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Cellular changes in lipid metabolism in response to altered energy balance and heat stress in lactating dairy cows

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Cellular changes in lipid metabolism in response to altered energy balance and heat stress in lactating dairy cows

by

Ma. Pia P. Faylon

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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Iowa State University

Ames, Iowa

2015
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>A$_1$R</td>
<td>ADENOSINE A$_1$ RECEPTOR</td>
</tr>
<tr>
<td>ACC</td>
<td>ACETYL CoA CARBOXYLASE</td>
</tr>
<tr>
<td>ADA</td>
<td>ADENOSINE DEAMINASE</td>
</tr>
<tr>
<td>ATGL</td>
<td>ADIPOSE TRIACYLGLYCERIDE LIPASE</td>
</tr>
<tr>
<td>ATP</td>
<td>ADENOSINE TRIPHOSPHATE</td>
</tr>
<tr>
<td>BAR</td>
<td>BETA-ADRENERGIC RECEPTOR</td>
</tr>
<tr>
<td>BCA</td>
<td>BICINCHONINIC ASSAY</td>
</tr>
<tr>
<td>BCS</td>
<td>BODY CONDITION SCORE</td>
</tr>
<tr>
<td>BLAST</td>
<td>BASIC LOCAL ALIGNMENT SEARCH TOOL</td>
</tr>
<tr>
<td>BSA</td>
<td>BOVINE SERUM ALBUMIN</td>
</tr>
<tr>
<td>cAMP</td>
<td>CYCLIC ADENOSINE MONOPHOSPHATE</td>
</tr>
<tr>
<td>CGI-58</td>
<td>COMPARATIVE GENE IDENTITY-58</td>
</tr>
<tr>
<td>CH$_4$</td>
<td>METHANE</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>CARBON DIOXIDE</td>
</tr>
<tr>
<td>CPT-1</td>
<td>CARNITINE PALMITOYLTRANSFERASE-1</td>
</tr>
<tr>
<td>DAG</td>
<td>DIACYLGlycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>DULBECCO’S MODIFIED EAGLE MEDIUM</td>
</tr>
<tr>
<td>DMI</td>
<td>DRY MATTER INTAKE</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>HALF MAXIMAL EFFECTIVE CONCENTRATION</td>
</tr>
<tr>
<td>EDTA</td>
<td>ETHYLENEDIAMINETETRAACETIC ACID</td>
</tr>
<tr>
<td>FA</td>
<td>FATTY ACID</td>
</tr>
<tr>
<td>FR</td>
<td>FEED RESTRICTION</td>
</tr>
<tr>
<td>FSH</td>
<td>FOLLICLE-STIMULATING HORMONE</td>
</tr>
<tr>
<td>G-3-PDH</td>
<td>GLYCEROL-3-PHOSPHATE DEHYDROGENASE</td>
</tr>
<tr>
<td>G-6-PDH</td>
<td>GLUCOSE-6-PHOSPHATE DEHYDROGENASE</td>
</tr>
<tr>
<td>GH</td>
<td>GROWTH HORMONE</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-HYDROXYETHYL)-1-PIPERAZINEETHANESULFONIC ACID</td>
</tr>
<tr>
<td>HS</td>
<td>HEAT STRESS</td>
</tr>
<tr>
<td>HSL</td>
<td>HORMONE SENSITIVE LIPASE</td>
</tr>
<tr>
<td>HSP27</td>
<td>HEAT SHOCK PROTEIN 27</td>
</tr>
<tr>
<td>HSP70</td>
<td>HEAT SHOCK PROTEIN 70</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>HALF MAXIMAL INHIBITORY CONCENTRATION</td>
</tr>
<tr>
<td>IgG</td>
<td>IMMUNOGLOBULIN G</td>
</tr>
<tr>
<td>ISO</td>
<td>ISOPROTERENOL</td>
</tr>
<tr>
<td>LD</td>
<td>LIPID DROPLET</td>
</tr>
<tr>
<td>LH</td>
<td>LUTEINIZING HORMONE</td>
</tr>
<tr>
<td>MAG</td>
<td>MONOACYLGLYCEROL</td>
</tr>
<tr>
<td>MGL</td>
<td>MONOACYLGLYCEROL LIPASE</td>
</tr>
<tr>
<td>N$_2$O</td>
<td>NITROUS OXIDE</td>
</tr>
<tr>
<td>NaCl</td>
<td>SODIUM CHLORIDE</td>
</tr>
</tbody>
</table>
NADPH = NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
NEBAL = NEGATIVE ENERGY BALANCE
NEFA = NON-ESTERIFIED FATTY ACID
NE\text{L} = NET ENERGY FOR LACTATION
P-ACC = PHOSPHORYLATED ACETYL CoA CARBOXYLASE
P1 = PHOSPHATASE 1
PDE3B = PHOSPHODIESTERASE-3-B
PFTN = PAIR FED THERMAL NEUTRAL
PHSL\text{Ser}563 = PHOSPHORYLATED HORMONE SENSITIVE LIPASE AT SERINE RESIDUE 563
PHSL\text{Ser}565 = PHOSPHORYLATED HORMONE SENSITIVE LIPASE AT SERINE RESIDUE 565
PKA = PROTEIN KINASE A
PLIN = PERILIPIN
PPLIN = PHOSPHORYLATED PERILIPIN
R_{\text{max}} = MAXIMAL RESPONSE
RPL32 = RIBOSOMAL PROTEIN L32
TAG = TRIACYLGLYCERIDE
THI = TEMPERATURE HUMIDITY INDEX
TMR = TOTAL MIXED RATION
TN = THERMAL NEUTRAL
TNZ = THERMAL NEUTRAL ZONE
ABSTRACT

The dairy industry has evolved through the years. There is a need to continuously improve on selective breeding programs and management strategies in order to produce superior animals that could meet the increasing demands for food as well as adapt to the changing environment. Currently, a substantial amount of research has focused on the metabolic and physiological adaptations observed in dairy cows, particularly involving changes in energy balance during lactation. Studies described herein are designed to elucidate metabolic changes in the lactating cow, with particular regard to adipose tissue metabolism, and determine how these changes observed at the cellular level could potentially contribute to the animal’s adaptive process. Study 1 focused on the regulation of lipolytic proteins in 2 different models of altered energy balance achieved via feed restriction and growth hormone administration. Data show that multiple mechanisms regulate lipolysis, resulting in changes in abundance and phosphorylation of various lipolytic proteins. Study 2 evaluated the direct impacts of heat stress on lipid metabolism in vitro, relative to the presence of a beta-agonist and insulin. Results suggest that acute heat stress directly increases the response of primary bovine adipocytes to lipolytic but not lipogenic stimuli, and that increased insulin sensitivity observed in heat-stressed cells may potentially contribute to the diminished lipolytic response observed in vivo.
CHAPTER 1: INTRODUCTION

Background

Energy balance is one of many factors that influence the dairy cow’s physiological activities. It is well established that dairy cows enter a state of negative energy balance (NEBAL) at the onset of lactation wherein energetic demands for milk production are not met by feed intake. This results in the mobilization of body reserves for energy necessary to support milk synthesis (Butler and Smith, 1989). The ability of the lactating dairy cow to adapt to these natural changes in energy balance is essential, as it influences the animal’s productivity and survivability in the herd.

Dairy farming worldwide is undergoing changes driven primarily by supply and demand. Innovations in selective breeding programs and feeding systems have led to a dramatic increase in milk production, with records showing that milk yield per cow has more than doubled in the last 40 years (Oltenacu and Algers, 2005). As cows are selected for higher production, they partition a greater portion of available energy to milk rather than body tissue accretion (Bines and Hart, 1982; Bauman et al., 1985; Veerkamp and Emmans, 1995), leaving the perception that higher producing cows mobilize more tissue and are in greater and more prolonged periods of NEBAL than lower producing cows. Furthermore, NEBAL, although more commonly associated with the transition period, can also occur late in lactation during insufficient supply and quality of feed, particularly among pastured high-yielding dairy cows, as well as those that are fed a TMR without taking into account the different performance levels and individual requirements of the cow (Gross et al., 2011). It is important to determine how extensive NEBAL affects the
dairy cow especially since it is often associated with lower rates of reproduction, increased incidence of health problems and declining longevity in modern dairy cows (Oltenacu and Algers, 2005).

Perhaps one of the major reasons for altering existing production systems in the dairy industry is climate change. Organisms can maintain a constant body temperature under a wide range of environmental conditions, but fail to function properly once their body temperature reaches a point outside this regulated thermal zone. With changes in the global climate adding to the high demands for food, environmental stress proves to be one of the greatest stressors challenging animal production today. In particular, heat stress, to which ruminants are highly susceptible, is a significant problem worldwide, costing farmers millions of dollars in lost productivity annually. An individual animal’s ability to cope with, and thrive in extreme climatic conditions can often spell the difference between high and low productivity. However, selection for more thermotolerant animals often leads to a decline in efficiency (Aggarwal and Upadhyay, 2013). This poses a problem since lactating cows by themselves already create a large amount of metabolic heat, leaving their immune systems compromised and increasing their risk of suffering from thermal stress. Rhoads et al. (2009) reported that differential tissue mobilization in heat-stressed cows is independent of energetic state; nevertheless, the physiological changes observed during conditions of heat stress and/or undernutrition are highly similar, from decreased dry matter intake to suppressed immunity and reproductive ability. As agricultural production systems have increasingly become more intensive as well as efficient, so too has the desire to improve productivity and profitability. Furthermore, society dictates that animals be treated as humanely as
possible, which in turn has altered the manner in which production systems operate. Therefore, advances in our knowledge of the adaptive responses of animals, as well as the methods they employ to cope with such stressors will generate new practices that will improve not only production but also animal welfare.

Recent discoveries on the active role of adipose tissue as an endocrine organ have generated much interest particularly in the association between lipid metabolism and production efficiency in dairy cows. The role of adipose tissue throughout the lactation cycle is highly variable as well as critical. As mentioned previously, energy balance status at the onset of lactation is often compromised because at this stage, the dietary intake is insufficient to meet the body’s high energy demands, leading to elevated rates of tissue mobilization (lipolysis). During the later stages of lactation, lipogenic capacity increases, leading to replenishment of fat reserves once feed intake becomes adequate. If the balance between fat breakdown and synthesis is not maintained, milk yield depression may result, or the animal may become more susceptible to disease. Clearly, adipose tissue metabolism is an essential component of the homeostatic mechanisms involved in milk production, which in turn is influenced by several other metabolic processes. Regardless of energy balance, an understanding of the regulation of adipose tissue metabolism is essential in developing strategies to improve the adaptive capacities and productivity of the dairy cow, particularly under conditions of environmental stress.

Objectives

Homeorhetic and homeostatic controls are important for adapting to various alterations in physiological and environmental conditions. The overall goal of this study was to investigate changes in lipid metabolism, in terms of substrate interactions and
responses to hormones (i.e. epinephrine and insulin) at the cellular level, and determine how these could potentially contribute to the adaptive process of the dairy cow.

Changes in lipolytic proteins relative to energy balance throughout lactation have been described previously. Hence, the first objective of this study was to further explore regulation of these lipolytic proteins in 2 other models of altered energy balance achieved via feed restriction and growth hormone administration. We hypothesized that abundance as well as phosphorylation of the various lipolytic proteins are altered when energy mobilization is stimulated by different pathways.

The second objective was to evaluate the direct impacts of heat stress on lipid metabolism during lactation in the dairy cow, by looking at changes in vitro in the abundance and phosphorylation of various lipolytic proteins, relative to the presence of a beta-agonist and insulin. We further explored the effects of heat stress on fatty acid synthesis in terms of the incorporation of acetate into lipids of bovine primary adipocytes and changes in the lipogenic enzyme acetyl-CoA carboxylase (ACC).
CHAPTER 2:
LITERATURE REVIEW

Climate Change and Agriculture

Causes of Climate Change

Evidence of changes in the global climate indicates that the planet is warming. Several indicators, including increased temperatures at the surface, in the troposphere and in oceans measured globally over the past 50 years are accompanied by increasing trends in extremes of heat and heavy precipitation events, as well as declines in extreme cold (NCA, 2013). Moreover, long-term temperature records from glaciers, corals, tree rings and other historical documents demonstrate that every decade in the late 20\textsuperscript{th} century has been warmer than the preceding decades (NOAA, 2011; Hansen et al., 2012; Jones et al., 2012). As reported by the U.S. Environmental Protection Agency (2014), the Earth’s average temperature has risen by 1.4°F over the past century, and is projected to rise another 2 to 11°F over the next 100 years.

There is broad scientific agreement that climate conditions are being altered on a global scale by human activities. Overall emissions of greenhouse gases from the burning of fossil fuels for energy, deforestation and other industrial processes contribute to the observed climatic changes (FAO, 2014). Emissions from human influences include heat-trapping gases like carbon dioxide (CO\textsubscript{2}), methane (CH\textsubscript{4}) and nitrous oxide (N\textsubscript{2}O), as well as particles such as black carbon (soot) and sulfates, which have warming and cooling influences, respectively (Wigley et al., 2013). Furthermore, the Consultative Group on International Agricultural Research (CGIAR) reported that agriculture is responsible for one-third of the total greenhouse gas emissions generated, with intensive
livestock farming contributing greatly to both CO$_2$ and CH$_4$ emissions in the global food system (2012).

As greenhouse gases build up in the atmosphere, they cause the Earth to trap extra energy from the sun, making the planet warmer. Temperature plays a significant role in how nature works, such that even a slight change in the average environmental temperature can have tremendous impacts on all living things, as well as most natural processes. Agriculture systems worldwide have developed in relatively stable climatic conditions, and while different regions will be impacted on differently, climate change projections will put pressure on the global capacity to produce food. It is therefore important to develop strategies that will help agricultural systems to adapt to increasing global temperatures, changing weather patterns and extreme events such as floods, droughts, heat waves and the like.

Heat Stress and Its Effects on Animals

Just as livestock contributes directly and indirectly to global warming through the emissions of greenhouse gases, livestock production systems are likewise vulnerable to environmental stresses brought about by climate change. There are 4 primary ways by which animal production is affected by changing climatic conditions: 1) feed-grain production, availability and costs; 2) livestock pastures and forage crop production and quality; 3) distribution of diseases, and that of vectors and parasites causing these; and 4) animal health, growth and reproduction (Scholtz et al., 2013; Hatfield et al., 2014). Depending on the region as well as the variety of breeds used for food production, the impacts of temperature stress often vary, thus generating great interest in understanding how domestic animals adapt to climate stressors.
Heat stress is one of the most important stressors and is considered to be a major threat to livestock sustainability because it often leads to poorer health and performance of animals. By definition, heat stress pertains to all forces related to high temperature that induce adjustments occurring from the subcellular to the whole-animal level to help organisms avoid physiological dysfunction and adapt to their environment (Aggarwal and Upadhyay, 2013). A term that is often associated with heat stress is the thermal neutral zone (TNZ), within which animals achieve homeostasis with the minimum use of thermoregulatory mechanisms (Du Prezz et al., 1990). Temperatures below or above the TNZ can trigger physiological and behavioral responses that affect an animal’s development and performance, or under extreme cases, lead to death. Knowledge of a particular organism’s comfort zones is of crucial importance, so that producers can take steps to minimize the harmful effects brought about by environmental stress and allow for optimum production.

High temperature, high humidity, low airflow and direct sunlight collectively can result in heat stress. The temperature-humidity index (THI) is a bioclimatic index that is often used to assess the degree of heat stress in livestock (Hahn et al., 2003). In response to increased THI, mammals set physical, biochemical and physiological processes into play to try and counteract the negative effects of heat stress and maintain thermal equilibrium. The immediate reaction to increased heat load is increased respiration rates, decreased feed intake and increased water intake (Bernabucci et al., 2010). Apart from these, other general homeostatic responses to thermal stress in mammals include increased sweating, reduced fecal and urinary water losses and a major decline in production (Baumgard and Rhoads, 2012). Clearly, adaptation of mammals to heat stress
requires the physiological integration of many organs and systems including the endocrine, cardiorespiratory and immune systems (Altan et al. 2003).

Heat Stress in Dairy Cattle

Dairy cattle are homeothermic animals, maintaining a relatively constant internal body temperature when exposed to a wide range of ambient conditions. During the summer months, intense radiant energy is experienced for an extended period of time, usually in the presence of high relative humidity. Lactating cows by themselves already create a large amount of metabolic heat, and the prevailing environmental conditions leave their body cooling mechanisms compromised (Nardone et al., 2010). Heat production and accumulation cause heat load in the cow to increase to the point that body temperature rises, feed intake becomes low, and ultimately milk production declines.

In order to adapt to heat conditions, dairy cows undergo a number of behavioral as well as physiological changes, mostly to mitigate environmental effects. Apart from reduced intake, these changes also include: decreased general activity, increased shade selection and water intake, lower growth and conception rates, increased respiratory and heart rates, panting activity, increased peripheral blood flow and sweating (Kennedy, 1999). Collectively, these changes indicate that there is major strain to the animals that ultimately results in reduced health status. It is important to note that these activities differ depending on time of exposure to HS conditions, and exhibit large variations across animals within groups. Although these effects are well documented, the resulting changes in cellular function and gene regulation are still poorly understood.
Heat Stress Effects on Lactation

Reduced level and quality of milk production is one of the major impacts of HS on the dairy industry. Approximately 35% of the decline in milk yield observed during HS is attributable to reduced feed intake, while the remaining 65% is due to the direct effects of hyperthermia (Rhoads et al., 2009). However, quantifying the direct effects of thermal stress on milk production is often difficult, as it is also strongly influenced by other non-environmental factors such as nutrition and management. Nevertheless, many studies have demonstrated possible mechanisms that explain how milk yield and quality is reduced under HS conditions. During HS, decreased synthesis of hepatic glucose coupled with lower levels of non-esterified fatty acids (NEFAs) in the blood leads to a decline in the glucose supply to the mammary glands (Wheelock et al., 2010; Rhoads et al., 2010), ultimately leading to low lactose synthesis which in turn results in low milk yield (Nardone et al., 2010). Furthermore, when HS is experienced close to calving, the cow’s ability to produce high quality colostrum is reduced as a result of impaired transfer of maternal IgG’s to the colostrum (West, 2003). A significant drop in percentages of milk fat and protein was also observed during HS, perhaps resulting from a shift in nutritional partitioning that is necessary for the animal to cope with hyperthermia (McDowell et al., 1976; Bernabucci et al., 2014).

A cow’s response to climatic change is affected by many factors. Certainly, reduction in milk yield is intensified by a decrease in feed consumption by the animals to compensate for the high environmental temperature (Blackshaw and Blackshaw, 1994). In addition to nutrition and the caloric content of feed, the intensity by which HS reduces
milk yield is further influenced by other factors such as breed, animal size, stage of lactation and level of production (Collier et al., 2012; Sharma et al., 1983).

Early research suggested that the Jersey breed may be more heat tolerant than Holsteins (Harris et al., 1960; Collier et al., 1981). However, more recent studies on breed differences have produced mixed results, making it more difficult to determine which breed is most heat tolerant. One breed comparison reported no breed differences between lactating Jersey, Holstein-Friesian and Holstein-Friesian x Jersey cows in terms of mean or maximum body temperature or BCS (Kendall and Webster, 2009). On the other hand, West (2003) reported that Jerseys had a tendency to have cooler rectal temperatures across different THI ranging from 72 to 84, which is consistent with earlier findings by Collier et al. (1981). West further stated that Holstein milk yield declined much more rapidly than that of Jerseys across the same range of THI. Additionally, in a retrospective study by Smith and colleagues (2013), they found that Holstein milk yield declined during moderate and severe HS, whereas Jersey milk yield declined only during severe HS. Overall, they concluded that although Jersey cows appeared to be more heat-tolerant, this didn’t change the fact that Holsteins still produced larger volumes of milk. Other recent comparisons between Jersey and Holstein breeds exist (Garcia-Peniche et al., 2006; Heins et al., 2008), but none of these studies have used lactating cows to allow for a direct comparison of the effects of HS on milk yield of these breeds. Indeed, the discovery of any difference among cattle breeds in terms of heat tolerance will be very beneficial to dairy producers, as this will allow them to choose the most suitable animals for successful production.
Selection geared towards higher milk production has also impacted the average body weight of animals in most herds. Large cows have larger gastrointestinal tracts that allow them to consume and digest more feed, thus generating more substrates for milk synthesis (Aggarwal and Upadhyay, 2013). Concomitantly, changes in the physical and genetic constitution of these cows may have affected their thermoregulatory capabilities such that larger animals have the tendency to become less efficient, as more energy is partitioned towards body maintenance rather than milk production (Kadzere et al., 2002).

Moreover, climatic conditions appeared to have maximum influence during the first 60 days of lactation, such that HS during early lactation negatively impacts total milk production (Aggarwal and Upadhyay, 2013). During this time, cows are in negative energy balance, and make up the deficit by mobilizing body reserves. Higher-producing cows are less likely able to cope when exposed to HS during this period because of the higher metabolic rate of these animals (Sharma et al., 1983). High-yielding cows are affected more than low yielding ones (Barash et al. 2001) because the upper critical temperature shifts downwards as milk production, feed intake and heat production increase (Silanikove, 2000).

Heat Stress Effects on Reproduction

HS induces several physiological and biochemical changes in body functions that negatively affect the reproductive efficiency of dairy cattle. Hyperthermia has been observed to drastically reduce breeding efficiency and conception rates, as well as increase embryonic loss (Gwazdauskas et al., 1981 ; Hansen, 2005) . The detrimental effects of high ambient temperature and humidity on the animals’ reproductive processes may be summed up into these four aspects: 1) disruption of the reproductive pattern; 2)
impaired endocrine interactions and altered follicular development pattern; 3) reduced quality of sperm, oocytes and embryos; and 4) changed nutritional and energy balance state.

Under the influence of HS, the duration and intensity of estrus is reduced (Younas et al., 1993). Furthermore, the decline in motor activity and other behavioral manifestations of estrus (e.g. mounting) lead to increased incidence of anestrus and silent ovulation (Hansen, 1997), which in turn dramatically reduces estrus detection and chances of pregnancy.

The main factors regulating ovarian activity are gonadotrophin-releasing hormone from the hypothalamus and the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. The effects of heat stress on these hormones and the mechanisms through which HS influences the function of the hypothalamic-hypophyseal-ovarian axis may be observed to be inconsistent due to variable reasons related to physiology and state of health of the animal (Aggarwal and Upadhyay, 2013). In one study, FSH secretion from the pituitary gland does not appear to be impaired in animals exposed to high ambient temperatures (Gilad et al., 1993). However, a clear reduction in the pulse and amplitude of LH release has been observed in cows exposed to HS (Gilad et al., 1993; Gauthier, 1986). In these instances, estradiol secretion is reduced, expression of estrus is poor and fertility declines (Rensis et al., 2003). Furthermore, the reduction in LH pulse and amplitude leads to delayed ovulation and decrease in the number and quality of oocytes which ultimately lower pregnancy rates (Diskin et al., 2002). In terms of other hormones, progesterone levels are markedly
reduced, resulting in compromised reproductive function (Mann et al., 2001; Wolfenson et al., 2002).

The formation of gametes and development of early embryonic stages have been shown to be highly temperature sensitive. Hansen (1997) reported that the deterioration of bull fertility with respect to heat stress stems from the fact that hyperthermia leads to poorer morphological and functional semen quality. Moreover, HS, through delayed ovulation and follicular persistency can lead to the ovulation of an aged, poor quality oocyte which is associated with low fertilization rate and embryonic mortality (Sartori et al., 2000; Al-Katanani et al., 2001; Roth et al., 2000).

It is very clear that the negative effects of heat stress on reproduction is the result of the direct influence on reproductive functions and embryonic development and indirect influences mediated through changes in energy balance. In heat stressed dairy cows, dry matter intake is reduced prolonging the period of negative energy balance and negatively influencing the plasma concentrations of insulin, IGF-1 and glucose (Jonsson et al., 1997; Ronchi et al., 2001). These often result in poor follicular development, low estrus expression and poor quality of oocytes.

Heat stress effects on metabolism

Heat stress induces changes in post-absorptive metabolism that are independent of reduced feed intake and whole-animal energy balance (Baumgard and Rhoads, 2013; Wheelock et al., 2010). These changes may be considered as adaptive strategies, as the animal tries to maintain normal body temperature under extreme environmental HS. One of the major differences observed between hyperthermic and heat tolerant animals involves glucose metabolism. Baumgard and Rhoads (2011) described heat-stressed
animals to be “metabolically inflexible” because of their inability to employ glucose-sparing mechanisms to prioritize milk synthesis. Meanwhile, the effect of HS on protein metabolism is apparent in changes in the mammary α- and β-casein synthesizing machinery, in which summer milk was observed to have lower contents of crude proteins and caseins (Bernabucci et al., 2002). These α- and β-casein units represent approximately 90% of total caseins and contain high numbers of phosphate groups (Schmidt, 1980), the phosphorylation of which requires the presence of ATP (Mercier and Gaye, 1983). Under energy deficit conditions, this phosphorylation is significantly impaired, such that it has been hypothesized that lower α- and β-casein content observed in summer milk may be attributed partially to a reduction in energy and protein availability consequent to HS (Lacetera et al., 1996; Nardone et al., 1997). Additional evidence indicating that HS directly affects protein metabolism is increased skeletal muscle breakdown, which has been reported in several species, including cattle (Baumgard and Rhoads, 2011; Wheelock et al., 2010). Skeletal muscle (amino acids in particular) is mobilized during periods of inadequate nutrient intake or disease to provide substrates necessary for energy metabolism. It is still not clear, however, whether the increased breakdown of skeletal muscle is a result of catabolism or heat-induced muscle damage and proteolysis (Rhoads et al., 2013). As far as lipid metabolism is concerned, heifers exposed to HS and in negative energy balance do not lose as much body weight as their pair-fed thermal neutral counterparts (Ronchi et al., 1999). In addition, hyperthermic cows elicited a much lower NEFA response to an epinephrine challenge as compared to their pair-fed thermal neutral counterparts (Baumgard and Rhoads, 2011). The lack of an elevated NEFA response is unexpected, as acute HS causes an increase in circulating
cortisol, norepinephrine and epinephrine levels (Collier et al., 2005; Bernabucci et al., 2010), which are known to be catabolic signals that normally stimulate lipolysis and adipose tissue mobilization. The fact that heat-stressed cows fail to enlist this ‘shift’ in post-absorptive energetic metabolism especially during periods of inadequate nutrition may be indicative of the direct impact of heat stress on energetics that is independent of feed intake.

Adipose Tissue Metabolism

Prior to the discovery of leptin and subsequent identification of other adipose tissue-derived mediators like adiponectin and resistin, adipocytes were considered to be no more than just passive storage cells for fat – a metabolically inactive tissue whose main functions were thermal insulation and mechanical support (Frayn, 1989). However, a clearer understanding of the roles of these “adipokines”, as they are collectively known (Kadowaki et al., 2006), dramatically revised this traditional view of adipose tissue, which is now considered as an important endocrine organ, with multiple roles in the maintenance of energy homeostasis and regulation of whole body physiology (Kershaw and Flier, 2004).

The primary role of adipose tissue is to allow storage and liberation of triacylglyceride reserves to provide fatty acids (FAs) that serve as oxidative fuels for other tissues during crucial periods of energy deprivation, a process known as lipolysis. Under conditions of energy surplus, dietary non-esterified fatty acids (NEFAs) are esterified to triacylglycerols (TAGs), which are subsequently stored in cytosolic lipid droplets (LDs) within adipocytes. In response to increased energy demands, these FAs
are mobilized for use by peripheral tissues for β-oxidation and ATP production. Although 
other non-adipose tissues are able to esterify FAs into TAGs and re-hydrolyze them 
depending on energy requirement, lipolysis for the provision of FAs as an energy source 
for other organs is a unique function of adipocytes (Ahmadian et al., 2007).

The hydrolysis of stored intracellular triacylglycerides during times of negative 
energy balance is made possible through the action of several proteins involved in a 
complex series of interrelated cascades mediated through the protein kinase A (PKA) 
pathway (Figure 2.1). The current model is that adipose triglyceride lipase (ATGL), 
which exhibits high substrate affinity for TAGs and is predominantly expressed in 
adipose tissue, performs the rate-limiting step in lipolysis (Zimmermann et al., 2004; 
Granneman et al., 2007), generating diacylglycerol (DAGs) and NEFAs in the process. 
Hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL) then break down 
DAGs and monoacylglycerol (MAG) respectively (Lass et al., 2011; Karlsson et al., 
1997), thereby releasing additional FAs and glycerol. Indeed, the molecular machinery 
involved in TAG hydrolysis and FA release works in a highly orchestrated fashion, 
allowing adipocytes to respond to various feeding conditions and energy demands. In 
dairy cattle, the abundance and phosphorylation states of these different lipolytic proteins 
are altered with changing energy balance in order to facilitate coordinated metabolic 
changes necessary to support lactation (Faylon et al., 2014).

Binding of catecholamines (epinephrine, norepinephrine, etc.) to β-adrenergic 
receptors (β-AR) activates adenylate cyclase via G-proteins and converts adenosine 
triphosphate (ATP) to cyclic adenosine monophosphate (cAMP; Campagna et al., 2008), 
which consequently leads to the activation of PKA. In rats, PKA phosphorylates HSL at
serine residues 563, 659 and 660 (Anthonsen et al., 1998; Shen et al., 1998; Gaidhu et al., 2009), allowing its translocation from the cytosol to the lipid droplet (LD). The phosphorylation of another LD-associated protein, perilipin (PLIN), by PKA has also been demonstrated to be necessary for the activation of HSL (Greenberg et al., 1991). Under basal conditions, PLIN surrounds the LD, preventing HSL-LD interaction. Phosphorylation of PLIN, however, triggers a conformational change that provides HSL access to the LD and its lipid substrate, thereby allowing catecholamine-induced lipolysis to occur. Conversely, the cellular energy sensor AMP-activated protein kinase (AMPK) phosphorylates serine-565 of HSL, inhibiting PKA-mediated phosphorylation of this enzyme, thus preventing lipolysis (Garton et al., 1989). It was proposed that this impairment of lipolysis via the action of AMPK may be important to prevent lipotoxicity in peripheral tissues as well as reduce the costly process of re-esterifying FAs in adipose tissues (Anthony et al., 2009; Gauthier et al., 2008).

ATGL activity is likewise stimulated by catecholamines (Gaidhu et al., 2009), although the molecular mechanism by which this occurs is still not clear. Unlike HSL, phosphorylation of ATGL at various serine residues has no effect on its activity. However, there is compelling evidence that the maximal activity of ATGL depends on its association with its co-activator comparative gene identity 58 (CGI-58; Schweiger et al., 2006), drastically enhancing ATGL-mediated lipolysis without affecting HSL activity (Lass et al., 2006). Normally, CGI-58 is also localized to the LD in association with PLIN. Upon hormonal stimulation and subsequent phosphorylation of PLIN, CGI-58 dissociates from PLIN and translocates to the cytosol where it interacts and potently activates ATGL, resulting in TAG breakdown (Lass et al., 2006).
The relative importance of ATGL is demonstrated by studies of gene ablation and functional loss of the enzyme. ATGL-deficiency in mice is associated with reduced lipolysis resulting in excessive fat deposition in several tissues including fat, liver, muscle, kidney and lung (Lass et al., 2011). Furthermore, siRNA directed against ATGL has been shown to decrease glycerol and FA release in 3T3-L1 adipocytes (Zimmermann et al., 2004). This decrease was more pronounced when adipocytes from HSL-null mice were utilized, suggesting that cooperativity exists between these enzymes (Duncan et al., 2007). Indeed, optimal rates of lipolysis have been observed in the presence of both ATGL and HSL.

The other important aspect of the regulation of lipolysis is its inhibition. Refeeding attenuates adipocyte lipolysis, primarily through the anti-lipolytic action of insulin, which involves both cAMP-dependent and cAMP-independent mechanisms. Decreased levels of cAMP in the adipocyte result in the suppression of lipolysis. Insulin binding causes autophosphorylation of its receptor, initiating a signaling cascade that consequently leads to the phosphorylation of phosphodiesterase 3B (PDE3B; Langin et al., 2006). PDE3B degrades cAMP, preventing the phosphorylation and activation of PKA that is necessary for HSL-mediated lipolysis. Meanwhile, cAMP-independent regulation of lipolysis by insulin involves the stimulation of protein phosphatase 1 (P1), which is responsible for dephosphorylating and deactivating HSL, thereby causing a decrease in the rates of lipolysis (Londos et al., 1985; Olsson et al., 1987; Stralfors et al., 1989).

Autocrine/paracrine regulation of lipolysis has also been observed. The concept of the adipose tissue being an active endocrine organ was established with the discovery of
cytokines. Among these is adenosine, which is abundantly released from adipose tissue e.g. during sympathetic nerve activation (Fredholm et al., 1976). Several studies showed that the adenosine A₁ receptor is responsible for the inhibition of lipolysis (Lonnroth et al., 1989). In rat adipocytes, the antilipolytic effect of adenosine was inhibited by the addition of adenosine deaminase (ADA), which converts adenosine to the much less active inosine, allowing catecholamine-induced lipolysis to proceed (Johansson et al., 2007). Similarly, inhibition of lipolysis is attenuated by the addition of theophylline, known to be an adenosine A₁ receptor antagonist and a nonselective inhibitor of phosphodiesterase which lets the cAMP cascade to remain fully active (Flechtner-Mors et al., 2005). Together, adenosine and insulin mediate additive antilipolytic effects that contribute to the regulation of lipolysis in adipocytes.

Adipose tissue is also the site for fatty acid synthesis in ruminants, with acetate as the main carbon source for lipogenesis in adipose tissue (Ballard and Hanson, 1967). Ballard and Hanson (1967) were the first to explain that glucose was a poor precursor for long-chain fatty acids because of very low activities of citrate cleavage enzyme (ATP:citrate lyase) and malic enzyme (NADPH-malate dehydrogenase) in ruminant lipogenic tissues in comparison to those in rats. Therefore, acetate and not glucose was considered the principal generator of cytosolic acetyl-CoA for de novo fatty acid synthesis, which occurs in the cytosol. Key enzymes involved in de novo fatty acid synthesis include acetyl Co-A carboxylase, fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G-6-PDH), and glycerol-3-phosphate dehydrogenase (G-3-PDH; Zhao et al., 2010).
Indeed, lipid metabolism is maintained by a complex interaction of hormonal and nutritional systems (McMurray et al., 2005), and is further complicated by environmental factors that could also affect energy intake and expenditure. Results of previous research suggest that among these, hyperthermia may be an important regulator of lipid metabolism, due in part to its effect on insulin sensitivity. Alterations in the endocrine and metabolic status induced by hyperthermia have been reported in numerous animal species (Bernabucci et al., 2002; 2006; Rhoads et al., 2009), instigating a growing concern over the negative effects of heat stress on the viability and sustainability of livestock production systems. Ronchi and colleagues (1999) demonstrated that heat stress has a direct effect on energy, lipid metabolism and liver enzymatic activities of Holstein heifers. Additionally, a reduction in energy intake combined with increased energy expenditure for maintenance lowers energy balance, and partially explains why lactating cattle lose substantial amounts of body weight during severe heat stress (Rhoads et al., 2009).

Clearly, alterations in lipid metabolism are tantamount to many pathophysiological consequences that are highly linked to the metabolic syndrome. Although to date, several pieces of information regarding the effects of thermal stress on metabolism are available, the intracellular components and mechanisms that participate, directly or indirectly, in the control of lipid trafficking in response to hyperthermia are less well known.
Figure 2.1. Lipolysis mediated through the protein kinase A (PKA) pathway. In adipose tissues, β-adrenergic stimulation of lipolysis leads to the consecutive hydrolysis of triacylglycerols (TAGs) and the formation of fatty acids (FAs) and glycerol. The process requires 3 main enzymes: adipose triacylglyceride lipase (ATGL) cleaves the first ester bond in TAGs, hormone sensitive lipase (HSL) preferentially breaks down diacylglycerols (DAGs) and monoacylglycerol lipase (MGL) finally hydrolyzes monoacylglycerols (MAGs) into FA and glycerol. For full hydrolytic activity, ATGL interacts with its co-activator protein comparative gene identity 58 (CGI-58), while HSL is phosphorylated, translocates to the lipid droplet (LD) where it associates with phosphorylated perilipin (PLIN).
CHAPTER 3:
REGULATION OF LIPID DROPLET-ASSOCIATED PROTEINS FOLLOWING GROWTH HORMONE ADMINISTRATION AND FEED RESTRICTION IN LACTATING HOLSTEIN COWS

Abstract

Lipid metabolism plays a crucial role in the adaptation of dairy cows to periods of energy insufficiency. The objective of the current study was to determine if lipolytic proteins are consistently regulated when energy mobilization is stimulated by different factors. We evaluated two models of altered energy balance in mid-lactation Holstein cows, including feed restriction (FR) and administration of bovine growth hormone (GH), by quantifying the abundance and/or phosphorylation of hormone sensitive lipase (HSL), perilipin (PLIN) and adipose triglyceride lipase (ATGL). For GH administration, adipose tissue and blood samples were collected 4 d prior to, and 3 and 7 d post administration of GH (N=20 cows). Similarly, adipose and blood samples were obtained 6 d prior to, and 1 and 4 d after initiation of FR (N=18 cows). Estimated net energy balance decreased and NEFA increased in both experimental models. Decreased ATGL and PLIN protein abundance was observed with GH administration and FR. Additionally, the abundance of phosphorylated HSL-Ser565 decreased in both models. Decreased abundance of phosphorylated PLIN was observed with GH administration, but not FR. Decreased ATGL protein abundance appears to be a consistent response to energy insufficiency in lactating cows, as this response was also described with negative energy balance at the onset of lactation. In contrast, the abundance of PLIN protein and phosphorylation of HSL-Ser565 were altered in the current research, but not at the onset of lactation. Our findings demonstrate that lipolysis is altered through the regulation of
multiple proteins, and that this regulation differ according to physiological state in lactating cows.

Introduction

Lipid metabolism is critical to the balance between production and fitness traits that contribute to the survival and reproduction rates of lactating dairy cattle. During times of energy insufficiency, lipid metabolism favors lipolysis and body fat energy reserves in the form of triacylglycerides are mobilized to support lactation. Proper regulation and coordination of lipolysis are essential to ensure the availability of adequate energy for lactation, maintenance and reproduction.

Lipid mobilization is a highly conserved process involving multiple enzymes which are regulated at the post-translational level (Watt et al., 2008; Lampidonis et al., 2011). For many years, hormone sensitive lipase (HSL) was believed to be the rate-limiting enzyme of lipolysis (Duncan, 2007). The cAMP-dependent activation of protein kinase A (PKA) leads to phosphorylation of HSL, which in turn allows the translocation of HSL to the lipid droplet (LD) and enhances enzyme activity (Haemmerle et al., 2002). Perilipin (PLIN) is a protein found on the surface of LDs and is also a target of PKA-mediated phosphorylation in 3T3-L1 adipocytes (Moore et al., 2005; Brasaemle, 2010). Under basal conditions, PLIN protects the LD from HSL-mediated lipolysis. Once phosphorylated, PLIN undergoes a conformational change, allowing HSL access to the lipid droplet and its lipid substrate (Miyoshi et al., 2006). More recently, the characterization of HSL-deficient mice provided compelling evidence that revised the concept that HSL is uniquely responsible for the hydrolysis of triacylglycerols and diacylglycerols of stored fat (Ryden et al., 2007). Adipose triglyceride lipase (ATGL) is
predominantly expressed in mouse adipose tissue and exhibits high substrate specificity for triacylglycerides (Villena et al., 2004; Zimmermann et al., 2004). ATGL becomes activated by another LD-associated protein, comparative gene identity protein 58 (CGI-58), following its dissociation from and upon phosphorylation of PLIN (Granneman et al., 2007; Schweiger et al., 2008). It has been demonstrated in human and animal models such as pigs and mice that ATGL abundance increases in response to glucocorticoids and caloric restriction, suggesting that its protein abundance becomes elevated with an increasing demand for lipid mobilization due to metabolic or pharmacologic stimuli (Deiuliis et al., 2008). However, little is known about the regulation of ATGL or other lipolytic proteins in dairy cattle.

The underlying hypothesis of the current research is that the abundance and phosphorylation of HSL, PLIN and ATGL are altered with changing energy balance in order to facilitate coordinated metabolic changes necessary for lactation. We previously described changes in lipolytic proteins relative to changes in energy balance that occur throughout a lactation cycle (Koltes and Spurlock, 2011). The objective of the current study was to determine if lipolytic proteins are consistently regulated when energy mobilization is stimulated by different pathways. To accomplish this objective, we evaluated two additional models of altered energy balance in mid-lactation Holstein cows: feed restriction and administration of bovine growth hormone. We then quantified the abundance or phosphorylation or both of HSL, PLIN and ATGL in response to these models of altered energy balance.
Materials and Methods

All animal experiments in this study were performed under a protocol approved by the Iowa State University Institutional Animal Care and Use Committee.

Animals

The animals and experimental treatments used in this research have been described previously (Koltes and Spurlock, 2011; 2012). Briefly, cows were trained and acclimated to the Calan Broadbent feeding system (American Calan, Northwood, NH) for 4 to 6 d prior to initiation of each experiment. Cows were fed ad libitum, and received a basal diet formulated using National Research Council (NRC, 2001) recommendations to meet or exceed the nutritional requirements of lactating cows. Individual feed intake was measured on a daily basis. Dry matter and net energy content (NE\textsubscript{L}) were determined from TMR samples. Body weights were recorded weekly, following milking in the morning. Milk yield was recorded at each milking, and the percentage of fat, protein and lactose were determined from weekly samples collected at a morning milking. Net energy balance was estimated as the difference between NE\textsubscript{L} consumed and utilized for milk production and maintenance (NRC, 2001).

Three adipose tissue biopsies were taken from each cow. During each biopsy, four 1-g samples of subcutaneous adipose tissue were collected from the tailhead region using a minimally invasive procedure as previously described (Koltes and Spurlock, 2011; 2012). The adipose tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until protein analysis. Samples were obtained from alternating sides of the cow, and the third biopsy sample was obtained approximately 5 cm anterior to the previous biopsy location.
To confirm the experimental treatments stimulated lipolysis, blood samples were obtained via jugular venipuncture following each biopsy for measurement of non-esterified fatty acids (NEFA). The concentration of NEFA was determined using a commercially available kit following the manufacturer’s protocol (Randox Laboratories, Co. Antrim, N. Ireland, UK).

*Feed restriction (FR).* Eighteen multiparous Holstein cows between 175 and 210 d postpartum were used in two replicates (N=9, N=9) which were done one month apart. Baseline feed intake measurements were obtained for 5 d prior to initiation of treatment. In order to simulate energy insufficiency similar to that of the transition period, feed intake was restricted to achieve a targeted energy balance level of -10 Mcal, with a maximum restriction of 50% of the baseline feed intake. On the third day of feed restriction, the quantity of feed provided to each cow was readjusted to account for changes in milk production. Cows were fed four times daily throughout the feed restriction period. Adipose tissue samples were collected 6 d prior to (control), and 1 and 4 d after initiation of restricted feeding. Likewise, blood samples were taken immediately following each biopsy for analysis of NEFA, as previously described. In this experimental design, biopsy 1 serves as the control for biopsies 2 and 3, within cow.

*Growth hormone (GH) administration.* Twenty multiparous Holstein cows between 175 and 210 d postpartum were evaluated in two replicates, separated in time by 2 days. As with the previous experiment, baseline feed intake was measured for 5 d prior to administration of GH. Bovine GH was administered as a single dose of Posilac® (Elanco, 500mg sometribove zinc), given via a subcutaneous injection in the neck. Adipose tissue was sampled 4 d pre- (control), and 3 and 7 d post-administration of the
GH. Similarly, blood samples were collected immediately following each biopsy for NEFA analysis. As with the Feed Restriction treatment, biopsy 1 serves as the control for biopsies 2 and 3, within cow.

Semi-quantitative Western Blotting

Total abundance of HSL, ATGL, and PLIN was determined by semi-quantitative Western blotting. Additionally, phosphorylation of HSL using antibodies targeting serine residues 563 and 565 (HSL-Ser563 and HSL-Ser565) was investigated because these sites are targeted by PKA (HSL-Ser563) and adenosine monophosphate-activated protein kinase (AMPK; HSL-Ser565) to stimulate and inhibit HSL activity, respectively, in both human skeletal muscle and adipose tissue (Watt et al., 2006). These antibodies are predicted to target serine residues 552 and 554 of the bovine HSL amino acid sequence (P16386), but will be referred to as HSL-Ser563 and HSL-Ser565 for consistency with the literature. Phosphorylation of perilipin (PPLIN) was determined using the total PLIN antibody, but the phosphorylated form of the protein was revealed by size separation during gel electrophoresis. The PLIN antibody is specific for bovine, and while the rest of the primary antibodies used in this study were produced based on human sequence, BLAST searches revealed high sequence homology (91%) and identity (92%) with the bovine sequence. The protocol for the extraction and preparation of proteins from adipose tissue for use in Western blotting has been previously described (Elkins and Spurlock, 2009). Bicinchoninic assay (BCA; Pierce Protein Research, Rockford, IL) was performed to determine protein concentrations, which were used to standardize the quantity of total protein loaded onto each gel. In addition, arbitrarily chosen samples were loaded onto lanes flanking the samples from each cow to serve as standards for normalization across
gels. Proteins were separated overnight in 10% [for detection of phosphorylated perilipin (PPLIN)] or 8% (for detection of all other proteins) SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Following transfer, membranes were blocked with 5% milk in TBS-T for at least 1 hour. Blots were incubated with the primary antibodies (Table 1), followed by incubation with anti-rabbit IgG horseradish peroxidase-linked secondary antibody (GE Healthcare, Pittsburgh, PA). Protein bands were detected with the ECL Plus Western Detection Kit (Amersham, Pittsburgh, PA) using an Alpha Innotech Imager (FluorChem FC2, Cell Biosciences, Santa Clara, CA) and quantified using Totallab software for 1D gel analysis (TL100, v2009; Totallab Ltd., Newcastle upon Tyne, UK). Data for each sample were normalized using the average value of the two standard lanes flanking the samples from each cow. Protein abundance was expressed in relative units for subsequent statistical analyses.

Data Analysis

All data were analyzed as a completely randomized design using the Proc Mixed procedure of SAS (SAS Institute, 1999). The model included biopsy day as a fixed effect, with cow and replicate as random factors. When the main effect of biopsy day was significant ($P<0.05$), differences among the means were determined using the p-diff procedure. The interaction effect between biopsy day and replicate was initially tested but was non-significant and subsequently removed from final analyses. All values are presented as means ± SE, with statistical significance set at $P < 0.05$. 
Results

Milk Production, Energy Balance, and NEFA Concentration

Changes in milk production, energy balance and circulating NEFA with growth hormone and feed restriction have been described previously (Koltes and Spurlock, 2012). Briefly, production data collected during the feed restriction experiment showed a steady decrease in milk yield starting from the initiation of restricted feeding. In contrast, milk production of cows administered GH increased throughout the 7-day experiment. Energy balance of the feed restricted cows decreased upon treatment, and was negative throughout the four days of restriction. This was accompanied by an increase in plasma NEFA concentration on days 1 and 4, relative to the control. Energy balance also decreased with the administration of growth hormone, but average energy balance across all cows remained slightly positive throughout the experimental period. Increased plasma NEFA concentration confirmed elevated lipid mobilization in GH treated cows.

Lipolytic Proteins

Protein abundance of ATGL decreased following feed restriction (P=0.03, Fig. 3.1A). The abundance of phosphorylated HSL\textsubscript{Ser565} also decreased (P<0.001, Fig. 3.1B), whereas HSL\textsubscript{Ser563} and total HSL protein were unchanged (P=0.57 and P=0.41 respectively). Protein abundance of total PLIN decreased (P=0.034), but no significant change in PPLIN was observed (P=0.13, Fig. 3.1C) following feed restriction.

Similarly, treatment with GH was also associated with a decline in the abundance of ATGL protein (P<0.001, Fig. 2A) and PHSL\textsubscript{Ser565} (P=0.02, Fig. 3.2B). The effect of treatment day on HSL and PHSL\textsubscript{Ser563} (P=0.263 and P=0.486 respectively) were not
significant (Fig. 3.2A and B). Total PLIN decreased after GH treatment (P=0.004), as did PPLIN (P=0.04, Fig. 3.2C).

Discussion

Dairy cattle typically undergo extensive remodeling of adipose tissue throughout a lactation cycle. Appropriate regulation of lipolysis and lipogenesis is essential for the maintenance of energy supplies for both lactation and fitness traits. Although a number of proteins have been recognized to participate in lipolysis (Kolditz and Langin, 2010; Contreras and Sordillo, 2011; Lass et. al., 2011), the regulation of these proteins in response to changing energy balance, particularly in the dairy cow, is poorly understood. We previously reported that ATGL, HSL and PLIN are dynamically regulated in subcutaneous adipose tissue throughout the lactation cycle of multiparous Holstein cows (Koltes and Spurlock, 2011). The objective of the present study was to evaluate the regulation of these lipolytic proteins when lipolysis is stimulated by physiological factors including feed restriction and administration of GH during mid-lactation. A summary of the response of HSL, ATGL, and PLIN to the three experimental models evaluated by our group (lactation cycle, feed restriction, and GH administration) is provided in Table 2.

The most consistent result across experimental models is the decrease in abundance of ATGL with increasing lipolytic activity. This result is unexpected because ATGL protein abundance increases with enhanced lipolytic activity during fasting in pigs, rodents, and humans (Deiuliis et al., 2008; Caimari et al., 2008; Kershaw et al., 2010; Nielsen et al., 2012). However, our protein data from the transition period (Koltes
and Spurlock, 2011) are consistent with ATGL mRNA expression also described for early lactation cows (Ji et al., 2012). The reason for the discrepancy between results in lactating cows and published results from other species is unclear. To our knowledge, our research is the first to investigate the modulation of ATGL protein in lactating animals. It is currently unknown if the homeorhetic changes associated with lactation, especially in high milk producing dairy cows, influence the physiological signals regulating ATGL. The abundance of ATGL increased following feeding of a high fat diet in mice (Gaidhu et al., 2010). This regulation may have occurred to balance the excessive adiposity due to the high fat diet, and is consistent with the proposed role of ATGL as a regulator of basal lipolysis (Mairal et al., 2006; Ryden et al., 2007). As such, ATGL may be down-regulated in our experimental models as an adaptive mechanism to prevent excessive hydrolysis of lipids during periods of energy insufficiency during lactation.

Alternatively, the observed decrease in ATGL protein abundance may reflect a lack of correlation between protein abundance and ATGL activity. Most notably, ATGL is activated by CGI-58 (Yamaguchi et al., 2004; Granneman et al., 2007) and inhibited by the G(0)/G(1) switch gene 2 (Lu et al., 2010). Additionally, localization of ATGL to the LD is influenced by the presence of perilipins (Wang et al., 2011). Therefore, the decreased abundance of ATGL observed in our experimental models may not accurately reflect changes in ATGL activity. The timeline of investigation may also impact observed changes in ATGL protein. Yang et al. (2013) proposed that ATGL may play an important regulatory role during long-term stimulation of lipolysis but is less involved in the acute lipolytic response. Our studies investigated ATGL following several days of altered energy balance, whereas prior studies focused largely on changes within 6-8 hour
period (Kim et al., 2006; Jocken et al., 2007). Differences in the response of ATGL to experimental treatments may reflect differences between acute and chronic regulation of the lipase.

Furthermore, differences in the morphology and composition of adipose tissue have been reported as a result of triglyceride breakdown in response to a change in physiological state (Pujol et al., 2005). It may be argued that the changes observed in ATGL may have been affected by the probable presence of other cell types in the biopsy sample which may have diluted and decreased protein abundance. Martin et al. (2009) and Greenberg et al. (1991) reported that HSL and PLIN are predominantly expressed in the adipose tissue, such that variability in the levels of these phosphoproteins may be indicative of any change in the state of the adipose tissue sample. The fact that in the current study, no significant changes in total HSL and PHSL were observed suggests that the variation in ATGL abundance reflects what is happening in adipose tissue during altered energy balance. Moreover, although a significant decrease in PLIN was observed after feed restriction and GH administration, we believe that the time lapse between treatment and biopsy was short enough that major changes in the composition of the samples were unlikely. In a related study, Koltes and Spurlock (2013) found that following feed restriction and GH treatment, mRNA abundance of 60S ribosomal protein L32 (RPL32) in cows did not change with sampling day, a possible indication that the proportion of protein from adipose tissue did not differ across biopsy times.

A decline in total PLIN protein abundance was observed following feed restriction and GH administration, but not with stage of lactation (Koltes and Spurlock, 2011). Perilipin regulates lipolysis via multiple mechanisms. It was first described as
having a ‘barrier effect’ whereby the presence of PLIN on the LD limited access of lipases to their lipid substrate (Garcia et al., 2004). It was subsequently discovered that upon phosphorylation of PLIN by PKA, a conformational change to PLIN occurs that allows the release of CGI-58 for activation of ATGL, and interaction of PLIN with HSL to facilitate lipolysis (Subramanian et al., 2004). Following this latter discovery, majority of the research has focused more on this important role of PLIN as facilitator of PKA-stimulated lipolysis rather than just as a lipid-droplet barrier. Our data suggest the abundance of total PLIN is important in the regulation of lipolysis in response to both FR and GH administration during mid-lactation, and that this regulation may reflect the potential of PLIN to act as a barrier to lipolysis. Phosphorylation of PLIN and HSL-Ser563 is accomplished by PKA, and this is a primary mechanism for lipolytic stimulation following catecholamine activation of beta adrenergic receptors (Anthonsen et al., 1998; Jocken et al., 2008). Increased phosphorylation of PPLIN, HSL-Ser563, and HSL serine residue 660, another PKA targeted site, has been described in cattle with the onset of lactation (Koltes and Spurlock, 2011; Locher et al., 2011). In the current study, FR and GH administration in mid-lactation did not alter phosphorylation of PLIN or HSL-Ser563. Chronic administration of GH is known to stimulate lipolysis in lactating cattle in part through decreased responsiveness to adenosine, a critical lipolytic inhibitor (Bauman and Vernon, 1993). Thus, increased phosphorylation of HSL may not be necessary for GH to elicit its lipolytic effect. The degree of FR used in this study was chosen to mimic the level of energy insufficiency typical of the first week of lactation, and was expected to stimulate PKA-mediated lipolysis. Our results demonstrate that even though lipolysis is stimulated by energy insufficiency in the transition period and with FR in mid-lactation,
the physiological context in which energy insufficiency occurs has an impact on the mechanism by which lipolysis is regulated. In particular, our data indicate the classic mechanism of lipolytic stimulation by β-adrenergic receptor activation and PKA phosphorylation does not have a primary role in the regulation of lipolysis following FR and GH administration in mid-lactation cows.

Regulation of lipolysis by HSL can also be achieved through phosphorylation of HSL by kinases other than PKA. In 3T3-L1 as well as rat adipocytes, phosphorylation of HSL$_{Ser565}$ is mediated by AMPK (Garton et al., 1989; Chaves et al., 2011). This phosphorylation does not affect the activity of HSL per se, but may prevent subsequent phosphorylation of HSL$_{Ser563}$ by PKA (Anthony et al., 2009). Activation of AMPK as observed in mice is associated with the depletion of energy reserves (Gauthier et al., 2008), which is expected with feed restriction and GH treatment. However, we found that feed restriction and GH elicited a significant decrease in HSL$_{Ser565}$, which may be consistent with a decline in AMPK activation. Activation of AMPK has also been associated with increased expression of ATGL in rat adipocytes (Gaidhu et al., 2010). Thus, our results showing decreased ATGL and HSL$_{Ser565}$ following both feed restriction and GH treatments are consistent with an expected decrease in activation of AMPK in these models. Phosphorylation of AMPK has been studied in transition cows, where its activation increased with the onset of lactation (Locher et al., 2012). However, further research is warranted to fully understand the complex interactions among AMPK, ATGL, HSL and lipolysis throughout lactation.
Conclusions

Adaptation to negative energy balance (NEBAL) is particularly critical because its severity and duration often translate to declines in reproduction and fertility (Patton et al., 2006; Wathes et al., 2007; Fenwick et al., 2008), as well as increased incidence of health problems (Leslie et al., 2003; Kadokawa and Martin, 2006). The lipolytic response in dairy cows is affected by many physiologic and metabolic states, and is regulated by multiple mechanisms. Importantly, stimulation of lipolysis by PKA phosphorylation of HSL and PLIN are likely critical during early lactation, but less important in response to other lipolytic stimuli later in lactation. The significant down-regulation of total PLIN abundance in response to FR and GH administration suggest the barrier effect of PLIN may have a significant effect on lipolysis under certain physiological conditions. Finally, the unexpected decrease in ATGL abundance across all models of energy insufficiency may suggest a unique mechanism for regulation of lipolysis in lactating dairy cows. Our findings demonstrate that lipolysis is regulated by multiple mechanisms, and this must be taken into account when evaluating strategies to manipulate energy utilization and partitioning in lactating cows.
Table 3.1. Antibodies used for semi-quantitative Western blotting. Antibody, protein quantity (µg), antibody dilutions, time of exposure to primary antibody, time of exposure for imaging are provided.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pro:tein Concentration (µg)</th>
<th>Antibody Concentration</th>
<th>Species Reactivity</th>
<th>Exposure to Primary Antibody</th>
<th>Exposure Length</th>
<th>Antibody Company (Catalog #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-perilipin</td>
<td>10</td>
<td>1:1000</td>
<td>Cow</td>
<td>1 O/N</td>
<td>2 min</td>
<td>Chemicon (AB10200)</td>
</tr>
<tr>
<td>HSL</td>
<td>100</td>
<td>1:500</td>
<td>Mouse</td>
<td>2 O/N</td>
<td>10 min</td>
<td>Cell Signaling (4107)</td>
</tr>
<tr>
<td>PhosphoHSL at Serine 563</td>
<td>400</td>
<td>1:500</td>
<td>Mouse</td>
<td>2 O/N</td>
<td>10 min</td>
<td>Cell Signaling (4139)</td>
</tr>
<tr>
<td>PhosphoHSL at Serine 565</td>
<td>400</td>
<td>1:500</td>
<td>Mouse</td>
<td>2 O/N</td>
<td>10 min</td>
<td>Cell Signaling (4137)</td>
</tr>
<tr>
<td>ATGL</td>
<td>400</td>
<td>1:500</td>
<td>Mouse</td>
<td>4 O/N</td>
<td>10 min</td>
<td>Cell Signaling (2138)</td>
</tr>
</tbody>
</table>

1 Phosphorylated perilipin was detected using the same antibody as was used for perilipin, but a second band was detected by size separation of phosphorylated to non-phosphorylated perilipin proteins on a 10% gel and 100:1 acrylamide to bisacrylamide.

2 X:Y, X µl antibody for every Y µL of 5% dry milk in tris buffered saline-Tween20.

Abbreviations: HSL, Hormone Sensitive Lipase; ATGL, Adipose Triglyceride Lipase; O/N, overnight(s); min, minutes;
Table 3.2. Changes in abundance or phosphorylation of lipolytic proteins in different models of altered energy balance in dairy cows.

<table>
<thead>
<tr>
<th>Response</th>
<th>Transition</th>
<th>Feed Restriction</th>
<th>Growth Hormone</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGL</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>HSL</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PHSLSer563</td>
<td>↑</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PHSLSer565</td>
<td>NS</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>PLIN</td>
<td>NS</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>PPLIN</td>
<td>↑</td>
<td>NS</td>
<td>↓</td>
</tr>
</tbody>
</table>

1 NS – no significant differences among treatments ($P \geq 0.05$); ↓ or ↑ indicates a decrease/increase in abundance of protein

2 data obtained from Koltes and Spurlock, 2011.
Figure 3.1. Abundance of lipid droplet associated proteins in subcutaneous adipose tissue (AT) in response to feed restriction. AT samples were obtained 6 d (solid white) prior to, and 1 (gray) and 4 d (solid black) after restricted feeding. Protein abundance of (A) adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL); (B) phosphorylated HSL at Serine-563 and 565 (PHSL-563 and PHSL-565, respectively); and (C) perilipin (PLIN) and phosphorylated perilipin (PPLIN) was determined by semi-quantitative Western blotting and expressed in relative units per quantity of total protein (10-400 µg) after normalization across gels to a common standard (STD). Each inset shows a representative Western blot. For perilipin (C), the upper band was quantified as PPLIN, and the lower band as PLIN. Different letters indicate differences among means, P<0.05.
Figure 3.2. Abundance of lipid droplet associated proteins in subcutaneous adipose tissue in response to growth hormone administration determined via semi-quantitative Western blotting. Adipose tissue samples were obtained 4 d (solid white) pre- and 3 (gray) and 7 d (solid black) post-treatment. Protein abundance of (A) adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL); (B) phosphorylated HSL at Serine-563 and 565 (PHSL563 and PHSL565, respectively); and (C) perilipin (PLIN) and phosphorylated perilipin (PPLIN) is expressed in relative units per quantity of total protein (10-400 µg) after normalization across gels to a common standard (STD). Each inset shows a representative Western blot. For perilipin (C), the upper and lower bands represent PPLIN and PLIN, respectively. Different letters indicate differences among means, $P<0.05$.
CHAPTER 4: EFFECTS OF HEAT STRESS ON LIPID METABOLISM OF BOVINE PRIMARY ADIPOCYTES

Abstract

Heat stress (HS) impacts numerous physiological processes, and recent research indicates lipid metabolism is altered in lactating cows experiencing HS. The objective of this study was to evaluate how HS affects lipid metabolism in subcutaneous adipose tissue (AT) of dairy cattle, particularly in terms of responsiveness and sensitivity to lipolytic and lipogenic stimuli. AT biopsies were performed on randomly selected multiparous, late lactation Holstein cows from which bovine primary adipocytes were isolated, and cultured at either 42°C (HS) or 37°C (thermal neutral, TN). For the first part of the lipolysis experiment (n=5), isoproterenol (ISO) was administered at varying concentrations and glycerol release was measured as an indicator of lipolytic response. A dose response curve to ISO was determined under HS and TN conditions from adipocytes isolated from each cow. In another experiment (n=5), cells were incubated with varying concentrations of insulin (0 – 2.5 mU) in combination with isoproterenol (ISOP, 4.5 x 10^{-7} M), after which glycerol release was measured to indicate lipolytic activity. Meanwhile, the incorporation of radioactive acetate into lipids relative to different concentrations of insulin (0, 0.5 and 1 mU) was measured to assess lipogenic activity in 8 randomly chosen Holstein cows. For these experiments, the effects of temperature, ISO and/or insulin concentration, as well as their interaction on AT lipolysis and lipid synthesis were evaluated. Likewise, the abundance and phosphorylation of several lipolytic and lipogenic proteins in relation to HS was analyzed. Lipolysis assay results show that
adipocytes exposed to HS had an elevated maximal response to ISO \((P=0.024)\), and were more sensitive to lipolytic stimulation by ISO \((P=0.04)\) compared to cells cultured at TN. At a high ISO concentration, a decrease in lipolytic response was observed for HS but not TN cells, suggesting potential down-regulation of the lipolytic response in HS but not TN cells. Furthermore, no significant temperature by insulin concentration interaction was observed between TN and HS cells, although the latter was found to significantly reduce the amount of glycerol released \((P<0.001)\), indicating greater inhibition by insulin to lipolytic stimulation. Meanwhile, in the absence of insulin, adipocytes cultured under HS exhibited an elevated response to ISOP \((P<0.001)\) relative to their TN counterparts. Basal lipolytic (-ISOP/-insulin) response was not different between HS and TN cells \((P>0.05)\). A significant decrease in the phosphorylation of hormone sensitive lipase (HSL) at Serine 563 (PHSL\textsubscript{Ser563}, \(P=0.03\)) and perilipin (PLIN, \(P=0.04\)) with respect to increasing insulin concentrations was observed for cells cultured under HS but not TN conditions. Meanwhile, lipogenesis data indicate that temperature by insulin interaction was not significant \((P=0.25)\), but insulin increased the amount of acetate incorporated into lipids in TN cells \((P<0.001)\). As for the effect of HS on the phosphorylation of acetyl CoA carboxylase (ACC), a significant temperature by insulin interaction was observed \((P=0.01)\), wherein P-ACC abundance decreased significantly for TN \((P=0.02)\) but not HS cells, suggesting that less fatty acids are being transported to the mitochondria for fatty acid breakdown. Results of the study suggest that HS affects lipid metabolism by altering the response to insulin of bovine primary adipocytes, which may contribute to the lack of adipose tissue mobilization observed \textit{in vivo}.
Introduction

Climate change is fast becoming a major threat to the viability and sustainability of livestock production systems in many regions of the world. A direct effect of climate change, heat stress, is an important challenge facing the dairy industry. Heat stress (HS) is a physiological condition when the core body temperature of an animal exceeds its range for normal activity, resulting from a total heat load that is more than its capacity for heat dissipation, thus prompting physiological and behavioral responses to reduce the strain (Bernabucci et al., 2010).

The increasing concern with the thermal comfort of dairy animals is justifiable not only for countries occupying tropical zones, but also for nations in temperate regions in which high ambient temperatures are an issue (Nardone et al., 2010). It was reported that in the U.S. alone, HS continues to cost the dairy industry an estimated $1 billion annually (St-Pierre et al., 2003). Although improvements in management strategies have been put in place, these only partly alleviate the impact of thermal stress on dairy cow performance during the hotter seasons. Moreover, genetic improvement programs that enhance production traits may increase an animal’s susceptibility to high environmental temperatures due to a correlation between production and metabolic activity (West, 1994; Settar et al., 1999).

In the past decade, alterations in the endocrine and metabolic status under hot environments have been reported in dairy cattle (Ronchi et al., 2001; Bernabucci et al., 2002, 2006; Rhoads et al., 2009). Ronchi and colleagues (1999) demonstrated that HS has a direct effect on energy, lipid metabolism and liver enzymatic activities of Holstein heifers. In addition, changes in the oxidative status and circulating adipocytokine levels
were observed in periparturient cows exposed to hot environments (Bernabucci et al., 2006). Furthermore, reductions in energy intake combined with increased energy expenditure for maintenance lower energy balance and only partially explain why lactating cattle lose substantial amounts of body weight during severe HS (Rhoads et al., 2009). This was shown in a series of pair-feeding experiments wherein plasma NEFA concentration in lactating dairy cows exposed to HS remained constant, suggesting that these animals did not mobilize as much adipose tissue as their pair-fed, thermal neutral (PFTN) counterparts despite decreased DMI and loss of body weight. Also, plasma insulin concentrations were measured to further determine if the blunted NEFA response observed in HS cows may have resulted from the presence of overriding antilipolytic factors such as insulin. Basal insulin concentrations did not differ in PFTN cows, but gradually increased by 37% in lactating cows exposed to HS. Additionally, it has been demonstrated that heat-stressed cows have an increased insulin response to a glucose tolerance test (Wheelock et al., 2010; O’Brien et al., 2010), which may be essential in their adaptation mechanism to HS.

In recent years, adipose tissue has received much attention due to the discovery of its role as a complex and highly active metabolic and endocrine organ (Kershaw and Flier, 2004). Moreover, new discoveries have significantly changed the traditional view of adipose tissue as a passive energy depot and highlighted its importance in the regulation of lipid metabolism. Adipose tissue plays a crucial role by storing or liberating triacylglyceride reserves via lipolysis to provide fatty acids that are important oxidative fuels for other tissues during times of energy deprivation. A dysregulation of lipid metabolism may lead to several abnormalities and so appropriate regulation is critical for
the maintenance of body energy homeostasis as well as for the prevention of metabolic diseases (Jaworski et al., 2013).

An accurate understanding of the biological mechanism(s) by which thermal stress reduces milk synthesis is critical for developing novel approaches (i.e. genetic, managerial and/or nutritional) to optimize dairy cow performance, particularly during the summer months. Furthermore, identifying additional mechanisms that govern the regulation of adipose tissue metabolism would be helpful in elucidating the processes affected under other physiological states, including lactation and disease. Therefore, the objective of this study was to evaluate the direct impact of HS on lipid metabolism in the lactating dairy cow, particularly on the molecular regulators of lipolysis and lipogenesis in cultured bovine adipocytes. In our initial study, we looked at the sensitivity to epinephrine stimulation as well as responsiveness to insulin of bovine primary adipocytes to assess if HS 1) alters their lipolytic response to beta-adrenergic receptor (BAR) agonists, and 2) enhances inhibition of lipolysis by insulin. For the second part of our study, we looked at changes in the rate of incorporation of radioactive acetate in bovine cells to determine if HS enhances insulin-stimulated lipogenesis.

Materials and Methods

Animals and adipose tissue biopsy

The maintenance of the animals and the experimental procedures performed on them were carried out in accordance with the Iowa State University Animal Care and Use Committee guidelines and regulations.
Experiments were conducted during the spring months (late February until April) of years 2012-2014 when cows were not exposed to environmental heat stress. Second to 3rd lactation Holstein cows (230 to 400 d postpartum) with a body condition score (BCS) > 3.75 were randomly selected for the lipolysis and lipogenesis studies. Subcutaneous adipose tissue biopsies were taken from between the pin and tail bone (ischial tuber and coccyx, respectively) of each cow using a minimally invasive procedure under local anesthesia. Adipose tissue was placed in buffered saline solution with glucose, kept at 37°C and transported to the laboratory for immediate analysis.

Isolation of adipocytes

The protocol for harvesting primary adipocytes was adapted from Liu et al. (1999) with some modifications. Samples were subjected to 2 rounds of 40-minute collagenase digestion at 37°C in a water bath with shaking (100 cycles/min). Adipocytes were isolated using nylon filter mesh and washed with warmed cocktail buffer (Krebs-Ringer solution [20 mM sodium bicarbonate, 20 mM HEPES, 20 mM D-glucose], 1 M sodium bicarbonate, 1 M HEPES, 1 M glucose, 6% bovine serum alumin (BSA), 1mg/mL collagenase type 1a from Clostridium histolyticum (Sigma Aldrich)). After removal of the infranatant, adipocytes were suspended in warmed explant media (1000 mg/L low glucose Dulbecco’s Modified Eagle Medium (DMEM), 4.4 mM sodium bicarbonate, 5 mM HEPES, 3% BSA), and approximately 900 µL of this cell suspension was aliquotted into 20 mL scintillation vials.

Experimental treatments

*Validation studies.* Primary adipocytes from lactating cows (N=4) were used to evaluate cell viability using the PrestoBlue® Cell Viability Reagent from Life
Technologies™ according to the manufacturer’s protocol. The abundance of the heat shock protein HSP27 (Cell Signaling) was evaluated via semi-quantitative western blotting to confirm an effect of thermal treatment.

Lipolysis experiments. The cell suspensions were exposed initially and at 2-hr intervals to a mixture of 95% air and 5% CO₂ and incubated in a gyratory incubator at 37°C. Following a 1 hr acclimation period, cells were incubated under 2 experimental conditions for 1 hr: 42°C (heat stress, HS) and 37°C (thermal neutral, TN). Core body temperature of lactating dairy cows is approximately 38°C. A slightly lower temperature was used to represent thermal neutral conditions because adipocytes were isolated from the subcutaneous adipose depot, which is expected to be maintained at a temperature slightly less than the core body temperature. Culturing cells at 42°C was done to represent a severe heat stress event in lactating cattle. Subsequently, adipocytes were administered isoproterenol (ISO; 1.0 x 10⁻⁵.⁵ M to 1.0 x 10⁻⁹ M) for determination of a lipolytic dose-response curve and incubated for 90 min under HS or TN conditions. Each ISO dose was evaluated in 3 vials of cells for each thermal treatment, and this experimental protocol was replicated using adipocytes from 5 different cows. The incubation medium was aspirated after a 90-min incubation with ISO and stored at -20°C until analysis. Approximately 400 μL of protein homogenization buffer (10% SDS, 1 M sodium fluoride, 0.5 M EDTA, 1 M HEPES, 1 μL/mL protease inhibitor (Sigma), 1 μL/mL phosphatase inhibitor (Sigma)) was added to the remaining cells and kept at -80°C for subsequent protein extraction.

To further evaluate the effects of HS on lipolytic activity in bovine adipose tissue, the response of isolated primary adipocytes to lipolytic stimuli relative to varying
concentrations of insulin was tested. As with the previous experiment, cells were exposed to the 2 temperature treatments (37°C and 42°C for TN and HS condition, respectively) following an hour-long acclimation period at 37°C. Insulin (0 to 2.5 mU) was administered 30 min after exposure to the thermal treatments. After 30-min incubation with insulin, ISO (4.5 x 10^-7 M) was added into the cell suspension, and the adipocytes were incubated for 90 min before cell harvest. At this concentration, the half maximal effect of ISO was observed previously and was therefore used in this study. The incubation medium was carefully pipetted out and kept at -20°C until further analysis. Protein homogenization buffer was added to the remaining cells and kept at -80°C until protein extraction. Each insulin-temperature treatment combination was evaluated in triplicates, and this experiment was repeated independently across 5 different cows.

**Lipogenesis experiment.** The adipocyte cell suspensions were exposed to a mixture of 95% air and 5% CO₂ prior to incubation, and every 2 hours thereafter throughout the experimental period. The amount of acetate incorporation was measured to assess lipogenic activity of bovine adipocytes in response to insulin. Cells were acclimatized at 37°C for 1 hr, after which they were exposed to the thermal treatments (37°C for TN and 42°C for HS) and allowed to acclimate for another hr. A final concentration of 1 µCi of [1-^{14}C]-acetate was added together with varying concentrations of insulin (0, 0.5 and 1 mU) to the cells. After a 3-hr incubation period, reactions were terminated by the addition of 100 µL of 17% perchloric acid to each vial. Each insulin dose per temperature treatment was evaluated in 3 vials of cells extracted from 8 cows.

The method for the extraction of total lipids from cells was adapted from Folch (1957) with some modifications. Briefly, acidified media was carefully aspirated from the
bottom of the vial, followed by the addition of Folch’s reagent (chloroform/methanol, 2:1, v/v). The cell-reagent mixture was agitated by placing the vials on top of a platform mixer for 15 minutes, after which the crude extract was washed twice with 1 N NaCl. The solution was centrifuged at 2000 rpm for 20 min, and the lower, lipid-containing chloroform phase was transferred to a new glass scintillation vial and allowed to evaporate to dryness overnight. Subsequently, 5 mL of scintillation cocktail (Sigma) was added to resuspend the lipids prior to quantification using a liquid scintillation counter.

Glycerol assay

Glycerol content in the incubation medium served as an indicator of lipolysis and was determined by use of a colorimetric assay with free glycerol reagent following manufacturer’s protocol (Sigma Aldrich, St. Louis, MO). Samples were run in triplicates in a 96-well format, and absorbance values were obtained using a Tecan Spectrafluor Plus (Tecan Group Ltd.). Glycerol concentrations were normalized to total protein extracted from cells cultured in each vial. The average of triplicate glycerol concentration values representing each of 3 replicate vials of cells per treatment combination was used in statistical analyses.

Protein extraction and semi-quantitative Western blotting

The protocol for the extraction and preparation of proteins from adipose tissues for use in Western blotting has been previously described (Elkins and Spurlock, 2009). Bicinchoninic assay (BCA; Pierce Protein Research, Rockford, IL) was performed to determine protein concentrations, which were used to standardize the quantity of total protein loaded onto each gel. Semi-quantitative Western blotting was performed to determine protein abundance. Following transfer, membranes were blocked with 5% milk
in TBS-T for at least 1 hr. Blots were incubated with specific primary antibodies at 4°C followed by incubation with anti-rabbit IgG horseradish peroxidase-linked secondary antibody from GE Healthcare (Pittsburgh, PA). Proteins were detected with the ECL Plus Western Detection Kit (Amersham, Pittsburgh, PA) using an Alpha Innotech Imager (FluorChem FC2, Cell Biosciences, Santa Clara, CA) and quantified using Totallab software for 1D gel analysis (TL100, v2009; Totallab Ltd., Newcastle, Tyne, UK). All proteins were evaluated on duplicate gels, and average values across the gels were used in statistical analyses. A single arbitrarily chosen sample was included on each gel for normalization across gels.

Calculations and statistical analysis

The lipolytic response to isoproterenol of isolated bovine adipocytes was evaluated by comparing the dose response curve of adipocytes under HS versus TN conditions. A glycerol dose response curve was generated for cells representing each cow using the GraphPad Prism 5.00 software (GraphPad Prism for Windows, 2007). A Student’s t-test was used to evaluate the effect of thermal treatment on several parameters including half maximal effective concentration (EC₅₀), slope, maximal response (Rₘₐₓ) and baseline response. Proc Mixed of SAS (1999) was used to evaluate the main effects and interaction between thermal treatment and insulin concentration. Accordingly, a separate analysis was done per thermal treatment to test for the main effects of insulin on the response of adipocytes to ISO stimulation.

For the first part of the lipolysis experiment, differences in abundance of lipolytic proteins according to thermal treatment and ISO concentrations were evaluated using Proc Mixed. The model statement included thermal treatment and isoproterenol
concentration as fixed effects, and cow as a random variable. The interaction of thermal treatment with ISO concentration was evaluated initially. This effect was non-significant ($P>0.05$) for all proteins and was excluded from final analyses. When the overall effect of thermal treatment or ISO concentration was significant ($P<0.05$), mean differences were evaluated using the p-diff procedure of SAS. Phosphorylated perilipin was often undetectable at lower concentrations of isoproterenol. Therefore, phosphorylated perilipin was compared across isoproterenol concentrations using a Chi-square test to determine if its presence or absence differed between thermal treatments.

For the lipogenesis study, Proc Mixed was used to test for the main effects and interaction between thermal treatment and insulin concentration. After which, analyses was done separately according to thermal treatment to evaluate the main effects of insulin on the response of cells. All values are presented as means ± SE, with the statistical significance set at $P<0.05$.

Results

Analysis of HSP27 abundance and cell viability

The abundance of HSP27 was determined via semi-quantitative western blotting to confirm that a physiological response was invoked at a temperature of 42°C. Protein abundance of HSP27 increased with time of incubation for all cells, and this increase was significantly greater in cells cultured under HS compared to TN conditions after culturing for 60 and 120 min. The increase in HSP27 protein abundance with time likely reflects a stress response to the culture conditions, but the significant increase in HSP27 protein observed in HS relative to TN conditions confirms that additional stress was experienced
by cells cultured at higher temperature. In addition, to assess whether heat stress influenced glycerol release and protein abundance through a decrease in cell viability, we performed a resazurin-based assay that monitored the number of live cells in suspension. Neither thermal treatment nor culture time had a significant effect on cell viability ($P>0.05$) (Fig. 4.1A and B).

Effects of heat stress on isoproterenol response

The effect of HS on the lipolytic response of bovine adipocytes to ISO was evaluated by determining the glycerol dose response curve for each cow (Fig. 4.2). Adipocytes cultured under HS had an elevated response to ISO, as indicated by increased $R_{\text{max}}$ values in HS versus TN cells (40.35 mM glycerol/mg protein versus 19.52 mM glycerol/mg protein, $P=0.02$). Additionally, HS cells were more sensitive to lipolytic stimulation by isoproterenol, depicted by lower EC$_{50}$ values relative to cells cultured at TN conditions (1.19 x $10^{-7}$ M versus 4.51 x $10^{-7}$ M, $P=0.04$). However, neither basal lipolysis (glycerol release with no added isoproterenol) nor the slope of the dose response curve differed between cells exposed to HS or TN environments ($P>0.05$). A dramatic decrease in glycerol release was observed for HS cells at an isoproterenol concentration of 1 x $10^{-5.5}$ M relative to lower isoproterenol concentrations. However, this was not seen among cells incubated at TN temperature (Fig. 4.2). This decline in glycerol concentration with HS treatment was consistent across cells from all 5 cows investigated.

Heat stress effects on stimulated lipolysis relative to insulin

Consistent with the previous experiment, the overall lipolytic response to ISO was higher among HS-treated cells (Fig. 4.3). In the absence of insulin, adipocytes cultured under HS conditions exhibited an elevated response to ISO (4.5 x $10^{-7}$ M; ISO-EC$_{50}$)
relative to their TN counterparts (24.8 mM glycerol/mg protein versus 12.59 mM glycerol/mg protein, \( P<0.001 \)). Likewise, HS cells had a greater response when exposed to a higher concentration of ISO (1 \( \times 10^{-6} \) M) without insulin (43.6 mM glycerol/mg protein versus 22.2 mM glycerol/mg protein, \( P<0.001 \)). The statistical evaluations did not demonstrate significant interaction between insulin concentration and temperature relative to ISO-\( EC_{50} \) and to a higher concentration of ISO (\( P=0.83 \) and \( P=0.12 \), respectively). When analyzed separately by thermal treatment, a significant inhibitory effect of insulin in the presence of ISO-\( EC_{50} \) was observed in both TN (\( P=0.04 \)) and HS (\( P=0.02 \)) cells. However, when cells were treated with a higher ISO concentration, the inhibitory effect of insulin was significant only for HS (\( P<0.001 \)) but not TN (\( P=0.34 \)) cells. The basal (-ISO/-insulin) lipolytic response was not different between HS and TN cells (\( P>0.05 \)) regardless of ISO concentration.

Changes in lipolytic proteins in response to heat stress

Between HS and TN cells, phosphorylation of hormone sensitive lipase (HSL) at serine 563 (PHSL\(_{\text{Ser563}}\)) differed significantly (\( P<0.001 \)) at an ISO concentration of 1 \( \times 10^{-6} \) M (Fig. 4.4) where maximal glycerol release was observed. Differences in total HSL levels in response to thermal stress and across different isoproterenol concentrations were not significant (\( P>0.05 \)). Thermal stress did not elicit a difference in total perilipin protein abundance (Fig. 4.5B). Phosphorylation of perilipin was not always detected, particularly at lower concentrations of ISO. However, chi squared test results showed that phosphorylated perilipin was detected more often (\( P=0.02 \)) with higher concentrations of ISO as compared to control (no ISO) in both HS and TN cells. Comparison of phosphorylated perilipin at these higher ISO concentrations indicated a significant effect
of thermal treatment ($P=0.03$), with greater abundance of PPLIN in HS compared to TN cells (Fig. 4.5C). Heat stress did not affect adipose triglyceride lipase (ATGL) abundance in isolated bovine adipocytes (Fig. 4.5A). Likewise, ATGL abundance did not change significantly across different ISO concentrations.

Meanwhile, in the presence of high ISO ($1 \times 10^{-6}$ M), a significant decrease in the phosphorylation of hormone sensitive lipase (HSL) at Serine 563 ($\text{PHSL}_{\text{Ser563}}, P=0.03$; Fig. 4.6A) and total perilipin (PLIN, $P=0.04$; Fig. 4.6B) with respect to increasing insulin concentrations was observed for cells cultured under HS but not TN conditions. No significant differences in terms of abundance were observed for HSL, ATGL and phosphorylated PLIN ($P>0.05$).

Effects of heat stress on lipogenesis

The effect of HS on lipogenic activity was evaluated by determining the amount of acetate incorporated into lipids of bovine primary adipocytes. The temperature by insulin interaction did not reach statistical significance ($P=0.25$; Fig. 4.7A). However, the amount of acetate incorporation increased significantly in TN cells ($P<0.001$) but only approached significance in the presence of HS ($P=0.053$).

A significant temperature by insulin interaction was observed for the abundance of phosphorylated ACC ($\text{P-ACC}, P=0.01$) (Fig. 4.7B). A significant decrease in P-ACC abundance was observed for TN ($P=0.02$) but not HS cells ($P>0.05$), suggesting that less fatty acids are being transported to the mitochondria for fatty acid oxidation.
Discussion

An in-depth analysis of the economic effects of heat stress (St. Pierre et al., 2003) indicated that in the U.S. alone, billions of dollars of lost productivity and impaired animal health in the dairy industry are attributed to heat stress. A prerequisite to developing mitigation strategies to minimize heat stress-associated metabolic abnormalities is a better understanding of how environmentally induced hyperthermia affects post-absorptive metabolism and energy partitioning. Heat stressed mammals undergo changes in thermoregulatory mechanisms designed to promote body heat loss, which ultimately impact metabolic and hormonal responses as well as alter substrate utilization. *In vivo* data indicate that lactating cattle experiencing HS have a diminished lipolytic response to changes in energy status (Rhoads et al., 2009; Schwartz et al., 2009) and have reduced responsiveness to norepinephrine (Baumgard and Rhoads, 2013). Thus, we hypothesized that HS directly impacts adipose tissue by altering its response to lipolytic and lipogenic signals.

For the present study, we used isolated primary adipocytes from lactating dairy cattle to evaluate the direct effects of heat stress on lipolysis stimulated by isoproterenol. Our findings indicate that exposure of isolated adipocytes to heat stress significantly increased their maximal response and sensitivity to isoproterenol. This result was unexpected, as previous studies *in vivo* have reported that heat-stressed dairy cattle are refractory to lipolytic signals (Rhoads et al., 2009; Schwartz et al., 2009; Baumgard and Rhoads, 2013). It should be mentioned that on days when the biopsies were performed, the environmental temperature ranged from 34 to 48°F (1-9°C), and that cows involved in this study were not environmentally heat-stressed. Furthermore, our validation
experiments confirm that cell viability was not impacted by HS, suggesting that the temperature-related differences observed in the lipolytic response to isoproterenol were the result of a direct effect of heat stress on the metabolic activity of adipocytes.

It is important to note that the maximal lipolytic response diminished at the highest dose of isoproterenol evaluated in cells cultured under HS, but not TN conditions. Isoproterenol initiates a lipolytic response by activation of beta-adrenergic receptors (BAR) (Morimoto et al., 2001). BAR undergo decreased sensitivity to agonists following prolonged exposure (Benovic et al., 1988) and this down-regulation of BAR is particularly apparent in adipose tissue (Spurlock et al., 1994). Previous studies have reported that acute heat stress causes a marked increase in circulating cortisol, norepinephrine and epinephrine levels (Bernabucci et al., 2010; Collier et al., 2005; Farooq et al., 2010), catabolic signals that normally stimulate lipolysis via BAR activation. However, increased circulating concentrations of non-esterified fatty acids were not observed. One potential explanation is that adipocytes initially experience increased sensitivity to BAR agonists during heat stress, and this increased sensitivity contributes to desensitization of the receptor. Continued exposure to heat stress may lead to the down-regulation of BAR, such that a diminished response to BAR agonists is observed in vivo. Alternatively, the decline in lipolytic response with high concentration of isoproterenol may be associated with the activation of adenosine receptors. There is substantial evidence of adenosine accumulation in response to metabolic stress (Linden, 2001; Fredholm et al., 2001), and activation of adenosine A1 receptor (A1R) has been linked to G1-mediated inhibition of adenylyl cyclase (Hasko et al., 2008). In fact, basal levels of endogenous adenosine were found to be sufficient in inhibiting lipolysis in
subcutaneous adipose tissue in humans (Lonnroth et al., 1989). Johansson and colleagues (2007; 2008) recently showed that there is substantial A1R reserve in mouse adipose tissue, and that adenosine and insulin mediate additive, but not synergistic antilipolytic effects. It is possible that increased levels of epinephrine and insulin in vivo during HS may have contributed to the decline in lipolytic response observed in bovine adipocytes by activating A1R and negating BAR response.

To further investigate the mechanisms by which heat stress impacts the response of adipocytes to lipolytic signals, the abundance and phosphorylation of several proteins involved in the regulation of lipolysis were evaluated. The role of HSL in the lipolytic pathway is well documented, and its activity is regulated by phosphorylation and translocation to the lipid droplet (Koltes and Sprlock, 2011; van der Drift et al., 2012; Yin et al., 2003). In particular, HSL is activated when phosphorylated at serine residue 563 by protein kinase A (PKA) (Ronchi et al., 1999; Roepstorff et al., 2004) although a number of other phosphorylation sites may also be involved (Anthonsen et al., 1998; Holm et al., 2000). Together with ATGL, HSL is responsible for about 95% of the triglyceride hydrolase activity in murine white adipose tissue (Schweiger et al., 2006). Our results demonstrating increased phosphorylation of HSL-Ser563 in cells cultured under HS conditions are consistent with increased sensitivity of BAR and activation of the PKA pathway with heat stress. The protein abundance of total HSL and ATGL did not differ with thermal stress, providing further evidence that the observed lipolytic changes were due to post-translational signals. These results are consistent with those described for rat adipocytes in response to endoplasmic reticulum (ER) stress, in that ER stress contributed to increased PKA activation resulting in increased phosphorylation of HSL at Ser 563 and
moderately elevated translocation of HSL from the cytosol to lipid droplets, without significant changes in HSL and ATGL protein abundance (Deng et al., 2012).

Furthermore, we report that a moderate increase in PPLIN with high concentrations of ISOP was observed with HS, relative to TN conditions. Phosphorylation of perilipin was numerically greater in heat stressed cells, but pairwise comparisons between HS and TN cells did not reach statistical significance. This response was unexpected, because perilipin is one of the most abundant PKA phosphoproteins in adipocytes (Egan et al., 1990; Greenberg et al., 1991) and its function on lipolytic regulation has been firmly established. However, Jiang et al. (2007) previously showed that departure or phosphorylation of perilipins on intracellular lipid droplets is associated with heat shock protein HSP70. Furthermore, they reported that heat stress facilitated the departure of perilipins, though low in level, from the lipid droplet surface and slightly increased the translocation of HSL from the cytosol to the lipid droplets in rat adipocytes. To our knowledge, the role of PKA mediated phosphorylation of perilipin in relation to the association of perilipin with HSP70 is unknown. Thus, associations between heat shock proteins and perilipin may impact the phosphorylation of perilipin by PKA during times of heat stress. However, further experiments are warranted.

Our results also showed that insulin inhibition of lipolysis in the presence of ISO at EC50 concentration was similar for TN and HS cells, suggesting that the inhibitory effect of insulin remains intact during heat stress. When combined with increased levels of circulating insulin in vivo, this hypersensitivity of dairy cows to insulin during heat stress may be a coping mechanism by which these animals counteract high lipolytic stimulation and/or decrease metabolic heat production (Baumgard and Rhoads, 2013). Additionally,
since heightened catabolic stimulation during heat stress has been reported previously (Bernabucci et al., 2010; Collier et al., 2005; Farooq et al., 2010), we further investigated the inhibitory effects of insulin on the lipolytic response of adipocytes to a higher concentration of ISO. Our data showed that HS cells appear to be more responsive to lipolytic inhibition by insulin. In relation to this, results of our Western blot analysis demonstrated that insulin caused a significant decrease in PHSL_{Ser563} and total PLIN in HS but not TN cells, suggesting that adipocytes may be more sensitive to the antilipolytic effects of insulin under conditions of HS and BAR activation. Our data is consistent with those of other investigators who have reported increased insulin sensitivity in cows, mice and rats during heat stress (Rhoads et al., 2013; Morera et al., 2012; Gupte et al., 2009). This increased sensitivity to insulin, combined with the high levels of circulating insulin observed \textit{in vivo}, likely explains the lack of NEFA response during HS.

Further research is warranted to fully explain the inconsistencies between our results and existing \textit{in vivo} data with respect to lipolytic response. The dichotomy between models clearly demonstrates that heat stress indirectly prevents \textit{in vivo} adipose tissue mobilization, potentially as a result of heat-induced increase in insulin sensitivity, and/or an increase in circulating insulin levels. Insulin is a well-characterized anti-lipolytic agent (Morimoto et al., 1998) and therapeutic heat treatment increases glucose uptake and intracellular insulin signaling (Gupte et al., 2011). It has been demonstrated previously that heat stress increases basal and glucose stimulated insulin secretion in multiple models (Baumgard and Rhoads, 2013). The rationale for why and the mechanism by which heat stress (a hypercatabolic state) increases insulin (a potent anabolic hormone) is unknown, although it is likely that this contributes to differences
observed in production parameters between heat-stressed and pair-fed thermal neutral animals (Baumgard and Rhoads, 2013).

The effect of HS on lipogenesis in bovine adipocytes was also investigated in this study. Based on the results of our lipolytic study with insulin, we initially hypothesized that cells cultured under HS conditions would have higher lipogenic activity, as indicated by the amount of incorporated acetate into lipids of cells. However, our findings show that the amount of acetate incorporation increased significantly with insulin treatment in TN but not HS cells. It is important to note that during HS, dry matter intake (DMI) of cows is dramatically decreased; in essence, it would make sense to assume that the contribution of the available acetate to fatty acid synthesis would also be reduced. Moreover, Western blot analysis of the abundance of ACC revealed that phosphorylation was significantly decreased in TN cells. ACC catalyzes the first step in fatty acid synthesis, and phosphorylation events render the enzyme to be less active. Inactivity of ACC in turn affects the inhibitory action of another enzyme, malonyl CoA, on carnitine palmitoyltransferase 1 (CPT-1) which controls the transfer of fatty acyl moieties from the cytosol to the mitochondria for beta-oxidation. The decrease in the abundance of phosphorylated ACC observed in TN but not HS cells suggests that less fatty acids are being transported to the mitochondria for fatty acid breakdown. Moreover, the significant treatment interaction for phosphorylated ACC could indicate that HS cells are less responsive to insulin’s lipogenic signals, as compared to adipocytes cultured in TN conditions.

In conclusion, hyperthermia has the potential to influence adipose metabolism due to environmental challenges or disease state. Results of our study suggest that acute heat
stress directly increases the response of primary bovine adipocytes to lipolytic signals, and that this response is mediated in part through increased PKA phosphorylation of HSL and PLIN. However, increased sensitivity to BAR agonists may also contribute to increased down-regulation of BAR during heat stress, potentially contributing to the diminished lipolytic response that has been described during heat stress in vivo. Furthermore, increased sensitivity to insulin during HS influenced lipolytic but not lipogenic response of adipocytes to HS. Further investigation is needed to further evaluate the effects of HS on specific signaling cascades involved in insulin regulation in lipid metabolism.
Figure 4.1. Cell viability assay and analysis of HSP27 protein abundance. A) Primary adipocytes from lactating cows (N=4) were used to evaluate cell viability using the PrestoBlue® Cell Viability Reagent from Life Technologies™ according to the manufacturer’s protocol. Absorbance readings were taken at 560 nm. B) The abundance of HSP27 protein was determined via semi-quantitative Western blotting to confirm an effect of thermal treatment. Primary adipocytes were incubated at 42°C (HS) or 37°C (TN) and harvested at 0, 30, 60, 120 min. Inset shows a representative Western blot. Lowercase letters correspond to differences across time point under TN conditions; Uppercase letters indicate differences across time points under HS. Bars indicate significant differences between temperature treatments (* P≤0.05, **P ≤0.001)
Figure 4.2. Effect of heat stress (HS) on the lipolytic response. A glycerol assay was performed to determine the effects of HS on the lipolytic response of bovine primary adipocytes to isoproterenol. Data represent the average glycerol concentration across cells from 5 cows for each isoproterenol by temperature (HS or thermal neutral, TN) treatment combination.
Figure 4.3. Effect of heat stress (HS) on inhibition of stimulated lipolysis by A) low (4.51 $\times$ 10^{-7} M) and B) high (1 $\times$ 10^{-6} M) levels of insulin. A glycerol assay was performed to determine the inhibitory effect of insulin on the lipolytic response of bovine primary adipocytes to isoproterenol. Data represent the average glycerol concentration across cells from 5 cows for each insulin by temperature (HS or thermal neutral, TN) treatment combination.
Figure 4.4. Protein abundance of hormone-sensitive lipase phosphorylated at Serine residue 563 (PHSL\textsubscript{Ser563}) relative to increasing isoproterenol concentration. Phosphorylation of HSL at Serine 563 upon treatment of bovine primary adipocytes with varying concentrations of isoproterenol (ISOP) and after incubation at either 42°C (HS) or 37°C (TN) was evaluated. Protein abundance was determined via semi-quantitative Western blotting, expressed as relative units and normalized to a common standard. Inset shows a representative Western blot. Lowercase letters correspond to differences across ISOP concentrations under TN conditions; Uppercase letters indicate differences across ISOP concentrations under HS. Bars indicate significant differences between temperature treatments (* $P \leq 0.05$, ** $P \leq 0.001$)
Figure 4.5. Protein abundance of A) adipose triglyceride lipase (ATGL), B) perilipin (PLIN) and phosphorylated perilipin (PPLIN) relative to increasing isoproterenol concentration. Protein samples used for semi-quantitative Western blotting were extracted from bovine primary adipocytes treated with varying concentrations of isoproterenol (ISOP) and incubated at either 42°C (HS) or 37°C (TN) conditions. Amount of protein is expressed as relative units after normalization with a common standard. All values are presented as means ± SE. Thermal treatment significantly altered the abundance of phosphorylated perilipin (PPLIN, $P=0.03$) but not total PLIN or ATGL.
Figure 4.6. Protein abundance of A) hormone sensitive lipase phosphorylated at Serine residue 563 (PHSL-Ser563) and B) perilipin (PLIN) with increasing insulin concentration. Protein samples used for semi-quantitative Western blotting were extracted from bovine primary adipocytes treated with varying concentrations of insulin (0-2.5 mU) and isoproterenol (ISOP, $10^{-6}$) and incubated at either 42°C (HS) or 37°C (TN) conditions. Amount of protein is expressed as relative units after normalization with a common standard. All values are presented as means ± SE. A decrease in the protein abundance of PHSL-Ser563 ($P=0.03$) and PLIN ($P=0.04$) was observed in cells exposed to heat stress.
Figure 4.7. Effect of insulin on A) rate of incorporation of radiolabeled acetate, and B) phosphorylation of acetyl CoA carboxylase in primary bovine adipocytes.
CHAPTER 5: INTEGRATIVE SUMMARY

The dairy industry has evolved tremendously through the years. Current selective breeding programs and farm management practices are geared towards producing animals whose production levels exceed those considered normal several decades ago. Moreover, climatic conditions have drastically changed through time, posing additional challenges to dairy farming worldwide. A substantial amount of research has focused on understanding the physiological and metabolic adaptations observed in dairy cows, particularly during the lactation period or in times of stress, which is vital in developing novel strategies (i.e. genetic, managerial or nutritional) to optimize dairy cow performance.

One of the many aspects that influence the physiology of the lactating dairy cow is energy balance. At the onset of lactation, dairy cows enter a state of NEBAL because the energetic demands for milk production and body maintenance are not met by feed intake. These animals partition a greater portion of available energy to milk production rather than body tissue accretion, mobilizing body energy reserves in the process. The ability of the dairy cow to adapt to these natural changes in energy balance often influences its productivity and survivability in the herd, as NEBAL has been linked to lower rates of reproduction and increased incidence of health problems. Adaptation to this condition therefore calls for coordination of the biological processes in the different tissues resulting in metabolic changes to ensure that the cow’s genetic potential for milk yield is exploited but at the same time physiological homeostasis is maintained.
Beyond storage of energy reserves in the form of triacylglycerides, the discovery of the role of adipose tissue as an active endocrine organ has generated much interest to elucidate the association between lipid metabolism and milk production. Clearly, a balance between fat synthesis and breakdown is necessary to optimize an animal’s production potential.

In this study, we investigated 2 different models of altered energy balance achieved via feed restriction (FR) and growth hormone (GH) administration, and specifically looked at cellular changes in lipid metabolism in terms of substrate interactions as well as abundance and phosphorylation of several lipolytic proteins. Our results showed that stimulation of lipolysis via PKA-mediated phosphorylation of HSL and PLIN is likely crucial during early lactation, but is less important during the later stages of lactation. The fact that decreased abundance of phosphorylated PLIN was observed after GH administration but not FR suggests that lipolysis is regulated by multiple mechanisms, and that regulation may vary according to physiological state. Another important finding in this study is that abundance of ATGL unexpectedly decreased across all models of energy insufficiency, which may indicate a unique regulatory mechanism of lipolysis in lactating dairy cows.

Adaptation to changes in energy balance and regulation of adipose metabolism are further complicated by environmental factors that could also affect energy intake and expenditure. Among these is heat stress (HS), which has become a costly industry problem in recent years. Results of the current research showed that acute heat stress directly increases the lipolytic response of primary bovine adipocytes, which is contrary to existing \textit{in vivo} data demonstrating that hyperthermic cows elicited a much lower
NEFA response to an epinephrine challenge. Our results also showed that adipocytes cultured under HS conditions were more responsive to the antilipolytic effects of insulin. This increased insulin sensitivity, combined with the elevated levels of circulating insulin during HS, may explain the lack of NEFA response observed in vivo. Contrastingly, HS cells were less responsive to lipogenic stimuli, as indicated by the lower amount of incorporated acetate into lipids of cells as well as increased phosphorylation of the lipogenic enzyme ACC. Our findings suggest that multiple signaling pathways may be involved in the regulation of lipid metabolism during HS.

Further research is needed to evaluate how specific changes related to altered energy balance affect lipid metabolism, and how these collectively influence the adaptive behavior of lactating dairy cows. A better understanding of what goes on during adipose metabolism may be obtained by investigating enzyme activity levels as well as mRNA and protein abundance of the various components involved in the lipolytic and lipogenic pathways, and how these change in response to different physiological states. Although mRNA and protein levels are reportedly not well correlated, this data could provide information about conserved gene transcripts that would allow for a better understanding of gene regulation during these times of altered energy balance or stress.
LITERATURE CITED


United States Environmental Protection Agency. 2014. [www.epa.gov](http://www.epa.gov)


