METABOLIC AND ENDOCRINE ADAPTATIONS TO HEAT STRESS IN LACTATING DAIRY COWS

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Abstract

Heat stress (HS), a stress response in homeotherms mainly due to elevated ambient temperature and failure of effective heat dissipation, causes a substantial negative economic impact to livestock industry worldwide. Reduced feed intake, a typical phenomenon observed during HS, was thought to be the primary driver for the milk production loss. However, accumulating evidence indicates that HS influences animal metabolism and endocrine profiles independent of reduced feed intake. Previous studies comparing heat-stressed lactating cows with control group pair-fed (PF) to the intake of HS group but housed in thermoneutral conditions, in order to eliminate the confounding factors result from differentiated feed intakes, showed that HS increased circulating insulin and decreased plasma non-esterified fatty acid (NEFA) in lactating cow, the opposite responses typical of PF cohorts. Therefore, the present studies were performed in order to elucidate the mechanism(s) underlying these counterintuitive changes. In response to a glucose tolerance test (GTT), both HS and PF decreased whole body glucose disposal rate, a sign of insulin resistance. Only PF decreased skeletal muscle insulin sensitivity in terms of reduced protein kinase B (PKB/AKT) phosphorylation, a downstream protein of insulin receptor (IR), while HS group maintained similar intact insulin responsiveness in the liver and skeletal muscle as thermoneutral conditions. There was a global reduction in gene expression of the enzymes related to lipid

metabolism in adipose tissue of heat-stressed cows. Similarly, β-adrenergic signaling, a major stimulator of lipid mobilization, was suppressed in terms of NEFA release response during a chronic epinephrine challenge in HS group. After the challenge, phosphorylations of cAMP-response element binding protein (CREB) and hormone sensitive lipase, both located downstream of β-adrenergic receptor, were decreased in HS, but not in thermoneutral conditions, another indicator of impaired adrenergic signaling. In contrast, IR and AKT phosphorylation were increased in HS conditions indicating insulin signaling may be elevated during HS in adipose. Collectively, HS reduces lipid mobilization and appears to favor glucose utilization via alterations of lipid metabolism and hormones signaling pathways. These unique alterations in HS might shed some light on developing counter-HS approaches in the future.

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Chapter 1. Literature Review

Introduction

Over 50% of total world meat and 60% of milk are produced from tropical and subtropical areas and future growth of livestock industry is going to be strongly driven by developing countries located in these areas (Renaudeau et al., 2012). Heat stress (HS) has long been recognized as a limiting factor of farm animal productivity (Fuquay, 1981) and causes a significant economic impact ($2.4 billion/year in U.S., St-Pierre et al., 2003b). This impact is expected to be aggravated in the future as it is predicted that global average surface temperature will rise by 1.8-4 ^{o}C by 2100 (Renaudeau et al., 2012). The major concern regarding heat stress in the dairy industry is its negative effect on milk yield and quality and this effect is potentiated by genetic improvement of dairy cows due to the inevitable connection between production level and metabolic heat generation. Despite the great loss caused by HS, the underlying mechanism(s) leading to changes in animal metabolism is still unclear.

Heat stress

Domesticated animals, classified as endotherms, maintain their body temperature (T_b) within a relatively small range in order to obtain an optimal inner environment for survival and reproduction despite dwelling in variable environmental conditions. A thermoneutral zone (TNZ) is defined as a range of ambient temperature (T_a) in which endotherms do not need to adjust their metabolic heat generation or dissipation to counter heat loss or gain (Kingma et al., 2012). The lower critical temperature (LCT) and the upper critical temperature (UCT) define the bottom and top boundaries of the TNZ. Instead of being a fixed range, TNZ is dynamic and both LCT and UCT are influenced by relative humidity, solar radiation, air flow velocity, hair coat thickness, etc.

(Hillman, 2009). Two essential approaches are recruited by endotherms to counter elevated heat load: sensible heat loss and evaporative heat loss. The former can be acquired by behavioral adjustments (e.g. seeking shades, switching posture) utilizing the principles of conduction, radiation and convection and usually is first enlisted because they become effective quickly and energy requirement is low. These heat transfer methods are largely dependent on the temperature gradient between the animal's surface type (e.g. skin, feather) and the surrounding environment (e.g. air) (Bakken, 1976). Therefore, when extreme hot weather occurs, the temperature gradient can be reversed and heat flow is directed into the animal. In that case, evaporative heat loss is the last resort and an animal has to increase cutaneous evaporative heat loss (e.g. sweating) and respiratory evaporative heat loss (e.g. panting) to maintain homeothermy. In addition, vapor pressure difference between an animal and the surrounding environment is the driving force of evaporative heat loss (Gomes de Silva and Campos Maia, 2012). Cooling systems used in livestock industry are based on utilizing principles of either facilitating heat transfer or reducing heat gain (Renaudeau et al., 2012).

The predominant concern about heat stress is that it negatively impacts animal's health and performance and causes a substantial economic burden in the livestock industry worldwide (Renaudeau et al., 2012). When T_a exceeds or drops below the TNZ, an animal has to spend additional energy to cool or warm the body. The temperature-humidity index (THI) combines both T_a and relative humidity into one index (NOAA, 1976) and has been used to assess the intensity of thermal stress. Igono et al. (1992) proposed that the minimum, mean, maximum THI for milk production of Holstein cows are 64, 72 and 76 respectively. Dry matter intake (DMI) and milk yield were found to decrease linearly with increase of either air temperature or THI when THI ranged from 72.1 to 83.6 (West et al., 2003). A negative correlation between THI and DMI and a

positive correlation between milk yield and DMI were reported in studies carried out in the southeastern U.S. (Holter et al., 1997). Bouraoui et al. (2002) reported similar results: daily THI negatively correlated to milk yield and feed intake. In addition, high producers generate more metabolic heat than non-lactating or low producers because of their metabolic processes associated with production and elevated digestive requirements (West, 2003). Therefore, high producers have a lower tolerance to THI increase and they are more susceptible to heat stress than low producers.

Suppressed feed intake is one of the markers in heat-stressed animals and has been blamed as the major culprit causing the devastating impact of heat stress on milk production in dairy industry (Beede and Collier, 1986; Kadzere et al., 2002). Genetic progress of dairy cows selected for high milk production is closely related to increased feed intake (Kadzere et al., 2002) and thus, decline in feed intake would predict the drop in milk yield. However, how HS induces intake decrease and what molecule(s) is used to transmit the stress signal within the body remain elusive. Since elevated T_b is another sign of HS, studies addressing the link between appetite and T_b might provide some insights. An early study showed that rats with lesions in rostral area of hypothalamus were less sensitive to high T_a and did not decrease food intake (Hamilton and Brobeck, 1964) and this indicates that appetite and T_b may both be governed by the hypothalamus. Leptin is a 16kDa protein primarily synthesized by adipose tissue and known as an important energy regulating molecule (Meier and Gressner, 2004). Both intracerebroventricular and peripheral injection of leptin can induce elevated T_b and depressed appetite in a dose-dependent manner (Luheshi et al., 1999). Heat stressed mice increase leptin and adiponectin secretion accompanied by decreased food consumption (Morera et al., 2012). Neuropeptide Y was also reported to play a role in T_b regulation and have opposite effects when injected into different areas in hypothalamus despite a consistent up-regulating effect on feed intake in the rat (Jolicoeur et al., 1995).

An interesting question is: can high milk producers sustain their milk production during HS if normal feed intake is maintained. This type of research has not been performed but can potentially be realized by infusing a signal molecule, which can stimulate HS responses without triggering appetite depression, if these reactions are separable. Studies have been performed based on the reverse thought process: limiting feed intake of animals housed in thermal neutral conditions to the level of heat-stressed counterparts in order to differentiate the direct effect of HS from those mediated by suppressed energy supply from feed. Rhoads et al. (2009) reported that the reduction in feed intake associated with HS only accounts for ~40% of decreased milk synthesis and the remaining loss should be a direct impact of HS. Another surprising observation from this study and others is that heat-stressed cows did not appear to mobilize lipid reserves since their plasma non-esterified fatty acid (NEFA) remained constant before and after entering HS (Sano et al., 1983; Itoh et al., 1998), while the pair-fed group progressively increased plasma NEFA concentration as nutrient restriction continued (Rhoads et al., 2009; Wheelock et al., 2010). Carcass data from chickens (Geraert et al., 1996) and pigs (Collin et al., 2001) showed that heat-stressed animals had a higher fat retention compared to pair-fed controls. Meanwhile, a human study showed that HS increased muscle glycogen oxidation and reduced exogenous glucose utilization during exercise (Jentjens et al., 2002). Human subjects exercising in a hot chamber had a higher plasma glucose compared with thermal neutral control when both groups were being infused with constant concentration of glucose (Yaspelkis et al., 1993; Hargreaves et al., 1996). Elevated blood glucose might be due to increased gluconeogenesis, increased glycogenolysis or reduced glucose uptake. In addition, HS has potent effects on the endocrine system. For example, it altered the secretion of prolactin, cortisol, progesterone in heifers (Ronchi et al., 2001), elevated basal and stimulated insulin concentration in cows (Itoh et al., 1998), and induced higher glucagon, cortisol, growth

hormone, epinephrine and norepinephrine in exercising human (Hargreaves et al., 1996). Accumulating evidence show HS, besides changing nutrient intake, is affecting a variety of metabolites and hormones in a wider and more profound manner than originally thought. These effects will be explored in greater depth in subsequent sections of this review.

Lactating cows

Evolution granted ruminants a unique capability to utilize the most abundant biomass in nature, cellulose, and other fibrous feed components, which are usually not digestible to nonruminants. Ruminants rely on microbial protein synthesis for their amino acid supply (Russell and Rychlik, 2001). As a trade-off, readily digestible carbohydrates, like starches and sugars, are almost thoroughly fermented by microbes in reticulorumen and become unavailable for small intestine absorption. Short-chain fatty acids (SCFA), predominantly acetate, propionate and butyrate, are microbial fermentation end products used to power ruminant metabolism. Therefore, glucose available in the ruminant body is derived almost exclusively from gluconeogenesis in the liver and a small portion from kidneys (Bell and Bauman, 1997). Propionate, the primary precursor of hepatic gluconeogenesis, is extracted almost completely (90%) by the liver from portal blood and can be efficiently converted into glucose (Brockman, 2005). Acetate, on the other hand, is the major building block for lipogenesis and the principle substrate for oxidation (Brockman, 2005). Such nutrient availability changes likely required metabolically active tissues to evolve corresponding adjustments in the ruminant. In fact, most ruminal tissues, such as cardiac and skeletal muscles, developed the capability of utilizing SCFA and their derivatives as oxidative fuel and reserve glucose for tissues, such as the brain, and functions that have strict glucose requirements (Bell and Bauman, 1997). The amount and composition of SCFA are highly correlated to the dietary intake of fermentable materials (Bell and Bauman, 1997), thus, feed

constituents and processing methods have profound impacts on determining the availability of various nutrients. Hay-fed cattle were proposed to have a higher acetate-propionate ratio than those on corn-based diet and have a higher efficiency of acetate utilization (Annison and Bryden, 1999). Although corn and sorghum-based diets were supposed to increase the energy supply in the small intestine than ruminal fermentation (Owens et al., 1986), this method is challenged by the concern of suppressing microbial protein synthesis which is fueled by starch fermentation (Huntington, 1997). Feed type also presents direct influences on ruminal microbes, which have a symbiotic relationship with the host (Russell and Rychlik, 2001), and thereby affect the health and performance of their ruminant host. Excessive feed rich in highly-fermentable carbohydrate can induce acidosis caused by the fast growth of starch-fermenting, lactic acid-producing bacteria, which are normally not competitive (Owens et al., 1998). Some pathogens can pass through rumen wall damaged by acidosis, reach the liver via circulation and cause liver abscesses (Nagaraja and Chengappa, 1998).

The time frame between late gestation and early lactation is well known as the transition period. During this period, mammary metabolism and energy requirements increase markedly to preapare mammary gland for copious milk secretion (Bell, 1995). Lactation is also a physiological process where homeorhesis occurs to support milk production (Bauman and Currie, 1980). Synthesis of lactose, the most critical osmotic solute in milk, requires glucose, thus enough glucose supply ensures copious milk production (Davis et al., 1979). Glucose demand in Holstein cows can double or even triple at 21 d postpartum compared with that in the last 21 d of gestation (Drackley et al., 2001a). This sudden change poses a great challenge to lactating animals as their voluntary feed intake is usually suppressed immediately before parturition (Grummer et al., 2004), causing animals to enter severe negative energy balance. Nonmammary metabolic adaptations

mobilizing body energy and protein reserves are made to meet the nutrient shortfall. Extensive lipid mobilization is a hallmark of metabolism during transition period. Plasma concentration of NEFA is positively correlated to the milk fat percentage and yield, and whole body fatty acid oxidation in lactating cows (Pullen et al., 1989). Around 35% of NEFA that enter into blood is consumed by rapid oxidation and another 17% is integrated into milk fat (Bell, 1995). This increased flux of NEFA is believed to result from reduced lipogenesis and fatty acid esterification, and increased adrenergic stimulation of lipolysis in adipose tissue (Bell, 1995). A high level of plasma NEFA is associated to elevated hepatic uptake of fatty acid (Emery et al., 1992). According to the calculation of Drackley et al. (2001a), less than half of the NEFA taken in liver are oxidized, and the remainder enter into ketogenesis or triacylglyceride generation and accumulate in liver because of the low hepatic capacity of exporting triacylglyceride in very low-density lipoprotein (Grummer et al., 2004). Allen et al. (2009) proposed an interesting theory stating that the net lipolysis and elevated hepatic uptake of NEFA leads to peripaturient hypophagia rather than the opposite because plasma NEFA concentration increase precedes hypophagia (Vazquez-Anon et al., 1994) In support of this notion, peripaturient cows with decreased plasma NEFA and β-hydroxybutyric acid induced by 2,4-thiazolidinedione tend to increase feed intake (Smith et al., 2007). As stated above, a massive increase of glucose uptake in mammary is supported by increased hepatic glucose output and the glucose originates almost exclusively from gluconeogenesis in the liver. Calculated glucose production from digestible energy intake is apparently not enough for actual glucose output during the periparturient period and leaves a large glucose deficit in cows (Drackley et al., 2001a). To make up for this discrepancy, periparturient cows exhibit decreased whole-body glucose oxidation, which is likely mediated by increased circulating somatotropin (growth hormone, GH) (Drackley et al., 2001a). Ovarian and placental

hormones, such as prolactin and estrogen, also participate the down regulation of glucose utilization mainly by suppressing insulin sensitivity and responsiveness in peripheral tissues (Bell and Bauman, 1997). Glucogenic amino acids are another important source for gluconeogenesis. Bell (1995) postulated that protein mobilized from skeletal muscle compensates the glucose shortfall as much as 500g/d during the first week of lactation. In support of this theory, Overton et al. (1998) showed that the conversion of alanine to glucose was upregulated in liver soon after calving, with a greater magnitude compared with that of propionate.

In contrast, heat-stressed lactating animals do not seem to follow similar adaptation strategies. The important hallmark of marked increased NEFA is missing in heat-stressed lactating cows (Rhoads et al., 2009), and they tend to have reduced plasma GH concentration (McGuire et al., 1991). At the same time, insulin secretion stimulated by glucose and glucose disposal rate are promoted in lactating cows under HS conditions (Itoh et al., 1998; Wheelock et al., 2010). The mechanism of GH's lactogenic effect is via rearranging energy partition routes in a whole-body scale including increased mammary nutrient uptake, decreased muscle glucose uptake, increased adipose mobilization, decreased insulin inhibition on lipolysis and gluconeogenesis (Bauman and Vernon, 1993). Decreased hepatic GH receptor abundance is observed in heat-stressed lactating cattle compared with the pair-fed group (Rhoads et al., 2010), indicating HS might affect nutrient partitioning via altering GH signaling effectiveness. On the other hand, lactate, an anaerobic glycolysis end product, is consistently elevated in various HS models from different species, and skeletal muscle is likely the main source of this rise because lactate originated from mesenteric organs would be filtered by the liver (Baumgard and Rhoads, 2013). In nonruminant model, only a small fraction of blood lactate is recycled back to glucose via the Cori cycle and the majority of lactate clearance is through oxidation (Brooks, 2007). And this should hold true for heat-stressed

animal with a negative energy balance because the Cori cycle requires net ATP input: the gluconeogenesis from lactate requires 6 moles ATP to generate 1 mole glucose whereas glycolysis releases 2 moles ATP from 1 mole glucose (Nelson and Cox, 2005). Accelerating the Cori cycle might exacerbate the energy shortfall in animals suffering from negative energy balance. As an oxidative energy source, on the other hand, lactate shuttles between glycolytic and oxidative tissues and this might serve as a glucose-sparing approach to reserve adequate glucose for cells in the central nervous system and the immune system, which have a strict reliance on glucose under the circumstance that NEFA-derived ketone is limited in HS (Baumgard and Rhoads, 2013). Therefore, the combined effect of metabolic inflexibility and increased maintenance energy requirement might be responsible for the drastic milk yield decrease occurring in heat-stressed cows. Current evidence supports the view that HS disturbs "normal" metabolic and hormonal adaptations enlisted by animals during lactation.

Metabolic adaptation of lactating cows under heat stress

Glucose

Ruminal carbohydrate metabolism is unique in that most dietary starches are fermented by microbes in reticulorumen and generate SCFAs. However, 20-25% of starch escapes microbial fermentation and enters into the small intestine, where digestion efficiency decreases when the amount of starch increases (Huntington et al., 2006). Intestinal starch digestion resembles the degrading process in nonruminants. Pancreas-secreted α-amylase cleaves starches into dextrins and linear oligosaccharides of 2 or 3 glucose units, and then glucose is generated by further hydrolysis catalyzed by maltase or isomaltase, but not sucrase which is not expressed in ruminants (Huntington, 1997). The major avenue of glucose entry into enterocytes is through the sodium-dependent glucose transporter (SGLT1) which is located at the brush border of intestine and

couples glucose transportation with inward sodium flow due to the sodium gradient maintained by Na^+-K^+-ATPase, while the predominant transporter for glucose exit is GLUT2 located at basolateral membrane (Harmon et al., 2004).

Gluconeogenesis is of primary importance for ruminants due to the limited availability of dietary glucose and moderate intestinal glucose digestion and absorption (Aschenbach et al., 2010). Carbon sources for gluconeogenesis are mainly from propionate, while lactate and glucogenic amino acids contributions are smaller in magnitude (Annison and Bryden, 1999). Propionate needs to be activated in the form of propionyl-CoA and this activation is catalyzed by hepatic propionyl-CoA synthetase (Kristensen and Harmon, 2006). Propionyl-CoA enters into the mitochondria, and is converted into succinyl-CoA via the reactions catalyzed by propionyl-CoA carboxylase (PCoAC) and methylmalonyl-CoA mutase (MCM) (Aschenbach et al., 2010). Succinyl-CoA, as an intermediate of the TCA cycle, is converted into oxaloacetate (OAA). The latter exits mitochondria via reduction-oxidation conversion, transforms into phosphoenolpyruvate (PEP), an irreversible reaction catalyzed by the rate-limiting enzyme, PEPCK in the cytosol (PEPCK-C), and further to glucose through a reverse glycolysis pathway (Aschenbach et al., 2010; Houtkooper et al., 2012). On the other hand, both lactate and amino acids can be converted into pyruvate to enter gluconeogenesis. In mitochondria, pyruvate can be converted into OAA by pyruvate carboxylase (PC), a rate-limiting enzyme in this conversion, and therefore, PC is proposed as a regulating enzyme that can fine-tune the entry of precursors into gluconeogenesis (Aschenbach et al., 2010). Additionally, hepatic mRNA abundance and enzyme activity of PC are elevated by feed restriction (Ballard et al., 1968; Velez and Donkin, 2005), while hepatic PEPCK mRNA is upregulated by GH but not feed withdrawal (Velez and Donkin, 2004), indicating different regulatory mechanisms relating to the contributions of carbon sources between different planes of nutrition.

Blood glucose is taken up by peripheral tissues, such as skeletal muscle and adipose, mainly through facilitated diffusion to contribute to cellular metabolism (Sasaki, 2002). The fates of glucose vary between different tissues and depend on animal energy status. Glycolysis is a common catabolic process in all mammalian cells (Pilkis and Granner, 1992). It generates 2 moles of pyruvate, 2 moles of ATP and 2 moles of NADH as end products from each mole of glucose. The detailed process has been elucidated (Nelson and Cox, 2005). Briefly, there are 9 consecutive reactions catalyzed by different enzymes and the pathway can be separated into 2 phases: 1) a chemical priming phase converts glucose into fructose 1,6-bisphosphate (F1,6BP) at the expense of 2 equivalents of ATP; and 2) an energy yielding phase converts F1,6BP to pyruvate with the production of 4 equivalents of ATP and 2 equivalents of NADH. Three of the reactions are accompanied by a large free energy decrease and therefore, they are non-equilibrium conversions catalyzed by hexokinase, phosphofructokinase-1 (PFK-1) and pyruvate kinase (PK), respectively. The major rate-controlling step is the reaction catalyzed by PFK-1, which undergoes allosteric regulation by its substrate and fructose 2,6-bisphosphate. Glucose transport in bovine skeletal muscle is speculated as a rate-limiting step since hexokinase, the enzyme catalyzing the initial reaction of glycolysis, has a higher rate than the glucose transporter(s) (Hocquette et al., 1996).

Due to the low activities of ATP:citrate lyase and NADP malate dehydrogenase in ruminant liver and adipose tissue (Hanson and Ballard, 1967) , glucose does not substantially contribute to cytosolic acetyl-CoA supply and subsequent lipogenesis. But this theory is challenged by the high rate of lipogenesis from lactate both in vivo and in vitro, suggesting that pyruvate kinase, which regulates glycolysis, is a pivotal control point for glucose contribution to lipogensis (Robertson et al., 1982). High intracellular level of acetyl-CoA is also proposed as a reason because acetyl-CoA can inhibit pyruvate dehydrogenase and block the incorporation of pyruvate or lactate into fatty

acid (Forsberg et al., 1985; Palmquist, 2006). Interestingly, the percent glucose contribution markedly increases in intramuscular adipocytes compared with that in subcutaneous adipocytes, although the absolute glucose incorporation rate is substantially lower in intramuscular adipose tissue than subcutaneous adipose tissue (Smith and Crouse, 1984). In lipogenic tissues, such as adipose and mammary, glucose is instead an important source of reducing equivalent production (Palmquist, 2006). After glucose is converted to glucose 6-phosphate (G6P) by hexokinase, this activated form generates NADPH via the pentose phosphate pathway (PPP). Two oxidation steps on G6P catalyzed by G6P dehydrogenase and 6-phospho-gluconate dehydrogenase generate 30%-50% NADPH for lipogensis in ruminants (Laliotis et al., 2010). The products of PPP can merge back into glycolysis or participate in the production of nucleotides and amino acids. Pyruvate generated from glycolysis lies at the intersection of numerous pathways, such as the TCA cycle, gluconeogenesis and lipogenesis. In ruminants, considerable amounts of pyruvate can be converted to and released as lactate by lactate dehydrogenase in skeletal muscle and adipose (Brockman, 2005), and hepatic lactate uptake is elevated during transition period, indicating an increase contribution of lactate as a precursor of gluconeogenesis (Reynolds et al., 2003).

Lactose production accounts for 50-60% glucose uptake in bovine mammary gland (Brockman, 2005). The synthesis of lactose is completely dependent on glucose uptake in the mammary gland because mammary tissue cannot produce glucose from glucogenic precursors (Scott et al., 1976). The galactose unit in lactose derives from glucose via the conversion into UDP glucose in the cytosol by a series of enzymes (Jones, 1978). When UDP glucose is transported into golgi lumen, it combines with another unit of glucose via lactose synthetase which is comprised of galactosyltransferase and α-lacalbumin (Jones, 1978). As the golgi membrane is impermeable

to lactose, the latter becomes the osmotic driving force to water influx and the determinant of milk production volume (Peaker, 1977).

Evidence from HS studies indicates that glucose metabolism is influenced by thermal factors. Hepatic glucose output and carbohydrate oxidation are enhanced in human athletes exercising at high temperature (Febbraio, 2001) and this augmented output is not inhibited by exogenous glucose infusion (Angus et al., 2001). Previous data showed that both HS and restricted feeding increased the abundance of PC mRNA in liver while only restricted feeding increased that of cytosolic PEPCK (Rhoads et al., 2011). An *in vitro* study also showed that upregulated PC expression and unchanged cytosolic PEPCK mRNA abundance in bovine hepatocytes while PC and PEPCK were not responsive to HS in rat hepatoma cells (White et al., 2012). The entry of lactate into gluconeogenesis is regulated by PC and blood lactate is consistently increased in various heat stress models (Baumgard and Rhoads, 2013). The augmented PC expression might be associated with the rise of lactate in HS. The intestinal glucose transporter, SGLT-1, is upregulated in terms of activity and mRNA abundance in heat-stressed chicks but not pair-fed cohort (Garriga et al., 2006). Data from a recent pig study showed that HS increased intestinal glucose transport by increasing GLUT-2 protein expression and Na^+-K^+-ATPase (Pearce et al., 2013b). Insulin, the potent glucose homeostasis regulator, is increased in heat-stressed animals (Itoh et al., 1998; Wheelock et al., 2010; Pearce et al., 2013a), indicating HS might affect glucose balance via insulin signaling. Multiple lines of evidence from animal HS studies containing feed-restriction control groups indicates that HS alters glucose uptake and turnover independently of reduced feed intake.

Fatty acid

Fluctuations in food supply have led to evolutionary adaptations where animals employ strategies to store energy in cells when food sources are abundant and extracting stored energy from cells during scare food supplies. Triacylglycerol (TAG) is the major storing form of cellular energy and most are compartmentalized in the lipid droplets which can take up a large portion of volume in white adipose tissue (WAT), a specialized cell type for this energy banking process (Farese and Walther, 2009).

The sources of fatty acid (FA) in animals are mainly from dietary lipid and *de novo* biosynthesis. In nonruminants, lipid digestion predominantly occurs after digesta enters the small intestine, while in adult ruminants, digestion starts in the reticulorumen (Bauchart, 1993). Rumen microbes actively participate in the digestion process of lipid, including the hydrolysis, biohydrogenation and microbial FA synthesis (Jenkins, 1993). Ester links on TAGs, phospholipids and glycolipids are hydrolyzed by rumen bacteria, releasing glycerol, sugar, and unsaturated FA (Bauman et al., 2003). Glycerol and sugar are fermented immediately to volatile FAs (Bauman et al., 2003), while unsaturated free FAs enter the process of biohydrogenation and turn into saturated FA after isomerization and reduction carried out by ruminal microbes (Jenkins, 1993). A small portion (10-15%) of FAs in digesta originates from microbial *de novo* synthesis, consisting mainly of $C_{18:0}$ and $C_{16:0}$ (Jenkins, 1993).

Highly saturated FFAs, mainly palmitic and stearic acids, pass through the omasum and abomasum without significant absorption or change and enter the small intestine where substantial absorption occurs (Bauman et al., 2003). Due to the complete hydrolysis of TAG, monoacylglycerol, a key factor for micelle formation and intestinal FA absorption (Doreau and Chilliard, 1997), is missing in ruminant digesta. Bile secretion and pancreatic juice compensate in

this part. Phospholipid from bile juice is hydrolyzed by pancreatic phospholipase and converted into lysophospholipids, which forms micelles with bile salt and FA. Micelles are absorbed into intestinal epithelial cells where free FA is re-esterified to TAG and packaged into chylomicrons (Demeyer and Doreau, 1999). Chylomicrons are transported in plasma and lymph and delivered to peripheral tissues (Bauchart, 1993). Lipoprotein lipase (LPL) located at the endothelium of lipid metabolic active tissues (lipid, muscle, liver, mammary) hydrolyze TAG and release FFA which is taken up by cells via passive diffusion and protein-mediated transport (Fielding and Frayn, 1998; Bionaz and Loor, 2008).

Due to the limited percentage (< 5% of dry matter weight) of lipid content in ruminant feed (Bergen and Mersmann, 2005), *de novo* biosynthesis of FA constitutes a major portion of lipid of milk fat (Lock and Bauman, 2004). For humans, the liver is the dominant site of *de novo* FA synthesis using glucose as a major precursor, whereas for ruminants, lipogenesis mainly occurs in adipose tissue with acetate as a major substrate (Bergen and Mersmann, 2005). During lactation, the mammary gland also possesses the ability of producing endogenous FA and 50% of milk fat originates from *de novo* synthesis (Lock and Bauman, 2004). Lipogenesis is a process of converting acetyl-coA into FA by the addition of two carbon units step by step in cytosol. As mentioned in the glucose section, glucose serves as a poor carbon source for lipogenesis and instead provides reducing equivalents. Acetate is converted into acetyl-CoA by acetyl-CoA synthetase and is a subsequent substrate for malonyl-CoA generation by acetyl-CoA carboxylase (ACC). Malonyl-CoA, besides being a building block of FA, acts as an allosteric inhibitor of carnitine palmityltransferase (CPT), leading to a blunted flux of long-chain FA into mitochondria and therefore, is an inhibitor of FA oxidation (Tong, 2005). The addition of malonyl-CoA onto acetyl-CoA is catalyzed by a multifunctional enzyme complex, fatty acid synthase (FAS), and the

overall reaction can be described as the following, using palmitate, a common fatty acid in animals, as an example: CH_3COS-CoA (Acetyl-CoA) + $7HOOCCH_2COS$-CoA (Malonyl-CoA) + $14NADPH + 14H^+ \rightarrow CH_3CH_2(CH_2CH_2)_6CH_2COOH$ (Palmitate) + $14NADP^+$ + 8CoA-SH + $7CO_2 + 6H_2O$ (Wakil, 1989). Another essential component allowing this reaction to proceed is a large amount of reducing power provided by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Three major pathways contribute to the production of NADPH: pentose phosphate, malate dehydrogenase, and isocitrate dehydrogenase pathways (Nafikov and Beitz, 2007). Pentose phosphate and isocitrate dehydrogenase pathways are the major constituents for NADPH consumed in FA synthesis (Vernon, 1980). Glycerol used for esterification of FA in ruminants is proposed to be supplied by the glyceroneogenesis pathway because dietary glucose is metabolized by gut microbes (Reshef et al., 2003). Glycerol 3-phosphate is generated from substrates other than glucose under the regulation of pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK), two enzymes thought to be only involved in gluconeogenesis (Ballard et al., 1967; Reshef et al., 1970). The stepwise additions of acyl unit onto glycerol 3-phosphate are catalyzed by glycerophosphate acyltransferase (GPAT), acylglycerophosphate acyltransferase (AGPAT), and diacylglycerol acyltransferase (DGAT) consecutively (Coleman and Lee, 2004). Synthesized TAG is stored in an organelle called the lipid droplet, which is surrounded by a monolayer of surface phospholipids (Walther and Farese, 2012). However, how this organelle is formed and how TAG is incorporated into preformed lipid droplet are not fully understood. Evidence from electron microscopy shows that lipid droplets are tightly associated with the endoplasmic reticulum (ER), where neutral lipid synthesizing enzymes resides, indicating that lipid droplets may be originated from the ER (Walther and Farese, 2012).

During food deprivation, or times when animals enter negative energy balance (e.g. early lactation), lipid reserves are mobilized by release of FA in a reverse manner of esterification carried out by a set of lipases. A monolayer membrane and membrane proteins protect the lipid droplet. Perilipin A is a predominant isoform of perilipin, which is located at the lipid droplet membrane and regulates basal and stimulated lipolysis (Duncan et al., 2007; Brasaemle et al., 2009). When catabolic signals are received by the adipocyte, perilipin A is phosphorylated and triggers the release of comparative gene identity 58 (CGI-58), a coactivator of adipose triglyceride lipase (ATGL) (Koltes and Spurlock, 2011). The three acyl groups are hydrolyzed successively by ATGL, hormone sensitive lipase (HSL), and monoacylglycerol lipase (Farese and Walther, 2009). One of the vital metabolic fate of FA is being oxidized as fuel. In oxidative tissues, such as skeletal muscle, liberated FA with 14 carbons or more has to be activated in the form of acyl-CoA by long chain acyl-CoA synthetase and transported into mitochondria through the carnitine shuttle with the aid of carnitine palmitoyltransferase (CPT) -I, carnitine:acylcarnitine translocase, and CPT-II (Kerner and Hoppel, 2000; Nguyen et al., 2008). Acyl-CoA translocated into mitochondria undergoes a process called β-oxidation catalyzed by enzymes following a 4-step cycle, releasing one acetyl-CoA each cycle (Wanders et al., 2010). β-oxidation also generates reducing equivalents: reduced form of flavin adenine dinucleotide (FADH) and nicotinamide adenine dinucleotide (NADH) as end products (Nguyen et al., 2008). Acetyl-CoA can enter the tricarboxylic (TCA) cycle to produce ATP. If a large amount of FA is released in the blood stream, as seen during fasting or under catecholaminergic stress, or after the consumption of high-fat, low-carbohydrate diet, FA taken up by liver can undergo a pathway called ketogenesis to generate ketone bodies, which can be utilized by the brain, kidney and muscle as fuel (Fukao et al., 2004). Acetyl-CoA produced from β-oxidation accumulates in mitochondria and becomes the substrate of generating ketone bodies,

which are transported to extrahepatic tissues to go through ketolysis, a reverse set of reaction of ketogenesis, and ketone bodies are converted back to acetyl-CoA in those tissues to enter TCA cycle (Fukao et al., 2004).

The effects of HS on lipid metabolism have not been fully elucidated. Studies from different species suggest that HS may alter lipid turnover distinctly from expected responses, such as increased NEFA and insulin resistance, based upon calculated energy balance. Carcass data from chickens (Geraert et al., 1996) and pigs (Collin et al., 2001) showed that heat-stressed animals had a higher fat retention compared to pair-fed controls. Previous studies demonstrated that heat-stressed cows did not appear to mobilize lipid reserves as their plasma non-esterified fatty acid (NEFA) remain constant before and after entering HS (Sano et al., 1983; Itoh et al., 1998), while pair-fed group progressively increased plasma NEFA concentration as nutrient restriction continued (Rhoads et al., 2009; Wheelock et al., 2010). Studies targeting adipose tissue and critical enzymes that are responsible for lipolysis and lipogenesis are needed to fully understand the impact of HS on lipid metabolism and its role with whole-body nutrient partitioning. Genes and enzymes involved in lipolysis, lipogenesis, glyceroneogenesis, etc. may serve as important research targets in order to further understand the role of lipid metabolism in heat-stressed animals.

Amino acids

Amino acids are the building blocks of protein, which is required for most biological activities. For dairy cattle, this requirement can be more important because increased milk production obliges higher input of amino acid for milk protein synthesis. Microbial protein synthesized from crude protein in the rumen, dietary protein that escapes ruminal fermentation and endogenous protein are the primary sources of amino acids absorbed at the small intestine (Clark et al., 1992). Although the efficiency of amino acid anabolism can vary substantially between

different energy statuses, the conversion from ingested fed protein (N) to body mass is generally low (0-35%) in ruminants (Lobley, 1992). After entering the rumen, amino acids are either integrated into microbial protein or metabolized into SCFA, carbon dioxide, methane and ammonia (Tamminga, 1979). Ammonia is either taken up by microbes to support growth or enter into host blood stream (Kennedy and Milligan, 1980). Circulating ammonia is readily converted into urea by the liver, and later will either be excreted or recycled to digestive tract (rumen mainly) by salivary transport and transfer through the rumen epithelium (Kennedy and Milligan, 1980). Urea directed to the digestive tract can be converted to ammonia by microbial urease and reabsorbed by enterocytes, or contribute to microbial protein synthesis (Reynolds, 1992). Microbial, dietary and endogenous proteins that evade ruminal degradation flow into small intestine and are hydrolyzed by abomasal pepsin and later by pancreatic proteolytic enzymes to facilitate absorption (Kay, 1969).

Amino acids can enter enterocytes as free amino acids and peptide amino acids (Remond et al., 2000). The transport of amino acids into and out of epithelial cells rely on sodium-dependent symporters, proton-motive force, the gradient of other amino acids, and antiporters (Broer, 2008). The metabolic fate of absorbed amino acids largely depends on nutrient availability: amino acid channeled through catabolic pathways and later serves as a precursor of gluconeogenesis contributes 40% of total amino acid loss in the fasted animal (Lobley, 1992). As mentioned previously in the glucose section, the large deficit of glucose precursor observed during transition period in cows is believed to be compensated by amino acids mobilized from skeletal muscles (Bell, 1995). This carcass protein reserve mobilization is also supporting milk protein synthesis (Bequette et al., 1998). For the lactating animal, circulating amino acids and small peptides are important sources for milk protein production in the mammary gland. Mammary epithelial cells

are responsible for the massive biosynthesis of milk protein which mainly consists of casein and whey protein, although small amounts of serum albumin and immunoglobulin can be transported into milk transcellularly (Bequette et al., 1998).

The effects of HS on protein turnover are controversial but may be related to the magnitude and duration of the heat load producing either a detrimental or therapeutic effect. An indicator of muscle catabolism, 3-methyl-histidine is reported to increase in heat-stressed poultry, and this increase is independent of DMI (Yunianto et al., 1997). Data from a rat study showed that both HS and pair feeding reduce muscle mass; however, pair-fed animals have a significantly higher magnitude of protein degradation leading to a more severe loss in skeletal muscle and the authors speculated that protein preservation is triggered in heat exposure (Samuels et al., 2000). In another study, rats that received a bout of HS before muscle overload had attenuated muscle hypertrophy and lower protein concentration compared to non-HS group and heat shock protein 72 (HSP72) was found to be significantly higher in HS group, indicating that HS and subsequent elevated HSP might inhibit muscle mass increase, although this study did not report changes in feed intake (Frier and Locke, 2007). Glutamine appears to have a protective effect on heat-shocked skeletal myotubes by inhibiting protein degradation (Zhou and Thompson, 1997) and this influence might be mediated by HSPs (primarily HSP70 and HSP25/27), independently of glutamine metabolism because a non-metabolizable glutamine analog is sufficient to mimic the HSP enhancing effect (Wischmeyer, 2002). In lactating cows, HS increases plasma urea nitrogen level compared with the pair-fed group (Shwartz et al., 2009; Wheelock et al., 2010) although whether this elevation stems from increased protein degradation, reduced plasma volume or other reasons remains unknown.

Hormone regulation of metabolism

Insulin signaling pathway

In mammals, insulin is one the most important anabolic hormone because of its potent and multifunctional actions on vital metabolic pathways. Under insulin's effects, the synthesis and accumulation of carbohydrates, lipid and protein are triggered, meanwhile, the degradation of these materials are inhibited. This versatile characteristic requires insulin to act on multiple targets and carry out different roles at the same time. In fact, insulin has been found to be able to change vesicle trafficking, activate protein kinases and phosphatases, promote cellular growth and development as well as gene expression (Saltiel and Pessin, 2002). In order to coordinate anabolic reactions with proper nutrient supply, there must be a mechanism enabling insulin-secreting cells to sense body energy status. This sensing system is located in β-cells within pancreas and the process of glucose-stimulated insulin secretion is well studied and reviewed (Henquin, 2000). After glucose enters β-cell, it is broken down to generate ATP. The rise in the ATP:ADP ratio causes the closure of ATP-sensitive K^+ channels and K^+ starts to accumulate within the cell resulting in cell membrane depolarization. Voltage-gated Ca^{2+} channels opens when depolarization reaches the threshold and Ca^{2+} flows into the cell. This influx activates the process of exocytosis of insulin-containing vesicles and insulin is released into blood stream. Long-chain fatty acids are also reported to trigger insulin secretion via binding to a G-protein coupled receptor, GPR40, or have a synergistic effect with glucose to activate β-cells (Dobbins et al., 1998; Itoh et al., 2003).

Following insulin release and transport as a hormone, it will bind insulin receptors (IR) located on insulin-sensitive tissues, such as muscle and adipose. The main effect of insulin on these tissues is to increase glucose uptake via GLUT4 translocation and therefore accelerate blood glucose clearance. Although the exact route from IR to GLUT4 is not fully mapped out, critical

events along this path are extensively studied and reviewed (Saltiel and Kahn, 2001; Muoio and Newgard, 2008). Binding of insulin to IR induces IR autophosphorylation and subsequently leads to the recruitment of insulin receptor substrate (IRS) proteins. Multiple tyrosine residues on IRS are then phosphorylated by IR and phosphorylated IRS start to interact with the regulatory subunit of phosphatidylinositol 3-kinase (PI3K). This interaction activates the catalytic subunit of PI3K which converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane. The release of PIP3 and subsequent binding to the phosphoinositide-dependent protein kinase (PDK) result in the colocalization of PDK and protein kinase B (PKB, also known as AKT) and the activation of AKT. Subsequent phosphorylation is carried out by activated AKT on the AS160 Rab GTPase-activating protein and the latter in turn interacts with the small GTPase RAB10. This interaction activates RAB10 and enables it to mobilize the intracellular GLUT4 pool, stimulate the exocytosis of GLUT4-containing vesicles, and eventually leads to increased glucose uptake. In addition, insulin promotes glycogen synthesis, a process depositing glucose in the form of glycogen, by phosphorylating glycogen synthase kinase-3 (GSK3). Glycogen synthase (GS) is inhibited by GSK3 when the signal from insulin is not present (Patel et al., 2008). Activation of the IR-AKT axis phosphorylates and inactivates GSK3 and thus liberates GS from suppression, leading to increased glycogen synthesis (Patel et al., 2008). Bouskila et al. (2008) suggested a different theory with the use of mice carrying mutated GSK3 which is nonresponsive to insulin and constitutively suppresses GS. They found insulin was able to stimulate skeletal muscle glycogen synthesis in mutant mice to a level comparable to those of wild type and postulated that increased glucose 6-phosphate, as a result of elevated glucose uptake, allosterically activates GS and counteracts the inhibiting effect from constantly active GSK3. On the other hand, insulin suppresses gluconeogenesis and glycogenolysis in liver (Saltiel

and Kahn, 2001). Forkhead transcription factor FOXO1 can bind to the promoters of PEPCK and glucose-6-phosphatase genes, activate their expression, and promote gluconeogenesis (Hall et al., 2000; Schmoll et al., 2000). Activated AKT phosphorylates FOXO1 and prevents the latter from entering the nucleus (Puigserver et al., 2003). However, Edgerton et al. (2009) argue that decreased PEPCK expression has a moderate effect on gluconeogenic flux and insulin is limiting hepatic glucose production by restraining the uptake of glucose precursors such as lactate and glycerol (Edgerton et al., 2009). The main inhibiting effect of insulin on lipolysis is via phosphorylation of phosphodiesterase type 3B (PDE3B), which decreases cellular cAMP level, attenuates catecholamine signaling and consequently reduces the amounts of active HSL and perilipin (Holm et al., 2000).

Binding of insulin to IR can initiate its own signaling termination. Self-phosphorylated IR phosphorylates and activates protein tyrosine phosphatase (PTP), which in turn dephosphorylates IR and IRS (Dadke et al., 2001; Saltiel and Pessin, 2002). Dephosphorylation can also occur via PIP3 catalyzed by lipid phosphatases, such as SH2-containing 5'-inositol phosphatase and phosphatase homologous to tensin (Saltiel and Pessin, 2002). A feedback attenuation executed by GSK3, which is a downstream target of AKT, can terminate insulin signaling by means of phosphorylating IR and IRS at serine or threonine residues and thus inactivating these proteins (Saltiel and Pessin, 2002). Insulin signaling is also regulated by IR internalization, either positively or negatively (Fagerholm et al., 2009). Phosphorylated IR can be rapidly internalized via caveolae-mediated endocytosis in the adipocyte (Fagerholm et al., 2009). Internalization of ligand-bound IR results in clearance of insulin and attenuation of insulin signaling (Di Guglielmo et al., 1998). Alternatively, internalized IR is believed to remain activated and be able to phosphorylated downstream protein (Di Guglielmo et al., 1998).

Insulin has a similar important role in ruminants as a metabolic regulator. The signaling pathway employed by ruminants for regulating glucose uptake in insulin-sensitive tissues is the same as those in non-ruminants (Sasaki, 2002). However, hyperinsulinemic-euglycemic clamp experiments demonstrate that the glucose utilization rate is lower in ruminant peripheral tissues than those in rat and human (Hocquette et al., 1996). This discrepancy is believed to be due to a lower availability of GLUT4 and lower capacity of insulin signal transduction at several critical phosphorylation steps (Sasaki, 2002). Again, owing to the availability of postprandial glucose from feed in ruminants, a unique adaptation exists where insulin is unable to inhibit hepatic gluconeogenesis from propionate (Aschenbach et al., 2010). The mechanism of this ruminant-specific insulin resistance is not fully understood.

Evidence from various species demonstrate that HS can increase insulin secretion or insulin sensitivity [rodents (Morera et al., 2012), growing pigs (Pearce et al., 2013a), growing steers (O'Brien et al., 2010), and lactating cows (Itoh et al., 1998; Wheelock et al., 2010)], however the underlying mechanisms are unclear. Restoration of heat shock protein 70 (HSP70) expression improved glucose tolerance in spontaneous diabetic monkeys (Kavanagh et al., 2011). Artificially induced HSP72 significantly improved insulin sensitivity, glucose homeostasis and attenuated apoptotic signals and cellular stress markers in pancreatic β-cells of db/db mice (Kondo et al., 2012). Similarly, thermal therapies are reported to be able to boost insulin sensitivity to an extent comparable to those seen with exercise training in humans (McCarty et al., 2009). Increased expression of HSPs triggered by HS might have a role related to increased insulin signaling observed in heat-stressed animals. In turn, rescuing insulin action increases the expressions of HSPs probably via the inhibition on GSK3 (Hooper and Hooper, 2009). The interconnected relationship between insulin and HSPs suggests a critical role for insulin signaling in response to

stress factors. The increased insulin secretion and insulin sensitivity observed in heat-stressed lactating cattle might serve as a survival adaptation to ensure enough nutrient supply to vital organs, such as the central nervous system, at the expense of the mammary gland. However, insulin responsiveness in terms of glucose disposal is inconsistent in lactating cow trials (Baumgard et al., 2011; Cole et al., 2011). These discrepancies may be due to the effective insulin signaling (Baumgard and Rhoads, 2013) and the underlying mechanism(s) in specific tissue types, such as the liver, skeletal muscle and adipose, remains unknown and warrants further study.

β-Adrenergic signaling pathway

Catecholamines are hormones secreted in response to stressors, such as physical exercise, hypoglycaemia, hypoxia, acidaemia, glucagon, caffeine, as well as heat (Beede and Collier, 1986; Zouhal et al., 2008). Sources of circulating catecholamines arise from the sympathetic nervous system, chromaffin cells of the adrenal medulla, and spill-over from vascular neuro-muscular junctions (Cole and Sood, 2012). Epinephrine (adrenaline) and norepinephrine (noradrenaline) are the two main types of catecholamines. Norepinephrine acts primarily on α-adrenoceptors while epinephrine can act on both α- and β-adrenoceptors, but primarily on β type receptors (Zouhal et al., 2008). A marked increase in catecholamine secretion induces various physiological adaptations and prepares animals to react to environmental stressors.

In vertebrates, the β-adrenoceptor (BAR) belongs to the largest transmembrane protein family, G protein-coupled receptor (GPCR) (Ritter and Hall, 2009). There are three subtypes of BAR: BAR1, BAR2 and BAR3, and they are the best-known mechanisms of activating lipolysis in adipose tissue (Carmen and Victor, 2006). Members of GPCR have a conserved 7-transmembrane domain and the binding of ligand induces conformational changes in the transmembrane and intracellular domains (Ritter and Hall, 2009). Ligand-bound BAR recruits and

activates heterotrimeric G protein with guanine nucleotide exchange function, replacing GDP with GTP on the α subunit (Gα) of G protein (Ritter and Hall, 2009). The activated Gα disassociates from the other two G protein subunits (Gβγ) and GPCR, and binds to downstream effectors, in the case of BAR: adenylyl cyclase (Ritter and Hall, 2009). Interaction with Gα activates adenylyl cyclase (AC) and leads to production of intracellular cyclic AMP (cAMP) from ATP (Altarejos and Montminy, 2011). Increased cAMP binds to regulatory subunits of protein kinase A (PKA) and promotes the separation of regulatory and catalytic subunits of PKA, subsequently activating PKA (Altarejos and Montminy, 2011). Stimulated PKA phosphorylates HSL and perilipin A, leading to increased accessibility of lipid droplet to HSL and ultimately, an elevated release of NEFA (Collins et al., 2004). Another well-studied effect of β-adrenergic stimulation is to regulate energy expenditure through thermogenesis in brown adipose tissue (BAT). p38 mitogen-activated protein kinase (MAPK) is activated by PKA and subsequently phosphorylates activating transcription factor 2 (ATF2), which in turn promotes the transcription of PPARγ coactivator-1 (PGC-1α) (Collins et al., 2004). Newly transcribed PGC-1α participates in the next round of transcriptional activation with the PPAR family and triggers the expression of uncoupling protein 1 (UCP1) (Collins et al., 2004), which acts as a proton leak and releases energy in the form of heat (Ricquier and Bouillaud, 2000). β-agonist is used in the livestock industry for its muscle-hypertrophic and concomitant fat-reducing effects (Lynch and Ryall, 2008). In skeletal muscle, direct phosphorylation of cAMP response element (CRE) binding protein (CREB) catalyzed by PKA activates CREB and increases CREB's affinity to CREB-binding protein (CBP) and p300, both are transcriptional adaptors associated with RNA polymerase II, consequently leading to upregulated transcription of genes that carries CRE (Lynch and Ryall, 2008). A number of myogenic basic helix-loop-helix proteins and myocytes enhancer factor 2, another group of

transcriptional activator critical for myogenesis, can be coactivated by CBP and p300. (Sartorelli et al., 1997; Lynch and Ryall, 2008). In addition, the promoting effect of epinephrine on glycogenolysis is believed to be through the upregulation of glycogen phosphorylase activity in muscle and, to a less extent, in the liver (Berg et al., 2002). Upon the binding of epinephrine to BAR, PKA phosphorylates glycogen phosphorylase kinase, which in turn phosphorylates glycogen phosphorylase and render it a more active form to carry out glycogen breakdown (Berg et al., 2002). However, evidence indicates net muscle breakdown is not in concert with elevated glycogen phosphorylase in exercising muscle and one possible explanation is that epinephrine triggers more robust glycogenolysis in other tissues, such as non-contracting muscle, than in contracting muscle (Kjaer et al., 2000).

Termination of β-adrenergic signaling occurs at different nodes along its pathway. There is an inherent GTPase activity on G protein converting GTP to GDP causing signal transduction cessation (Berg et al., 2002). Termination at the receptor level is realized by phosphorylation catalyzed by GPCR kinase (GRK) and internalization mediated by β-arrestsins (Ritter and Hall, 2009). Both GRK and β-arrestin are proteins that can directly bind to GPCR and mediate signaling of GPCR other than the well-known desensitization (Reiter and Lefkowitz, 2006). Phosphorylation catalyzed by GRK can occur at serine and threonine residues on carboxy-terminal or the third cytoplasmic loop of GPCR (Reiter and Lefkowitz, 2006). β-arrestin can only bind to ligand-bound, GRK-phosphorylated GPCR (Shenoy and Lefkowitz, 2003). Binding of β-arrestin to GPCR uncouples the receptor from heterotrimeric G protein and guides receptor to clathrin-coated pits via the aid of adaptor protein 2 (AP2) and clathrin, eventually resulting in receptor internalization (Moore et al., 2007). Internalized receptors are then sorted to two fates: recycling and degradation. Receptors sorted to recycling endosome are transported back to the cytoplasm and become

resensitized, while those targeted to multivesicular late endosomes are passed to lysosomes for degradation (Moore et al., 2007). Another fast feedback inhibition on PKA is mediated by PDE4 family with A-kinase anchoring proteins (AKAP) playing an critical role in this process (Vandamme et al., 2012). Imaging studies showed cAMP change happens in a restricted cell compartment and does not diffuse to other areas, probably because AKAPs couples PKA to its upstream membrane receptor and AC, as well as its downstream feedback components, such as PDE and protein phosphatase (PP) (Perino et al., 2012). This spatial colocalization ensures the promptness and specificity of PKA signaling. Phosphorylation and activation of PDE4 by PKA degrades cAMP and terminates further signal transduction, while phosphorylation of PP2A catalyzed also by PKA dephosphorylates PDE4, serving as a second layer of feedback loop (Vandamme et al., 2012).

Thermal stress has long been known to be able to trigger catecholamine release in animals (Axelrod and Reisine, 1984). Prolonged heat exposure induces heat acclimation which can partly reflected by decreased levels of growth hormone, catecholamines and glucocorticoid (Bernabucci et al., 2010). However, physical fitness seems to have little effect on plasma catecholamine levels during uncompensable heat stress in humans (Wright et al., 2010). Heat shock proteins (HSP), consisting of several families of highly conserved proteins, was originally found to be induced by heat shock and later found to be triggered by various environmental stressors to protect cellular proteins from aggregation, misfolding and degradation (Gabai and Sherman, 2002; Kregel, 2002; Johnson and Fleshner, 2006). *In situ* hybridization study on heat-stressed rats showed that HSP70 is highly expressed in hippocampus, paraventricular nucleus, dorsomedial nucleus of the hypothalamus, and median eminence, regions that are involved in the neuroendocrine stress response in the brain (Blake et al., 1990). Isoproterenol, a BAR agonist, cannot induce HSP70

synthesis alone, but potentiates the expression of HSP70 after moderate exercise in rodent cardiac and skeletal muscles (Paroo and Noble, 1999). The exact relationship between HSP and β-adrenergic signaling is still unclear. It has been demonstrated that HS cows exhibit impaired epinephrine-induced lipolytic response compared to a pair-fed group (Baumgard et al., 2011). Increased carcass adipose retention compared with the pair-fed cohorts was observed in heat-stressed pigs (Collin et al., 2001) and chickens (Geraert et al., 1996). Whether this is due to an insulin-mediated anti-catabolic effect or the direct effect of HS is unknown. Therefore, the impact of HS on β-adrenergic signaling, especially in terms of its lipid mobilizing effect, warrants additional study.

Other hormones

Growth hormone (GH) exerts diverse anabolic and catabolic functions in multiple target tissues coordinating fuel partitioning to nutrient status including favoring nitrogen retention and protein synthesis (Moller and Jorgensen, 2009; Vijayakumar et al., 2010). To accomplish these roles, GH binds to GH receptor (GHR), a member of cytokine receptor superfamily, the latter dimerizes and activates adjacent Janus kinase 2 (JAK2), which is a tyrosine kinase (Vijayakumar et al., 2010). Following the activation, JAK2 phosphorylates GHR, which in turn recruits the members of signal transducer and activator of transcription (STAT) family (Vijayakumar et al., 2010). Upon phosphorylation by JAK2, STATs dissociate from GHR, dimerize and translocate into nucleus to regulate target gene expression (Vijayakumar et al., 2010). In transition cattle, GH has a profound homeorhetic effect to support metabolic adaptations in mammary gland during early lactation, such as promoting hepatic gluconeogenesis and suppressing glucose uptake in adipose and skeletal muscle (Bell and Bauman, 1997). Heat stress decreases the concentration of circulating GH, an outcome that appears to be mediated by concomitant feed intake reduction

(Rhoads et al., 2009). Despite the similarity of GH secretion, HS cattle decrease abundance of hepatic GHR and downstream STAT5 phosphorylation compared with pair-fed cohorts, suggesting a downregulation of GH signaling in the liver, which is responsible for the major production of insulin-like growth factor 1 (IGF1) under the control of GH (Rhoads et al., 2010). The negative effect of HS on GH signaling might be related to the metabolic alterations occurring in heat-stressed animals.

Thyroid hormone (TH) is well-known as a key regulator of energy storage and expenditure primarily through actions in important metabolic pathways, such as lipolysis, lipogenesis, gluconeogenesis, etc. (Mullur et al., 2014). The majority of TH in the circulation is in the form of a prohormone, thyroxine (T4), synthesized in the thyroid gland, while the more metabolically active form of TH, triiodothyronine (T3), is produced in several extrathyroidal tissues (Kahl et al., 2015). The signaling of TH initiates with the uptake of T3 or T4 into the cytoplasm via TH transporters (Sinha et al., 2014). The activation of TH, from T4 to T3, is catalyzed by type 1 or type 2 iodothyronine deiodinases (D1 or D2) in the cytoplasm (Sinha et al., 2014). After entering into the nucleus, T3 binds to TH receptors (TRs), leading to the recruitment of co-activator complexes which acetylates histone, generates a permissive chromatin environment, and consequently promotes gene expression (Sinha et al., 2014). The critical roles of THs in maintaining basal metabolic rate, promoting thermogenesis and regulating appetite and food intake (Mullur et al., 2014) render them an important consideration in HS research (Kahl et al., 2015). Reductions of THs are consistently recorded in multiple HS research [decreased T3 and T4 (Magdub et al., 1982); decreased T3 (Nardone et al., 1997); decreased thyrotropin, T3, and T4 (Kahl et al., 2015)]. A comparative study of cattle breeds showed that blood T3 reduction is related to the capacity of heat tolerance during heat stress: breeds with higher T3 reduction are better in

maintaining rectal temperature and feed intake (Pereira et al., 2008). Therefore, reducing THs might reflect an adaptation during HS in order to reduce endogenous heat production (Bernabucci et al., 2010).

Glucocorticoids (GCs) are stress hormones closely related to homeostasis maintenance during period of no stress and critical for emergency survival during acute stress (Alarifi et al., 2001). The actions of GC are mediated by GC receptor (GR), a nuclear receptor. Upon binding to GC, activated GR translocates into the nucleus and promotes or suppresses the expression of target genes by binding to glucocorticoid-response elements (GREs) with the aid of other transcription factors (Kadmiel and Cidlowski, 2013). Cortisol is the major active form of GC and 90% of cortisol in plasma appears in the form bound to corticosteroid-binding globulin (CGB) while only free GC is an active signal mediator (Kadmiel and Cidlowski, 2013). Therefore, CGB is an important regulator of GC's bioavailability and delivery to target tissues (Kadmiel and Cidlowski, 2013). Metabolic function of cortisol includes inducing hepatic gluconeogenesis, promoting lipolysis in adipose, suppressing glucose uptake in skeletal muscle and adipose, etc. (Kadmiel and Cidlowski, 2013). Discordant GC responses during HS occur in acute and chronic phases: elevation in the former while reduction in the latter, possibly due to acclimation (Collier et al., 1982). Despite the critical role of GC in stress adaptation and homeostasis, the mechanism(s) underlying the alteration of GC induced by HS is unknown and warrants further study.

Summary

Previous studies demonstrate that HS has a profound impact on the lactating cow's metabolic profile, but with regards to the molecular signaling pathways and cellular energetics in metabolically-active tissues, few studies have been performed and detailed mechanisms remain

ambiguous. For example, insulin signal transduction alterations in the liver, skeletal muscle and adipose might have roles in hyperthermic adaptations. As stated in this review, these tissues extensively participate in the whole-body metabolism and nutrient partitioning processes, corresponding to animal's body energy status and endocrine profile. They serve as good candidates to pinpoint the influence of HS in the intricate metabolic map. The importance of adipose tissue is recognized in the last several decades not only as a passive energy reservoir, but as an active regulator of nutrient homeostasis and a relay station of a wide range of homeostatic processes (Rosen and Spiegelman, 2006). However, our understanding of the role that adipose has in HS metabolic adaptation is scarce. The counterintuitive observations obtained from the previous HS studies indicate that adipose might perform surprisingly different functions in response to various environmental conditions and body nutrient statuses. In order to unravel these mysteries and obtain a potential means to counter the detrimental impacts of HS, the present sequence of studies were performed, focusing on metabolic and endocrine adaptations in lactating cows and with emphasis on adipose tissue.

Chapter 2. Effects of heat stress on insulin responsiveness in lactating Holstein cows

Abstract

Multiparous cows (n = 12; parity = 2; 136 ± 8 DIM, 560 ± 32 kg BW) housed in climate chambers were fed a TMR consisting primarily of alfalfa hay and steam-flaked corn. Cows were subjected to 2 experimental periods (P): 1) thermoneutral conditions (18 °C, 20% humidity) with *ad libitum* intake (TN for group 1, WF = well-fed for group 2) for 9d and 2) either heat-stress (HS) conditions (cyclical temperature 31.1 - 38.9°C, 20% humidity: min THI = 73, max THI = 80.5) fed for *ad libitum* intake (group 1, n = 6), or TN conditions, pair-fed (PF) with a HS animal (group 2, n = 6) for 9 d. Rectal temperature (Tre) and respiration rate (RR) were measured thrice daily at 0430, 1200 and 1630h. To evaluate muscle and liver insulin responsiveness, biopsies were obtained immediately before and after an insulin tolerance test (ITT) on the last day of each period. Insulin receptor β (IRβ), insulin receptor substrate 1 (IRS-1), AKT/protein kinase B (AKT) and phosphorylated AKT (p-AKT) were measured by Western blot analyses for both tissues. During P2, HS increased Tre and RR by 1.48°C and 2.4-fold, respectively ($P < 0.01$). HS reduced ($P < 0.01$) DMI by 8 kg/d and by design PF cows had similar intake reductions. Milk yield was decreased similarly (30%) in HS and PF cows and both groups entered into a similar (−4.5 Mcal/d) calculated negative energy balance during P2. Compared with P1 ($P < 0.05$), basal glucose levels increased (5%) in PF cows, but decreased (5%) in HS cows during P2. The ITT caused a more rapid glucose disposal in P1 compared with P2 ($P < 0.05$), but glucose clearance did not differ between environments in P2. In liver, insulin increased P-AKT protein content in each period ($P < 0.05$) and decreased AKT abundance in WF ($P < 0.05$). Phosphorylation ratio of AKT increased 120% in each period ($P < 0.05$) after insulin infusion. IR protein remained constant during each

period. The protein level of IRS was lowered ($P < 0.05$) by insulin in WF. In skeletal muscle, protein abundance of the IR, IRS, and AKT remained stable between periods and environment. Insulin increased p-AKT in each period ($P < 0.05$), but this response tended to decline in P2 for PF animals ($P = 0.094$), but not during HS. These results indicate that mild systemic insulin resistance during HS may be related to reduced nutrient intake but liver insulin responsiveness remained unchanged.

(**Keywords**: heat stress, insulin, hyperthermia, dairy cow)

Introduction

Although advances in environmental cooling systems ameliorate production losses during summer months, heat stress continues to cost the American dairy industry approximately $1 billion annually (St-Pierre et al., 2003a). Dairy cattle acclimation to elevated environmental temperature involves numerous mechanisms intended to reduce heat load. Physiological adaptations increase heat dissipation (i.e., enhanced respiration and sweating rates) and decrease heat production (i.e., reduced feed intake) at the expense of animal performance. Reduced feed intake during heat stress has traditionally been assumed to be primarily responsible for the decrease in milk yield (Collier et al., 1982; West, 2003). However, we have recently demonstrated that lactating heat-stressed dairy cows exhibit greater milk yield reduction per unit of feed intake than pair-fed thermal neutral counterparts (Rhoads et al., 2009; Wheelock et al., 2010). This result suggests that heat stress-induced changes in feed intake accounts for a minor portion of the overall reduction in milk synthesis. Although both pair-fed and heat-stressed cows appear to enter similar levels of negative energy balances, they experience different bioenergetic adaptations. Contrary to PF cows, HS animals have decreased plasma NEFA, increased plasma insulin and increased plasma urea nitrogen (Rhoads et al., 2009; Wheelock et al., 2010). These adaptations seem to demonstrate a shift in whole-body glucose metabolism in heat-stressed cows, independent of reductions in feed intake.

Metabolic adaptations are coordinated, in part, by the endocrine system to fulfill the ever-changing energy demands of mammals (Coll et al., 2007). It is well accepted that insulin plays a crucial role in fasting- to fed-state transition. Insulin is the most potent anabolic hormone known and involved in regulating the turnover of carbohydrates, lipids and proteins (Saltiel and Kahn, 2001). In relation to carbohydrate metabolism, a unique feature in ruminants is that their glucose

source(s) depend almost exclusively on gluconeogenesis in liver since dietary carbohydrates are subjected to microbial fermentation before they become available for absorption in the small intestine (Bell and Bauman, 1997). Insulin suppresses gluconeogenesis by down-regulating genes encoding gluconeogenic enzymes (Michael et al., 2000). Hepatic glycogen is another source for plasma glucose supply and is used as a glucose reservoir by animal. Glycogen accumulation can be stimulated by insulin via increasing glucose transport and glycogen synthesis (Saltiel and Kahn, 2001). Basal and stimulated plasma insulin concentrations are typically increased during periods of heat stress but how such changes may be sensed by the liver, e.g. insulin responsiveness, remain unclear (Rhoads et al., 2013). This is important, since the liver clearly exhibits alterations in gluconeogenic gene expression and growth hormone (GH)-dependent insulin-like growth factor I (IGF-I) gene expression, possibly related to insulin action (Rhoads et al., 2010; Rhoads et al., 2011).

Skeletal muscle can serve as a major site of bodily energy consumption given the sheer mass and has the capability of using different energy substrates selectively based upon nutrient availability. Insulin can play a crucial role in mediating this selectivity shift based on skeletal muscle responsiveness (Long et al., 2011).

Within intracellular insulin signaling pathways, there are several important proteins, including insulin receptor (IR), insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt), which function as important junctions of regulation, signal divergence and crosstalk nodes with other signaling cascades (Taniguchi et al., 2006). Insulin receptor is localized in insulin-responsive tissue at the surface of cell membrane to initiate downstream signaling (Karlsson and Zierath, 2007). Activated IR recruits and phosphorylates IRS, which is involved in maintaining glucose homeostasis between fed and fasting statuses (Guo et al.,

2009). Via PI3K and a series of second messengers, insulin signaling involves Akt, a kinase that carries out physiological functions by up- or down-regulating genes related to glycogen synthesis, gluconeogenesis and lipogenesis (Karlsson and Zierath, 2007).

Here we hypothesized that heat-stressed cattle fail to enlist glucose sparing mechanisms to preserve glucose for milk synthesis. To investigate the role of HS on insulin-dependent glucose metabolism, we established a model to examine insulin responsiveness of skeletal muscle and liver during a period of HS or a similar plane of reduced nutrition. An insulin tolerance test (ITT) was employed in conjunction with tissue biopsies to measure critical proteins in the insulin signaling pathway and estimate skeletal muscle and hepatic insulin responsiveness during heat stress.

Materials and Methods

Animals and Experimental Design

Twelve multiparous, lactating Holstein cows (parity = 2; 136 ± 8 DIM, 560 ± 32 kg BW) were randomly assigned to one of two environmental treatments during two experimental periods. The total length of the trial was 20 d with two experimental periods (P) consisting of: 1) 9 d of thermoneutral conditions and *ad libitum* feed intake (TN for group 1, WF = well-fed for group 2); 2) 9 d of either heat-stress (HS, group 1, n = 6) and *ad libitum* intake or pair feed (PF, group 2, n = 6) in thermoneutral conditions. Animals in the thermoneutral control conditions were pair-fed with HS animals to eliminate the confounding effects of dissimilar nutrient intake. During P2, the percent reduction in feed intake in heat-stressed cows was calculated and applied to the feed offered to the PF thermoneutral cows. The heat stress environment was designed with cyclical temperatures (31.1-38.9 °C, 20 % humidity: min THI = 73, max THI = 80.5) mimicking a normal Arizona summer day, with the PF animals remained in a constant environment of 20 °C. Cows

were housed in individual tie stall stanchions at the University of Arizona' William J. Parker Agricultural Research Complex, fed a TMR consisting primarily of alfalfa hay and steam-flaked corn (Table 2.1) and milked at 0500 and 1700 daily. Rectal temperature (Tre) and respiration rate (RR) were measured thrice daily at 0430, 1200 and 1630 h. All procedures were approved by the University of Arizona Institutional Animal Care and Use Committee.

Blood Sampling and Insulin Tolerance Test (ITT)

Bilateral indwelling jugular catheters were inserted in all cows on d 4 of each period. On d 6 of each period, an insulin challenge (1 µg/kg BW) was administered at 1400 h as performed previously (O'Brien et al., 2010). Bovine insulin (Sigma, St. Louis, MO) was initially dissolved to 1 mg/mL in 0.1 M HCl, then diluted into sterile saline and kept at -80 °C until utilization. After insulin I.V. infusion, the catheter was chased with 12 mL of sterile saline. Blood samples were collected at -30, -20, -10, 0, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 90, and 120 min relative to insulin infusion. Samples were collected into disposable glass tubes containing 250 U of sodium heparin and were immediately placed on ice. After centrifugation, plasma was split into 2 aliquots and stored at -20 °C; one aliquot was later analyzed for plasma glucose and the other for insulin concentrations.

Plasma glucose and insulin concentration was determined with commercially available kits (Autokit Glucose C2; Wako Chemicals USA, Richmond, VA and Mercodia Bovine Insulin ELISA, ALPCO Diagnostics, Salem, NH).

Western Immunoblot Analysis

To evaluate skeletal muscle and liver insulin responsiveness, biopsies were obtained immediately before (-15 min) and after (+15 min) the ITT. Skeletal muscle (semitendinosus)

biopsies were obtained by alternating hindlegs. Liver samples were collected by percutaneous needle biopsy as described previously (Rhoads et al., 2007). Both biopsies were snap-frozen in liquid nitrogen and kept at -80 °C until molecular analyses. Total protein extracts were prepared as described previously (Rhoads et al., 2007). Protein content of extracts was measured with the BCA protein assay (Pierce, Rockford, IL). Protein abundance of IR, IRS, AKT and phosphorylated AKT (p-AKT) were measured by Western blot analyses as previously described (Rhoads et al., 2010). Antibodies were rabbit anti-mouse AKT, rabbit anti-human p-AKT Ser 473 (Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-human IR and rabbit anti-human IRS (Santa Cruz Biotechnology, Inc, Santa Cruz, CA).

Statistical Analysis

Protein and plasma glucose data were analyzed with the Proc Glimmix procedure (SAS Institute, Cary, NC) using period, insulin infusion and period×insulin infusion interaction as fixed effects and animal as random effect. For Tr, RR, and plasma insulin, data were analyzed using the Proc Mixed procedure, time as a repeated effect, period, insulin infusion and period×insulin infusion interaction as fixed effects and animal as random effect.

Results

There were no group differences in rectal temperature or respiration rate during P1 (Table 2.2). Animals from group 1 (HS condition in P2) during P2 had significantly higher ($P < 0.05$) rectal temperature and respiration rate compared with group 2 (PF with thermoneutral condition during P2). During P2, PF animals had higher ($P < 0.05$) rectal temperature than they did in P1 at 0430 and 1630 h, but these differences (0.22 and 0.36 °C) are relatively small compared to those of HS.

Feed intake of group 1 animals decreased ($P < 0.01$) in P2 under HS conditions (Figure 2.1A). Heat stress treatment progressively lowered group 1 DMI until d 5 and DMI maintained at a similar level from d 5 to d 9. As stated in the Material and Method section, feed intake of group 2 mirrored the decreasing pattern of group 1 feed intake. Group 2 cows had lower overall DMI ($P < 0.01$) compared with those in group 1. This difference existed in P1 and by design remained in P2. There was no group difference observed in milk yield in either P1 or P2 (Figure 2.1B). Both groups progressively decreased milk production after initiation of P2.

Overall EBAL was lower ($P < 0.01$) in group 2 compared with group 1 and in P2 compared with P1 (Figure 2.1C). There was a treatment × period interaction indicating the extents of decrease in EBAL were different between groups. Both groups were in negative EBAL (NEBAL) condition during P2 and severity of NEBAL continued to progress for the first 5 d in P2 and stabilized for the rest of P2.

The ITT caused a more rapid glucose disposal in P1 compared with P2 ($P < 0.05$), but glucose clearance did not differ between groups in P2 (Table 2.3). Glucose concentration in group 1 was lower than group 2 in both P1 and P2 (Figure 2.2).

In muscle, protein abundance of IR, IRS and AKT remained stable between periods and environments (Figure 2.3 A & B). Insulin increased p-AKT in each period ($P < 0.05$), but this response tended to decline in P2 for PF animals ($P = 0.094$), but not during HS. After insulin infusion, phosphorylation ratio (abundance of phosphorylated protein/abundance of total protein) of AKT was significantly lowered ($P < 0.05$) by PF treatment (Figure 2.3 D) in group 2 while it remained unchanged in heat stressed animals (Figure 2.3 C).

In the liver, insulin increased p-AKT protein content and phosphorylation ratio of AKT in each period ($P < 0.05$) but they did not differ between periods (Figure 2.4 C & D). Protein abundances of IR and IRS remained constant during each period and were not altered by insulin challenge (Figure 2.4 A & B).

Discussion

Feed intake reduction is traditionally thought to be the major cause of milk yield decrease of dairy cows exposed to environmental heat load above their thermal comfort zone. With experiments designed to separate overlapping effects of high temperature and nutritional deprivation, we have reported that reduced feed intake can only explain part of milk yield reduction in dairy cows under HS conditions (Rhoads et al., 2009). In the present study, milk production did not differ between HS and PF during P2. However, HS cows consumed more feed and had a more severe decrease in terms of EBAL to produce similar amount of milk compared to PF group. Overall, this is consistent with the observation that heat stress, independent of reduced feed intake, directly contributes to milk yield decline.

It is well documented that malnourished lactating animals reduce plasma insulin and/or responsiveness as a homeorhectic mechanism to spare glucose for the mammary gland (Bauman and Currie, 1980; Escriva et al., 1992; Kemnitz et al., 1994; Bauman, 1999; Davis et al., 2010). During P2, both HS and PF cows reduced their glucose clearance rate during an ITT compared with P1, but there was no treatment difference. Dietary reduction appeared to sufficiently explain lowered systemic insulin sensitivity observed in P2 in terms of glucose disposal rate in the present study. However, at the molecular level, insulin responsiveness in skeletal muscle and the liver were maintained in the HS group, while the PF group tended to decrease insulin sensitivity in

skeletal muscle. If blunted glucose clearance stems from decreased skeletal muscle AKT phosphorylation in the PF animals, insulin resistance in HS appears to occur downstream of AKT phosphorylating event. In a previous study with a similar experimental design, Wheelock et al. (2010) demonstrated that PF animals decreased the level of accumulated insulin secretion during a glucose tolerance test (GTT) while HS animals did not. Other heat-stressed animal studies reported increased secretion of insulin during GTT in cattle (Itoh et al., 1998) or augmented basal insulin level in mice (Morera et al., 2012) under hyperthermic conditions. Overall, despite a marked decline in feed intake, heat stressed animals maintained or even increased insulin secretion while glucose disappearance rate is significantly suppressed. There are several possible explanations: 1) elevated intracellular Ca^{2+} is reported in malignant hyperthermia susceptible humans and enhanced Ca^{2+} suppresses insulin-mediated glucose uptake in skeletal muscle (Freymond et al., 2000); 2) plasma glucose is compensated by increased hepatic glucose output during HS from gluconeogenesis (O'Brien et al., 2010; Rhoads et al., 2011; White et al., 2012) or glycogenolysis (Febbraio, 2001) and these events become immune to insulin under HS; 3) unknown post-receptor signaling alterations occur downstream of IR in skeletal muscle or adipose tissue leading to reduced glucose uptake. In addition, insulin-mediated glucose uptake is not the only approach enlisted by animals to regulate glucose flux. Insulin-independent glucose transporters are expressed in tissue specific manner (Zhao and Keating, 2007) and HS may affect different routes of glucose in- and out-fluxes. The mechanism(s) by which HS increases insulin parameters is unknown. There is evidence indicating that hyperthermia improves insulin-resistance via HSP, which either protects pancreatic β-cells (Kondo et al., 2012) or counteracts stress kinases, which contribute to insulin-resistance, in skeletal muscle (Geiger and Gupte, 2011). Hooper and Hooper (2009) proposed a self-perpetuating cycle model for the relationship between

insulin and HSP: 1) obesity-driven inflammation triggers insulin resistance; 2) impaired insulin signaling in turn reduces the expression of HSPs, subsequently leading to vulnerable pancreas tissue and accumulation of inflammatory cytokines and their related proteins; and 3) damaged pancreas β-cells further lower insulin signaling and the lack of anti-inflammatory HSPs allows inflammation to expand unhindered. Together, it is reasonable to speculate that heat stress restores insulin sensitivity and stimulated insulin secretion in lactating cows under negative energy balance by up-regulating HSPs expression.

The insulin-sensitive glucose transporter isoform, GLUT4 is primarily expressed in skeletal muscle and adipose tissue. The redistribution of GLUT4 vesicles from the cytoplasm to the plasma membrane triggered by insulin is responsible for a net 10- to 40-fold increase in glucose influx into the cells and PI3K-AKT pathway plays an important role in GLUT4 translocation (Whiteman et al., 2002). Phosphorylation at residue Thr308 and Ser473 of AKT is necessary for the induction of GLUT4 vesicle exocytosis (Watson and Pessin, 2006). In the current study, phosphorylation ratio of AKT in skeletal muscle tended to be reduced by PF but not HS, indicating nutrient deprivation decreased AKT activation level, while direct effects of high ambient temperature was sufficient for maintenance of AKT activation. A possible explanation for the reduction in AKT phosphorylation observed in PF cows may be mediated by AMP-activated protein kinase (AMPK) energy sensing system. As an energy sensor, AMPK reacts to the fluctuations in the ratio of ATP/AMP as well as phosphocreatine/creatine (Winder, 2001) and it is able to attenuate the phosphorylation of AKT at Ser 473 (Bolster et al., 2002). In contrast, heat stress can dephosphorylate AMPKα, the catalytic subunit of AMPK, and therefore inhibit the physiological functions of AMPK (Wang et al., 2010). In liver, both HS and PF animals maintained the AKT phosphorylation ratio at the same level as in TN and WF period, respectively. Together, these

results indicate that heat stress prevents cows from sparing glucose for milk synthesis by maintaining AKT phosphorylation ratio in skeletal muscle. How AKT activation remained unchanged in skeletal muscle during heat stress is unclear and warrants further studies on the relationship between heat stress and AMPK signaling that may shed some light on understanding the underlying mechanisms.

Conclusion

We previously demonstrated that decreased feed intake can only explain 35% of the milk production decline during heat stress periods on dairy cows and hyperthermic conditions can lower milk yield via other mechanisms. In the present study, we focused on measuring systemic insulin sensitivity and insulin responsiveness on important metabolic organs since insulin signaling occupies a central role orchestrating whole body glucose metabolism. Both pair-feeding and heat stress reduced whole body insulin sensitivity in terms of glucose disposal. Insulin-activated downstream signaling performed equally well in skeletal muscle and liver during thermal neutral and heat stress periods but insulin responsiveness was blunted during pair-fed period only in muscle. The mechanism of shunting glucose into milk production enlisted by pair-fed animals may be mediated by reducing insulin post-receptor signaling, while heat stress cows did not appear to employ this mechanism. Future experiments are needed to investigate the signaling pathways underlying the maintained tissue insulin sensitivity despite an overall reduction in systemic insulin sensitivity during heat stress.

Table 2.1. Ingredients and chemical composition of diets.

Item, % of DM unless noted	Content
Ingredient	
Alfalfa hay	50.3
Steam-flaked corn	25.5
Whole cottonseed	8.7
Amino plus[1]	2.1
Beet pulp	4.8
Molasses	4.3
Supplement[2]	2.4
Calcium salts of palm oil[3]	1.9
Chemical analysis	
Moisture	8.5
Ash	13.4
NDF	32.8
ADF	28.4
CP	21.3

[1]Soybean-based supplement; 51.7% CP (Ag Processing Inc., Hasting, NE).
[2]Contained 1.34% fat, 6.23% Ca, 4.49% P, 3.10% Mg, 0.58% S, 0.23% K, 16.18% Na, 3,7% Cl, 2,290.59 mg/kg of Zn, 2,037.94 mg/kg of Mn, 1,109.46 mg/kg of Fe, 629.18 mg/kg of Cu, 75.02 mg/kg of Co, 13.83 mg/kg of Se, 10.40 mg/kg of Mo, 51.65 mg/kg of I, 360.16 IU/g of vitamin A, 35.67 IU/g vitamin D, and 1.16 IU/g of vitamin E.
[3]Maxxer; Tarome Inc., Eloy, AZ.

Table 2.2. Effects of heat stress (HS) or pair-feeding (PF) on rectal temperature (Tr) and respiration rate (RR) in lactating Holstein cows

	Period 1[1]		Period 2[2]			*P*		
Parameter	Group 1 (TN)	Group 2 (TN)	Group 1 (HS)	Group 2 (PF)	SEM	Group	PER	Group x PER
Tr,°C								
0430 h	38.23[a]	38.25[a]	39.17[c]	38.55[b]	0.07	<0.01	<0.01	<0.01
1200 h	38.26[a]	38.22[a]	39.65[b]	38.29[a]	0.08	<0.01	<0.01	<0.01
1630 h	38.30[a]	38.33[a]	40.14[c]	38.66[b]	0.06	<0.01	<0.01	<0.01
RR, BPM								
0430 h	36[a]	38[a]	73[b]	38[a]	2	<0.01	<0.01	<0.01
1200 h	38[a]	40[a]	90[b]	41[a]	2	<0.01	<0.01	<0.01
1630 h	36[a]	39[a]	83[b]	35[a]	2	<0.01	<0.01	<0.01

[1]During period 1, cows in both groups were treated identically (housed in thermal neutral [TN] conditions and allowed to eat ad libitum).

[2]During period 2, cows were either heat-stressed and allowed to eat ad libitum or pair-fed and kept in TN conditions.

[a, b, c] Values within row of each variable with differing superscripts indicate statistical difference ($P < 0.05$).

Table 2.3. Effects of heat stress (HS) or pair-feeding (PF) on glucose and insulin responses to an insulin tolerance test in lactating Holstein cows

Parameter	Period 1		Period 2		SEM	P		
	Group 1 (TN)	Group 2 (TN)	Group 1 (HS)	Group 2 (PF)		TRT	PER	TRT × PER
Glucose								
30 min AUC[1]	-530	-215	-121	-121	100	0.13	0.02	0.13
60 min AUC	-1224	-551	-443	-394	216	0.11	0.05	0.17
Δ^2, mg/dL	32.4	16.4	11.9	8.5	5.1	0.07	0.01	0.24
Insulin								
Basal, ng/mL	0.87	0.96	0.96	0.65	0.18	0.57	0.56	0.30
G:I[3]	108.7	133.9	111.7	156.0	36.2	0.32	0.69	0.75

[1]Area under the curve, mg*dL*min.
[2]Change in glucose concentrations between the start of insulin infusion and glucose response nadir.
[3]Glucose: insulin ratio.

FIGURE 2.1.

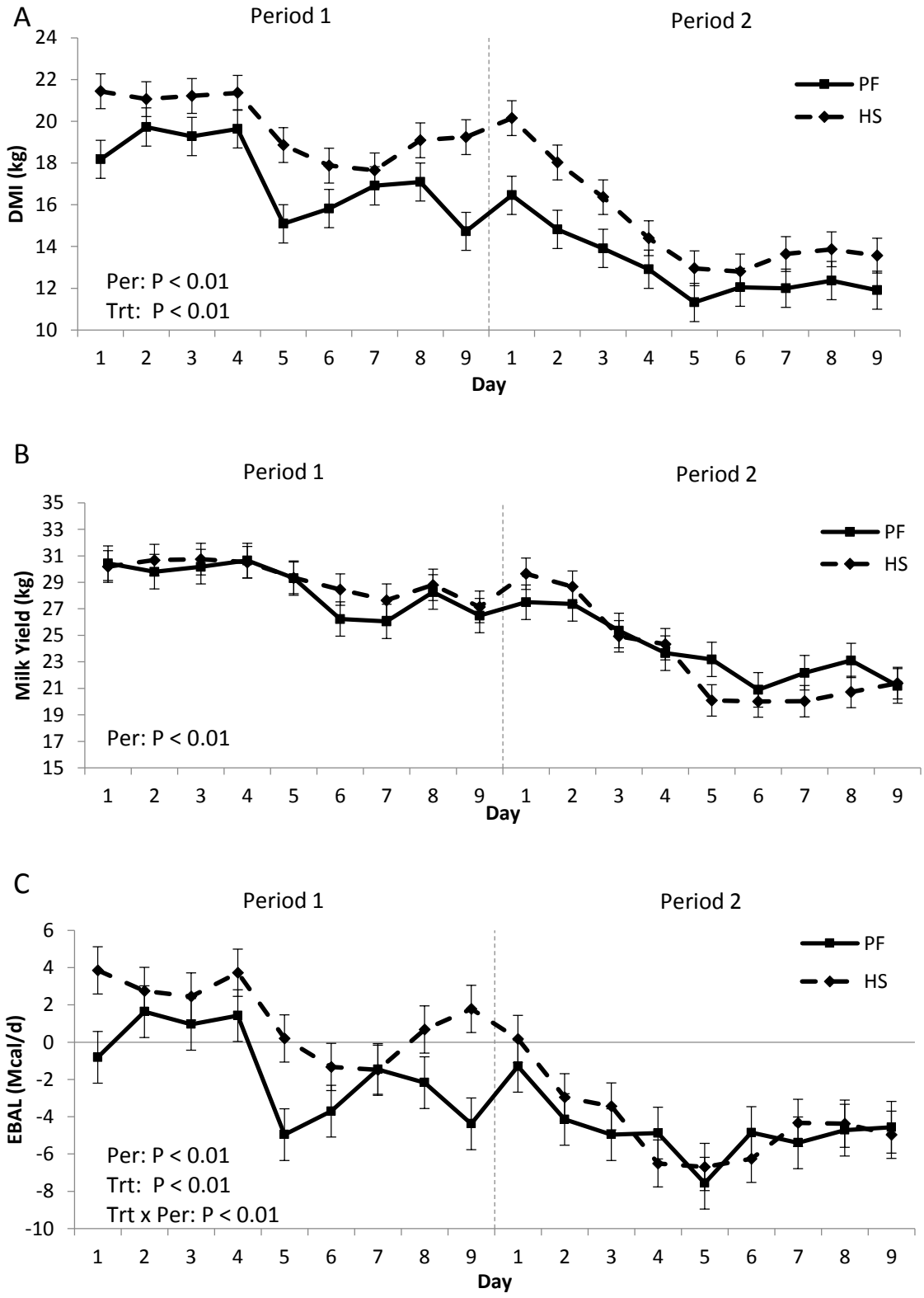

A
Period 1
Period 2
DMI (kg)
PF
HS
Per: P < 0.01
Trt: P < 0.01
Day
B
Period 1
Period 2
Milk Yield (kg)
PF
HS
Per: P < 0.01
Day
C
Period 1
Period 2
EBAL (Mcal/d)
PF
HS
Per: P < 0.01
Trt: P < 0.01
Trt x Per: P < 0.01
Day

Figure 2.1. Effects of heat stress (HS) or pair-feeding (PF) on A) DMI, B) milk yield and C) energy balance (EBAL) in lactating Holstein cows. Solid lines with squares represent PF cows and dashed lines with diamonds represent HS. Cows were in thermal neutral ad libitum condition during period 1 and either exposed to HS (cyclical temperatures ranging from 31.1 to 38.9 °C with constant humidity 20%) and fed ad libitum or exposed to thermal neutral conditions and pair-fed with HS cows during period 2.

FIGURE 2.2.

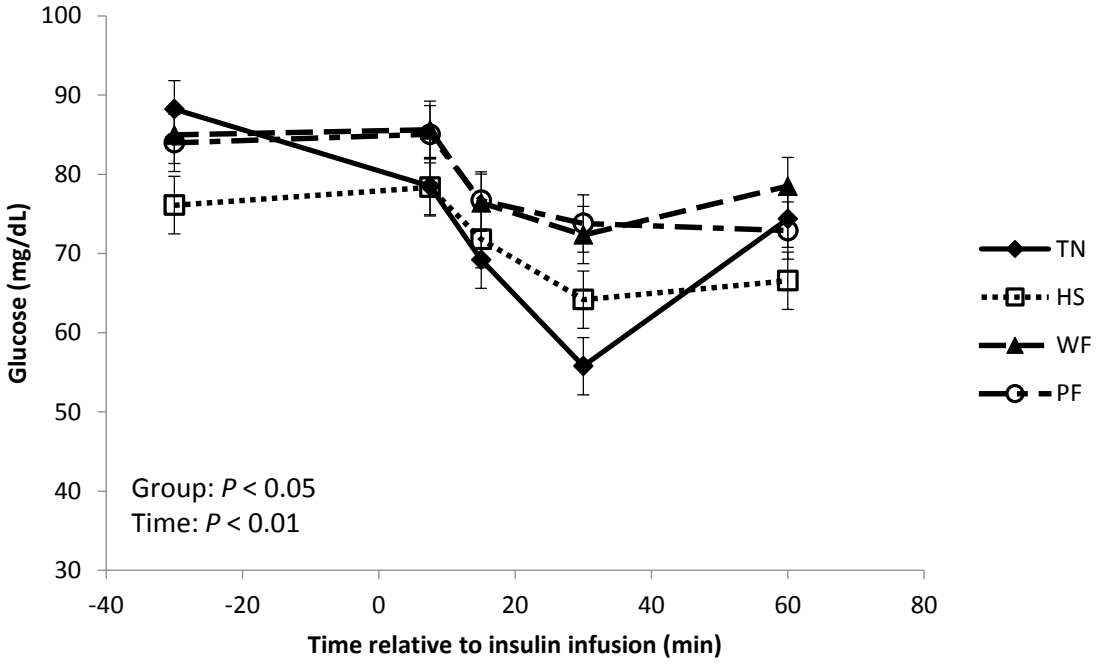
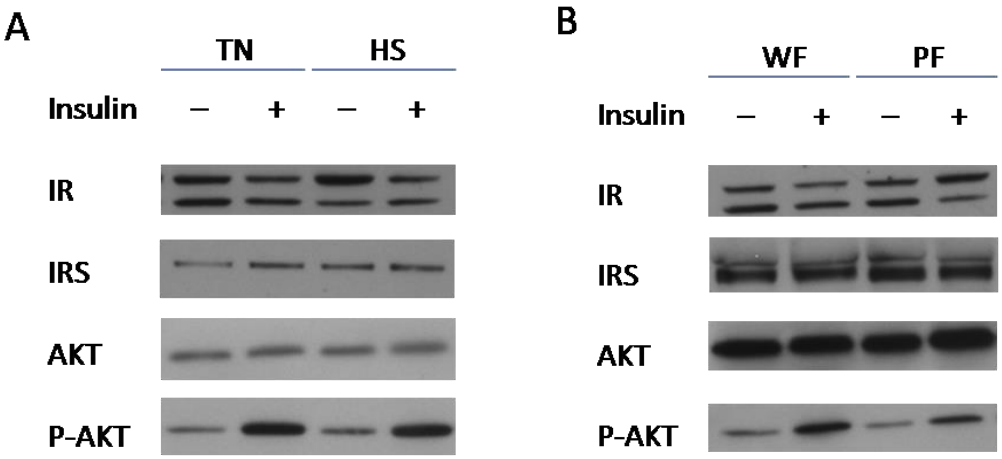
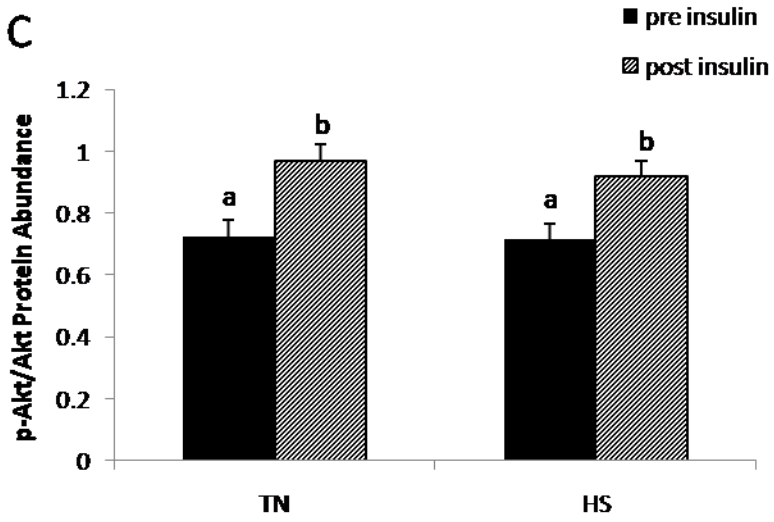


Figure 2.2. Effects of heat stress (HS) or pair-feeding (PF) on glucose disposal rate during insulin challenge lactating Holstein cows. Twelve lactating dairy cows were exposed to heat stress conditions or subjected to a reduced plane of nutrition. Conditions were constant thermal neutral conditions and ad libitum feed intake for 9 d (TN: solid line with diamonds & WF: dashed line with triangles) followed by exposure to cyclical temperatures ranging from 31.1 to 38.9°C with constant 20% humidity and were fed ad libitum (HS: dotted line with squares) or exposure to constant thermal neutral conditions and pair-fed to the feed intake of HS counterparts (PF: dashed-dotted line with circles). On d 6 each period, insulin was administered via jugular catheter and blood samples were collected at -30, 7.5, 15, 30, and 60 min relative to insulin administration.

FIGURE 2.3.


A
TN
HS
Insulin
–
+
–
+
IR
IRS
AKT
P-AKT
B
WF
PF
Insulin
–
+
–
+
IR
IRS
AKT
P-AKT


C
pre insulin
post insulin
p-Akt/Akt Protein Abundance
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1
0.8
0.6
0.4
0.2
0
a
b
a
b
TN
HS

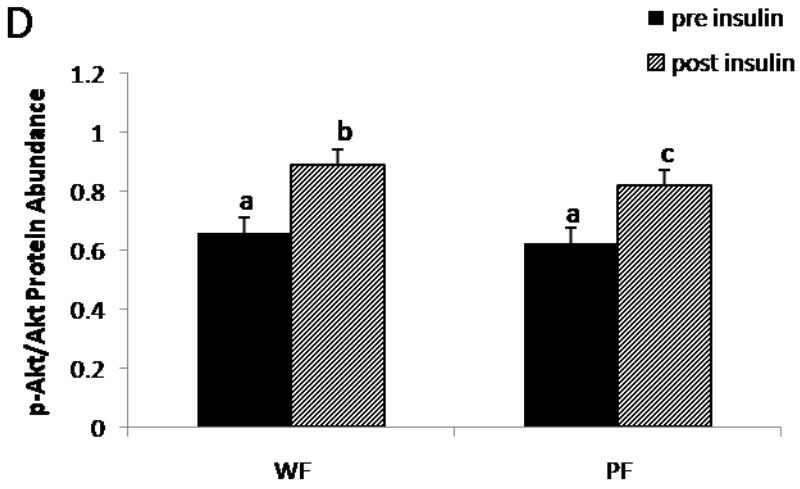
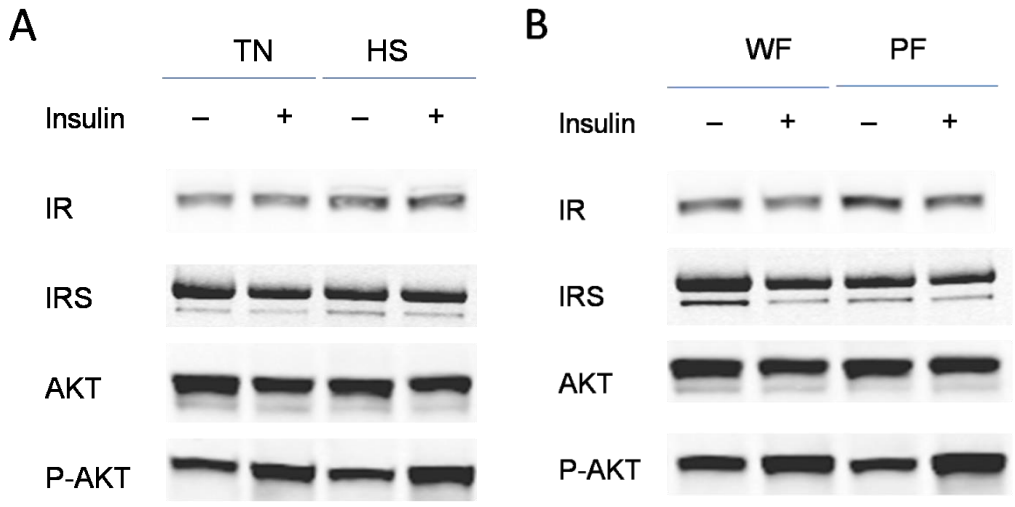
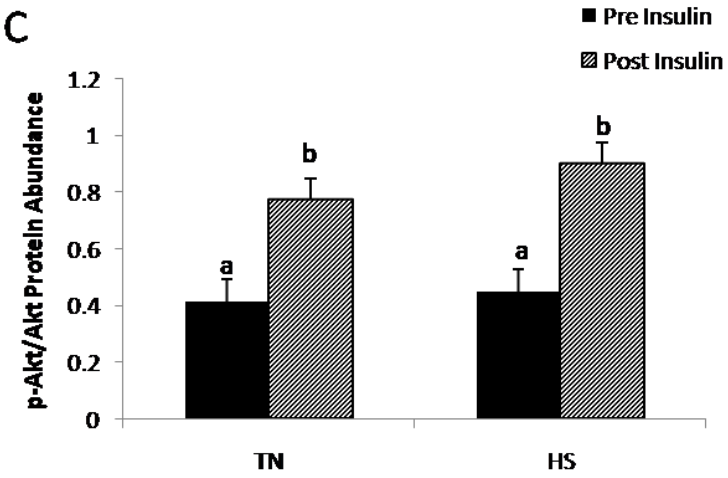

D
pre insulin
post insulin
p-Akt/Akt Protein Abundance
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
c
WF
PF

Figure 2.3. Effect of heat stress and insulin challenge on skeletal muscle components of insulin signaling. Twelve lactating dairy cows were exposed to heat stress conditions or subjected to a reduced plane of nutrition. Conditions were constant thermal neutral conditions and ad libitum feed intake for 9 d (TN & WF) followed by exposure to cyclical temperatures ranging from 31.1 to 38.9°C with constant 20% humidity and were fed ad libitum (HS) or exposure to constant thermal neutral conditions and pair-fed to the feed intake of HS counterparts (PF). On d 6 of each period, insulin was administered with skeletal muscle biopsies obtained at -15 (Pre) and 15 min (Post) relative to insulin administration. **A** and **B**: Muscle protein extracts were analyzed by western immunoblotting for the abundance of the insulin receptor (IR), insulin receptor substrate (IRS), AKT and phosphorylated AKT (p-AKT). Data are from one representative cow. **C** and **D**: Bars represent means ± SE of AKT protein phosphorylation ratio (abundance of p-AKT/ abundance of AKT). Bars with different letters differ at $P < 0.05$.

FIGURE 2.4.


A
TN
HS
Insulin
–
+
–
+
IR
IRS
AKT
P-AKT
B
WF
PF
Insulin
–
+
–
+
IR
IRS
AKT
P-AKT


C
Pre Insulin
Post Insulin
p-Akt/Akt Protein Abundance
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
b
TN
HS

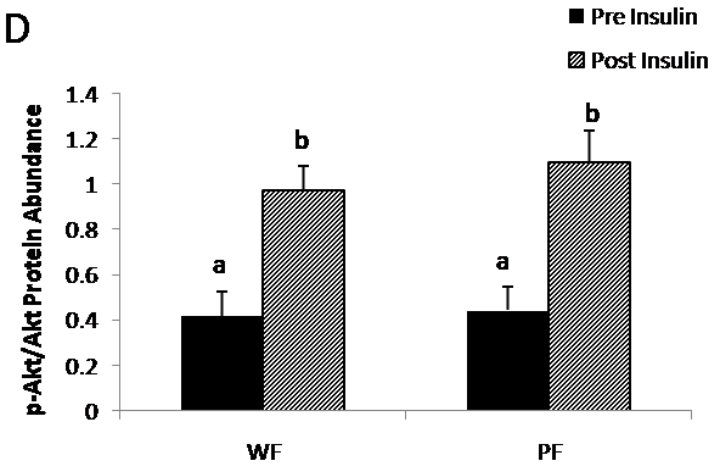

D
Pre Insulin
Post Insulin
p-Akt/Akt Protein Abundance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
b
WF
PF

Figure 2.4. Effect of heat stress and insulin challenge on liver components of insulin signaling. Twelve lactating dairy cows were exposed to heat stress conditions or subjected to a reduced plane of nutrition. Conditions were constant thermal neutral conditions and ad libitum feed intake for 9 d (TN & WF) followed by exposure to cyclical temperatures ranging from 31.1 to 38.9°C with constant 20% humidity and were fed ad libitum (HS) or exposure to constant thermal neutral conditions and pair-fed to the feed intake of HS counterparts (PF). On d 6 of each period, insulin was administered with liver biopsies obtained at -15 (Pre) and 15 min (Post) relative to insulin administration. **A** and **B**: Liver protein extracts were analyzed by western immunoblotting for the abundance of the insulin receptor (IR), insulin receptor substrate 1 (IRS-1), AKT and phosphorylated AKT (p-AKT). Data are from one representative cow. **C** and **D**: phosphorylation ratio of AKT comparisons between periods; bars represent means ± SE of AKT protein phosphorylation ratio (abundance of p-AKT/ abundance of AKT). Bars with different letters differ at $P < 0.05$.

Chapter 3. Heat stress alters fatty acid turnover-related genes expression pattern in lactating dairy cows

Abstract

During heat stress (HS) in lactating dairy cows, adipose tissue appears to become refractory to lipolytic signals whereas pair-fed cows employ mechanisms allowing lipid mobilization to spare glucose utilization in peripheral tissues. Despite this, little is known regarding the effects of HS on metabolic gene expression in adipose tissue. Multiparous cows (n=11; parity=3.3 ± 0.4, 305 ± 33 DIM; 665 ± 18 kg BW) housed in climate chambers were fed a TMR consisting primarily of alfalfa hay and steam-flaked corn and subjected to 2 experimental periods: 1) thermoneutral conditions (18°C, 20% humidity) with *ad libitum* intake for 9 d and 2) either HS conditions (cyclical temperature 31–40°C, 20% humidity: min THI = 73, max THI = 86) fed for *ad libitum* intake (group 1, n = 5), or TN conditions, pair-fed (PF, group 2, n = 6) for 9 d. Rectal temperature (Tre) and respiration rate (RR) were measured thrice daily at 0600, 1400 and 1800h. To evaluate adipose tissue gene expression, biopsies were obtained at the end of each period and total RNA isolated for quantitative real-time PCR (qPCR) analyses. During P2, HS cows had a 1.8 °C increase in Tre and a 3-fold increase in RR compared with TN cows ($P < 0.01$). Pair feeding did not alter Tre or RR. Heat stress reduced DMI by 18% ($P < 0.01$) and by design PF cows had similar intake reductions. Milk yield was decreased by 10.8% during HS and 6.8% in PF cows. Total RNA was extracted from adipose tissue collected by biopsies at the end of each period. Heat stress decreased gene expression levels of hormone sensitive lipase (HSL, $P = 0.0539$), adipose triglyceride lipase (ATGL, $P < 0.05$), lipoprotein lipase (LPL, $P < 0.05$), pyruvate carboxylase (PC, $P = 0.0587$), peroxisome proliferator-activated receptor α (PPARα, $P < 0.01$), PPARγ ($P = 0.0685$), β2 adrenergic receptor (BAR2, $P = 0.0754$), while restrained feeding did not alter them. Both HS and

PF had significantly negative impact on fatty acid synthase (FAS, $P < 0.05$). Expression level of insulin-like growth factor I (IGF1) decreased ($P = 0.0514$) during HS but increased ($P = 0.0877$) in PF. Proteins extracted from adipose biopsies were subjected to Western Blot semi-quantification assay. The protein abundance of ATGL was increased in PF group ($P < 0.05$), but decreased in HS ($P < 0.1$), compared to their respective control condition. Feed restriction also increased protein abundance of BAR2 ($P < 0.05$) and perilipin (PLIN, $P < 0.1$), while HS had no effect on them. These results indicate that HS directly alters adipose tissue metabolism-related gene expression independently of reduced plane of nutrition.

(**Keywords**: heat stress, adipose, fatty acid metabolism, dairy cow)

Introduction

Heat stress (HS) negatively impacts a variety of dairy parameters, including milk yield and quality, and therefore causes a significant financial burden in many dairy-producing areas of the world (Renaudeau et al., 2012; Baumgard and Rhoads, 2013). Advances in management strategies (i.e. cooling systems) have alleviated some of the negative impact of thermal stress on dairy cattle, but production continues to markedly decrease during summer (Gaughan et al., 2010; Shiao et al., 2011). In addition, genetic improvement programs which enhance production traits increase the cow's susceptibility to environmental HS due to the close relationship between metabolic heat generation and production level (West, 2003; Spiers et al., 2004).

The dramatic reduction in feed intake is an important sign of HS and is generally accepted to be the main reason for the negative effects of HS on animal production (Rhoads et al., 2009; Renaudeau et al., 2012). However, under certain circumstances feed intake does not account for the entire reduction in milk yield during HS. To distinguish the direct and indirect (via changes in feed intake) effects of HS, experiments imposing HS or restricted nutrient intake matched to those of HS cows were conducted in lactating dairy cattle (Rhoads et al., 2009; Wheelock et al., 2010). These studies demonstrated that feed intake accounts for a portion (~50%) of the reduction in milk yield and that HS cattle employ metabolic alterations which are distinct from the PF cohorts. During fasting or periods of negative energy balance, animals mobilize adipose and non-esterified fatty acid (NEFA) is released into circulating system to serve as a fuel source (Hales and Randle, 1963; Lafontan and Langin, 2009; Karpe et al., 2011). In HS animals, despite suffering from a negative energy balance, adipose tissue appears to become refractory to catabolic signals and NEFA is maintained at similar level as TN conditions (Rhoads et al., 2009; Shwartz et al., 2009; Wheelock et al., 2010), indicating HS restricts the use of endogenous lipid reserves. In contrast,

the PF cows mobilize adipose tissue lipids and rely on fatty acids to support energy and mammary gland needs. Therefore alterations in adipose tissue metabolism under HS may account for a portion of the unexplained deleterious effect of HS on milk yield.

In the present study, our objective was to characterize the profiles of lipid metabolism-related genes in lactating dairy cows under heat stress conditions. Both RNA and protein abundance of critical genes associated with fatty acid turnover were examined.

MATERIALS AND METHODS

Animals and Experimental Design

Eleven multiparous, lactating Holstein cows (parity = 3.3 ± 0.4, 305 ± 33 DIM; 665 ± 18 kg BW) were randomly assigned to one of two environmental treatments during two experimental periods. The trial was composed of two experimental periods (P): 1) 9 d of thermal neutral conditions (18°C, 20% humidity) and fed *ad libitum* (TN = thermal neutral for group 1, WF = well-fed for group 2); 2) 9 d of either heat stress, *ad libitum* intake (HS for group 1, n = 5) or pair-fed (PF for group 2, n = 6) with restricted feed in TN conditions. The HS environment was designed with cyclical temperatures (31 - 40°C, 20% humidity) peaking at 2 pm to mimic hot summer days. Cows were housed in individual tie stall stanchions at the University of Arizona Agricultural Research Complex, fed with a TMR consisting primarily of alfalfa hay and steam-flaked corn and milked at 0600 and 1800 daily. The nutritional composition of the TMR met the predicted requirements (NRC, 2001) of energy, protein, minerals and vitamins (Table 1). Rectal temperature (Tre) and respiration rate (RR) were measured thrice daily at 0600, 1400 and 1800 h. All procedures were approved by the University of Arizona Institutional Animal Care and Use Committee.

Blood Collecting and Biopsies

Blood samples were collected twice daily at 0900 and 1400 in evacuated containers with EDTA (BD Vacutainer, Franklin Lakes, NJ). Plasma was separated from collected whole blood with centrifugation at 1,500 × g for 10 min, aliquoted into 2-ml microcentrifuge tubes and stored at -20 °C.

Adipose tissue biopsies were performed at the end of each period (d 9) as described previously (Rhoads et al., 2007). Biopsies were obtained from contralateral sites at the tail head area and samples were snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

Plasma Analyses

Plasma glucose, NEFA and β-hydroxybutyrate (BHBA) were measured with commercially available kits (Glucose Assay Kit, Sigma, St Louis, MO; NEFA-HR kit, Wako Chemicals USA; BHBA, RANBUT, Randox Laboratories Ltd.). For daily sampling, the intra- and interassay CV were 5.57% and 1.76% for glucose, 2.59% and 3.55% for NEFA, and 4.45% and 1.45% for BHBA. Daily plasma insulin were measured with commercial ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden) and intra- and interassay CV were 4.22% and 14.77% respectively.

Quantitative Real-Time PCR

Total RNA was isolated from adipose tissue biopsy with TRIzol reagent (Invitrogen, Carlsbad, CA) and Phase Lock Gels (Eppendof AG, Hamburg, Germany) following manufacturers' instructions. Purification of RNA was carried out with RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA concentration of each sample was determined by NanoPhotometer Pearl (Implen GmbH, Munich, Germany) and RNA quality was evaluated by Experion Automated

Electrophoresis System (Bio-Rad, Hercules, CA). Reverse transcription of 1 µg RNA was executed with iScript cDNA Synthesis Kit (Bio-Rad) in Thermo Cycler (Bio-Rad) and synthesized cDNA was stored at -20 °C.

Gene expression of β1-adrenergic receptor (BAR1), BAR2, hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), perilipin (PLIN), leptin (LEP), fatty acid synthase (FAS), lipoprotein lipase (LPL), pyruvate carboxylase (PC), phosenolpyruvate carboxykinase (PEPCK), peroxisome proliferator activated receptor α (PPARa), PPARγ (PPARc) were measured by real-time PCR using bovine specific-primers (Table 3.3). Previously synthesized cDNA (10 ng) was mixed with primers corresponding to genes of interest and SsoAdvanced SYBR Green Supermix (Bio-Rad) in a 25-µL reaction. Each assay was set to run 40 cycles and melt curve was performed at the end to verify the generation of single product using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Data were collected and analyzed with CFX Manager software (Bio-Rad). Standard curve and non-template control were included in each plate to calculate amplification efficiency. Data from each gene of interest were normalized using the geometric mean of cyclophilin, ribosomal protein S15 and 18S ribosomal RNA.

Western Immunoblotting

The protein abundance of ATGL, FAS, PLIN and BAR2 from adipose tissue biopsies were determined by Western Immunoblotting. Total protein extraction and membrane protein enrichment were previously described (Rhoads et al., 2007). Briefly, 1 g of pulverized frozen adipose tissue from biopsy was homogenized in 5 ml sucrose buffer [50 mM Tris (pH 7.6), 250 mM sucrose, 5 mM EGTA, 150 mM NaCl, 1X Halt Protease and Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL)], and subjected to centrifugation (1000 ×g for 5 min at 4 °C) to remove unsolubilized materials. Aqueous part of homogenate was divided into two parts (1 ml

and 4 ml). The 1-ml part was clarified twice by centrifugation (10,000 ×g for 20 min at 4 °C) and supernatant containing total protein was stored at -80 °C. The 4-ml part was subjected to high-speed centrifugation (100,000 ×g for 60 min at 4 °C) and precipitation was resuspended in 0.5 ml lysis buffer [10 mM Tris (7.6), 1% Triton X-100, 1 mM EGTA, 150 mM NaCl, 1X Halt Protease and Phosphatase Inhibitor Cocktail].

The protein concentration of extracts was measured using the BCA protein assay (Pierce). Protein extract was mixed with Laemmli Buffer and β-mercaptoethonal and heated at 95 °C for 10 min. Fixed amounts of protein were loaded into 4% - 15% Criterion TGX Precast Gel (Bio-Rad) for electrophoresis and then transferred to nitrocellulose membranes in Trans-Blot Turbo Transfer Starter System (Bio-Rad). The membranes were stained with Ponceau S (Santa Cruz Biotechnology Inc., Dallas, TX) to verify equal loading and destained with 0.1% NaOH. The membranes were then blocked with Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) and 5% w/v nonfat-dried milk for 1 h at room temperature and incubated in primary antibody diluted with blocking solution at 4 °C overnight. Antibodies were ATGL rabbit anti-human antibody (mAb) (1:1000; #2138 Cell Signaling Technology Inc, Danvers, MA), FAS rabbit anti-human Ab (1:1000; #3180 Cell Signaling Technology), PLIN rabbit anti-human Ab (1:100; sc-67164 Santa Cruz Biotechnology), and BAR2 rabbit anti-human (1:100; sc-569 Santa Cruz Biotechnology). Membranes were washed three times (5 min each) with TBST on the next day and incubated with the secondary antibody (IRDye 800CW Goat anti-Rabbit IgG; LI-COR Biosciences Inc, Lincoln, NE) at 1:20000 dilution in blocking solution for 1 h at room temperature. Fluorescence signal was detected in Odyssey Infrared Imaging System (LI-COR) and images were analyzed with Odyssey Imaging software (LI-COR).

Statistical Analysis

Data were analyzed with the Proc Glimmix procedure (SAS Institute, Cary, NC). Metabolites and insulin data from the daily plasma collection and milk yield were analyzed with group, period and interaction of both as fixed effects and day as a repeated effect, and additionally analyzed with P1 as a covariate and day as a repeated effect with cow nested within groups. For results of qPCR and Western Blot, group, period and group×period were used fixed effects; and cow nested within group was listed in Random option. Statistical results were considered significant if $P < 0.05$ and having tendency if $0.05 < P < 0.1$.

Results

When animals were housed in TN conditions, both groups had similar Tre throughout P1. Cows from group 1 exhibited increased Tre continuously from the onset of heat stress until d 3 of P2 and plateaued for the rest of P2. Feed restriction did not affect Tre or RR of group 2. Overall, HS cows had a 0.96 ^{o}C increase in Tre ($P < 0.01$) and a 3-fold increase in RR ($P < 0.01$) compared to TN period (Figure 3.1C & D).

Heat stress decreased animals' DMI by 18% ($P < 0.01$) and by design PF cows had the same percentage decrease (Figure 3.1A). Cows housed in HS conditions began to decrease their feed intake from the onset of P2 and reached nadir on d 3 of P2. Milk yield was reduced in both groups ($P < 0.01$) during P2, however, HS exhibited a 10.8% decrease while PF reduced by 6.8% (Figure 3.1B). When using milk yield from P1 as a covariate, interaction of group by day became a significant effect and the milk yield discrepancy between HS and PF reached significant level ($P < 0.05$) from d 7 to d 8.

Circulating glucose concentration remained stable throughout all groups and periods (Table

3.2). When using P1 as a covariate, PF had a significantly higher glucose concentration than HS ($P < 0.05$) indicated by a significant interaction of group by day after d 7 ($P < 0.05$). Both groups had elevated plasma NEFA after entering P2 ($P < 0.05$), however, there was no group effect (Table 3.2). Another negative energy balance indicator, BHBA, did not change between groups or periods (Table 3.2). Although plasma insulin concentration differed between groups, neither HS nor PF treatments caused changes during P2 (Table 3.2).

Heat stress decreased gene expression (Figure 3.2) of HSL ($P = 0.0539$), ATGL ($P < 0.05$), LPL ($P < 0.05$), PC ($P = 0.0587$), PPARa ($P < 0.01$), PPARc ($P = 0.0685$), BAR2 ($P = 0.0754$), while PF had no effect. Both HS and PF reduced FAS mRNA abundance ($P < 0.05$). Gene expression of IGF1 decreased ($P = 0.0514$) during HS but tended to increase ($P = 0.0877$) during PF.

The protein abundance of ATGL was increased in the PF group ($P < 0.05$), but decreased in HS ($P < 0.1$), compared to their respective baseline period (Figure 3.3). Feed restriction also increased protein abundance of BAR2 ($P < 0.05$) and PLIN ($P < 0.1$), while HS had no effect..

Discussion

In the present study, animals housed in HS condition exhibited common signs of hyperthermia (elevated Tre and markedly increased RR), which is consistent with previous heat stress cow studies (Rhoads et al., 2009; Wheelock et al., 2010; Rhoads et al., 2011) and confirmed that animal were in fact experiencing heat stress. Milk yield loss magnitude in HS exceeded the portion attributable to depressed feed intake and this observation agrees with our previous reports (Rhoads et al., 2009; Wheelock et al., 2010). This is again indicating, aside from the negative effect of reduced nutrient supply, heat stress is causing additional detrimental impacts on animal

production. Our separating experimental design should therefore enable us to minimize the confounding effects from dissimilar feed intake and better evaluate the direct effects from hyperthermia.

Evolutionary adaptations to inconsistent feed supply involve various strategies to enable organisms to store extra energy when food is sufficient and to mobilize energy reservoirs when facing scarce food supplies. Triacylglyceride (TAG) is the form that animals, seed plants, and fungi commonly employed to store excessive energy and become the primary energy source when animals encounter food deprivation (Zimmermann et al., 2004). In response to signals sent from energy sensing systems during starvation, TAG stored in adipose tissue is liberated into circulation in the form of NEFA and this causes the rise of NEFA concentration in the blood (Eaton et al., 1996). The surprising observations from previous studies on HS lactating cows is that despite insufficient nutrient supply, HS cows appeared to maintain their plasma NEFA concentration at the similar level as that under well-fed conditions (Rhoads et al., 2009; Shwartz et al., 2009; Wheelock et al., 2010). In the present study, however, plasma NEFA increased in both HS and PF group and did not differ between groups. This differentiated response might be due to differences in the lactation stage of the cows in this study versus the previous studies. In our previous serial studies, mid-lactating cows were chosen in the experiments, while in the present study, in order to obtain enough adipose tissue for later analysis, we chose late lactating cows for the trial. The late-lactating cows in this study produced substantially lower levels of milk compared to previous mid-lactation cows. The energy shortage might not reach the magnitude to enable us to find marked NEFA difference between groups. A second key difference is that cows in this study did not reach the same magnitude of HS as the mid-lactation cows likely related to a lower level of metabolic heat production in late-lactation associated with a lower feed intake and production level. Another

interesting discrepancy is that when using P1's data as a covariate, circulating glucose concentration in the HS group was significantly lower than the PF cohorts.

Despite the apparent similarity of plasma NEFA concentrations, HS animals exhibited a remarkably dissimilar mRNA expression profile compared to PF. Key lipolysis-related genes, ATGL, HSL were downregulated by HS but remained unchanged in PF. Hydrolysis of TAG is initiated by ATGL and TAG is converted into diacylglyceride (DAG) and NEFA (Duncan et al., 2007). Following the release of the first free fatty acid, HSL continues to hydrolyze DAG and converts them into monoglyceride (MAG) and free fatty acid (Duncan et al., 2007). In addition, HSL is a multi-functional enzyme and catalyzes the hydrolysis of TAG and MAG (Lass et al., 2011). One of the major regulators of HSL is catecholamine-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) axis (Jaworski et al., 2007). When stored energy is needed, catacholamines are released and activate specific G-protein coupled receptors (GPCR) known as BAR. Following a series of signaling events, cAMP-bound PKA phosphorylates and activates HSL. Phosphorylation also facilitates the translocation of HSL from cytosol to lipid droplet surface (Jaworski et al., 2007). Kershaw et al. (2007) reported that ATGL is under the direct regulation of PPARc in mouse adipocytes and the gene of ATGL contains putative PPARc binding sites. This is consistent with our finding that HS but not PF decreased PPARc mRNA abundance exhibiting a similar regulatory trend to ATGL. Genetic knockdown of ATGL was also reported to be able to decrease gene expression involving fatty acid oxidation and overexpression of ATGL increased PPARa activity in rat hepatocytes (Ong et al., 2011). In adipocytes, PPARa agonist did not change the mRNA level of HSL or ATGL, but it induced the expression of genes related to fatty acid oxidation (Goto et al., 2011). Based on these observations, it seems logical that the activity of lipolysis is correlated to fatty acid oxidation in white adipose tissue, and PPARa, a sensor of

endogenous fatty acid and their derivatives (Lefebvre et al., 2006), acts as a link between these two processes. Although PPARa agonist can upregulate mRNA expression of adiponectin and its receptors in human and rodent models (Tsuchida et al., 2005; Hiuge et al., 2007), whether adipocyte PPARa is closely correlated to the whole body metabolism, especially fatty acid oxidation needs further confirmation. Study from brown adipose tissue demonstrated that lipolytic products induce gene transcription of PPARa (Mottillo et al., 2012). The decreases of HSL and ATGL indicate animals are less dependent on fatty acid oxidation for energy supply. Our data showed that HSL, ATGL, PPARa and PPARc had similar changing patterns: reduced in HS and unchanged in PF. Taken together, HS appears to tamp lipolytic activity leading to an inhibition of fatty acid oxidation.

Interestingly, the mRNA abundance of subcutaneous adipose tissue β3-adrenergic receptor (BAR3) was extremely low in our animals (data not shown). Among adrenergic receptor subtypes, BAR3 is predominantly expressed in adipocytes (Collins et al., 2004) and BAR3 specific agonist exhibits potent anti-obesity effect on rodents (Granneman et al., 2005). Studies on ruminants reported that BAR3's activation reduces visceral fat mass but had no effects on subcutaneous fat depots (Samadi et al., 2013) and the infusion of BAR3 agonist does not trigger lipolytic effect on ewes (Ferlay et al., 2001). This evidence, combined with ours, indicate that BAR3 might be expressed in visceral fat specifically and its activation might not be a major contributor of whole body lipolysis in ruminants. Human studies showed that the activation of BAR2 is able to increase plasma NEFA and fat oxidation (Hoeks et al., 2003) and promotes lipolysis (Du et al., 2014). In the present study, BAR2 tended to decrease when animals entered the HS period and this reduction might contribute to lowered lipolysis-related gene expression. In contrast, the PF exhibited the opposite regulation of BAR2 and lipolytic-related gene expression. Future work is needed to

determine the regulation of adipocyte BAR2 during HS and the associated role on expression of lipolytic genes.

Leptin (LEP) acts an essential regulator of body energy homeostasis and it is mainly produced in white adipose tissue (WAT) (Chilliard et al., 2005). Previous studies showed a close association between LEP mRNA level in adipose and nutritional status; fasting decreases adipose LEP mRNA abundance (Tsuchiya et al., 1998; Amstalden et al., 2000; Zhang et al., 2002). According to our qPCR results, however, LEP remained constant regardless of periods or group, which might indicate cows in our experiment did not reach sufficient negative energy balance to trigger the down-regulation of LEP.

Lipogenesis, the reversal of fatty acid β-oxidation, is the process by which acetyl-CoA or malonyl-CoA is converted into fatty acids. Acetyl-CoA is assembled into saturated fatty acids step by step by FAS, a multi-functional enzyme complex (Smith et al., 2003). Adipose FAS-specific knockout mice exhibit increased energy expenditure and brown fat-like adipocytes (Lodhi et al., 2012). In our case, both HS and PF significantly reduced the mRNA level of FAS and this is a sign that energy is shunted away from lipogenesis and acetyl-CoA is tunneled through other routes to compensate lower feed intake. Another study on mice reported that FAS inhibitor induced lower feed intake and weight loss (Loftus et al., 2000) and therefore, there might be a causal relation between FAS depression and voluntary reduction of feed intake in our HS group.

Aside from *de novo* lipogenesis, lipid accumulation in adipose can result from re-esterifying free fatty acids. Cellular fatty acid uptake is governed by LPL, a rate-limiting protein expressed in the endothelium of extrahepatic tissues, mainly in adipose and muscle (Weinstock et al., 1997). A previous study demonstrated that reducing LPL expression by siRNA transfection decreased

intracellular lipid level by 80% compared to the control in mouse adipocytes (Gonzales and Orlando, 2007). The significantly decreased LPL mRNA level observed in our HS animals but not PF group indicates a lower uptake and accumulation in adipose during hyperthermia. The genes of LPL, PEPCK, acyl-CoA synthase and adipose fatty acid binding protein are under the regulation of PPARc, which is known to be involved in adipose differentiation, and thus, PPARc is considered to have a lipogenic effect in WAT (Kersten, 2001). Fatty acid uptake by adipocyte can either enter fatty acid oxidation or reesterification. Quantitative estimates of triglyceride/fatty acid cycle showed that a substantial portion of fatty acid was reesterified back to triglyceride and only a small fraction of fatty acid released from lipolysis is oxidized (Reshef et al., 2003). With the increase in lipolysis, there must be a concomitant increase in the source of glyceride-glycerol to support reesterification. Glyceroneogenesis was proposed as a pathway to generate glyceride-glycerol from precursors other than glucose (Ballard et al., 1967; Reshef et al., 1970). This pathway is supported by the fact that PC and PEPCK, once thought to be only pertinent to gluconeogenesis, were expressed in WAT (Reshef et al., 2003), also confirmed by our qPCR data. The specific deletion of PEPCK in WAT abolished the generation of glyceride-glycerol from pyruvate, and mice carrying this deletion became lipodystrophic (Olswang et al., 2002). Under HS, the reductions of HSL, ATGL, PC and PEPCK mRNA abundances indicate a global suppression on triglyceride/fatty acid cycling, which differed from PF group. The mechanism underlying this suppression is unclear. Animals under HS might decide that reducing metabolic heat from triglyceride/fatty acid cycle outweighs the need of increasing energy support from adipose. But how this decision is made and where this signaling competition happens warrant further studies.

Heat stress, according to our data, is causing direct effects on animals separately from nutritional insults. Lipolysis-related genes were down regulated in the HS group while the same

set of genes remained unchanged in PF group. Attenuated lipolysis gene expression might be responsible for relatively low plasma NEFA level observed in our previous studies (Rhoads et al., 2009; Shwartz et al., 2009; Wheelock et al., 2010). Interestingly, genes responsible for lipogenesis were also blunted by HS while the majority remained unchanged in PF group. Although the net effect of lipolysis and lipogenesis on fat accumulation was not measured in this study, one would speculate that energy fluxes towards and away from adipose were both decreased during HS, indicating that the increased dependence on adipose for energy supply was attenuated.

Table 3.1. Ingredients and chemical composition of diets

Item	% of DM
Ingredient	
Alfalfa hay	62.49

Steam-flaked corn	27.25
Whole cottonseed	6.87
Maxxer[1]	0.91
Supplement[2]	2.49
Chemical analysis	
CP	17.67
NDF	25.39
ADF	19.75
NE_L, Mcal/kg of DM	1.76

[1]Calcium salts of palm oil (Tarome Inc., Eloy, AZ).
[2]The supplement contained 3.81% fat, 5.52% Ca, 6.42% P, 4.95% Mg, 0.59% S, 0.34% K, 1.87% Na, 6.84% Cl, 1881.82 mg/kg of Zn, 1869.44 mg/kg of Mn, 1171.58 mg/kg of Fe, 1108.18 mg/kg of Cu, 75.49 mg/kg Co, 12.91 mg/kg Se, 9.83 mg/kg of Mo, 47.61 mg/kg of I, 327.24 IU/g of vitamin A, 32.41 IU/g of vitamin D, and 1.03 IU/g of vitamin E.

Table 3.2. Effect of heat stress (HS) or pair-feeding (PF) on blood parameters in lactating Holstein cows

	Group 1			Group 2			P-Value		
Blood Parameter	TN	HS	SEM	WF	PF	SEM	Group	Period	Group × Period

Glucose, mg/dL	86.90	83.58	2.30	86.62	92.17	2.26	0.14	0.56	0.039
NEFA[1], ueq/L	234.87	293.78	44.09	144.77	187.10	43.74	0.12	0.047	0.72
BHBA[2], mg/dL	6.99	7.58	0.63	7.01	7.06	0.62	0.76	0.41	0.48
Insulin, µg/L	0.61	0.63	0.12	1.16	1.06	0.11	<0.01	0.80	0.55

[1]Non-esterified fatty acid.
[2]β-hydroxybutyric acid.

Table 3.3. Oligonucleotide primers used for real-time quantitative PCR (SYBR Green assay).

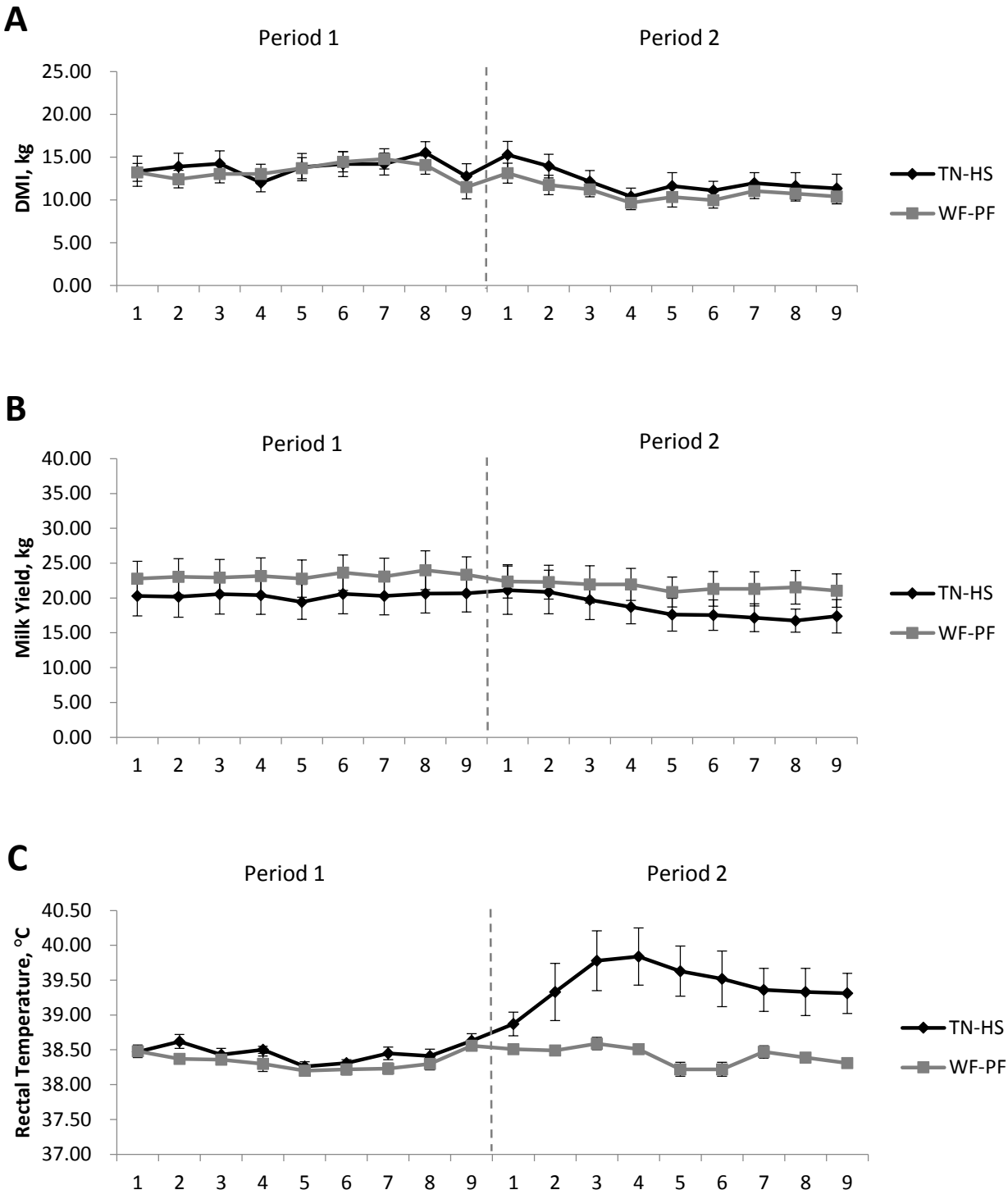
Transcript[1]	Sequence, 5'-3'	Reference
BAR1	F: CGAGCAGAAGGCACTCAAGAC	Sumner and McNamara, 2007

	R: CCAGGTCGCGGTGGAA	
BAR2	F: CCCCAGGCACCGAAAACT	Sumner and McNamara, 2007
	R: TCCCTTGTGAATCAATGCTATCA	
HSL	F: GAGTTTGAGCGGATCATTCA	Sumner and McNamara, 2007
	R: TGAGGCCATGTTTGCTAGAG	
ATGL	F: GTGGACGGTGGCATCTCAGA	Deiuliis et al., 2010
	R: AAGCGGAGGCCATCCCTGTA	
PLIN	F: AGACACTGCCGAGTATGCTG	Sumner and McNamara, 2007
	R: TGGAGGGAGGAGGAACTCTA	
LEP	F: TCACCAGGATCAATGACATCTCA	Thorn et al., 2006
	R: ACCAGTGACCCTCTGTTTGGA	
FAS	F: CTGATGAGCTGACGGACTCCA	Harvatine and Bauman, 2006
	R: GCGATTGGGCAGGGCT	
LPL	F: GAACTGGATGGCGGATGAAT	Harvatine and Bauman, 2006
	R: GGGCCCCAAGGCTGTATC	
PC	F: CGTCTTTGCCCACTTCAAGG	Bradford and Allen, 2005
	R: GGCGCGTATTGAGGCTG	
PEPCK	F: GTCCTGGCCCTGAAGCAGA	Bradford and Allen, 2005
	R: TCCTGCTCCTGGTGCGTTG	
PPARa	F: CGGTGTCCACGCATGTGA	Carriquiry et al., 2009
	R: TCAGCCGAATCGTTCTCCTAAA	
PPARc	F: AAGAGCCTTCCAACTCCCTCA	Harvatine et al., 2009
	R: CCGGAAGAAACCCTTGCAT	
IGF1	F: CCAGACAGGAATCGTGGATG	Carriquiry et al., 2009
	R: ACTTGGCGGGCTTGAGAG	

CYC	F: GTGGTCATCGGTCTCTTTGG	Deiuliis et al., 2010
	R: CACCGTAGATGCTCTTACCTC	
RPS15	F: ATCATTCTACCCGAGATGGT	Self-designed
	R: TGCTTCACGGGCTTGTAAGT	
18S	F: AGAAACGGCTACCACATCCA	Goossens et al., 2005
	R: CGCCAGACTTGCCCTCCA	

[1]Abbrevations: BAR1: β-adrenergic receptor 1; BAR2: β-adrenergic receptor 2; HSL: hormone sensitive lipase; ATGL: adipose triacylglycerol lipase; PLIN: perilipin; LEP: leptin; PC: pyruvate carboxylase; PEPCK: phosphoenelpyruvate carboxykinase; PPARa: peroxisome proliferator-activated receptor α; PPARc: peroxisome proliferator-activated receptor γ; IGF1: insulin-like growth factor 1; CYC: cyclophilin; RPS15: ribosomal protein S15; 18S: ribosomal RNA.

FIGURE 3.1.


A
Period 1
Period 2
DMI, kg
25.00
20.00
15.00
10.00
5.00
0.00
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
TN-HS
WF-PF
B
Period 1
Period 2
Milk Yield, kg
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
TN-HS
WF-PF
C
Period 1
Period 2
Rectal Temperature, °C
40.50
40.00
39.50
39.00
38.50
38.00
37.50
37.00
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
TN-HS
WF-PF

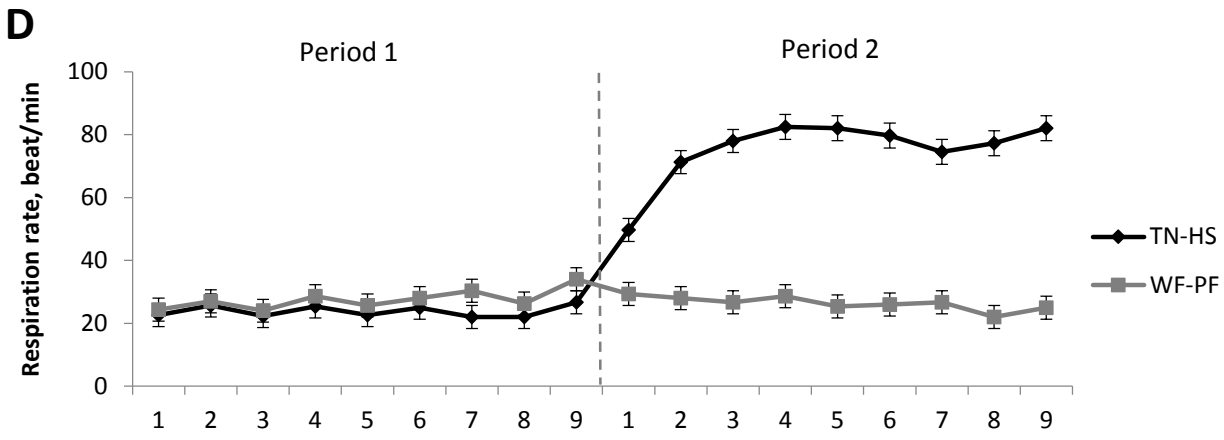


Figure 3.1. Effects of heat stress (HS) or pair-feeding (PF) on A) DMI, B) milk yield, C) rectal temperature and D) respiration rate of lactating dairy cows. Black lines with diamonds represent group 1 (TN-HS) and grey lines with squares represent group 2 (WF-PF). Group 1 cows were in thermal neutral, ad libitum conditions during period 1 (TN) for 9 d and heat stress, ad libitum during period 2 for 9 d. Group 2 cows were in thermal neutral throughout the study, fed ad libitum during period 1 (WF) for 9 d and pair-fed with HS group during period 2 (PF) for 9 d.

FIGURE 3.2.

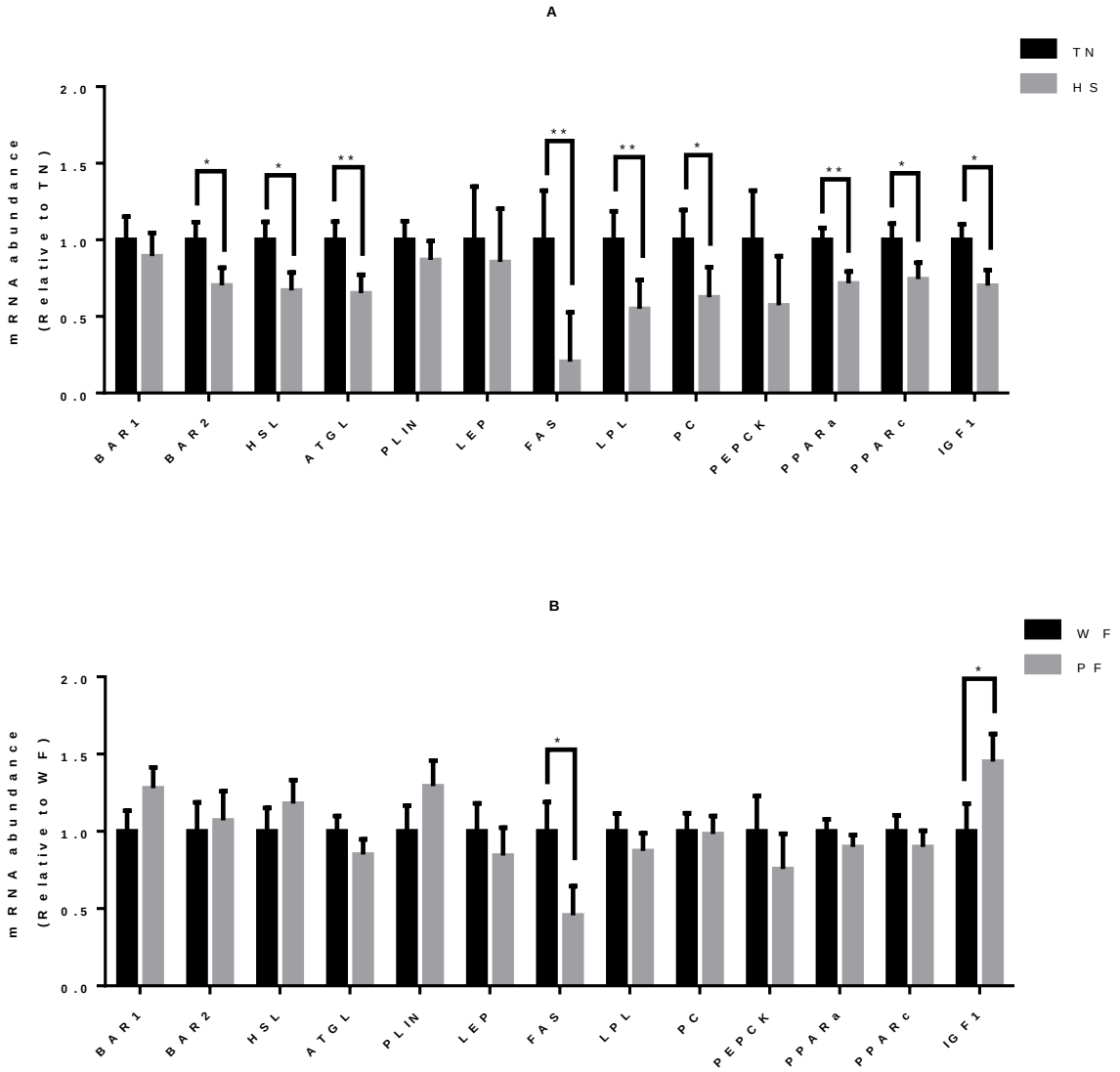
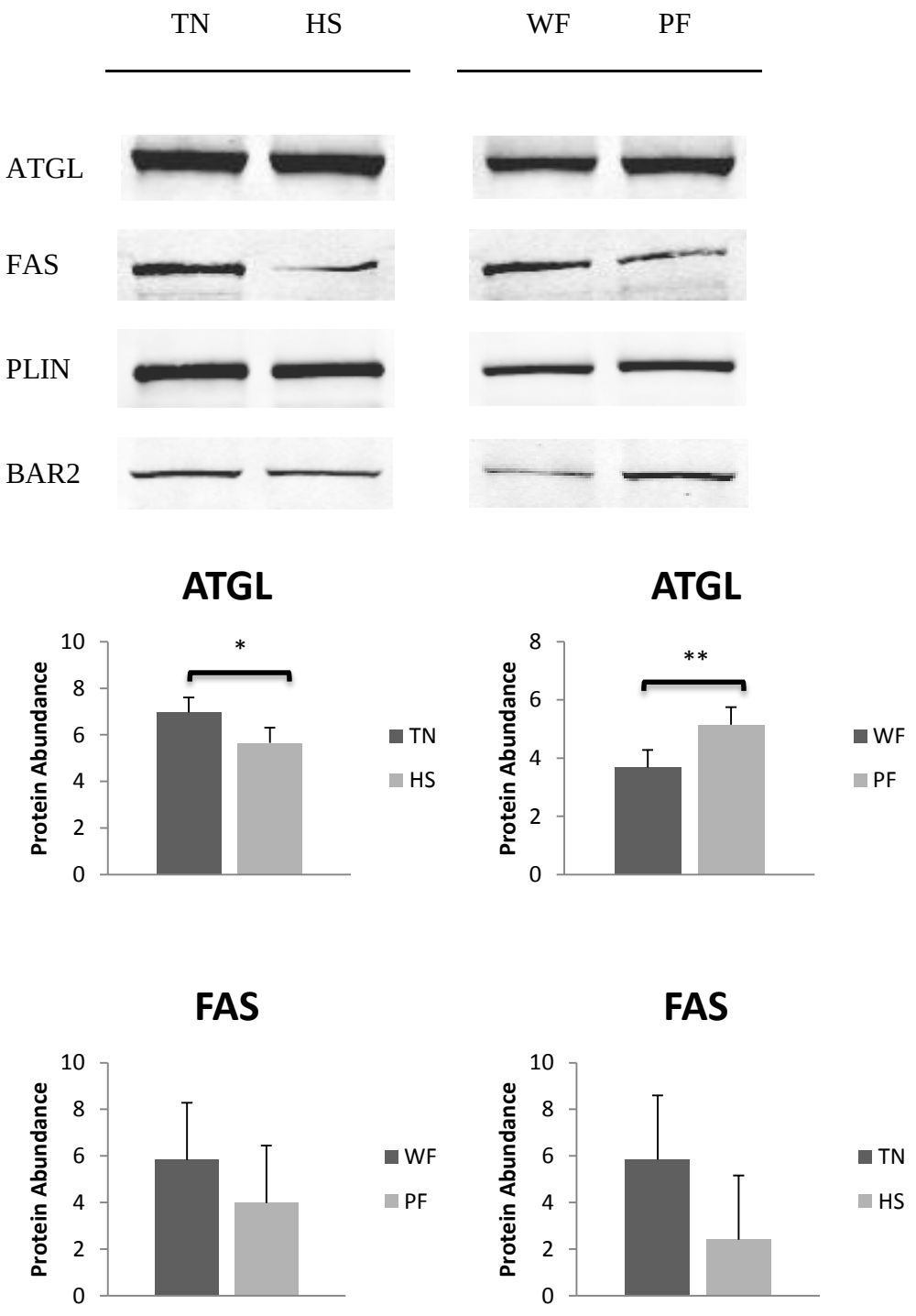


Figure 3.2. Effect of A) heat stress or B) plane of nutrition on lipid metabolism-related gene expression. Abundances of mRNA were measured by real-time quantitative PCR. Twelve late lactating dairy cows were exposed to heat stress conditions (group 1) or subjected to a reduced plane of nutrition (group 2). Group 1 cows were in thermal neutral, ad libitum conditions during

period 1 (TN) for 9 d and heat stress, ad libitum during period 2 for 9 d. Group 2 cows were in thermal neutral throughout the study, fed ad libitum during period 1 (WF) for 9 d and pair-fed with HS group during period 2 (PF) for 9 d. Results are represented relative to expression of genes in subcutaneous adipose tissue from TN or WF. Asterisk (*) signifies $0.05 < P < 0.1$, and double asterisks (**) indicate $P < 0.05$.

FIGURE 3.3.


TN
HS
WF
PF
ATGL
FAS
PLIN
BAR2
ATGL
Protein Abundance
10
8
6
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2
0
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TN
HS
ATGL
Protein Abundance
8
6
4
2
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WF
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Protein Abundance
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Protein Abundance
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TN
HS

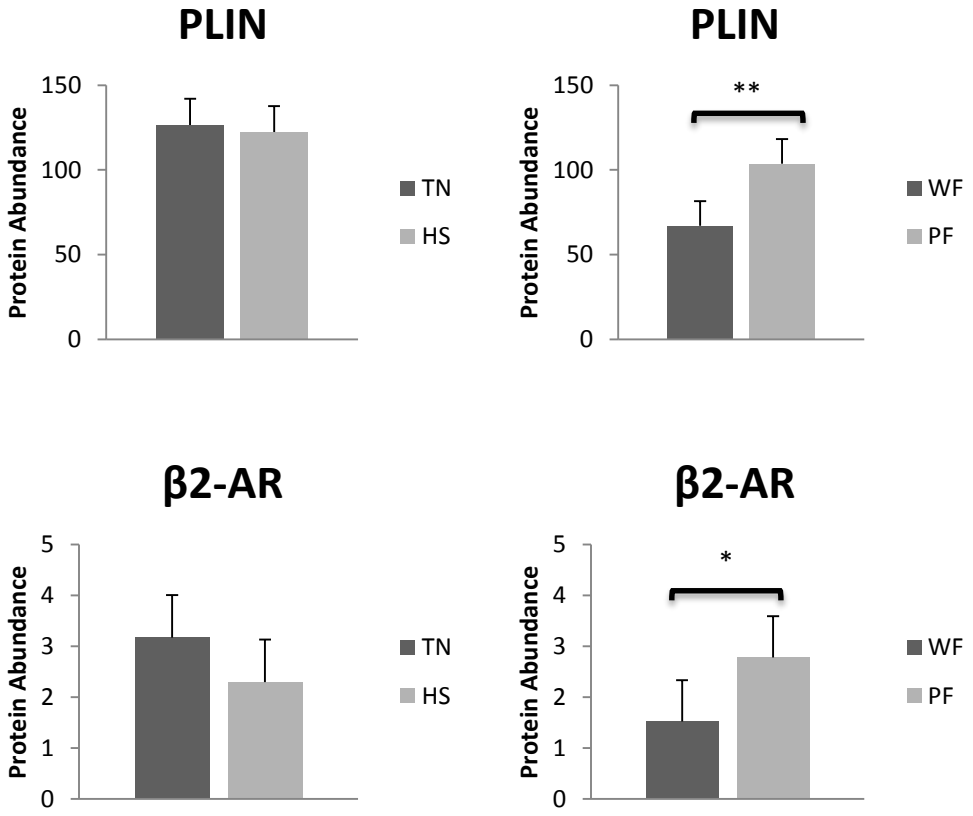


Figure 3.3. Effect of heat stress or plane of nutrition on lipid metabolism-related protein abundances. Twelve late lactating dairy cows were exposed to heat stress conditions or subjected to a reduced plane of nutrition. Group 1 cows were in thermal neutral, ad libitum conditions during period 1 (TN) for 9 d and heat stress, ad libitum during period 2 for 9 d. Group 2 cows were in thermal neutral throughout the study, fed ad libitum during period 1 (WF) for 9 d and pair-fed with HS group during period 2 (PF) for 9 d. Asterisk (*) signifies $0.05 < P < 0.1$, and double asterisks (**) indicate $P < 0.05$.

Chapter 4. Adaptation of lipid mobilization in lactating dairy cows under heat stress

Abstract

Malnourished animals mobilize adipose tissue to alleviate the impact of energy deficiency on galactopoiesis, but lactating dairy cows under heat stress (HS) lessen their dependence on this strategy. One possibility is that lipid-mobilizing response becomes refractory to adrenergic signaling during heat stress. To test this hypothesis, multiparous dairy cows (n = 6; parity = 4 ± 0.9; 436 ± 93 DIM; 721 ± 39 kg BW) housed in climate chambers were fed *ad libitum* with a TMR consisting primarily of alfalfa hay and steam-flaked corn and subjected to 2 experimental periods: 1) thermoneutral conditions (TN; 18°C, 20% humidity) for 9d and 2) HS conditions (cyclical temperature 31–40°C, 20% humidity) for 9 d. Rectal temperature (Tre) and respiration rate (RR) was measured thrice daily. During each period, cows were administered with epinephrine (Epi) intravenously on d 6 and intramuscularly (0.02 mg/kg) twice daily from d 7 to d 9. Serial blood collections were performed around Epi administration and plasma glucose, non-esterified fatty acid and insulin were measured. Before and after epinephrine administration, adipose biopsies were obtained from contralateral tailhead areas. Lipolysis and lipogenic-related proteins from adipose were measured by Western immunoblotting. Heat stress caused significant increases in Tre ($P < 0.05$) and RR ($P < 0.05$) and reduced DMI by 18% ($P < 0.05$) and milk yield by 10% ($P < 0.05$) compared to TN. Fatty acid release responding to Epi was reduced by HS ($P < 0.05$). Adrenergic stimulation increased the phosphorylatioin ratio of cyclic-AMP response element-binding protein ($P < 0.05$) and hormone sensitive lipase ($P < 0.05$) during TN but not in HS. Protein abundance of β2-adrenergic receptor maintained at the same level throughout all periods and treatments. Adipose triglyceride lipase protein expression was blunted ($P < 0.05$) by

epinephrine in both TN and HS. Fatty acid synthase, acetyl-CoA carboxylase (ACC) and phosphorylated ACC protein abundance were decreased ($P < 0.05$) by HS but were not altered by epinephrine challenge. Insulin receptor increased ($P < 0.05$) in HS regardless of epinephrine administration. Protein kinase B phosphorylation tended to increase ($P < 0.1$) in response to epinephrine during HS. These observations provide evidences showing that heat-stressed animals limit their lipid mobilization by tailoring key proteins as an adaptation for survival.

(**Keywords**: heat stress, adipose, lipid mobilization, adrenergic signaling, dairy cow)

Introduction

Although heat stress (HS) has been a subject of research for decades and various approaches (i.e. feed additives, cooling systems) had been implemented in dairy industry, it remains a threat to milk production and quality, and might be exacerbated by potential global warming (Gaughan et al., 2010; Shiao et al., 2011; Renaudeau et al., 2012; Peters et al., 2013). The voluntary feed intake decrease of dairy cattle under HS was traditionally considered as the major reason of milk yield decline. However, accumulating evidence indicates that HS has direct impacts, other than suppressing appetite, on animals' metabolism, especially on the glucose-sparing mechanisms involving fatty acids (Rhoads et al., 2009; Shwartz et al., 2009; Wheelock et al., 2010; Pearce et al., 2013a).

One of the puzzling observations from previous HS studies is that elevated circulating concentration of non-esterified fatty acid (NEFA) typically observed in feed restricted controls was not seen in heat stressed animals (Rhoads et al., 2009; Shwartz et al., 2009; Wheelock et al., 2010). Meanwhile, increased insulin level or glucose disposal rate were reported in previous HS animal studies (Itoh et al., 1998; Wheelock et al., 2010; Morera et al., 2012). It is tempting to speculate that HS affects metabolism by altering signaling pathways involved in glucose and fatty acid turnover processes, or reversely speaking, animals adjust their metabolic strategy to alleviate deleterious effects from elevated temperature. Among systemic energy regulation networks, insulin is the most potent anabolic hormone (Saltiel and Kahn, 2001) and adrenergic signaling is responsible for major catabolic reactions in adipose (Zechner et al., 2009). Enzymes involved in insulin and adrenergic signaling pathways are potential targets of HS.

In the present study, we hypothesized that HS blunted fatty acid mobilization by desensitizing β-adrenergic signaling and maintaining the normal insulin signaling functions. Enzymes that involved in lipolysis and lipogenesis were investigated, as well as those participate in β-adrenergic and insulin signaling.

Materials and methods

Animal and Experimental Design

Six multiparous, lactating Holstein cows (parity = 4 ± 0.9; 436 ± 93 DIM; 721 ± 39 kg BW) underwent a two-experimental-period (P) trial consisting of: 1) 9 d in thermal neutral (TN) conditions and ad libitum and 2) 9 d in HS and ad libitum. The HS environment was designed with cyclical temperatures (cyclical temperature 31-40 °C, 20% humidity). Cows were housed in individual tie stall stanchions at the University of Arizona Agricultural Research Complex, fed a TMR consisting primarily of alfalfa hay and steam-flaked corn and milked at 0600 and 1800 daily. Rectal temperature (Tre) and respiration rate (RR) were measured thrice daily at 0600, 1400 and 1800h. The nutritional composition of the TMR met the predicted requirements (NRC, 2001) of energy, protein, minerals and vitamins (Table 1). All procedures were approved by the University of Arizona Institutional Animal Care and Use Committee.

Acute Epinephrine Challenge and Plasma Collecting

An acute epinephrine challenge was performed on d 7 morning of each period. Each cow was administered an intravenous bolus of epinephrine (1.4 µg/kg BW) and blood samples were withdrawn from catheter at fixed time points (-45, -40, -30, -20, -10, -5, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 120, 125 and 130 min relative to epinephrine administration) as described previously (Rhoads et al., 2007). Samples were collected in evacuated containers with EDTA (BD Vacutainer, Franklin Lakes, NJ). Plasma was separated from collected whole blood with centrifugation at

1,500 × g for 10 min, aliquoted into 2-ml microcentrifuge tubes and stored at -20 °C.

Chronic Epinephrine Challenge and Adipose Biopsies

On d 7-9 of each period, animals were given epinephrine intramuscular (IM) injection (0.02 mg/kg, once in d 7 afternoon and d 9 morning, twice on d 8). Biopsies were performed on d 6 (before IM epinephrine challenge) and d 9 (after IM epinephrine challenge) to collect adipose tissue from contralateral tailhead areas of each animal as described previously (Rhoads et al., 2007). Adipose tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C. Blood samples were collected on d 9 at 30 min before I.M. epinephrine injection and 0, 1, 2, 3, 4 h after injection. Plasma was obtained as mentioned in last paragraph.

Plasma Analyses

Plasma glucose and NEFA were measured with commercially available kits (Glucose Assay Kit, Sigma, St Louis, MO; NEFA-HR kit, Wako Chemicals USA). Plasma insulin was measured with commercial ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden). The intra- and interassay coefficients of variation (CV) for acute epinephrine challenge samples were 2.56% and 4.40% for glucose, and 9.66% and 13.2% for NEFA. For chronic epinephrine plasma samples, the intra- and interassay CV were 1.94% and 3.36% for glucose, 5.51% and 7.97% for NEFA, and 1.98% and 4.21% for insulin.

Western Immunoblotting

Total protein extraction was previously described (Rhoads et al., 2007). Briefly, 1 g of pulverized frozen adipose tissue biopsy was homogenized in 5 ml sucrose buffer [50 mM Tris (pH 7.6), 250 mM sucrose, 5 mM EGTA, 150 mM NaCl, 1X Halt Protease and Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL)], and subjected to centrifugation (1000 × g for 5

min at 4 °C) to remove unsolubilized materials. Aqueous part was clarified twice by centrifugation (10,000 × g for 20 min at 4 °C) and supernatant containing total protein was stored at -80 °C.

Extract protein concentration were measured using the BCA protein assay (Pierce). Protein extract was mixed with Laemmli Buffer and β-mercaptoethonal and heated at 95 °C for 10 min. Fixed amounts of protein were loaded into 4% - 15% Criterion TGX Precast Gel (Bio-Rad, Hercules, CA) for electrophoresis and transferred to nitrocellulose membranes with Trans-Blot Turbo Midi Transfer Pack (Bio-Rad) in Trans-Blot Turbo Transfer Starter System (Bio-Rad). The membranes were stained with Ponceau S (Santa Cruz Biotechnology Inc., Dallas, TX) to verify equal loading and destained with 0.1% NaOH. The membranes were then blocked with Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) and 5% w/v nonfat-dried milk for 1 h at room temperature and incubated in primary antibody diluted with blocking solution at 4 °C overnight. Antibodies (Ab) were purchased from Cell Signaling Technology Inc. Danvers, MA unless otherwise specified. They were cyclic AMP response element-binding protein (CREB) Ab (1:100, sc-186 Santa Cruz Biotech.), phospho-CREB (Ser133) Ab (1:500, ab32096 Abcam Biotech Co., Cambridge, England), 5' AMP- activated protein kinase (AMPK) Ab (1:1000, #5831), phospho-AMPK (Thr172) Ab (1:1000, #2535), adipose triglyceride lipase (ATGL) Ab (1:1000, #2138), hormone sensitive lipase (HSL) (1:100, sc-25843 Santa Cruz Biotech.), phospho-HSL (Ser563) Ab (1:1000, #4139), fatty acid synthase (FAS) Ab (1:1000, #3180), perilipin (PLIN) Ab (1:100, sc-67164 Santa Cruz Biotech), acetyl-CoA carboxylase (ACC) Ab (1:1000, #4190), phospho-ACC (Ser79) Ab (1:1000, #3661), β2-adrenergic receptor (BAR2) Ab (1:100, sc-569 Santa Cruz Biotech), insulin receptor (IR) Ab (1:200, sc-711, Santa Cruz Biotech), protein kinase B (PKB/AKT) Ab (1:1000, #9272), phospho-AKT (Ser473) Ab (1:2000, #4060) and α-tubulin (1:1000, #2144). Membranes were washed three times (5 min each) with TBST on the next day

and incubated with the secondary antibody (IRDye 800CW Goat anti-Rabbit IgG; LI-COR Biosciences Inc, Lincoln, NE) at 1:20000 dilution in blocking solution for 1 h at room temperature. Signals from membranes were measured by Odyssey Infrared Imaging System (LI-COR) and analyzed with Odyssey Imaging software (LI-COR). Protein abundance was normalized using α-tubulin protein abundance.

Data and Statistical Analysis

Data were analyzed with Proc Glimmix of SAS 9.3. Plasma glucose, NEFA and insulin from acute and chronic epinephrine challenges and their area under the curve (AUC) were compared between periods with time as a repeated fixed factor. Protein abundance obtained from Western immunoblotting were analyzed with period, time relative to injection (pre or post), and period×time relative to injection as fixed effects; cow listed in Random statement; and Tukey adjustment. Significance was declared when $P < 0.05$ and $0.05 < P < 0.1$ was considered a tendency.

Results

Exposure to high ambient temperature increased rectal temperatures by 1.3 °C ($P < 0.01$) and respiration rate ($P < 0.01$) indicating experimental animals were experiencing HS (Figure 4.1C & D). Heat stress conditions decreased DMI by ~18% ($P < 0.05$) and milk yield by ~10% ($P = 0.05$) (Figure 4.1A & B).

Despite a lower plane of nutrition during HS, basal circulating glucose, NEFA and insulin remained unchanged (Figure 4.2 & 4.3). Both acute and chronic epinephrine challenges were able to stimulate a rapid increase of glucose and NEFA in both TN and HS. Plasma insulin was elevated by IM epinephrine injection in both periods and there was a numerical increase at 2 h in HS compared to the same time point of TN (Figure 4.3C). In addition, HS increased the circulating

NEFA response to acute ($P < 0.05$) (Figure 4.2B) but reduced that to chronic (AUC: $P < 0.05$) epinephrine challenges compared to TN (Figure 4.3B). Circulating glucose exhibited a higher response ($P < 0.05$) to acute epinephrine infusion in TN compared to HS (Figure 4.2A) but the response did not differ in response to the IM epinephrine administration (Figure 4.3A).

Protein abundance (Figure 4.4) of HSL and phospho-HSL were increased in HS compared to TN ($P < 0.05$, $P < 0.05$ respectively), while CREB and phospho-CREB protein abundance were reduced in HS compared to TN ($P < 0.1$, $P < 0.05$ respectively). Phosphorylation ratio of AMPK, CREB and HSL were augmented ($P = 0.056$, $P < 0.05$, $P < 0.05$ respectively) after IM epinephrine injection in TN while their basal abundance was upregulated during HS and was not increased by IM injection. Neither period nor epinephrine injection changed the expression of BAR2. Protein abundance of ATGL was suppressed by epinephrine challenge in both periods ($P < 0.05$). Fatty acid *de novo* synthesis rate-limiting enzyme FAS, ACC and phospho-ACC was down-regulated by HS ($P < 0.05$) and was not altered by epinephrine injection. Among insulin signaling proteins, IR increased during HS ($P < 0.05$) but was not affected by epinephrine injection. Phosphorylated of AKT tended to increase after epinephrine challenge during HS compared to that in TN ($P = 0.07$).

Discussion

Accumulating evidence suggest that HS, aside from reducing feed intake, impacts the metabolic profile in a more direct and profound manner (Rhoads et al., 2009; Shwartz et al., 2009; O'Brien et al., 2010; Rhoads et al., 2010; Wheelock et al., 2010; Rhoads et al., 2011). The most counterintuitive and curiosity-invoking observation is the relatively low circulating NEFA concentration (Rhoads et al., 2009; Shwartz et al., 2009; Wheelock et al., 2010) and relatively high

plasma insulin concentration (O'Brien et al., 2010; Wheelock et al., 2010) typically observed in heat-stressed animals compared to feed-restricted groups. During HS, calculations reveal that animals are in a state of negative energy balance and this situation is more severe in early- and mid-lactating animals. To offset the deleterious impact of energy deprivation, the dairy cow will typically enlist numerous metabolic adjustments in a homeorhetic manner. Normally, fatty acid mobilization is a major adjustment to provide energy in support of normal body functions and contribute to glucose and protein preservation (Drackley et al., 2001b; Lam et al., 2003; Norrelund et al., 2003). However, the HS dairy cow is paradoxical in this regard and in order to understand the mechanism underlying the low plasma NEFA concentration seen in heat-stressed animals, the present study sought to investigate changes in key lipolytic and lipogenic enzymes as well as key nodes of the β-adrenergic and insulin signaling axes. Ultimately, we would like to capture this information to create a model illustrating how HS exerts its influence on lactating cows, especially in terms of fatty acid metabolism.

Before an animal can mount any response to environmental fluctuations, such fluctuations have to be detected and interpreted by animal. With respect to energy homeostasis, AMPK is believed to be the major energy sensor in eukaryotes using a sensing mechanism that relies on AMP: ATP and ADP: ATP ratios (Hardie, 2011). Studies demonstrate that cAMP-inducing agents activate AMPK in adipocytes as a consequence of an increase in the AMP: ATP ratio due to the energy depletion caused by acylation of fatty acids from lipolysis (Koh et al., 2007; Gauthier et al., 2008). In this study, the ratio of phosphorylated AMPK, the activated form, increased in response to the epinephrine injection only in TN. During HS, the basal abundance of phosphorylated AMPK ratio was slightly increased and epinephrine administration was unable to stimulate phosphorylation markedly. A possible explanation may be the fact that heat-stressed

animals suffer from a negative energy balance (Wheelock et al., 2010) and the AMP: ATP would rise even without epinephrine stimulation. It has been reported that AMPK can phosphorylate HSL at Ser565 and render the latter a less active form (Anthony et al., 2009). One explanation for the seemingly contradictory roles played by AMPK is that the AMPK-mediated suppression of HSL would alleviate oxidative stress and prevent excessive release of NEFA. Subsequently, NEFA could later return to adipose and undergo reesterification, which is an ATP-costly process and exacerbates energy deprivation (Gauthier et al., 2008).

A direct result of epinephrine administration is the activation of adrenergic receptors and their downstream signaling pathways. In adipose, activation of β-adrenergic receptors by catecholamines leads to stimulation of lipolysis via G-protein coupled receptor (GPCR) -cAMP-PKA signal transduction (Duncan et al., 2007). Phosphorylations of PLIN and HSL resulting from PKA activation facilitate fatty acid mobilization in adipose (Miyoshi et al., 2006). At the same time, CREB is another phosphorylation target of activated PKA and is suspected to induce insulin resistance in adipose tissue (Mersmann, 1998; Qi et al., 2009). In the present study, phosphorylations of CREB and HSL follow a highly similar pattern across different treatments, indicating they might fall under the same mechanism of regulation, most likely through PKA. Both CREB and HSL phosphorylation ratio increased dramatically after epinephrine administration in TN. Basal phosphorylation ratios of these two proteins in HS period were as high as the stimulated status in TN and epinephrine treatment did not further increase their phosphorylation. There are two plausible explanations for these observations during HS: 1) phosphorylation was at its maximum and could not be augmented by epinephrine; 2) HS suppressed phosphorylation of these proteins by impeding phosphorylating or triggering dephosphorylating machineries, or uncoupling cAMP-PKA chain from β-adrenergic receptor, or a combination of different mechanisms

mentioned above. Data from our acute epinephrine challenge showed that during HS, administration of epinephrine triggered a more robust response of circulating NEFA compared to those in TN. Since the concentration of a substance in circulation is a function of both appearance and removal, one could speculate that either the lipolytic response is greater or tissue uptake of NEFA is reduced during HS. Certainly during TN, it is likely epinephrine caused a lipolytic response consistent with greater total amounts of HSL and phosphorylation of HSL in subcutaneous adipose tissue. However, HS activated basal HSL and CREB and exogenous epinephrine had no further effect indicating a lack of lipolytic stimulation. Perhaps the acute rise in circulating NEFA had more to do with epinephrine altering peripheral tissue metabolism away from fatty oxidation allowing plasma NEFA to accumulate. Alternatively, a positive effect of epinephrine on lipolysis during HS might be the result of increased access to lipid droplets because activated PKA can also phosphorylate PLIN and convert the latter to a pro-lipolytic status (Duncan et al., 2007). Regardless, additional studies are needed to determine the mechanisms responsible for the rise in NEFA during an acute epinephrine challenge.

The protein expression of another important lipolytic contributor, ATGL, was surprisingly attenuated by epinephrine regardless environmental conditions. Gene expression of ATGL has been reported to be down-regulated by insulin and forskolin, a cAMP inducing regent, in a dose-dependent manner in porcine preadipocytes (Deiuliis et al., 2008). To our knowledge, this is the first observation showing the suppressing effect of epinephrine on ATGL in adipose tissue. Diacylglycerol (DAG) and NEFA are the main products of ATGL. When DAG exceeds the rate of muscle and liver use, it can directly inhibit insulin-mediated glucose transport activity (Erion and Shulman, 2010) and a considerable portion of NEFA undergoes re-esterification (Reshef et al., 2003), leading to more severe energy deprivation. Based on the explanation regarding AMPK's

inhibition of HSL presented by Gauthier et al. (2008), the impact of epinephrine on ATGL suppression in the present study might serve as a preventative mechanism to avoid excessive release of NEFA and DAG, which might exaggerate the negative energy balance in heat-stressed animals.

De novo fatty acid synthesis is an ATP- and NADPH-costly process and we would anticipate that the protein abundance of lipogenic enzymes would decline in a calorie restriction state. However, this regulatory pattern is true only in liver but not in adipose (Mulligan et al., 2008). Calorie restriction enhanced mRNA and protein abundance of FAS and ACC and endogenous synthesized palmitate in adipose tissue (Bruss et al., 2010; Hancock et al., 2011), and these counterintuitive phenomena might be a result of matching the requirement of elevated fatty acid oxidation rate in calorie restricted animals (Bruss et al., 2010). In contrast, protein abundance of FAS and ACC were substantially reduced after animals entered HS in the present study. Phospho-ACC, a less active form of ACC, declined during HS and the phosphorylation ratio of ACC remained unchanged throughout the study, leading to a reduction in active ACC as a result. This apparent discrepancy is another unique influence seen during HS and it might be associated with the reduced NEFA response compared with pair-fed group observed in previous studies.

The mechanisms by which HS suppresses appetite and lipogenesis in adipose are unclear but leptin may be a promising candidate that mediates these HS effects. Leptin is known as a hormone that suppresses appetite mediated by serotonin (Yadav et al., 2009) and downregulates lipogenesis by decreasing the protein abundance of FAS, ACC and phospho-ACC (Buettner et al., 2008). Decreased plasma leptin is considered a sign of calorie restriction (Weigle et al., 1997; Wadden et al., 1998). Increased lipogenesis in adipose observed in calorie restriction might be a result of reduced leptin signaling. In addition, Buettner et al. (2008) demonstrated that phosphoinositide 3-

kinase (PI3K) was required to achieve the anti-lipogenesis effect of leptin and this effect was independent of insulin signaling because AKT phosphorylation ratio was unchanged after brain leptin infusion. A study by Scherer et al. (2011) confirmed that the lipogenesis in white adipose tissue (WAT) induced by brain insulin infusion was independent of peripheral insulin signaling. Therefore, peripheral insulin sensitivity may not relate to lipogenesis in WAT and may both be the outcomes of increased systemic leptin signaling under HS conditions.

While β-adrenoceptors govern the release of stored energy, insulin acts as a potent anabolic regulator supporting glucose uptake, lipogenesis and inhibition of gluconeogenesis, glycogenolysis and lipolysis (Saltiel and Kahn, 2001). In the present study, insulin signaling appeared to be altered by HS. Leptin and adiponectin, both known for their insulin sensitizing effects, were reported to be upregulated by HS in terms of mRNA abundance in WAT of mice (Morera et al., 2012). In the present study, HS increased protein abundance of IR, which might be associated with previously reports of potential increased insulin sensitivity (Wheelock et al., 2010). However, IRS1 remained unchanged and this is in agreement with mRNA data from a mouse study (Morera et al., 2012). As discussed earlier, insulin signaling in WAT may not necessarily relate to lipogenesis. Leptin signaling, on the other hand, might have a more dominant effect regulating fatty acid metabolism. Meanwhile, AKT/PKB increased after epinephrine injection in both periods. Cross activation of adrenaline on PKB was reported in rat muscle via cAMP (Brennesvik et al., 2005). This cross activation might be responsible for the inhibition of ATGL since incubation of 3T3-L1 cells with insulin decreased both mRNA and protein abundance of ATGL and administration of inhibitor of PI3K, the immediate upstream protein of AKT, completely reversed insulin's effect on ATGL (Chakrabarti and Kandror, 2009). The cross activation of AKT/PKB

mediated by epinephrine and insulin might act as a negative feedback on lipolysis to prevent excessive releases of NEFA and DAG as mentioned in previous paragraph.

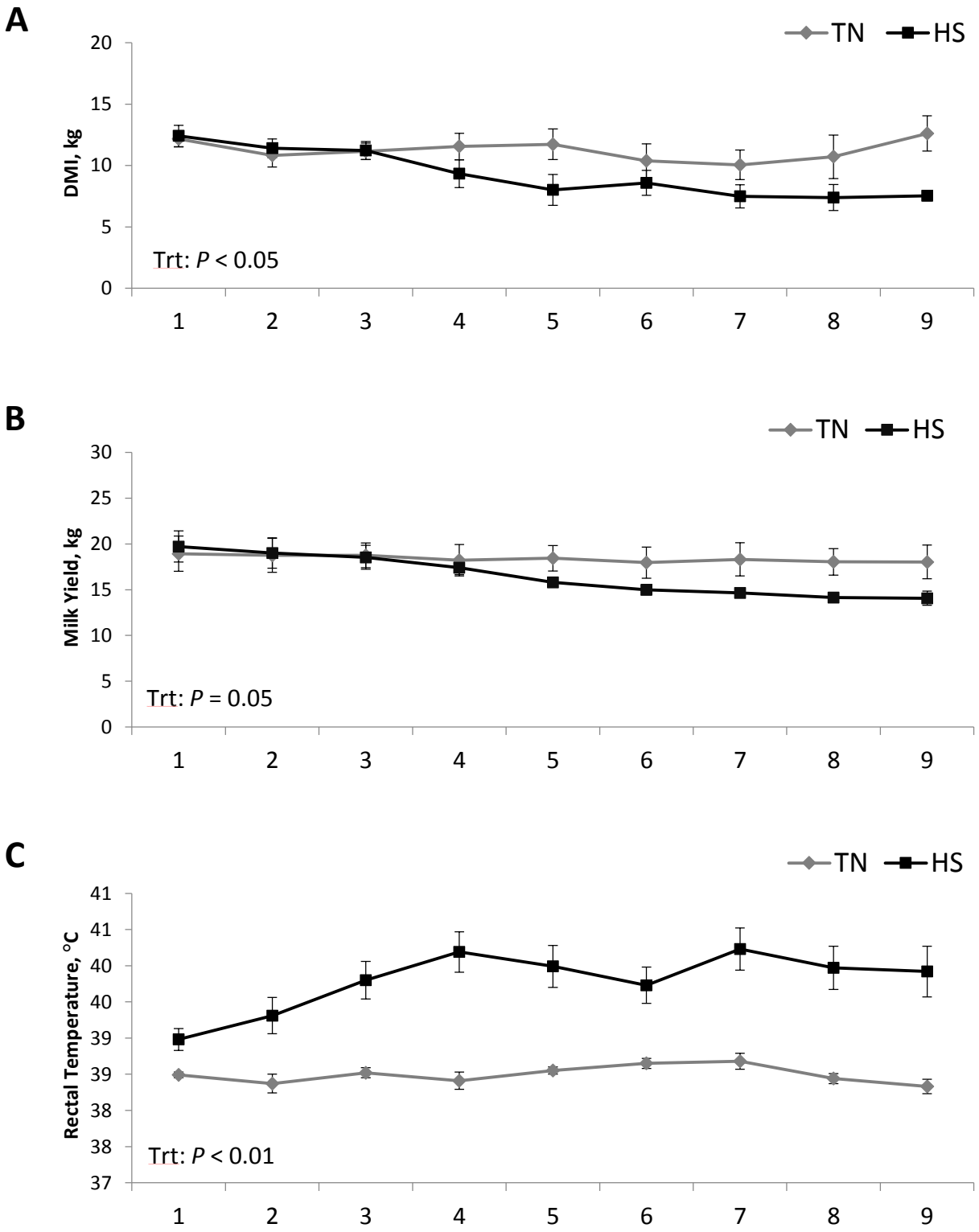
Lipid mobilization is a universal method employed by animals to counter energy fluctuations. However, this strategy can short-circuited by 1) futile cycle of lipolysis and reesterification: 30%-40% of NEFA is reesterified during fasting and the rate of reesterification can increase proportionally with the rate of lipolysis (Gauthier et al., 2008) and 2) increased *de novo* lipogenesis to match the increased rate of lipolysis (Bruss et al., 2010). These anabolic reactions accompanying lipid mobilization lower energy efficiency and produce waste heat, which would be an undesirable product for animals suffering HS. Therefore, heat-stressed animals may employ other approaches, such as reducing milk yield, promoting glucose absorption (Wheelock et al., 2010), elevating hepatic glucose output (Febbraio, 2001), etc. to fill the gap of blunted lipid mobilization. The blunting effect of HS on lipolysis might stem from limiting the availability of lipid via suppressing *de novo* lipogenesis. Controlling heat production by fine-tuning energy influx and efflux from lipid might outweigh maintaining milk production for animals under HS.

Table 4.1. Ingredients and chemical composition of diets.

Item	% of DM
Ingredient	
Alfalfa hay	62.49
Steam-flaked corn	27.25
Whole cottonseed	6.87
Maxxer[1]	0.91
Supplement[2]	2.49
Chemical analysis	
CP	17.67
NDF	25.39
ADF	19.75
NE_L, Mcal/kg of DM	1.76

[1]Calcium salts of palm oil (Tarome Inc., Eloy, AZ).
[2]The supplement contained 3.81% fat, 5.52% Ca, 6.42% P, 4.95% Mg, 0.59% S, 0.34% K, 1.87% Na, 6.84% Cl, 1881.82 mg/kg of Zn, 1869.44 mg/kg of Mn, 1171.58 mg/kg of Fe, 1108.18 mg/kg of Cu, 75.49 mg/kg Co, 12.91 mg/kg Se, 9.83 mg/kg of Mo, 47.61 mg/kg of I, 327.24 IU/g of vitamin A, 32.41 IU/g of vitamin D, and 1.03 IU/g of vitamin E.

FIGURE 4.1.


A
TN
HS
DMI, kg
Trt: P < 0.05
B
TN
HS
Milk Yield, kg
Trt: P = 0.05
C
TN
HS
Rectal Temperature, °C
Trt: P < 0.01

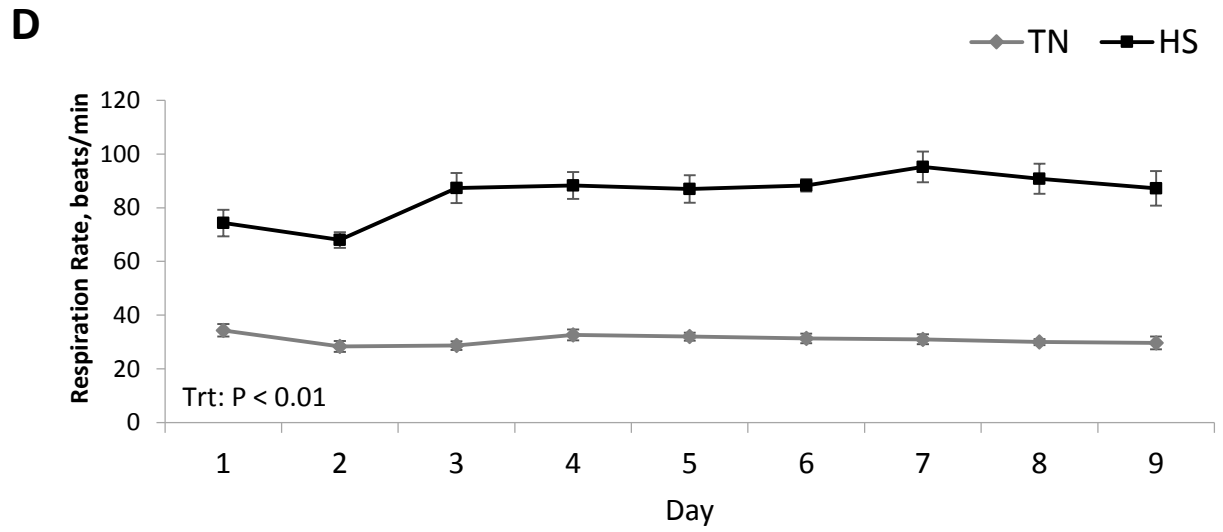


Figure 4.1. Effects of heat stress (HS) on A) DMI, B) milk yield, C) rectal temperature and D) respiration rate of lactating dairy cows. Cows were in treatment 1) thermal neutral (TN), ad libitum conditions for 9 d and then on treatment 2) in HS, fed ad libitum for 9 d.

FIGURE 4.2.

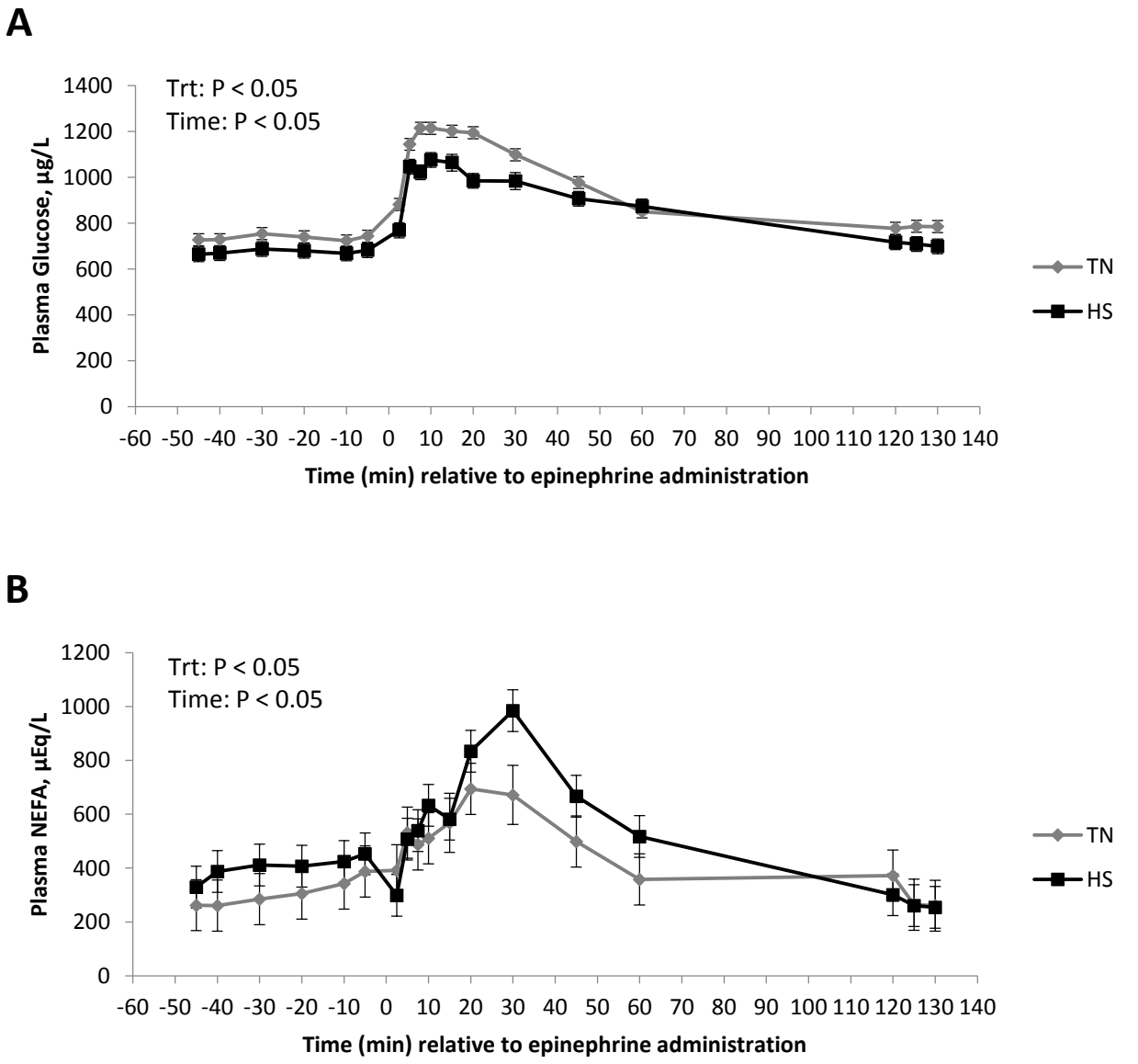


Figure 4.2. Effects of heat stress (HS) on A) plasma glucose, and B) plasma NEFA in response to intravenous epinephrine administration on lactating dairy cows. Cows were on treatment 1) thermal neutral (TN), ad libitum conditions for 9 d and then on treatment 2) in HS, fed ad libitum for 9 d. On d 7 morning of each treatment, epinephrine was administered via jugular catheter and blood samples were collected at -45, -40, -30, -20, -10, -5, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 120, 125 and 130 min relative to epinephrine administration.

FIGURE 4.3.

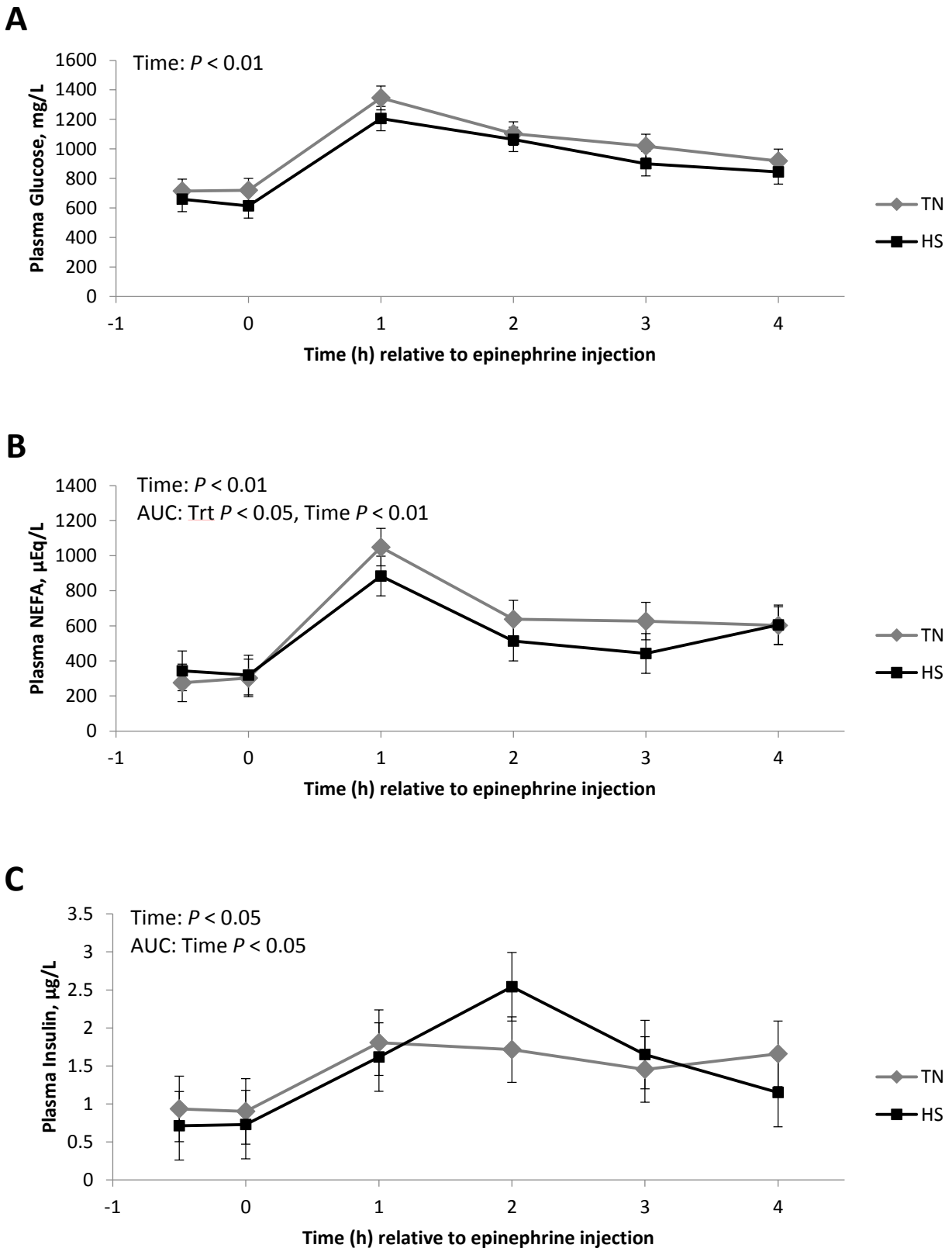
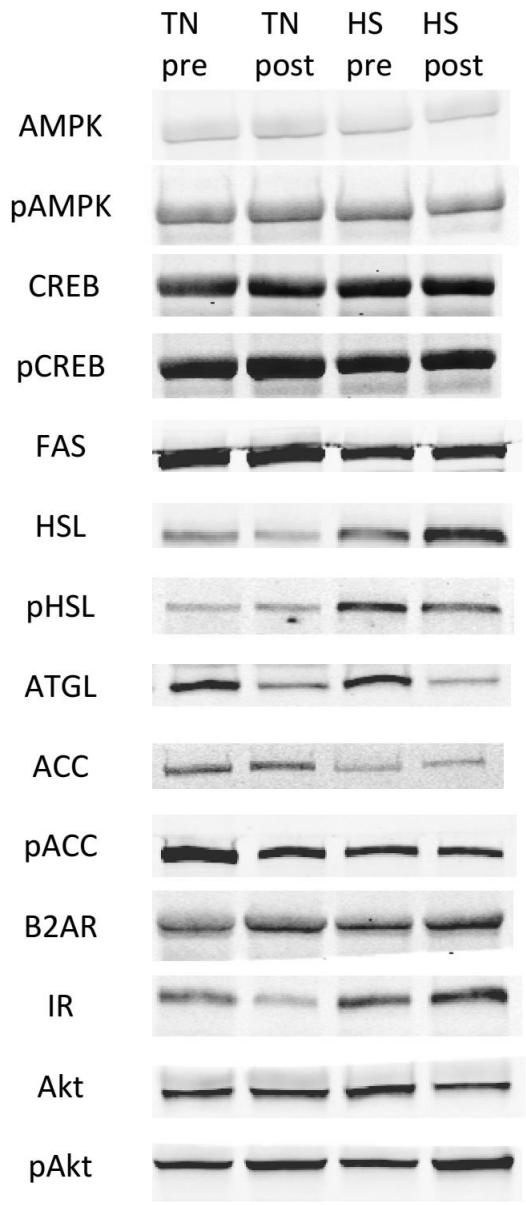

A
Time: P < 0.01
Plasma Glucose, mg/L
1600
1400
1200
1000
800
600
400
200
0
-1
0
1
2
3
4
Time (h) relative to epinephrine injection
TN
HS
B
Time: P < 0.01
AUC: Trt P < 0.05, Time P < 0.01
Plasma NEFA, µEq/L
1400
1200
1000
800
600
400
200
0
-1
0
1
2
3
4
Time (h) relative to epinephrine injection
TN
HS
C
Time: P < 0.05
AUC: Time P < 0.05
Plasma Insulin, µg/L
3.5
3
2.5
2
1.5
1
0.5
0
-1
0
1
2
3
4
Time (h) relative to epinephrine injection
TN
HS

Figure 4.3. Effects of heat stress (HS) on A) plasma glucose, B) plasma NEFA, and C) plasma insulin in response to intramuscular epinephrine administration on lactating dairy cows. Cows were on treatment 1) thermal neutral (TN), ad libitum conditions for 9 d and then on treatment 2) in HS, fed ad libitum for 9 d. On d 9 of each treatment, epinephrine was administered intramuscularly and blood samples were collected at -0.5, 0, 1, 2, 3, 4 h relative to epinephrine administration.

FIGURE 4.4.


TN pre
TN post
HS pre
HS post
AMPK
pAMPK
CREB
pCREB
FAS
HSL
pHSL
ATGL
ACC
pACC
B2AR
IR
Akt
pAkt

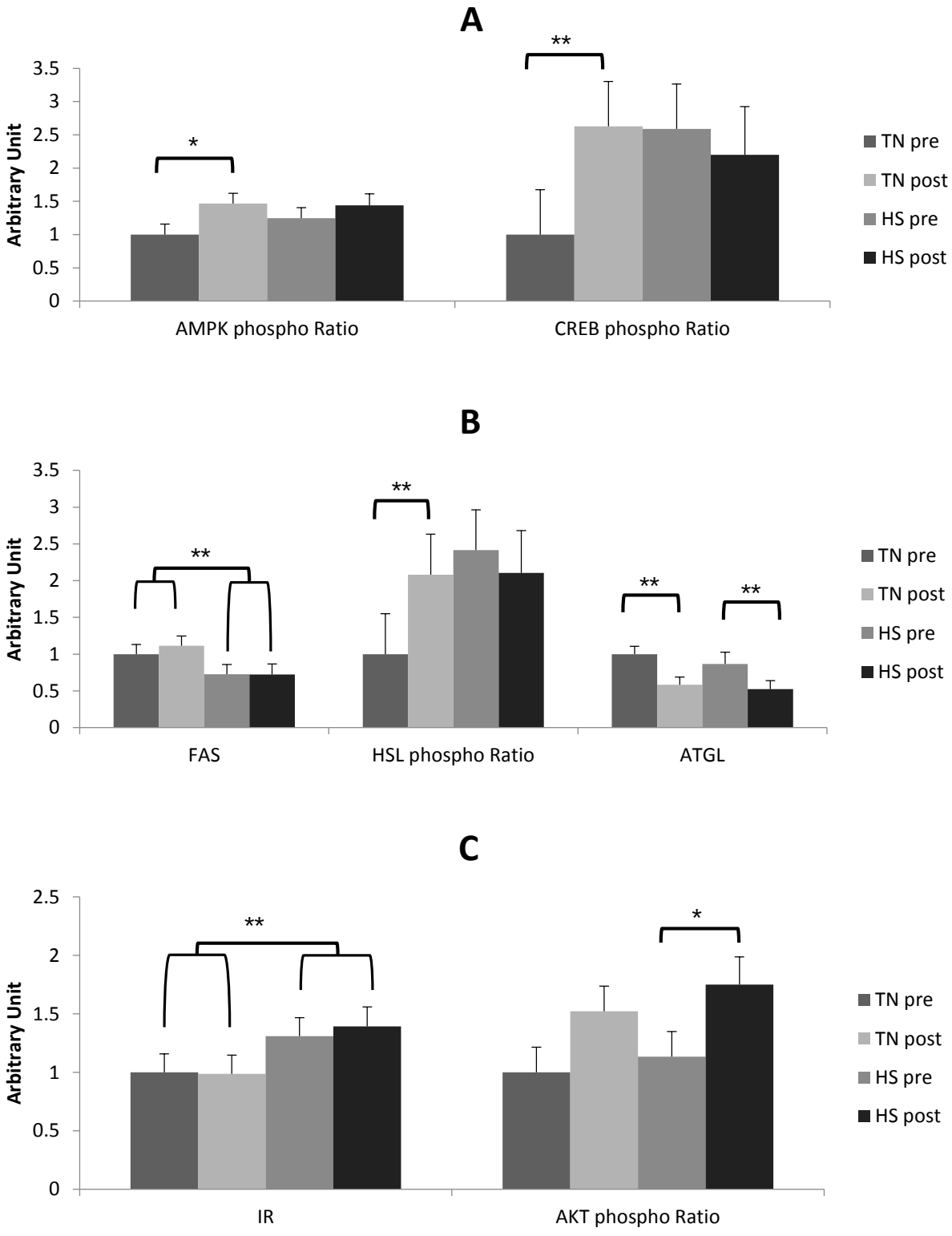


Figure 4.4. Effect of heat stress lipid metabolism-related protein abundances in subcutaneous adipose tissue. Fixed amount of protein extracted from adipose biopsies were

analyzed by Western immunoblotting for the abundances of 5' AMP-activated protein kinase (AMPK), phospho-AMPK (Thr172), cyclic AMP response element-binding protein (CREB), phospho-CREB (Ser133), adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), phospho-HSL (Ser563), fatty acid synthase (FAS), perilipin (PLIN), acetyl-CoA carboxylase (ACC), phospho-ACC (Ser79), β2-adrenergic receptor (BAR2), insulin receptor (IR), protein kinase B (AKT), phospho-AKT Cows were on treatment 1) thermal neutral (TN), ad libitum conditions for 9 d and then on treatment 2) in HS, fed ad libitum for 9 d. All protein abundances were normalized by α-tubulin protein abundance. Asterisk (*) signifies $0.05 < P < 0.1$, and double asterisks (**) indicate $P < 0.05$.

Chapter 5. Summary and Future Perspectives

Glucose homeostasis in HS

In this series of studies, basal plasma glucose level remained stable in our animals under both TN and HS conditions. These results are inconsistent with our previous lactating cattle studies (Shwartz et al., 2009; Wheelock et al., 2010) and rodent study (Morera et al., 2012), in which HS groups had lower blood glucose compared with TN, but in line with our growing bull data (O'Brien et al., 2010). When accounting for a potentially higher pack cell volume in heat-exposed animals, likely due to dehydration, the unchanged blood glucose concentration would indicate a virtual decrease in blood glucose concentration. The basis for this loss of glucose is unclear. Decreased glucose concentration is usually accompanied by increased circulating insulin level (Itoh et al., 1998; Wheelock et al., 2010; Morera et al., 2012). Insulin-mediated glucose uptake is believed to rely on the recruitment of GLUT4 in skeletal muscle and adipose tissues (Whiteman et al., 2002). Elevated expression of GLUT4 mRNA in these tissues has been demonstrated in a rodent model under HS (Morera et al., 2012). We investigated the upstream events of GLUT4 redistribution. Protein abundance of IR was upregulated in adipose tissue but unchanged in skeletal muscle and liver. Abundance of IRS1 and phosphorylation of AKT were maintained in skeletal muscle, adipose and liver in both TN and HS. Plasma glucose can also be derived from gluconeogenesis and glycogenolysis. Both in vivo (Rhoads et al., 2011) and in vitro (White et al., 2012) studies showed that gluconeogenic gene expression were upregulated by HS. Therefore, a diminished plasma glucose reservoir is unlikely due to suppressed gluconeogenesis. Alternatively, reduced glucose output from glycogen might contribute to decreased blood glucose. In our acute epinephrine challenge study, epinephrine administration triggered a smaller spike of plasma glucose in HS than TN. Glycogenolysis, can be stimulated by activation of BAR, which triggers

glycogen breakdown by activating glycogen phosphorylase (Berg et al., 2002). Glycogen kinetics is regulated by insulin as well, which suppresses net hepatic glycogenolysis through activating glycogen synthase (Petersen et al., 1998). Because muscle has low glucose 6-phosphatase activity (Gamberucci et al., 1996), glycogenolysis in muscle does not contribute substantially to the release of blood glucose and liver should be the predominant glucose source in response to an epinephrine challenge. The impact of HS on glycogen balance in liver deserves further investigation, especially on the fluxes through glycogenolysis and glycogen synthesis, as well as the related pivotal enzymes regulating pathways.

Why and how heat stress suppresses appetite

Animal eat more to support heat production at a temperature below the TNZ, and eat less to reduce heat generation when the environment exceeds the TNZ (Wansink, 2004). However, given that the energy expenditure for maintenance increases during heat stress (McDowell et al., 1969; Morrison, 1983; Beede and Collier, 1986), decreasing feed intake when suffering from high ambient temperature sounds counterintuitive. It remains unknown that whether suppressed appetite is an adjustment for better survival, or a side-effect of an effort to lower core temperature. Brobeck (1948) suggested that feed intake is neither determined by body energy status nor by body temperature, but rather by the capability of animal to dissipate heat generated by feed digestion and thus, feed intake might serve as an approach to regulate body temperature. Ingram and Legge (1974) suggested feed intake might be inhibited when an animal is trying to establish a new thermal equilibrium and eating might disturb this process because body temperature starts rising immediately with the onset of eating and evaporative heat loss from panting can be totally eliminated by the act of eating. The feed intake response that we observed in our series of dairy cattle trials indicates that the appetite control center might be over-reacting to the temperature

change and can be self-adjusted to some extent, but they never returned to the same level as those in thermoneutral conditions. More recent studies revealed that feed intake is regulated by complex central nervous and endocrine systems involving a long list of neuronal structures and signal molecules (Schwartz et al., 2000; Morton et al., 2006). Molecules such as leptin, ghrelin, neuropeptide Y, agouti-related protein, etc. might have potential roles in regulating appetite in thermal stress and their synthesis and release profiles under these environmental circumstances are largely unknown and warrant further study.

NEFA Metabolism

HSL phosphorylation level and ATGL abundance do not directly predict plasma NEFA level, because high phosphorylated HSL is not necessarily associated with NEFA release while low ATGL did not prevent NEFA rise. The control of plasma NEFA is more complicated than we expected. This control might be on trafficking route of cytosol HSL to lipid droplet, perilipin and/or other proteins that control the accessibility of lipid, transporters that control the exiting of NEFA from cell to blood, other less well-known compensatory lipase, or different responses to epinephrine that appear in different adipose depots (visceral fat, intramuscular fat). Alternatively, increased NEFA might be a result of restricted uptake of FA in FA-utilizing tissues, e.g. skeletal muscle and mammary gland, and thus slower clearance of NEFA from blood stream.

Due to the rapid NEFA response (< 5 min) that we observed in our acute epinephrine challenge study, the series of actions: activation of cytosol lipases, traveling to the surface of droplet, executing hydrolysis and eventually leading to an increase of plasma NEFA that is detected have to occur in a very short period. The colocalization of GPCR, AC, cAMP, PKA and PDE mediated by AKAP has been confirmed (Perino et al., 2012). Thus, similar anchoring

machinery for PKA, HSL, perilipin and other lipid droplets proteins would be a reasonable strategy that could again be utilized by adipocytes. In fact, optic atrophy 1 (OPA1), a protein related to autosomal OPA, known to be present in inner mitochondrial membrane to regulate mitochondrial dynamics during apoptosis, has been recently identified as an AKAP associated with lipid droplets (Greenberg et al., 2011; Pidoux et al., 2011). To overcome the concomitant decrease of perilipin with the knockdown of OPA1, Pidoux et al. (2011) constructed and introduced wild-type OPA1 and a mutated form that could not bind to PKA, both were not sensitive to shRNAi, in adipocytes with endogenous OPA1 knockdown. What they found is that adipocytes with wild-type OPA1 construct exhibited normal PKA-stimulated lipolysis, while mutated OPA1 abolished PKA's lipolytic function and perilipin phosphrylation without affecting cAMP level and HSL phosphorylation. This may serve as a possible explanation for the HSL hyperphosphorylation without concomitant NEFA elevation that we observed in HS group. Heat stress might be affecting OPA1 and perilipin while leaving upstream PKA activation and HSL phosphorylation intact.

The debate about whether NEFA transport across the plasma membrane is predominantly by free-diffusion or protein-mediation is still ongoing. However, it is clear that membrane proteins are involved in trafficking and facilitate NEFA entry in heart and skeletal muscle cells (Glatz et al., 2010). The entry of NEFA can also be possibly suppressed by accumulated intracellular NEFA as a result of depressed fatty acid utilization, because NEFA movement across membrane is ultimately driven by gradient (Glatz et al., 2010). The competition between glucose and fatty acids as respiratory fuel has been described as a glucose-fatty acid cycle and the mechanism about how fatty acids inhibit glucose oxidation is well established (Randle, 1998). Moreover, fatty acids are able to reduce muscle glucose uptake despite hyperinsulinemic conditions (Kelley et al., 1993). If the epinephrine-induced NEFA elevation was due to reduced uptake of NEFA by fatty acid-

oxidative tissue, e.g. skeletal muscle, HS would serve as a rare model in which glucose oxidation is preferred over fatty acid and something other than increased circulating insulin is mediating this effect. There is evidence from our unpublished pig trial showing that skeletal muscle from HS group had a lower metabolic flexibility and thus less fatty acid utilization compared with control.

Another possibility is that epinephrine-triggered NEFA elevation is a consequence of lipolysis other fat depots instead of subcutaneous fat. In humans, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) have distinct metabolic characteristics. Differential responses to catecholamines and insulin appear between VAT and SAT: VAT is more sensitive to catecholamine activation than SAT and this might be a result of higher BAR density and less insulin and α2-adrenergic signaling-mediated antilipolytic effects in VAT compared with SAT (Engfeldt and Arner, 1988; Wahrenberg et al., 1989). Differential effects of HS on SAT and VAT depots could account for the discordant NEFA response observed between the acute and chronic epinephrine challenges.

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