaling in adipose tissue. However, during times of stimulated lipolysis both HSL and ATGL may work in tandem to maximize lipolysis.

A.4.2 Translocation of phosphorylated STAT3 to the nucleus with leptin stimulation

Pearson’s correlation coefficients between phosphorylated STAT3, as measured by FITC, and nuclear DNA, as measured by DAPI, for all positive pixel (Rtotal) within a field of SVC-derived adipocytes treated with leptin differed across time points (P = 0.01, Figure 5 A). Colocalization between phosphorylated STAT3 and nuclear DNA increased after 5, 10, 120 and 240 minutes of exposure to leptin compared to 0 minutes of exposure (P < 0.03). Peak colocalization between phosphorylated STAT3 and nuclear DNA occurred at 10 minutes of leptin exposure (P < 0.02). This measurement determines the overlap of all pixels for each wavelength. Since background staining of one or both channels can greatly influence this correlation, methods have been developed to reduce the influence of background staining (12). When correlations were adjusted to only include those that passed the Manders’ threshold for the whole image (Rcoloc), phosphorylated STAT3 tended to colocalize with nuclear DNA (P = 0.06,
Figure 5C). Peak colocalization occurred after 10 minutes of exposure to bovine leptin. Additionally, the overlap of phosphorylated STAT3 with nuclear DNA tended to be altered by leptin exposure (tM1, P = 0.06), but the overlap between nuclear DNA and phosphorylated STAT3 was not altered by leptin exposure (tM2, P = 0.13). As with colocalization of the whole image, phosphorylated STAT3 pixels overlapped with nuclear DNA pixels at 10 of leptin exposure compared to all other time points (P < 0.01). These results were confirmed by the observation that visual colocations was significantly altered with leptin treatment (P = P 0.05; Figure 5D), and peak visual colocalization was observed after 10 minutes of treatment compared to all other time points (P < 0.01). Phosphorylated STAT3 was expected to rapidly translocate to the nucleus following leptin exposure. However, the lack of colocalization between phosphorylated STAT3 and nuclear DNA after 10 minutes of leptin exposure was not expected based on previous work in islets (13).

**A.4.3 Leptin induced translocation of ATGL to the lipid droplet after 2 hours**

Translocation of ATGL to the lipid droplet was altered with leptin administration across time points (P = 0.004). Translocation of ATGL increased with 120 minutes of exposure to bovine leptin compared to all other time points (P < 0.03). Adipose triglyceride lipase did not translocate to the lipid droplet at 5, 10, or 20 minutes of leptin exposure compared to control samples (Figure 6). However, its cofactors, CGI-58 and G0S2, did not translocate to the lipid droplet following leptin administration (P = 0.084, Figure 6; P = 0.169, Figure 7; respectively).
After two hours of leptin exposure, ATGL translocation to the lipid droplet, but CGI-58 did not significantly translocated from the lipid droplet. This supports our previous conclusion that leptin induces ATGL translocation to the lipid droplet through a PKA-independent mechanism. The inhibition of the leptin induced increase in ATGL protein abundance by STATIC suggested that STAT3 may regulate ATGL activity. Porcine SVC-derived adipocytes exposed to leptin also exhibited a JAK and mitogen activated protein kinase (MAPK) dependent increase in ATGL transcript abundances (10). However, Li et al. (10) did not determine the interaction of the JAK/STAT3 or MAPK pathways with the translocation of ATGL.

In conclusion, ATGL may participate in lipolysis through two different mechanisms. One mechanism by which ATGL participates in lipolysis is through the PKA pathway. Following activation of PKA, HSL and perilipin are phosphorylated. Phosphorylated HSL translocates to the lipid droplet where it is expected to hydrolyze tri- and diacylglycerides, and phosphorylated perilipin releases CGI-58 from the lipid droplet, allowing for the translocation of ATGL to the lipid droplet. A second mechanism by which ATGL may participate in lipolysis is through a leptin induced increased in total protein abundance. The increase in ATGL protein abundance may increase its translocation to the lipid droplet due to decreased availability of its inhibitor, G0S2. However, it remains unknown if leptin stimulates STAT3 to directly increase ATGL transcription through binding the promoter region.
### Table 1. Primary and secondary antibodies used for immunofluorescence

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<th>Antibody</th>
<th>Company</th>
<th>Catalog number</th>
<th>Secondary Antibody</th>
<th>Fluoroform</th>
<th>Catalog number a</th>
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<td>PSTAT3 b</td>
<td>Cell Signaling</td>
<td>9145</td>
<td>Rabbit IgG</td>
<td>FITC</td>
<td>111-095-003</td>
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<tr>
<td>HSL</td>
<td>Cell Signaling</td>
<td>8719</td>
<td>Rabbit IgG</td>
<td>Dylight 405</td>
<td>711-475-152</td>
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<tr>
<td>ATGL</td>
<td>Cell Signaling</td>
<td>2138</td>
<td>Rabbit IgG</td>
<td>Dylight 405</td>
<td>711-475-152</td>
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<tr>
<td>CGI-58</td>
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<td>Goat IgG</td>
<td>Dylight 649</td>
<td>705-495-003</td>
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<tr>
<td>G0S2</td>
<td>Novus Biologicals</td>
<td>4137</td>
<td>Mouse IgG</td>
<td>Dylight 594</td>
<td>115-515-146</td>
</tr>
</tbody>
</table>

*All secondary antibodies were purchased from Jackson Immunoresearch in West Grove, PA*

Abbreviations: PSTAT, phosphorylated signal transduction and activator of transcription 3 at tyrosine 705; HSL, hormone sensitive lipase; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identity-58; G0S2, G0/G1 switch protein 2; FITC, fluorescein isothiocyanate
Table 2. A list of Correlation Coefficients calculated by the Manders' Coefficient plug-in from ImageJ.

<table>
<thead>
<tr>
<th>Correlation Coefficient</th>
<th>Definition</th>
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<tr>
<td>$R_{total}^a$</td>
<td>Pearson' correlation coefficient for the whole image using only positive pixels</td>
</tr>
<tr>
<td>$R_{coloc}$</td>
<td>Returns correlation coefficients where both channel $1^b$ and $2$ are above their threshold</td>
</tr>
<tr>
<td>$tM1$</td>
<td>Returns the percentage of above-background pixels in channel $1$ that overlap with above-background pixels in channel $2$</td>
</tr>
<tr>
<td>$tM2$</td>
<td>Returns the percentage of above-background pixels in channel $1$ that overlap with above-background pixels in channel $2$</td>
</tr>
</tbody>
</table>

$^a$ Abbreviation given by the plugin.

$^b$ Channel $1$ represents the 490 nm wavelength (FITC) and channel $2$ represents the 358 nm wavelength (DAPI).
A.6 FIGURES

Figure 1. Demonstrates how images were assessed. A) Illustrates how the diameters of assessed lipid droplets were measured. The white line represents where the fluorescence was measured. The upper box represents the relative fluorescent abundance. B). Illustrates how translocation was measured for each lipid droplet. The white lines represent how the fluorescence was measured. The upper box represents the relative fluorescent abundance. The values along the line were exported and utilized for statistical analysis. C). Represents expected colocalization of phosphorylated STAT3 (FITC) and nuclear DNA (DAPI) fluorescence. The top left images is the phosphorylated STAT3 image, and the lower left image is the nuclear DNA image. The larger image is the merged images. Green represent phosphorylated STAT3, blue represents nuclear DNA, and white represents areas of colocalization. D). Represents abnormal colocalization of phosphorylated STAT3 and nuclear DNA fluorescent. The top left images is the phosphorylated STAT3 image, and the lower left image is the nuclear DNA image. The larger image is the merged images. Green represent phosphorylated STAT3, blue represents nuclear DNA, and white represents areas of colocalization.

Figure 2. Average diameter for lipid droplets used to determine fluorescence for hormone sensitive lipase and adipose triglyceride lipase. A) Average diameter of lipid droplets treated with media, 100nM isoproterenol, or 100ng/mL of leptin for 2 hours for SVC-derived adipocytes used to detect HSL and ATGL translocation. B) Average
diameter of lipid droplets treated with 100 ng/mL of leptin through the 2 hour time course for SVC-derived adipocytes stained with ATGL and CGI-58 or G0S2.

Abbreviations: HSL, hormone sensitive lipase; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identity-58; G0S2, G0/G1 switch protein 2

*Figure 3. Translocation of adipose triglyceride lipase (ATGL) to the lipid droplet after treatment with isoproterenol or leptin.* A) Representative images of SVC-derived adipocytes treated with media (Control), 100nM of isoproterenol, or 100 ng/mL of leptin. DyLight 405 was used to detect ATGL antibody, and bodipy was used to detect neutral lipids in the lipid droplet. In the merged image, ATGL is blue and bodipy is green. B) Translocation of ATGL to the lipid droplet. The white line in the lower right hand image is 0.2 inches relative to the view. Differences in subscripts were given when the overall P < 0.05, and indicated pairwise differences of P < 0.05.

*Figure 4. Translocation of hormone sensitive lipase (HSL) to the lipid droplet after treatment with isoproterenol or leptin.* A) Representative images of SVC-derived adipocytes treated with media (Control), 100nM of isoproterenol, or 100 ng/mL of leptin. DyLight 405 was used to detect HSL antibody, and bodipy was used to detect neutral lipids in the lipid droplet. In the merged image, HSL is blue and bodipy is green. B) Translocation of HSL to the lipid droplet. The white line in the lower right hand image is 0.2 inches relative to the view. Differences in subscripts were given when the overall P < 0.05, and indicated pairwise differences of P < 0.05.
**Figure 5. Colocalization of phosphorylated signal transducer and activator of transcription (STAT) 3 with the nucleus.** A) Representative images of SVC-derived adipocytes treated with 100 ng/mL over the course of 4 hours. Time of exposure is listed on the left hand side. DAPI was used to stain for nuclear DNA and FITC was used to detect the antibody for phosphorylated STAT3. In the merged image, blue represents nuclear DNA and green represents phosphorylated STAT3. B) Average Pearson’s correlation coefficients for the colocalization nuclear DNA and phosphorylated STAT3 for all positive pixels within the image. C) Average Pearson’s correlation coefficients for the colocalization of nuclear DNA and phosphorylated STAT3 for pixels above Manders’ threshold. D) Average number of visual colocalization. Arrows in 10 minutes images represent the increased fluorescence in phosphorylated STAT3 that would count as a visual colocalization. Arrows in 120 minute images represent an area that would not be counted as visual colocalization. The white line in the lower right hand image is 0.2 inches relative to the view. Differences in subscripts were given when the overall P < 0.05, and indicated pairwise differences of P < 0.05.

**Figure 6. Translocation of adipose triglyceride lipase (ATGL) and comparative gene identity -58 (CGI-58) to the lipid droplet after leptin exposure over the course of 2 hours.** A) Representative images of SVC-derived adipocytes treated with 100 ng/mL of leptin over the course of 2 hours. Time of exposure is listed on the left hand side. DyLight 405 was used to detect the ATGL antibody, bodipy was used to stain
neutral lipids, and DyLight 649 was used to detect the CGI-58 antibody. In the merged image, blue represents ATGL, green represents bodipy, and red represents CGI-58. B) Translocation of ATGL to the lipid droplet for both images that contained CGI-58 and G0S2. C) Translocation of CGI-58 to the lipid droplet. The white line in the lower right hand image is 0.2 inches relative to the view. Differences in subscripts were given when the overall P < 0.05, and indicated pairwise differences of P < 0.05.

**Figure 7. Translocation of adipose triglyceride lipase (ATGL) and G0/G1 switch protein 2 (G0S2) to the lipid droplet after leptin exposure over the course of 2 hours.** A) Representative images of SVC-derived adipocytes treated with 100 ng/mL of leptin over the course of 2 hours. Time of exposure is listed on the left hand side. DyLight 405 was used to detect the ATGL antibody, bodipy was used to stain neutral lipids, and DyLight 594 was used to detect the G0S2 antibody. In the merged image, blue represents ATGL, green represents bodipy, and red represents G0S2. B) Translocation of ATGL to the lipid droplet for both images that contained CGI-58 and G0S2. C) Translocation of G0S2 to the lipid droplet. The white line in the lower right hand image is 0.2 inches relative to the view. Differences in subscripts were given when the overall P < 0.05, and indicated pairwise differences of P < 0.05.
Figure 1
Figure 2

A.

![Bar graph showing diameter in inches for HSL and ATGL with different treatments: Control, Isoproterenol, Leptin.](image)

B.

![Bar graph showing time course of diameter in inches with different time points: 0 min, 5 min, 10 min, 20 min, 120 min.](image)
Figure 3

A.

Control

Isoproterenol

Leptin

B.

![Bar graph showing translocation relative units for different treatments.](image)

- Control
- Isoproterenol
- Leptin

*Note: Bars with different letters (a, b) indicate statistical significance.*
Figure 4

A. Control

Isoproterenol

Leptin

B. Translocation, Relative Units

Control Isoproterenol Leptin

Graphical data showing the effects of control, isoproterenol, and leptin on translocation.
Figure 5
Figure 6

A.

0 minutes

ATGL
Lipid droplet
CGI-58
Merged

5 minutes

10 minutes

20 minutes

120 minutes

B.

Translocation of ATGL Relative units

Minutes of exposure

C.

Translocation of CGI-58 Relative Units

Minutes of exposure
Figure 7

A. 

0 minutes 

5 minutes 

10 minutes 

20 minutes 

120 minutes 

B. 

C.
A.7 REFERENCES


APPENDIX B. IDENTIFICATION OF A POTENTIAL SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 BINDING SITE IN THE PROMOTER REGION OF ADIPOSE TRIGLYCERIDE LIPASE

Preliminary Data

Dawn A. Koltes and Diane M. Spurlock

B.1 ABSTRACT

Previous work indicated that leptin induced lipolysis occurs through a signal transducer and activator of transcription (STAT) 3 mediated increase in adipose triglyceride lipase (ATGL) protein abundance. To determine if STAT3 regulates the transcription of ATGL, a DNA pull down assay was used to determine if STAT3 binds any of the computationally predicted transcription factor binding sites in the promoter region of ATGL. Following leptin administration, STAT3 binding increased for 1 of 5 STAT3 promoter regions which contained 2 putative transcription factor binding sites for STAT3. These results indicate that leptin may modulate ATGL transcription by increasing STAT3 binding in the ATGL promoter region.
B.2 INTRODUCTION

Previous research identified STAT3 as a potential mediator of leptin induced lipolysis in bovine adipocytes (Chapter 4). Adipose triglyceride lipase (ATGL) protein abundance increased with leptin exposure, and this effect was attenuated with the addition of a STAT3 inhibitor. Based on these results, we hypothesized that leptin increases lipolysis by STAT3 mediated regulation of ATGL transcript expression. Therefore, the objective of this study was to determine if STAT3 binds to the promoter region of ATGL in adipocytes.

B.3 MATERIALS AND METHODS

B.3.1 Prediction of STAT3 Binding Sites

Adipose triglyceride lipase is located on the reverse strand of bovine chromosome 29. Potential transcription factor binding sites for STAT3 were predicted in the promoter region of ATGL using two approaches. Eight predicted transcription factor binding sites were identified within 2 kilobase (kbps) of the ATGL start site in the UMD3.1 bovine genome build using TRANSFAC (Matys et al., 2006). The TRANSFAC database includes more than 37,000 computationally and experientially validated transcription factor binding sites from multiple species. The TRANSFAC algorithm uses a positional weighing matrix to predict transcription factor binding sites based on core matches and matrix matches to sites in the database. Core and matrix matches with correlation coefficients greater than 0.89 and 0.80, respectively were considered for validation. The eight potential transcription factor binding sites from TRANSFAC are listed in table 1, and predicted sequences are highlighted in red and purple font in figure 1. Additionally, TRANSFAC allows for tissue specific searches based on experimentally
validated data in human and mouse. These sites are identified in table 1. Consensus transcription factor binding sites were also identified using JASPER software (Bryne et al., 2008). The consensus site for *Mus musculus* STAT3 was utilized to search for potential transcription factor binding sites within 2 kb of the start site of *ATGL*. The corresponding bovine transcription factor binding sites predicted by JASPER are in blue in figure 1.

**B.3.2 Pull-down assay**

Adipocytes were harvested from 3 mid lactation (278-288 DIM) primiparous cows utilizing methods previously described in chapter 4. Following a thirty minute acclimation period, adipocytes were exposed to media only or media containing 100 ng/mL of leptin for 20 minutes. Protein-DNA complexes were crosslinked using 4% paraformaldehyde for 10 minutes, followed by quenching for 5 minutes with 1M glycine. Adipocytes were centrifuged and stored until the pull-down assays were performed. DNA pull-down was performed using the Chromatin- ImmunoPrecipitation (ChIP) agarose kit (26156, Pierce, Thermo-Scientific, Rockford, IL; Figure 2). Briefly, adipocytes were lysed and DNA was sheared by micrococcal nuclease digestion. Approximately 10% of the preparations were stored as input controls (IC). The remainder of the preparation was split and exposed to STAT3 (8719, Cell Signaling, Boston, MA) or IgG (included in the kit) antibodies for 72 hours at 4°C. The protein-DNA complexes were pulled down using protein A/G agarose beads. Protein was subsequently digested from STAT3 enriched (SE), IgG enriched (IE) and IC samples using Proteinase K. The DNA fraction was purified prior to real-time polymerase chain reactions (qPCR) using columns provided in the kit.
B.3.3 Primer design

Primers were designed to target all nine predicted STAT3 binding sites. Primers were also designed for a known STAT3 binding site upstream of hepatocyte growth factor (*HGF*) (Tomida and Saito, 2004) and within an exonic region of ATGL to act as positive and negative controls, respectively. Due to the affinity of micrococcal nuclease to cleave 5’ of adenosine (A) or thymine (T), primers were designed to avoid AT-rich regions. Primers for qPCR are listed in table 2 and the sequences amplified are shown in figure 2. For simplicity, we named the sequences to be amplified as promoter regions 1-5.

B.3.4 Real-time polymerase chain reaction

The standard protocol for qPCR was 95° C for 2 min followed by 40 repeated cycles of 95° C for 30 s, 50° C for 30 s, and 72° C for 45 s. The PCR products were then held at 95° C for 1 min, followed by 56° C for 1 min. Due to low abundance of input DNA, qPCR was increased to 50 cycles for the first cow (8156). Since, multiple melt curves were observed using 50 cycle with the first cow (8156) and threshold values (Ct) were between 25 and 35, the number of cycles was reduced to 40 cycles for the remaining cows (8144 and 8200). Quantitative PCR was run in a 96-well format on a MyiQ thermocycler (Bio Rad, Hercules, CA) using SYBR Green chemistry (Bio-Rad, Hercules, CA) according to manufacturer’s protocol, scaled to a total volume of 12.5 μl with 2.5 μl of the reaction being DNA. A serially diluted standard curve from input DNA was included with each assay as a positive control and for evaluation of PCR efficiency. Reactions devoid of template were included as negative controls for each set of primers. All samples and controls were run in duplicate, and the average of duplicate
threshold cycle (Ct) values was used in statistical analyses. Replicated qPCR reactions with a major peak that deviated from the average more than + 1.5°C from the average melt curve were removed from the analysis. Samples removed included three HGF replicates and one ATGL promoter region 4 replicate.

**B.3.3 Statistical analysis**

Threshold cycle values were normalized by subtracting the input Ct value and adjusted for PCR efficiency (ΔCt values) as previously described (Vandesompele et al., 2002). The average ΔCt values were analyzed using PROC MIXED of SAS (SAS/STAT User’s Guide, 1990). Treatment, antibody, and the interaction of treatment and antibody were fit as fixed effects and cow was fit as a random effect (SAS/STAT User’s Guide, 1990). A promoter region was considered enriched when the interaction between treatment and antibody was significant (P < 0.05). When a promoter region was significantly enriched by leptin treatment, correlations between the ΔΔCt for leptin treated adipocytes and normalized ATGL and phosphorylated STAT3 protein abundance were analyzed using PROC CORR of SAS (SAS/STAT User’s Guide, 1990). Protein abundances were normalized by subtracting protein abundances from adipocytes treated with 100ng/mL of leptin from control adipocytes (Chapter 4). Results are presented as LSmeans of the ΔCt (Table 3) and as fold change differences in amplification of the target sequence following pull-down assay using STAT3 relative to IgG antibodies ($2^{-(\text{SE DNA} - \text{IC DNA}) - (\text{IE DNA} - \text{IC DNA})}$) in figure 1.
B.4 RESULTS AND DISCUSSION

The promoter region of \textit{HGF} tended to be enriched with leptin treatment, while amplification of exon 4 and 5 of \textit{ATGL} was similar for leptin and control treated adipocytes. Promoter region 1 of \textit{ATGL} was enriched with leptin treatment. Amplification of promoter region 1 of \textit{ATGL} increased with leptin treated STAT3 enriched samples compared to all other treatments (P < 0.05). All other promoter regions examined were not enriched by treatment. See table 3 for LSmeans and P-values and figure 3 for ΔΔCt fold changes.

Enrichment of the promoter region of \textit{HGF} serves as an important positive control of STAT3 signaling. The low number of biological samples and high variation of enrichment between cows may have prevented this enrichment from reaching significance. Leptin treatment did not enrich STAT3 binding of exon 4 and 5 of \textit{ATGL} demonstrating the specificity of this assay. Enrichment of \textit{ATGL} promoter region 1 indicates that leptin increased \textit{ATGL} protein abundance via STAT3 increased transcription of \textit{ATGL}. Two putative transcription factor binding sites reside within the \textit{ATGL} promoter region 1, and this assay was not sensitive enough to determine which of the two transcription factor binding site is enriched by leptin treatment. Regardless, this is the first study to demonstrate potential binding of STAT3 to the promoter region of \textit{ATGL} under leptin treatment.

Correlations between the enriched promoter region 1 and protein abundance for \textit{ATGL} and phosphorylated STAT3 were not significant (Figure 4). This is not surprising since only 3 biological replicates were used in this analysis. Interestingly, both \textit{ATGL} and phosphorylated STAT3 abundance may be positively associated with enrichment of
ATGL promoter region 1 in the 2 samples (8200 and 8156) with similar enrichment, but the lowly enriched sample (8144) had higher protein abundance for both ATGL and phosphorylated STAT3. The inclusion of additional biological samples would allow for a better understanding of STAT3 enrichment of ATGL promoter region 1.

These preliminary data provide evidence that STAT3 binds a DNA element less than 150 bps upstream of the ATGL start site. The enriched amplicon contains two predicted STAT3 transcription factor binding sites, and the increased binding is dependent on leptin treatment. These results indicate that leptin may stimulate STAT3 binding to the promoter region of ATGL, potentially increasing transcription and subsequent translation of ATGL. These results in combination with Chapter 4 provide multiple lines of evidence indicating STAT3 mediates leptin induced lipolysis.
B.5 TABLES

**Table 1.** Predicted signal transducer and activator of transcription 3 binding sites from TRANSFAC with their corresponding core and matrix matches.

<table>
<thead>
<tr>
<th>Position (strand)</th>
<th>Core Match</th>
<th>Matrix Match</th>
<th>AT specific search&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seq.1 (+)</td>
<td>0.928</td>
<td>0.892</td>
<td>X</td>
</tr>
<tr>
<td>Seq.2 (+)</td>
<td>0.905</td>
<td>0.869</td>
<td>X</td>
</tr>
<tr>
<td>Seq.3 (+)</td>
<td>0.941</td>
<td>0.853</td>
<td></td>
</tr>
<tr>
<td>Seq.4 (+)</td>
<td>0.932</td>
<td>0.846</td>
<td></td>
</tr>
<tr>
<td>Seq.5 (+)</td>
<td>0.898</td>
<td>0.864</td>
<td></td>
</tr>
<tr>
<td>Seq.6 (-)</td>
<td>0.909</td>
<td>0.829</td>
<td>X</td>
</tr>
<tr>
<td>Seq.7 (+)</td>
<td>1</td>
<td>0.893</td>
<td></td>
</tr>
<tr>
<td>Seq.8 (+)</td>
<td>0.984</td>
<td>0.859</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>TRANSFAC allows for tissue specific prediction. Those marked with X were identified as adipose tissue specific.
Table 2. Primer sequences used for quantitative real-time polymerase chain reaction of predicted transcription factor binding site (TFBS).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Accession Number</th>
<th>TFBS(^a)</th>
<th>Amplicon Size (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGL PR 1 Forward</td>
<td>5'-CCT CTT TCA CTT GTG TGC GG-3'</td>
<td>NM_001046005.2</td>
<td>1,2</td>
<td>119</td>
</tr>
<tr>
<td>ATGL PR 1 Reverse</td>
<td>5'-GGC TGG CTG GCA GGC CCT GA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGL PR 2 Forward</td>
<td>5'-TCA GGG CCT GCC AGC CAG CC-3'</td>
<td>NM_001046005.2</td>
<td>3,4</td>
<td>138</td>
</tr>
<tr>
<td>ATGL PR 2 Reverse</td>
<td>5'-GCA AGG ATC TTG GTC CCC AA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGL PR 3 Forward</td>
<td>5'- TTG GGA CCA AGA TCC TTG C-3'</td>
<td>NM_001046005.2</td>
<td>5</td>
<td>91</td>
</tr>
<tr>
<td>ATGL PR 3 Reverse</td>
<td>5'-TCC AAC CGG GAA ATC ATC CC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGL PR 4 Forward</td>
<td>5'-CCT GCC CGA ATA TAG GAT GG-3'</td>
<td>NM_001046005.2</td>
<td>6,7</td>
<td>171</td>
</tr>
<tr>
<td>ATGL PR 4 Reverse</td>
<td>5'-CCC GTC CGC TCA GAA ACC CC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGL PR 5 Forward</td>
<td>5'-CCA CCC AGT TCT AGT TCA GC-3'</td>
<td>NM_001046005.2</td>
<td>8(^*)</td>
<td>155</td>
</tr>
<tr>
<td>ATGL PR 5 Reverse</td>
<td>5'-AGA AAA TGC CAG CTT CCT CC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGL exon 4 Forward</td>
<td>5'-AGC TCA AGA ACA CCA TCA CG-3'</td>
<td>NM_001046005.2</td>
<td>NBC(^b)</td>
<td>189</td>
</tr>
<tr>
<td>ATGL exon 5 Reverse</td>
<td>5'- GTT TGC ACA TCT CTC GAA GC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGF Forward</td>
<td>5'-GAG AGA GTA AAG GGC TGT TGT T-3'</td>
<td>NM_001031751.1</td>
<td>PBC(^c)</td>
<td>98</td>
</tr>
<tr>
<td>HGF Reverse</td>
<td>5'-GCG ATG AGC TAA GTT TGT TGT G-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
a Abbreviations: ATGL, adipose triglyceride lipase; ATGL PR, ATGL promoter; HGF, hepatocyte growth factor

b NBC refers to the negative binding control.

c PBC refers to the positive binding controls.

*Includes one consensus transcription factor binding site identified by JASPER.
Table 3. Least squared (ls) means and P-values for analyses of DNA enrichment from pull down assays.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Leptin</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>STAT3</td>
<td>IgG</td>
</tr>
<tr>
<td>ATGL PR 1</td>
<td>-0.702&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-2.302&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-0.007&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATGL PR 2</td>
<td>-7.443&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-4.918&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-4.363&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATGL PR 3</td>
<td>-2.222&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-2.255&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.155&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATGL PR 4</td>
<td>-3.940&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-2.803&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-2.197&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATGL PR 5</td>
<td>0.585&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.640&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-0.458&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>HGF</td>
<td>1.045</td>
<td>-0.142&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.298&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ATGL</td>
<td>-1.550&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-2.893&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-1.470&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: ATGL PR 1, Adipose triglyceride lipase (ATGL) promoter region 1; ATGL PR 2, ATGL promoter region 2; ATGL PR 3, ATGL promoter region 3; ATGL PR 4, ATGL promoter region 4; ATGL PR 5, ATGL promoter region 5; HGF, hepatocyte growth factor

<sup>a</sup> Control and Leptin represent either the untreated or leptin treatments adipocytes, respectively.

<sup>b</sup> IgG and STAT3 represent antibodies applied to DNA pull-down samples.

<sup>c</sup> Interaction refers to the interaction between treatment and antibody.

<sup>d</sup> LSmeans were calculated from the ΔCt.

Differences in subscripts indicated differences in pairwise comparisons.
B.6 FIGURES

Figure 1. The region upstream of the ATGL start site including predicted transcription factor binding sites from TRANSFAC and JASPER. TRANSFAC predicted are numbered according to their identification by TRANSFAC and are listed in table 1. Sequences identified in the 5’ to 3’ orientation are in red font and those in the 3’ to 5’ orientation are in purple font. Lowercase letters indicate nucleotides included in the core matches, and all other nucleotides in a different color were included in the matrix matches. Sequences predicted by JASPER are all in the 5’ to 3 prime orientation and are in blue font. Amplified products using primers listed in table 2 are in different colored boxes. The promoter regions (PR) were labeled 1-5 according to their location from the start site. PR 1 is outlined in a blue box, PR 2 is outlined in a green box, PR3 is outlined in a black box, PR 4 is outlined in a red box, and PR 5 is outlined in a purple box.

Figure 2. Experimental design for the DNA pull-down. A) Overview of laboratory techniques. 1) Harvested adipocytes were treated with leptin or media for 20 minutes. 2) Protein bound to DNA is crosslinked. 3) DNA is sheared using micrococcal nuclease. 4) A portion of the sheared DNA is saved as Input control (IC) and the remainder of the samples is exposed to either STAT3 (SE) or IgG (IE) antibody for 72 hours then DNA was pulled down using protein A/G beads. 5) All DNA is purified after proteinase K digestion. 6) Semi-quantitative PCR was performed using the purified DNA
from step 5. B) Formulas used to calculate ΔCt values used for statistical analyses and fold change calculated by ΔΔCt.

**Figure 3.** Fold change between DNA exposed to IgG and STAT3 antibodies for control and leptin treated adipocytes. A) and B) Fold change of the amplification of DNA exposed to the IgG and STAT3 antibodies. The dark gray box represents the fold change for the control adipocytes and the light gray box represents the fold change for the adipocytes treated with 100 ng/mL of leptin for 20 minutes. ATGL exon refers to primers designed to amplify exons in ATGL and serves as the negative binding control. HGF refers to the primers designed to amplify the known STAT3 transcription factor binding site in hepatocyte growth factor and serves as the positive binding control. ATGL PR1-5 refer to the promoter regions (PR) that were tested for enrichment as defined in figure 1 and table 2. These regions were designed to encompass eight predicted transcription factor binding regions. † represents tendencies in enrichment as defined by a P value < 0.1 for the interaction of antibody (IgG and STAT3) and treatment (control and Leptin), and * represents significance enrichment as defined by a P-value < 0.05 for the interaction of antibody (IgG and STAT3) and treatment (control and Leptin).

**Figure 4.** Scatterplots of the fold change of leptin treated adipocytes for adipose triglyceride lipase (ATGL) promoter region1 with A) ATGL protein abundance and B) phosphorylated STAT 3 protein abundance of leptin treated adipocytes compared to control adipocytes.
Figure 2

A. 

B. 

\[ \Delta C_{t_{\text{lgG}}} = C_{t_{\text{EDNA}}} - C_{t_{\text{CDNA}}} \]

\[ \Delta C_{t_{\text{STAT}}} = C_{t_{\text{EDNA}}} - C_{t_{\text{CDNA}}} \]

\[ \Delta \Delta C_{t_{\text{STAT}}} = 2^{(\Delta C_{t_{\text{STAT}}} - \Delta C_{t_{\text{lgG}}})} \]
Figure 3

A. 

B. 

Fold Change

Control
Leptin

ATGL exon  HGF  ATGL PR1  ATGL PR2  ATGL PR3

ATGL PR4  ATGL PR5
A.

![Graph A](image)

$\text{Fold change of ATGL promoter Region 1}$

$\text{Difference between leptin and control ATGL protein abundance}$

$r = -0.90081$

B.

![Graph B](image)

$\text{Fold change of ATGL promoter Region 1}$

$\text{Difference between leptin and control phosphorylated STAT3 protein abundance}$

$r = -0.31437$
B. 7 REFERENCES


ACKNOWLEDGEMENTS

The author would like to express her gratitude to all those who have helped make this dissertation possible. To the members of my committee, I would like to thank you for your time, guidance, and patience. In particular, I would like to thank Dr. Diane Spurlock for the opportunity to work with her and fostering my growth as a scientist. I would like to thank Dr. Chris Tuggle for joining my committee so late in the game, and Dr. Mike Spurlock for all his help preparing for preliminary exams and serving on my committee. Additionally, I would like to thank Dr. Steven Lonergan, Dr. Josh Selsby, Dr. Jason Ross, Dr. Nick Gabler, Dr. Michael Spurlock, Dr. Jim Reecy, and Mary Sue Mayes for their advice on laboratory techniques and/or use equipment to complete projects included in this dissertation. I would also like to thank Pia Faylon, Brittany Shonka, Katie Fobor, Jenny English, and Rebecca Schumacher for their assistance in the laboratory.

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