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I am submitting herewith a dissertation written by Emily Rebecca Cope entitled "Influence of Exogenous Beta-hydroxybutyrate on Metabolic Efficiency, Luteinizing Hormone, and Hypothalamic and Pituitary Gene Expression in Sheep." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

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Influence of Exogenous Beta-hydroxybutyrate on Metabolic Efficiency, Luteinizing Hormone, and Hypothalamic and Pituitary Gene Expression in Sheep

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Emily Rebecca Cope
May 2018

DEDICATION

This dissertation is dedicated to my family whose support, encouragement, and laughs gave me the strength to purse my Ph.D.

ACKNOWLEDGEMENTS

With great gratitude I thank my Lord, Jesus Christ, for all my many blessings; without whom, none of this would possible. With great sincerity, I thank my parents for instilling in me a work ethic that has no limits. To my father, Bill, who always told me to never skip the hills, even when the run is tough, because I was only cheating myself. To my mother, Kat, for your unconditional love, understanding, and proofreading. To my husband, Andrew, whose support, love, and sacrifices have been boundless. Thank you for always being my sounding board and voice of reason, in both science and life. Andrew, this whole journey would have been impossible without you and your constant encouragement; thank you.

To my advisors Dr. Travis Mulliniks and Dr. Brynn Voy for your guidance, mentorship, and pearls of wisdom. Dr. Mulliniks, thank you for always helping me to remember to look at the picture and not be bogged down with the unknowns. Also, thank you for sharing a love of doughnuts and ice cream that was free of judgement. Dr. Voy, thank you for your patience and willingness to teach, even if takes more than *just a minute*. Thank you both for your commitment to my success, for I am a better scientist because of your influence. Also, I thank you both for your contagious passion and dedication to your profession that is inspiring.

Thank you to my committee members Dr. Brian Whitlock, Dr. Meg Staton, and Dr. Dallas Donohoe for providing support, scientific consultation, and mentorship. Each of you have been an integral part of my success and for that, I am grateful.

I appreciate the support, help, and laughs from my lab mates and peers in the Animal Science department. Many thanks to Sarah Edwards, Jeremy Hobbs, and Zach McFarlane for being part of the original Mulliniks Mafia. Thank you to my friends in the department for always being willing to help, even if sheep were involved. Also, thank you to Suchita Das for being a superior lab technician and an all-around delightful person. Lastly, I want to express my extreme gratitude and thankfulness to the University of Tennessee, Knoxville Animal Science department for your support, both financially and professionally throughout my graduate studies. Overall, it has been a pleasure. Cheers!

ABSTRACT

Four studies were conducted to determine the effect of central administration of exogenous beta-hydroxybutyrate (BHB) on circulating metabolites, luteinizing hormone (LH), and hypothalamus and pituitary differential gene expression in sheep. In three of the studies, wethers were assigned to be centrally injected into the lateral ventricle of the brain through intracerebroventricular cannulas (ICV) with a single injection of BHB solution (BHB; 12,800 µmol/L) or saline solution (CON; 0.9% NaCl). In Exp. 1, between CON and BHB treated sheep, 11 and 44 genes were differentially expressed (adj. $P <$ 0.05) within the pituitary and hypothalamus, respectively. Functional enrichment analyses revealed BHB altered expression of genes in pathways related to stimulus perception, inflammation, and cell cycle control. In Exp. 2, wethers were injected through the ICV with one of 4 treatments: 0, 400, 800, or 1,600 µmol/L of BHB solution. Serum glucose concentrations tended ($P = 0.08$) to decrease linearly with increasing concentrations of BHB. Amplitude of LH peaks decreased linearly ($P = 0.03$) with increasing concentrations of BHB. In Exp. 3, wethers were centrally injected with either 12,800 µmol/L BHB or saline. Serum glucose and insulin concentrations increased ($P <$ 0.01) with BHB injection. Injection of BHB decreased ($P < 0.01$) circulating serum NEFA concentrations. Injection of BHB did decrease ($P < 0.01$) mean LH concentration. Wethers injected with BHB had decreased ($P < 0.01$) amplitudes of LH peaks. In Exp. 3, ovariectomized ewes were assigned to be fed either at BW maintenance (**MAINT**) or fed at a 30% feed reduction (**RES**). Ewes were randomly assigned to be centrally injected for 10 d with 300 µl into the lateral ventricle twice daily with one of two treatments of either

BHB or saline. Serum glucose concentrations decreased (P = 0.02*)* with infusion of BHB. Serum NEFA concentration increased (P < 0.01) with RES ewes. Luteinizing hormone exhibited a tendency (P = 0.06) for a diet × treatment interaction. Overall, elevated BHB in the brain may mimic a negative energy signal leading to alterations in serum concentrations of metabolites and LH.

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

In cow-calf systems, the inability for livestock to remain reproductively productive is a major limiting factor in production efficiency (Bellows and Short, 1994). With that in mind, optimizing reproductive efficiency is imperative for sustainability and profitability of cow-calf producers. To achieve this, nutrient supply has to meet nutrient requirements for maintenance, lactation, and reproduction. However, glucogenic precursors are often limited due to ruminal fermentation of forage-based diets to meet the glucose demand for metabolism (Cronje et al., 1991), impairing utilization efficiency of ruminal acetate and circulating NEFA (Preston and Leng, 1987). Thus, energy efficiency of grazing beef cows may be reduced and inadequate to meet the demand and needs. In addition, this imbalance is even more exacerbated after calving with the increase demand for glucose to support lactation requirements. Limited glucogenic precursors can lead to the depletion of oxaloacetate as key intermediate for gluconeogenesis; resulting in a rise in metabolic dysfunctions (Hawkins et al., 2000). Metabolic dysfunctions and imbalances can be defined as low blood glucose and insulin concentrations concomitantly detected with elevated beta-hydroxybutyrate (BHB) concentrations (Butler, 2003; Zarrin et al., 2013). Metabolic dysfunctions can negatively affect animal performance, such that overall reproductive efficiency is comprised. For instance, pre-calving and pre-breeding circulating BHB concentration has shown to delay conception date in young beef cow (Mulliniks et al., 2013; Hobbs et al., 2017). Metabolic dysfunctions influence on reproduction; however, the mechanisms of action are unclear. Therefore, this dissertation

discusses the role BHB has on nutrient metabolism and reproductive performance in ruminants.

ENERGY UTILIZATION

In ruminants, nutrients are partitioned for growth, maintenance, and establishment of body reserves such as lean and adipose tissue. The main priority of any ruminant is first to maintain basal metabolism. After maintenance needs are met, additional energy is allocated or partitioned accordingly. In a review, Short et al. (1990) suggested the order of nutrient partitioning priority is as follows: 1) basal metabolism, 2) activity, 3) growth, 4) basic energy reserves, 5) maintenance of pregnancy, 6) lactation, 7) additional energy reserves, 8) estrous cycles and initiation of pregnancy. The priority of partitioning can change depending on the current physiological state and can further be adjusted to maintain metabolic homeostasis. For example, postpartum cows prioritize metabolizable energy first towards milk production, then growth, and finally the regaining of adipose tissue (Lucy, 2003). However, under the same metabolic load, cows will partition and adapt to metabolic demands differently (Sundrum, 2015).

The inability to adjust during energy demanding periods can lead to distribution of homeostasis; therefore, it is important for coordinated metabolic adaptations to occur. Aside from attenuated homeostasis, homeorhetic controls are necessary to orchestrate metabolism of body tissues to support necessary physiological state (Bauman and Currie, 1980). While both are important for whole body metabolism regulation, homeostasis is critical for acute metabolic changes, while homeorhesis is involved in chronic metabolic changes (Bauman and Currie, 1980). Furthermore, homeostasis can be further defined by

metabolic adaptations through mobilization of body reserves, influence of endocrine control, and altered sensitivity to insulin (Bell, 1995). Key tissue regulators of metabolic homeostasis include the liver, adipose tissue, and skeletal muscle (Bell, 1995). For instance, Rauw et al. (2014) evaluated BW change of ewes during a Nevada winter in a resource-poor environment. The study determined the ewes that lambed the previous year were better adapted to harsh, nutrient poor conditions and lost less BW than the ewes that had not lambed the year before (Rauw et al., 2014). Homeorhetic animals are able to prioritize resources and are more efficient at mobilizing adipose tissue; thus, indicating enhanced adaptability among this group of animals (Rhoads et al., 2013).

Negative energy balance

Negative energy balance (NEB) can arise during altered metabolic states when increased energy requirements exceed those of dietary energy intake (Fenwick et al., 2008), which often times is caused by the inability to consume adequate energy to meet requirements for lactation, growth, and reproduction (Mulliniks et al., 2011). When dietary energy intake is limited or does not meet the energy demands of the animal, energy must be synthesized from body storage of lean and adipose tissue (Herdt, 2000). During periods of energy deficiency, adipose tissue provides a critical role to provide necessary energy by mobilizing stored fatty acids through lipolysis. This mobilization of stored fats and lean muscle to support growth and milk production can result in a loss of body condition, which is associated with blood metabolite and hormone alterations (Chandra et al., 2011). Under similar metabolic demands, some cows experiencing NEB are able to cope with NEB through mechanisms of metabolic adaptation (Herdt, 2000).

Failure to adapt to NEB has also been associated with ketosis, fatty liver, and insulin action impairment (Herdt, 2000; Tardif et al., 2001). If fatty acid oxidative rate declines during NEB, a large accumulation of liver triacylglycerides leads to fatty liver syndrome, thus potentially resulting in insulin sensitivity and increased ketogenesis (Grummer, 1993). Concentrations of serum metabolites such as glucose, NEFA, and BHB, along with the hormone, insulin, are commonly used as an indirect measure of the adaptation to NEB in cattle (Canfield and Butler, 1990).

One of the most critical aspects of metabolic homeostasis in ruminants is glucose metabolism. Ruminant metabolic homeostasis is regulated by glucose metabolism. Through microbial fermentation of carbohydrates, little glucose is directly absorbed, such that less than 10% of glucose from ruminal fermentation is utilized by the ruminant (Young, 1977). Therefore, glucose requirements have to be met by gluconeogenesis. However, ruminal fermentation of volatile fatty acid production from forages yields inadequate quantities of glucogenic precursors (Waterman et al., 2007). Additionally, forage-based diets have an increased ruminal production of acetate leading to an imbalance in acetate:propionate ratio (McCollum, 1983; Cronje et al., 1991). The imbalance in acetate:propionate ratio can lead to unfavorable alterations in energy metabolism (Waterman et al., 2006). As a result, propionate production can become limiting, subsequently decreasing gluconeogenesis (Aschenbach et al., 2010; Endecott et al., 2012). If glucose availability is decreased or glucose metabolism is inhibited, then oxaloacetate, a key intermediate for gluconeogenesis, can become depleted, as it is preferentially utilized for gluconeogenesis (Waterman and Butler, 2010). Furthermore,

the decrease in gluconeogenesis could potentially result in a reduction of acetate oxidation and utilization (Preston and Leng, 1987). For instance, increasing glucogenic potential of the diet has shown to increase energy utilization rate in young range beef cows grazing low-quality native range (Mulliniks et al., 2011). Insufficient supply of dietary glucogenic precursors result in other glucogenic substrates being utilized leading to catabolism of tissues to supply those glucogenic precursors (Waterman and Butler, 2010).

During periods of increased energy demands as such lactation, or when forage quality is limited, cows must utilize mobilization of stored adipose tissue to meet energy demands. Increased demands for tissue mobilization to support lactation results in increased circulating NEFA concentrations. During periods of increased lipid mobilization, circulating NEFA enter the liver and can exceed the capacity of the liver to oxidize fatty acids to acetyl CoA (Ospina et al., 2010); thus, a feedback mechanism may inhibit lipolysis through the regulation of fat mobilization (Metz et al., 1974). When the maximum ability of the liver to completely oxidize NEFA through beta-oxidation and triglyceride storage is exceeded, circulation concentrations of ketones increase (Walsh et al., 2007). Inadequate intracellular glucose availability can lead to inefficient acetate utilization which further contributes the futile production of ketone bodies (Waterman and Butler, 2010; Mulliniks et al., 2011). Ketone body production can occur by one of two pathways: 1) reduction of acetoacetate as a result of incomplete oxidation of fatty acids in hepatic mitochondria or 2) oxidation of butyrate in ruminal epithelial cells (Krehbiel et al., 1992). Within the liver, the predominate ketone body produced is BHB.

Though the liver is the main site of ketone synthesis, the liver cannot utilize ketone bodies directly for acetyl-CoA production due to lacking succinyl-CoA:3:ketoacid-coenzyme A transferase 1 (Goodridge and Sul, 2000).

Negative energy balance can be further exacerbated by increased concentrations of ketone bodies. Elevated BHB inhibits insulin secretion by changing mitochondrial metabolism (Yamada et al., 2010). In addition, Tardif et al. (2001) reported impaired insulin action in rat cardiomyocytes with elevated ketone bodies. Beta-hydroxybutyrate inhibits the activation protein kinase B (PKB) phosphorylation, thus blocking the translocation of GLUT-4 to the cell membrane (Tardif et al., 2001; Yamanda et al, 2010). Prolonged exposure to BHB resulted in inhibition of insulin-mediated glucose uptake by soleus muscle in mice (Yamada et al., 2010). Elevated insulin and BHB concentrations have been reported concomitantly with impaired glucose uptake (Zarrin et al., 2013); thus indicating a potential role of ketone body induced insulin resistance (Tardif et al., 2001).

Energy balance and reproduction

The relationship between energy balance and reproduction are maintained through energy sensing mechanisms. Furthermore, it has been suggested that there is an energy sensing mechanism within the central nervous system (CNS), specifically glucose-sensing, that connects energy balance to reproduction (Kinoshita et al., 2003; Martin et al., 2008). More specifically, alterations or disruptions of the neuroendocrine feedback axes are likely responsible for this relationship (Martin et al., 2008). Glucose is a key mediator for nutrition and reproduction, thus satisfying metabolic requirements (Short and Adams, 1988). Negative energy balance impairs fertility, but the mechanisms that

couple energy status to reproductive function are poorly defined. Reproductive function can be adversely affected by metabolic dysfunctions and nutrient imbalances (Mulliniks et al., 2013). The manifestation of metabolic dysfunctions, as through elevated BHB, can modulate peripheral metabolic status and reproductive incompetence in domestic ruminants (Herdt, 2000; Mulliniks et al., 2013; Hobbs et al., 2017).

The magnitude of negative energy balance varies between cows (de Vires and Veerkamp, 2000); therefore, making their response to the energy deficient important. When energy expenditure is greater than energy intake, alterations in energy balance can occur. Energy metabolism is the mechanism by which intermediary signals are used to convey the relations between energy status and physiological status in cows (van Knegsel et al., 2005). The effects of NEB have been associated with decreased fertility, subsequently leading to poor conception rates in ruminants (Wathes et al., 2003; Wathes et al., 2007; Mulliniks et al., 2013). The impact of NEB on reproduction has been reported to effect the uterus (Butler, 2000), ovary (Llewellyn et al., 2007), and oocyte (Leroy et al., 2004). Endogenous signals from NEB contribute to the inhibition or facilitation of reproduction (Beam and Butler, 1999; Mulliniks et al., 2013). Follicular growth is affected by hormones and metabolites whose secretion is dependent on the degree of NEB of the cow (Lucy et al., 1992). During NEB, metabolite changes occur such that there are delays in reproductive hormones that would otherwise initiate follicle stimulation (Butler, 2003). Hess et al. (2005) reported prolonged anestrous in cows experiencing extended periods NEB. Additionally, the negative feedback effect of estradiol to the hypothalamus extends anestrous in cows experiencing NEB (Short et al.,

1990). The extent and duration of NEB experienced by the cow is important for determining when normal ovarian cyclicity will resume after parturition (Pushpakumara et al., 2003). Excessive NEB has been reported to decrease pregnancy in lactating dairy cows 70 d postpartum (Ospina et al., 2010).

The time point of going from NEB to positive energy balance after calving is defined as BW nadir (Mulliniks et al., 2012). The occurrence of BW nadir is highly correlated to when estrus cyclicity resumes (Butler et al., 1981; Canfield and Butler, 1991). For instance, Mulliniks (2008) illustrated that BW nadir and reproduction can be uncoupled in young range cows if strategic dietary nutrients were supplied. However, in general, negative energy balance is negatively correlated to reproduction. Largely, central control via the hypothalamic-pituitary-gonadal (HPG) axis is likely influenced by energy deficits. The HPG axis is responsible for the development and regulation of the reproductive system, which operates through both positive and negative feedback mechanisms that control the secretion and release of hormones. The hypothalamus is charged with releasing hormones that cause the release of other hormones at target tissues (Asimakopoulos, 2012). The HPG axis produces hormones that are responsible for modulating reproduction and regulate central and peripherally produced hormones (Vadakkadath Meethal and Atwood, 2005). The hypothalamic neuropeptide, gonadotropin releasing hormone (GnRH), controls the release of luteinizing hormone (LH) by the pituitary in a pulsatile manner. Release of GnRH from the hypothalamus to the pituitary is carried by the hypothalamo-hypophyseal-portal system to gonadotrope cells in the anterior pituitary (Martin et al., 2008). This begins a feedback loop in the

peripheral with tissue activins that signal the hypothalamus to secrete GnRH (Vadakkadath Meethal and Atwood, 2005). Once GnRH binds to its receptor, signal transduction events occur such that subsequent hormones are released resulting in the formation of a neuroendocrine axis (Bliss et al., 2010). The intensity and frequency of the GnRH pulse influences the release of LH (Thackray et al., 2010). The LH pulses released by the pituitary are reflection of GnRH released into the hypophyseal portal circulation (Clarke and Cummins, 1985). Release of LH from the pituitary gland drives follicular maturation, estradiol production, and ovulation (Hileman et al., 1993; Schillo, 1992). After LH is secreted, through receptor binding, stimulation of gonadal sex hormone production, oogenesis, and spermatogenesis occur (Vadakkadath Meethal and Atwood, 2005). The sex steroids, through negative feedback inhibition, signal back to hypothalamus and pituitary to decrease gonadotroph production (Senger, 2003). Estradiol produced by the developing follicles in the ovaries decreases GnRH from the hypothalamus, through a negative feedback loop (Plant, 2015).

Cardoso et al. (2015) describes the dependence of fertility on the metabolic signaling by the hypothalamus through nutritional programming. Specifically, within the hypothalamus the preoptic area (POA) is responsive to neuronal signals, nutritional metabolites, and reproductive hormones (Waterman and Butler, 2010). Additionally, the hypothalamus medial basal (MBH) is responsive to signals from hormones, like insulin (Waterman and Butler, 2010). Together the POA and MBH from the hypothalamus interrupt neuroendocrine responses to regulate anterior pituitary secretions of

reproductive hormones (Waterman and Butler, 2010). Aside from the POA and MBH, the arcuate and ventromedial nuclei make up the tonic center for GnRH.

Central pathways sense and mediate the effects of NEB on fertility. Bridges et al. (2012) suggested reproduction is affected by nutritional deficiency through 1) directly altering GnRH secretion at the level of hypothalamus or gonadotrophs release from pituitary or 2) indirectly through changes in metabolic hormones. When oxidizable fuels are inadequate, hypothalamic neurons are unable to sustain GnRH pulse (Schneider, 2004), subsequently affecting LH concentrations. Reproduction may be influenced by alterations in circulating metabolites associated with metabolic dysfunctions that may indicate a signal elicited by shifts in fuel oxidation (Szymanski et al., 2007). Metabolic status impacts reproductive function through the modulation of the neural network of GnRH in the hypothalamus (Garcia-Garcia, 2012). For instance, central infusion of BHB decreased LH mean concentration and pulsatility in female, ovariectomized rats (Iwata et al., 2011). The effects of nutrient deprivation on reproductive performance and efficiency have been attributed to the absence and (or) decrease in pulsatile LH secretion in cattle and sheep (Thomas et al., 1990; Hileman et al 1992, Schillo, 1992). Due to a decreased LH concentration, anestrous can be induced (Schneider et al., 2012). Inadequate luteal stimulation can result from deficient LH secretion via impaired LH pulse amplitude (Jain et al., 2007). Previous reports have suggested a decrease in LH amplitude may be caused by decreased responsiveness by the pituitary; thus, potentially explaining LH amplitude sensitivity to energy status (Fitzgerald et al., 1987).

Availability of energy substrates, such as glucose, at the CNS may serve as potential signals to regulate reproductive success (Chilliard et al., 1998). Glucose is required as a metabolic fuel for the CNS and inadequate supply has been associated with reduced release of GnRH from the hypothalamus (Funston et al., 1995; Lucy, 1996; Wetteman et al., 2003). Decreased glucose availability and alterations in gluconeogenesis have been previous implicated in nutritional anestrous in cattle (McCann and Hansel, 1986; Short and Adams, 1988; Randle, 1990) as a potential regulator of GnRH release (Funston et al., 1995). Metabolic imbalances, especially those associated with a reduction in gluconeogenesis, result in a reduction in LH secretion (Hess et al., 2005). Glucose concentration may potentially be detected by hindbrain ependymocytes, controlling energy homeostasis and reproduction (Iwata et al., 2011). During NEB, when oxidizable fuels are inadequate, hypothalamic neurons are unable to sustain GnRH pulse (Schneider, 2004). Inadequate availability of utilizable glucose reduces the release of GnRH (Wettemann et al., 2003), subsequently affecting the secretion of LH. Funston et al. (1995) utilized a glucose antagonist, 2-deoxy-D-glucose, in ovariectomized ewes and reported LH releasing mechanisms were altered as result of antagonism of glucose uptake. In the same study, the authors determined the affected LH release action occurred at higher brain centers because the ewes maintained the ability to respond exogenous GnRH. It has been previous reported that the hindbrain possess glucose detectors through evidence supported by work with 2-deoxy-D-glucose to suppress LH secretion in rats (Murahashi et al., 1996), wethers (Bucholtz et al., 1996), goats (Ohkura et al., 2004), and

ewes (Funston et al., 1995); thus, further suggesting the influence of glucose availability on LH secretion in ruminants.

Other metabolites have been implicated in delayed conception, reduced pregnancy rates, and decreased LH. Elevated NEFA and BHB were concomitantly reported with reduced mean LH concentration and LH pulse in ovariectomized heifers (DiCostanzo et al., 1999). Direct effects of NEFA concentrations on ruminant hypothalamus and pituitary tissues have not been established (Wettemann et al., 2003). Early postpartum dairy cows have periods of suppressed LH pulse frequency concurrently with elevated BHB (Roche, 2006). Furthermore, female rats centrally infused with BHB had increased serum BHB concentrations with suppressed LH pulses (Iwata et al., 2011). The authors in the same study proposed the increased BHB may have elicited an energy deficit signal, thus inhibiting gonadal function through GnRH suppression. The same hindbrain ependymocytes that expression glucose sensing abilities also express monocarboxylate transporters; thus, suggesting hindbrain ependymocytes have the ability for energy sensor monitoring to control gonadotropin secretion (Iwata et al., 2011). Elevated concentrations of BHB prior to calving and breeding have been reported in late conception beef cows (Mulliniks et al., 2013). Early postpartum dairy cattle display suppressed LH pulse frequency concomitant with elevated BHB concentrations (Walsh et al., 2007). Ospina et al. (2010) reported decreased conception rates with elevated BHB concentrations. Additionally, early lactation dairy cows with decreased nutrient availability experience low LH pulse frequency (Roche, 2006) and decreased LH secretion (Randel, 1990) with increased BHB concentrations. Therefore, BHB concentrations may be an indicator of

metabolic status (Pushpakumara et al., 2003) and reproductive performance in ruminants (Hobbs et al., 2017).

The effects of BHB on the HPG axis are either direct effects or through intermediate mechanisms; however, the precise mechanism is unclear (DiCostanzo et al., 1999). The utilization rate of BHB by the brain is a direct reflection of circulating plasma concentrations (Hasselbalch, 1995). The delivery of BHB to brain is accomplished through monocarboxylate transporters (Laeger et al., 2010). The membrane G-protein, GPR109A, may allow the central nervous system to monitor circulating concentrations of BHB (Kammula, 1976). Furthermore, BHB may affect intracellular signaling without entering neurons and instead bind to the membrane receptor (Titgemeyer et al., 2011). Iwata et al. (2011) suggested ketone bodies detected in the hindbrain may serve as a negative energy signal to inhibit gonadotropin secretion, thereby altering reproduction. Ependymocytes may respond to changes in glucose availability (Iwata et al., 2011), which may be altered by elevated BHB concentrations. Furthermore, pyruvate oxidation is inhibited by BHB in cerebrocortical mitochondria, which may inhibit glucose metabolism in ependymocytes to sense availability of energy substrates (Iwata et al., 2011).

Elevated NEFA concentrations have been associated with decreased reproductive performance. A negative correlation was reported in days from calving to conception in multiparous cows with elevated NEFA concentrations (Wathes et al., 2007). Garverick et al. (2013) reported elevated serum concentrations of NEFA in cows that failed to conceive at first artificial insemination compared to cows that conceived by first

insemination. Low LH pulse frequency has also been associated with elevated NEFA concentrations in dairy cows (Roche, 2006). In agreement, Hileman et al. (1993) reported inhibition of fatty acid oxidation reduces LH secretion in sheep. In contrast, Mulliniks et al., (2013) reported NEFA concentrations were not different between cows that conceived early or late in the breeding season. Insulin-dependent glucose metabolism may be affected by elevated NEFA concentrations (Pires et al., 2008). It has been previously reported that serum elevated NEFA concentrations are negatively associated with NEB (Lucy et al., 1991); therefore, attenuating elevated NEFA concentrations to avoid subsequent rises in BHB may be instrumental for increasing fertility.

Insulin has been previous regarded as a metabolic moderator between nutrition and fertility in cattle, especially by positively influencing LH synthesis and release (Monget and Martin 1997). Furthermore, in rats insulin receptors have been localized in the arcuate nucleus, suggesting areas involved in LH release are sensitive to insulin (van Houten et al., 1980). Hileman et al. (1993) evaluated the central administration of insulin to enhance the secretion of LH in ovariectomized ewes and concluded insulin alone does not act a nutritional signal to regulate LH secretion. However, insulin-induced hypoglycemia has been indicated to suppress LH pulse frequency in sheep (Clarke et al., 1990; Medina et al., 1998), but can be reversed by administration of peripheral glucose (Clarke et al., 1990). Therefore, insulin in concert with glucose stimulate the release of hypothalamic GnRH (Arias et al., 1992), subsequently affecting the release of LH. For instance, Ohkura et al. (2004) reported low postpartum glucose and insulin

concentrations suppress GnRH secretion; therefore, consequently decreasing pituitary LH secretion.

Neuroendocrine pathways involving hypothalamic neurons such as agouti-related peptide (AgRP), hypothalamic neuropeptide Y (NPY), and proopiomelanocortin neurons have been implicated as components for pathway sensing and transmitting metabolic information for reproductive hormone control in ruminants (Amstalden et al., 2011). More specifically these neurons, located in the arcuate nucleus, are pathways by which nutritional signals are affected (Crown et al., 2007; Amstalden et al., 2011). In ruminants, NPY has been attributed to inhibitory effects on LH release (Gazal et al., 1998; Morrison et al., 2003). Metabolic and nutritional information, transmitted through signals, is largely perceived at the hypothalamus and is integrated into cellular networks for neuroendocrine controls (Schneider, 2004). The convergence of information governing reproductive success and overall metabolic homeostasis occurs within the HPG. Peripheral metabolic and hormonal factors play an integral role in controlling GnRH (Garcia-Garcia, 2012); therefore, subsequently affecting LH. Energy substrate availability may be responsible for mediating the metabolite-hormone interaction that governs the regulation of GnRH and LH in ruminants.

Reproduction is influenced by nutritional state and energy status. The integration of metabolic and hormonal peripheral cues into the hypothalamus modulates reproductive status. Internal and external stimuli regulate the control of interaction between metabolism and reproduction. However, the relationship between energy balance and

reproduction is not one dimensional as illustrated above, but rather likely encompasses a complex interaction of metabolic stimuli, hormonal mediators, and central neural control.

Gene regulation coupling metabolism and reproduction

Both the hypothalamus and pituitary are influential in the linking of energy metabolism and fertility (Nishida et al., 2005; St-Amand et al, 2011). The hypothalamus is a regulatory center responsible for food and water intake, stress responses, and energy metabolism (Nishida et al., 2004). The pituitary gland secretes distinctive hormones regulating reproduction, metabolism, and growth (Nishida et al., 2004). Maintenance homeostasis for metabolism, reproduction, and stress response are regulated and controlled by the hypothalamus and pituitary (St-Amand et al., 2012). Extensive research has been done to evaluate the mechanisms and pathways that integrate information regarding nutritional controls on reproduction (Lucy, 2003; Hess et al., 2005; Cardoso et al., 2013); especially with regard to the HPG axis. Gene regulation and expression studies are being utilized to investigate the relationship between metabolism and reproduction. Zarrin et al. (2013) reported intravenous infusion of BHB did not affect mRNA expression related to gluconeogenesis, glycolysis, pyruvate dehydrogenase complex, or citrate synthase in dairy cows. In addition, fatty acid oxidation was also unaffected by intravenous BHB infusions. In dairy cows experiencing NEB, RNA sequencing (RNAseq) of liver tissue reported altered gene expression of genes involved in inflammatory disease, lipid transport, catabolism, and fatty acid β-oxidation (McCarthy et al., 2010). Other evidence also suggests BHB is implicated in the promotion of stress responses by promoting hyperacetylation of histone proteins (Shimazu et al., 2013).

Using in situ hybridization, Adam and Findlay (1998) determined sheep in NEB have altered expression of the NPY gene. The NPY gene has an influential role in central control of food intake and energy balance (Martin et al., 2008), as nutrient comprised animals have increased levels of NPY (Waterman and Butler, 2010). Infusion of BHB in the carotid artery of mice increased expression of NYP and AgRP genes; thus suggesting a sensed energy deficit response to elicit physiological homeostasis (Carneiro et al., 2016). In cell cultured bovine mammary epithelial cells, an immunosuppressive effect was elicited in immune response genes following treatment of BHB; thus, indicating negative effect on gene expression of innate immune cells (Hillreiner et al., 2016) Unfortunately, little literature is available regarding the central effects of elevated BHB on gene expression, further necessitating the need for the investigation of coupled metabolic and reproductive pathways.

As demands for agricultural efficiency increase, research efforts with a focus on metabolic efficiency and reproductive competency are necessary. Characterization of genes involved in metabolic and reproductive phenotypes in ruminants would enhance understanding of the relationship between metabolism and fertility. Furthermore, functional genomics, such as gene expression and function studies would expand the knowledge regarding metabolism and fertility.

A complete set of transcripts and their abundance with regard to a specific developmental state or physiological condition is defined as a transcriptome (Wang et al, 2009). Through the utilization of RNAseq, a sample of RNA is converted to a complimentary DNA library of fragmented adapters, and the molecules are sequenced

(Jäger et al., 2011). Gene expression models are more accurately profiled through deep sequencing of transcriptomes (Zhang et al., 2013). Furthermore, gene expression level is often used a representation for determining if the RNA product is functional within a tissue or cell and for functional characterization (Hebenstreit et al., 2011). Transcriptome information, through RNAseq, allows for identifying differential regulation (Jäger et al., 2011). Beyond tissue transcriptome characterization, differential gene expression is a valuable technology for gene expression that responds to endogenous or exogenous signals. Differential gene expression analysis allows for identifying quantitative changes in gene expression or abundance (Oshlack et al., 2009). The importance of understanding gene expression is encapsulated in tissue level transcriptomes for functionality regulating metabolism and reproduction. Therefore, the studies within this dissertation may provide further insight into understanding the influence BHB has on reproduction in ruminants.

CONCLUSIONS

In summary, reproduction is directly influenced by metabolic changes; however, the mechanisms of action have not been elucidated. Understanding the interface between metabolism and reproduction will be important for increasing livestock efficiency. Elevated beta-hydroxybutyrate concentrations can influence processes related to metabolic dysfunction and reproductive incompetence in ruminants; therefore, making BHB a marker for altering the mechanisms involved in these processes. Beta-hydroxybutyrate may serve as a potential energy signal to modulate peripheral metabolic status, subsequently affecting reproduction through altering LH secretion. However, the

mechanism that BHB influences reproduction is unclear. Further research is needed to understand the interaction of BHB to decrease reproductive competence in ruminants.

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CHAPTER II

BETA-HYDROXYBUTYRATE ADMINISTRATION IDENTIFIES ACUTE DIFFERENTIALLY EXPRESSED GENES RELATED TO METABOLISM AND REPRODUCTION IN THE HYPOTHALAMUS AND PITUITARY OF CASTRATED MALE SHEEP

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ABSTRACT

To determine the effects on molecular pathways that couple metabolic imbalances and reproduction, 10 castrated male sheep were randomly assigned to be centrally injected into the lateral ventricle through intracerebroventricular cannulas with 1 mL of β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or saline solution (CON; 0.9% NaCl). Approximately two hours post injection, sheep were humanely euthanized, and hypothalamus and pituitary tissues were harvested for transcriptome characterization using RNA-sequencing. Ribonucleic acid (RNA) was extracted from the hypothalamus and pituitary and sequenced at a high depth (Hypothalamus: 468,912,732 reads; Pituitary: 515,106,092 reads) using the Illumina Hi-Seq 2500 platform and aligned to *Bos taurus* and *Ovis aries* genomes. Of the total raw reads, 87% (hypothalamus) and 90.5% (pituitary) mapped to the reference *Ovis aries* genome. Within these read sets, approximately 56% in hypothalamus and 69% in pituitary mapped to either known or putative protein coding genes. Fragments per Kilobase of transcripts per Million normalized (FPKM) counts were averaged and ranked to identify the transcript

expression level. Gene Ontology analysis (DAVID Bioinformatics Resources) was utilized to identify biological process functions related to genes shared between tissues, as well as functional categories with tissue-specific enrichment. Between CON- and BHB-treated sheep, 11 and 44 genes were differentially expressed (adj. $P < 0.05$) within the pituitary and hypothalamus, respectively. Functional enrichment analyses revealed BHB altered expression of genes in pathways related to stimulus perception, inflammation, and cell cycle control. The set of genes altered by BHB creates a foundation from which to identify the signaling pathways that impact reproduction during metabolic imbalances.

INTRODUCTION

Secondary to meat and wool production, sheep have been used extensively as models for ruminant research. They are particularly valuable for studies of fertility and nutrient metabolism (Baird, 1983; Gosden et al., 1994; Blache et al., 2000). The Ovis aries genome has been sequenced (Jiang et al., 2014) and continues to be characterized, and RNA sequencing has provided valuable transcriptome data for several sheep tissues (Zhang et al., 2013; Xiang et al., 2016; Suarez-Vega et al., 2017). A high resolution atlas of gene expression for sheep across multiple tissues has also recently been produced (Clark et al., 2017). The hypothalamus and pituitary play central roles in the coupling of energy metabolism and fertility (Nishida et al., 2005; St-Amand et al., 2011), which is critical for efficient livestock production. Characterization of the transcriptomes of the hypothalamus and pituitary in sheep would enhance the ability to associate metabolic and reproductive phenotypes with the underlying genes. As demands for agricultural

proficiency increase, research efforts with a focus on metabolic efficiency and reproductive competency are necessary.

In production agriculture, decreased reproductive success in beef cows is often associated with consumption of grazing low-quality forages coupled with increased energy demands during lactation (Hawkins et al., 2000). Ruminants rely heavily on hepatic gluconeogenesis to meet the majority of their glucose requirements because very little glucose is derived from their diet (Overton et al., 1999). Glucose requirements are increased after parturition due to the use of glucose in milk lactose synthesis. This increased demand for glucose is often compromised by an inadequate supply of glucogenic precursors derived from ruminal fermentation of the volatile fatty acid propionate (Mulliniks et al., 2011). Due to this increase in nutrient demand of lactation and inadequate glucogenic precursors from the diet, ruminants experience periods of nutrient imbalances. A metabolic imbalance can be defined as a condition in which nutrients, be it an abundance or insufficient supply, are not appropriate to meet the requirements for that animal (Herdt, 2000). With these metabolic imbalances and decreases in energy reserves, increases in body tissue mobilization occur, resulting in elevated non-esterified fatty acids (NEFA) concentrations. Increases in NEFA concentrations may overwhelm the ability of the liver to completely oxidize fatty acids to acetyl CoA and subsequently result in elevated circulating ketones, especially β-hydroxybutyrate (BHB). In ruminants, these periods of nutrient imbalance, specifically negative energy balance (Mulliniks et al., 2013), are associated with elevated NEFA and BHB concentrations (Butler, 2003; Zarrin et al., 2013), which ultimately can cause

metabolic dysfunctions (Tardif et al., 2001) and reduced reproductive performance (Mulliniks et al., 2013). In addition, elevated BHB concentrations are a good indicator of poor adaptation to NEB and adipose tissue mobilization (Herdt, 2000). Furthermore, elevated BHB concentrations have been indicated to potentially modulate reproductive competence in domestic ruminants (Mulliniks et al., 2013; Hobbs et al., 2017). However, the impact of increased central levels of BHB, which crosses the blood brain barrier (Hasselbalch et al., 1995), on hypothalamic and pituitary tissues is unknown. Intracerebral injection of BHB into the lateral ventricle of the brain may elicit a negative response on genes associated with reproduction and metabolism either through elevated BHB or signaling mechanisms. The objective of this study was two-fold: (1) use transcriptomics to characterize the tissue level response of both the hypothalamus and pituitary to an acute injection of BHB in the lateral ventricle; and (2) thoroughly characterize the transcriptomes of the male sheep hypothalamus and pituitary, as a means to enhance further studies of these two tissues.

MATERIALS AND METHODS

All animal experiments were approved by the Institutional Animal Care and Use Committee of The University of Tennessee and performed in accordance with the "Guide for the Care and Use of Agricultural Animals in Research and Teaching" (IACUC #2148).

Animals and tissue collection

Ten, ten-month-old Suffolk-crossed castrated male sheep (49 ± 2 kg) were housed at the Joe Johnson Animal Research and Teaching Unit at the University of Tennessee, Knoxville. Male castrated sheep were utilized, which is an accepted model that minimizes sex steroid variation associated with the use of cycling females (Riggs and Malven, 1974; Kesner et al., 1981; Miller et al., 2002; Matsuda et al., 2015). Sheep were individually housed and fed once daily, 1.1 kg/d of a 13.5% crude protein and 72.5% total digestible nutrients diet to maintain body weight. Wethers were fit individually with indwelling jugular catheters to facilitate blood collection. Ten-mL blood samples were collected and placed in Corvac serum separator tubes (Corvac, Sherwood Medical, St. Louis, MO). Blood samples were collected, cooled, and centrifuged at 2,000 × g at 4°C for 20-min. Serum was collected, transferred to conical tubes, and stored at -20°C for subsequent analyses for serum metabolites.

Treatments

Six months prior to the start of the study, sheep were fitted with intracerebroventricular (ICV) cannulas into the lateral ventricle of the brain as previously described (Whitlock et al., 2010) prior to experimental treatments. Sheep were randomly assigned to receive a single injection into the lateral ventricle with 1 mL of either β-hydroxybutyric acid (BHB; 12,800 µmol/L) or sterile, non-pyrogenic physiologic saline solution (CON; 0.9% NaCl; Hospira, Lake Forest, IL). The solution of 12,800 µmol/L β-hydroxybutyric acid (DL-β-hydroxybutyric acid sodium salt; H6501; Sigma-Aldrich, St. Louis, MO) was prepared in 0.9% NaCl and stored at 4°C until injection. Following

aseptic preparation, treatments were administered through the ICV cannula with a 25 gauge Huber Point needle followed by 250 µl of saline (0.9% NaCl). The dose of BHB injected was selected to mimic values that would be expected in sheep with circulating BHB levels indicative of subclinical ketosis, defined as serum concentrations above 800 µmol/L (Lacetera et al., 2001). Approximately 2 hours after administration of experimental treatments sheep were humanely euthanized by injecting a bolus of pentobarbital sodium (Euthasol; 100 mg/kg) intravenously. Whole pituitary and hypothalamus tissues were collected within 10 minutes postmortem and immediately frozen and stored at -80°C degrees for preservation until RNA extraction. Samples of perirenal adipose tissue and liver were also snap frozen for future use.

RNA extraction

Frozen whole pituitary (n=5 per treatment group) and hypothalamus (n=5 per treatment group) were pulverized under liquid nitrogen, and a small sample (~ 1 g; n= 5 per tissue, per treatment group) of tissue powder was collected and used for RNA extraction. Total RNA was isolated using a commercial kit (RNeasy® Micro; QIAGEN, Valencia, CA). Integrity and concentration of isolated RNA samples were determined using an Agilent 2100 Bioanalyzer (Agilent; Santa Clara, CA). RNA quality was determined based on sample RNA Integrity Number (RIN) value. All RNAs had RIN values of > 9.5.

QPCR

A subset of genes that were differentially expressed by BHB infusion was validated with quantitative real-time PCR (QPCR). The cDNA were synthesized from 500 ng of total RNA in 20 µl reactions using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Predesigned and validated primers for QPCR were purchased from Qiagen (Quantitect; Germantown, MD). In triplicate, QPCR was performed for each sample using iQ SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Expression levels for targeted genes of interest were normalized to expression of GAPDH, used as a housekeeping gene.

Illumina sequencing, data analyses, and bioinformatics

RNA library preparation and pair-end sequencing were performed by the Genome Sequencing and Analysis Facility at the University of Texas, Austin. Barcoded libraries (n = 5 per treatment for each tissue) from hypothalamus and pituitary were prepared and sequencing performed using a HiSeq 2500 (Illumina) according to standard protocols provided by the manufacturer. The read depth for each sample was greater than 100 million reads per sample. The read lengths for both the hypothalamus and pituitary were 91 base pairs. Transcriptome data for this study was deposited Sequence Read Archive under the accession number PRJNA422381.

Raw sequences were examined for quality with FastQC (version 0.10.1). The sequences were trimmed using Trimmomatic (version 0.32) using the pair-end mode parameters (6). TopHat (version 2.0.12) was used to align the processed reads to the *Ovis aries* v3.1 genome (60). Bowtie 2 (version 2.2.1) was used to build the index for the

reference *Ovis aries* genome on which the processed reads were aligned. Cufflinks (version 2.2.1) was used to perform alignment corrections (60), and Cuffmerge was used to assemble primary transcriptomes.

Cuffquant was used to analyze and quantify gene and transcript expression profiles. Gene counts were normalized in Cuffnorm using FPKM (expected *Fragments per Kilobase* of transcripts per *Million* fragments sequenced). Using the 75^{th} quartile fragment for upper quartile normalization, normalization FPKM counts to the average value of the 75^{th} quartile across all libraries were made for each tissue, (q1, q2, q3, q4, q5) then averaged and ranked from highest-to-lowest (Trapnell et al., 2010). Normalization was necessary for comparing gene expression measures between groups in order to account for varying lane sequencing depths as well as other technical effects (Bullard et al., 2010). Accordingly, a threshold FPKM value (0.05) was used to remove transcripts that may represent biological or technical noise from further analyses (Hart et al., 2013). Transcripts with FPKM values less than or equal to 0.05 were excluded from further analyses, an approach used by Toung et al (Toung et al., 2011). Hierarchical clustering was performed in MetaboAnalyst (3.5) to visualize relative differences between control hypothalamus and pituitary transcriptomes (Xia and Wishart, 2002), using only data for genes expressed all sheep. Samples were normalized using median values, log-transformed, and scaled using Pareto scaling prior to clustering.

Differential gene expression

The R packages of Limma (version 3.22.1) (Ritchie et al., 2015) and edgeR (version 3.8.6) (Robinson et al., 2010) were used to test for differential gene expression

in response to BHB treatment. Genes significantly affected by BHB infusion in hypothalamus and pituitary were identified by ANOVA after blocking on individual. P-values were adjusted using the Benjamini-Hochberg correction to control for false discovery rate. Raw data were normalized to scale using calNormFactors with methods set as TMM and the raw library sizes were transformed to log-2 counts per million. Differential gene expression data for this study was deposited in the NCBI Sequence Read Archive under the accession number PRJNA422381.

Gene ontology enrichment

Pituitary and hypothalamus transcriptomes were functionally annotated based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway membership using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang et al., 2007; Huang da et al., 2009). Uncharacterized, putative genes and those encoding ribosomal proteins were removed prior to GO enrichment analyses. To increase the depth of genes with GO annotations, bovine orthologs of sheep transcripts were used for GO enrichment analysis in DAVID (Ha et al., 2015). Orthologs were identified with the bovine genome (NCBI assembly UMD v 3.1.1) using BLAST (NCBI, v 2.2.26).

Serum metabolomics

Serum samples (50 μl) from each sheep were extracted for metabolomics using 0.1% formic acid in acetonitrile:water:methanol (2:2:1), as previously described (Clemmons, 2017). Electrospray ionization was used to introduce the samples into an

Exactive Plus Orbitrap MS, using an established method (Lu et al., 2010; Kamphorst et al., 2011). Metabolite identities were confirmed using the MAVEN software package (Clasquin et al., 2012), and peak areas for each compound were integrated using the Quan Browser function of the Xcalibur MS Software (Thermo Fisher Scientific, Waltham, MA). Metabolomics data were pre-processed and analyzed using Metaboanalyst (Xia et al., 2015). Peak areas were normalized by median values, transformed logarithmically, and scaled using Pareto scaling prior to statistical testing. Metabolites differing significantly ($P < 0.05$) between CON and BHB serum samples were identified using t-test, controlling for false discovery rate of 5% using the method of Benjamini-Hochberg.

RESULTS

Sequencing data summary

A total of 468,912,732 and 515,106,092 raw reads, obtained from five sheep that received only the saline (CON) treatment injection, were used to characterize the basal hypothalamus and pituitary transcriptomes, respectively (Table 1). Of these, 87% (hypothalamus) and 90.5% (pituitary) mapped to the reference sheep genome. Within these two sets of reads, approximately 56% in hypothalamus and 69% in pituitary mapped to either known or putative genes (genes with no known functional or structural homology; Table 1). The threshold that we used (FPKM < 0.05) was chosen to filter out very low abundance transcripts but retain the ability to detect rare transcripts that are expressed in only a subset of cells. After filtering, a total of 16,280 and 15,768 unique transcripts, based on NCBI RefSeq transcript IDs, were represented among the mapped

reads for hypothalamus and pituitary, respectively (Table 2). The majority (~94%) were detected in both tissues, with 1,009 (6.2%) and 501 (3.2%) of transcripts being tissue-specific expression in hypothalamus and pituitary, respectively. In the hypothalamus, the 16,280 expressed transcripts correspond to 15,717 unique genes, while 15,251 genes were represented by the 15,768 transcripts expressed in pituitary. A minor percentage (approximately 4% in both tissues) of genes were represented by more than one transcript. The majority of transcripts (~96%) in each tissue were annotated with predicted (XM) rather than curated (NM) annotations (Pruitt et al., 2014). More specifically, NM annotations confer known RNA products, while XM indicate model predictions for RNA (Pruitt et al., 2014). To increase functional annotation for downstream analyses, BLAST was used to identify the bovine orthologs of each sheep transcript. For example, 689 genes expressed in hypothalamus were annotated with an NM RefSeq ID in the Ovine genome, compared to 10,517 annotations for the corresponding bovine orthologs (Table 3). A total of 1,892 genes in hypothalamus and 1,846 genes in pituitary were uncharacterized, putative genes with an assigned symbol of LOC followed by a GeneID number, i.e. LOC101115442. Relative similarities and differences in gene expression between the two tissues are shown in Figure 1.

Relative expression analysis

For each tissue, FPKM values were averaged across each of the five libraries to calculate an average expression value for each gene. The averaged FPKM values were used as a metric to estimate expression levels of each gene within each tissue. As expected, the distributions of the gene expression levels for high expression genes peak

with a long-left tail for the low expression genes for both the hypothalamus and pituitary, indicating the majority of genes are expressed at a moderate or low level.

Gene ontology enrichment analysis

Gene Ontology annotations and KEGG pathway enrichment were used to compare the functional profiles of hypothalamus and pituitary transcriptomes. Genes were ranked according to expression and grouped according to percentile, from low to high expression. Genes in the 20^{th} and 80^{th} percentiles, representing relatively low and high levels of expression, were analyzed for GO enrichment and KEGG membership. Accordingly, the two sets of genes with highest levels of expression in each tissue share significant functional annotations. Of the 20 significantly enriched GO categories, 16 overlap between the two tissues (Table 4). Likewise, 17 of the 20 most-enriched KEGG pathways are shared between pituitary and hypothalamus. In contrast, GO and KEGG enrichment diverge between genes in the lowest quintile of expression, reflecting the presence of specialized, low abundance neurons and cell types in each tissue. This may be due to the more specificity in function of lower expression genes (Li et al., 2011).

Differential gene expression

The transcriptomes were queried to identify changes in gene expression in response to injection of BHB in the lateral ventricle. Expression of 44 genes differed significantly (adj. $P < 0.05$) in hypothalamus between CON and BHB animals (Table 5). Within the pituitary, BHB injection altered the expression of 11 genes. Three genes (FK506 binding protein 5, FKBP5; Zinc finger and BTB, ZBTB16 domain containing 16;

Eukaryotic elongation factor-2 kinase, EEF2K) were significantly altered by BHB in both tissues. A subset of genes affected by BHB in each tissue was validated independently by QPCR (Figure 2).

Metabolomics of differentially expressed metabolites

Central infusion of BHB may alter peripheral metabolism through effects on CNS signaling to tissues. The serum metabolomes of CON and BHB sheep were compared using untargeted metabolomics to identify other potential metabolic effects of BHB treatment. Ten metabolites differed between treatment groups ($P < 0.05$; allantoin, cysteine, deoxyinosine, glucose-6-phosphate, homoserine, hydroxyphenylacetic acid, N, carbamoyl-L-aspartate, threonine, thymidine, uridine; Figure. 3). Infusion of BHB significantly altered circulating levels of metabolites involved in pyrimidine metabolism (uridine, thymidine, and N-carbamoyl-L-aspartate), glycine/serine/threonine metabolism (threonine and homoserine), and cysteine/methionine metabolism (cysteine and homoserine), which all increased by BHB except for N-carbamoyl-L-aspartate.

DISCUSSION

The hypothalamus and pituitary are essential for integrating metabolism and reproduction. To expand our understanding of these tissues at the molecular level, transcriptomes of the *Ovis aries* whole hypothalamus and pituitary were characterized by deep RNA sequencing. A total of 10 sheep were used, providing a relatively robust sample that captured variation between individuals. A somewhat permissive filter was used to remove sequences with very low expression values, to optimize our ability to

capture low abundance transcripts, such as those that are specific to certain regions or nuclei. Approximately 260M (hypothalamus) and 350M (pituitary) reads were mapped to genes, resulting in ~ 15K expressed genes in each tissue after filtering for low expression. The majority of genes in each tissue are known or have putative identifications, based on assignment of gene ID terms in NCBI. However, each transcriptome also contained ~ 12% of genes that are supported by sequence inference but are currently uncharacterized (i.e., assigned LOC IDs). These transcriptomes should therefore be valuable in the ongoing process of annotating the sheep genome.

Beta-hydroxybutyrate is a multifunctional metabolite that both provides cellular energy in periods of deprivation and signals energy deficit to adaptively alter tissue metabolism. Circulating concentrations of BHB in ruminants can increase when cellular glucose concentrations are too low to supply sufficient oxaloacetate, producing an imbalance between acetate supply and oxidation (Yamashita et al., 2001). Consequently, serum BHB levels are an indicator of metabolic status (Pushpakumara et al., 2003) and reproductive competence (Mulliniks et al., 2013; Hobbs et al., 2017) in ruminants. The growing body of literature that describes signaling actions of BHB (rev. in (Newman and Verdin, 2017)) suggests that it could play a mechanistic role in the coupling of energy balance and fertility in ruminants, particularly due to BHB readily crossing the blood-brain barrier. Based on numbers of differentially expressed genes, the hypothalamus was more responsive to BHB infusion than the pituitary. Several genes that were induced by BHB are involved in hypothalamic energy metabolism. For example, BHB upregulated expression of pyruvate dehydrogenase kinase 4 (PDK4) in the hypothalamus. Pyruvate

dehydrogenase kinase 4 is one of four kinases that act as gatekeepers of the TCA cycle through their control of pyruvate dehydrogenase activity. We and others (Ji et al., 2012; Gudiksen and Pilegaard, 2017) have shown that expression of PDK4 is rapidly upregulated in response to fasting and associated with induction of fatty acid oxidation in other tissues (Torchon et al., 2017). Increased expression of PDK4 that we observed may reflect the influence of BHB on substrate metabolism in the hypothalamus. Expression of hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor; HIF1A) was also upregulated by BHB in the hypothalamus. In addition to its role in oxygen-sensing, HIF1A mediates control of hypothalamic glucose-sensing (Zhang et al., 2011). The combined effects of BHB on PDK4 and HIF1A may reflect acute alterations in substrate utilization within the hypothalamus. Within the pituitary, BHB significantly increased expression of 3-hydroxybutyrate dehydrogenase, type 1 (BDH1), which reversibly catalyzes the interconversion of BHB and acetoacetate in a substrate-dependent manner (Newman and Verdin, 2014).

Two of the three genes (FKBP5 and EEF2K) that were altered by BHB in both tissues play roles in the cellular response to stress, including the response to energy deprivation. FK506 binding protein 51 (encoded by the FKBP5 gene) is an inhibitory co-chaperone of the glucocorticoid receptor (Fries et al., 2017). In cooperation with Hsp90, FKBP51 restores homeostasis in the hypothalamic-pituitary-adrenal axis after stress (Criado-Marrero et al., 2018), prompting speculation that it could play a role in sex-specific effects of stress (Hoeijmakers et al., 2014). Expression of FKBP5 in the hypothalamus is induced by fasting in mice (Yang et al., 2012), which is consistent with

its upregulation by BHB in our study. Interestingly, FKBP5-null mice are resistant to diet-induced obesity, suggesting a potential metabolic role for this protein (Stechschulte et al., 2016). Its role in HPA activity and responsiveness to energy balance and acute increases in BHB suggest that FKBP5 may interconnect metabolic status and reproduction. During periods of nutrient deprivation, eukaryotic elongation factor 2 kinase (EEF2K) phosphorylates and inhibits EEF to prevent protein translation and preserve cellular ATP and resources (Leprivier et al., 2013). The upregulation of EEF2K in both hypothalamus and pituitary may reflect sensing of BHB as a signal of energy deprivation. The zinc finger family member ZBTB16 was also upregulated in both the pituitary and hypothalamus by BHB. This gene ZBTB16 has been reported to have a role in the regulation of puberty onset (Lomniczi et al., 2013). In addition, zinc finger family members may be involved in hypothalamic responses to progesterone in heifers (Fortes et al., 2016).

Sensing of BHB concentration by the hypothalamus has been linked to peripheral metabolic adaptations (Carneiro et al., 2016). The serum metabolome of BHB-treated sheep primarily showed effects on components of amino acid and pyrimidine metabolism, with most metabolites increased by BHB treatment. In addition, BHB significantly increased (by ~ 6-fold) serum levels of hydroxyphenylacetic acid, which is produced during metabolism of tyrosine and phenylalanine. The physiological significance of this metabolite in circulation is unclear; however, recent metabolomics studies in other species have linked it to depressive disorder (Chen et al., 2017), defects in amino acid metabolism (Kurko et al., 2016), and menopause (Ke et al., 2015), among

other conditions. Whether the serum metabolite changes that resulted from effects on peripheral nutrient mobilization in response to central sensing of a perceived energy deficit or to decreased utilization cannot be determined, but they demonstrate a relationship between central BHB and peripheral metabolism in sheep.

While lactating cows in negative energy balance is one of the most relevant applications of our study, we chose to use castrated male lambs (wethers) as a model. This model was chosen because it has been used in other studies to control the sex steroid variation associated with use of cycling females. In addition, due to the variety of mechanisms that negative energy balance influences reproductive processes, we modeled the acute and direct effect of BHB infusion. This approach was chosen to focus on the direct effects of BHB. While we identified metabolites in serum that were significantly affected by BHB infusion, we cannot determine the source of those metabolites or the local metabolic response to BHB in the brain. Comparable profiling of the ventricular fluid metabolome after BHB infusion may be useful in future studies. Finally, because our transcriptome profiles were obtained from the composite hypothalamus, our model lacked the sensitivity to detect region- or nuclei-specific effects of BHB on gene expression.

CONCLUSIONS

The hypothalamus and pituitary play critical roles in the coupling of energy balance to fertility. Deep transcriptome sequencing of the sheep pituitary and hypothalamus provided here will support further characterization of this process in ruminants. Our results also provide insight into the acute response to increased central

levels of BHB, which is both an energy source and a signaling metabolite that may connect peripheral energy status with reproduction in sheep and other species. In particular, identification of two genes that have been linked to nutrient stress sensing in other models and that were upregulated by BHB in both tissues provides a starting point from which to identify pathways that mediate the effects of BHB. While lactating cows in negative energy balance are a major application of our study, females in low-quality forage systems can also exhibit reproductive challenges due to elevations in BHB (Mulliniks et al., 2013). Though limitations exist, results from this study can help to further elucidate mechanisms through which central BHB influences reproduction in ruminants and potentially other species.

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APPENDIX

Table 2. 1: Summary of sequencing reads for each tissue

Tissue	Total*	Mapped†	Mapped to Genes‡
Hypothalamus	468,912,732	408,071,670 (87%)	264,870,861 (56.5 %)
Pituitary	515,106,092	466,385,964 (90.5%)	356,686,163 (69.2 %)

* Total number of combined sample reads (n=5).

† Reads that mapped to the known *Ovis aries* genome.

‡ Reads that mapped to known genes in each tissue.

Table 2. 2: Summary of tissue transcripts before and after thresholding for low expression

	Prior to thresholding		After thresholding[a]	
Parameter	Hypothalamus	Pituitary	Hypothalamus	Pituitary
Expressed genes[b]	17,172	16,953	15,717	15,251
Tissue-specific genes[c]	578	361	909	445
Known genes (%)[d]	87	87	88	88
Uncharacterized genes (%)[e]	13	13	12	12
Expressed transcripts[f]	18,017	17,798	16,280	15,768
Tissue-specific transcripts[g]	1,009	501	582	364
Expressed genes with >1 transcript (%)[h]	4	4	3	3
Confirmed transcript (NM)[i]	686	666	637	609
Predicted transcript[j]	17,331	17,132	15,643	15,159

[a] Thresholding was based on FPKM < 0.05.
[b]Expressed genes in each tissue.
[c]Tissue specific gene identified in each tissue.
[d] Genes with a unique gene symbol.
[e] Putative genes with an assigned symbol of LOC followed by a GeneID number, i.e. LOC101115442.
[f]Expressed transcripts within each tissue.
[g]Tissue specific transcripts are transcripts unique to either tissue.
[h] Genes with more than one sequenced transcript.
[i] Transcripts with a unique NM Refseq accession identifier.
[j] Transcripts with an XM Refseq accession identified.

Table 2. 3: Summary of Refseq accession numbers

Tissue	*Ovis aries*		*Bos taurus*	
	Known RefSeq Genes (NM)	Model RefSeq Genes (XM)	Known RefSeq Genes (NM)	Model RefSeq Genes (XM)
Hypothalamus	686	17,331	10,517	7,348
Pituitary	666	17,132	10,406	7,280

NCBI Refseq Accessions numbers for annotations based on the *Ovis aries* and *Bos Taurus* genomes.

Table 2. 4: Most abundant BP GO FAT terms related to top quintile of expression[a] in hypothalamus and pituitary genes

GO ID	GO Term	HYP genes mapped to terms (%)	PIT genes mapped to terms (%)	p-adj.[b] HYP[c]	p-adj. PIT[d]
GO:0043604	amide biosynthetic process	5.81	5.67	$2.12E^{-14}$	$7.06E^{-15}$
GO:0006412	Translation	5.35	5.40	$1.09E^{-14}$	$1.18E^{-16}$
GO:0043043	peptide biosynthetic process	5.42	5.44	$2.88E^{-14}$	$7.57E^{-16}$
GO:0006518	peptide metabolic process	5.88	5.78	$4.77E^{-13}$	$7.57E^{-16}$
GO:0043603	cellular amide metabolic process	6.72	6.58	$8.91E^{-13}$	$3.56E^{-13}$
GO:0034613	cellular protein localization	5.96	5.67	$1.09E^{-08}$	$1.45E^{-07}$
GO:0070727	cellular macromolecule localization	6.00	5.70	$1.20E^{-08}$	$1.51E^{-07}$
GO:1901566	organonitrogen compound biosynthetic process	7.30	7.20	$1.15E^{-05}$	$1.16E^{-06}$
GO:0034622	cellular macromolecular complex assembly	4.62	4.33	$1.26E^{-05}$	$1.26E^{-04}$
GO:0008104	protein localization	8.21	8.00	$4.15E^{-05}$	$1.42E^{-05}$
GO:0022618	ribonucleoprotein complex assembly	1.83	1.91	$4.23E^{-05}$	$7.65E^{-07}$
GO:0046907	intracellular transport	6.30	6.51	$6.45E^{-05}$	$1.48E^{-07}$
GO:0033036	macromolecule localization	9.21	8.88	$6.63E^{-05}$	$7.46E^{-05}$
GO:0022613	ribonucleoprotein complex biogenesis	2.90	3.10	$1.17E^{-04}$	$2.25E^{-07}$
GO:0071826	ribonucleoprotein complex subunit organization	1.87	1.95	$1.33E^{-04}$	$3.42E^{-06}$
GO:0006886	intracellular protein transport	3.74	3.71	$5.00E^{-04}$	$1.23E^{-04}$
GO:0061024	membrane organization	3.48	-[e]	$2.02E^{-04}$	-
GO:0050804	modulation of synaptic transmission	1.11	-	$2.72E^{-04}$	-
GO:0031175	neuron projection development	2.25	-	$4.82E^{-04}$	-
GO:0044802	single-organism membrane organization	3.17	-	$4.89E^{-04}$	-
GO:0044257	cellular protein catabolic process	-	3.29	-	$1.30E^{-04}$
GO:0006457	protein folding	-	2.03	-	$3.49E^{-07}$
GO:0010467	gene expression	-	16.00	-	$3.70E^{-06}$
GO:1902582	single-organism intracellular transport	-	5.59	-	$5.92E^{-06}$

[a] Genes ranked by average expression value in each tissue and top 20% were used for GO analyses. [b]p-adj. = p-value adjusted for false discovery. [c]HYP: Hypothalamus. [d]PIT: Pituitary. [e] "-" Indicates particular GO term not observed in specific tissue.

Table 2. 5: Differentially expressed genes of interest in hypothalamus and pituitary after lateral ventricle injection of BHB

Hypothalamus			
Gene Symbol	Description	FC*	p-adj.†
FKBP5	FK506 binding protein 5	4.44	$1.77E^{-03}$
ZBTB16	Zinc finger and BTB domain containing 16	3.27	$1.77E^{-03}$
CA6	Carbonic anhydrase VI	0.21	$2.36E^{-03}$
CCL23	Chemokine (C-C motif) ligand 23	4.53	$3.21E^{-03}$
KLF9	Kruppel-like factor 9	1.66	$1.25E^{-02}$
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	2.51	$1.30E^{-02}$
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.32	$2.71E^{-02}$
PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	0.49	$2.90E^{-02}$
LOC101119731	Signal transducer CD24-like	2.93	$3.58E^{-02}$
LOC101107675	Phospholipase A2, membrane associated-like	14.12	$4.59E^{-02}$
EEF2K	Eukaryotic elongation factor-2 kinase	1.51	$4.79E^{-02}$
Pituitary			
BDH1	3-hydroxybutyrate dehydrogenase, type 1	2.28	$2.28E^{-03}$
PREP	Prolyl endopeptidase	1.67	$1.39E^{-02}$
FKBP5	FK506 binding protein 5	2.43	$1.39E^{-02}$
ZBTB16	Zinc finger and BTB domain containing 16	2.35	$1.61E^{-02}$
PDZRN4	PDZ domain containing ring finger 4	0.37	$3.19E^{-02}$
FBXO15	F-box protein 15	1.77	$3.19E^{-02}$
LOC100037695	Osteoprotegerin	0.57	$3.19E^{-02}$
EEF2K	Eukaryotic elongation factor-2 kinase	1.68	$3.58E^{-02}$
NR4A1	Nuclear receptor subfamily 4, group A, member 1	0.19	$3.74E^{-02}$
FAM78A	Family with sequence similarity 78, member A	1.75	$3.74E^{-02}$
HORMAD2	HORMA domain containing 2	8.4	$4.68E^{-02}$

* FC = Fold-change, BHB/control

† p-adj. = p-value adjusted for false discovery

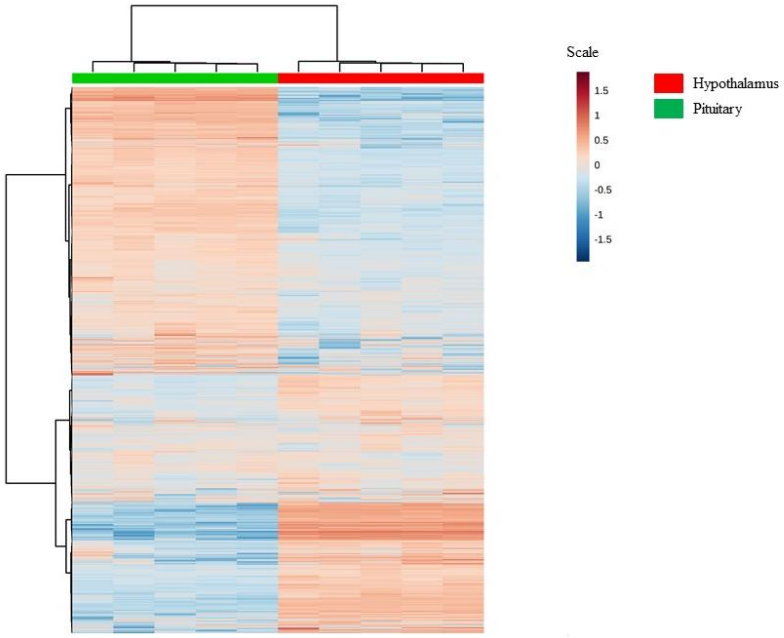

Figure 2. 1: Hierarchical cluster analysis of hypothalamus and pituitary genes
Normalized expression values for the set of genes detected above the threshold for low expression (FPKM ≥ 0.05) in all samples were used for hierarchical clustering. Data were clustered using Metaboanalyst (V3.5), using Euclidean distance as a metric for dissimilarity and clustering based on average linkage. Color indicates relative expression level of each gene in each sample, from high (red) to low level.

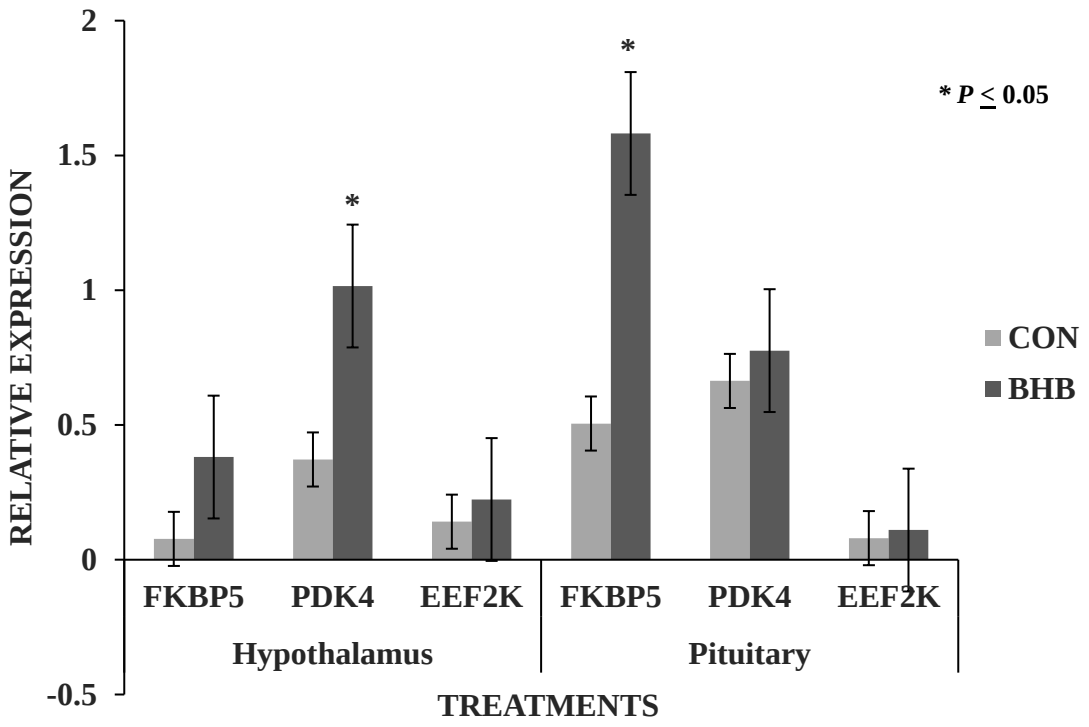

Figure 2. 2: Validation of genes affected by intracerebroventricular BHB injection in both hypothalamus and pituitary by QPCR

The three genes affected by BHB in both tissues were validated by QPCR to confirm RNAseq analyses. Samples (n=5/treatment) are from sheep subjected to a single injection into the lateral ventricle of 1 ml of either saline solution (CON; 0.9% NaCl) or β-hydroxybutyric acid sodium salt solution (BHB; 12.8 mM). Relative expression (means ± std. dev.) indicates expression of gene of interest normalized to that of GAPDH, which was used as a housekeeping gene. Expression levels were compared between BHB and CON using T-test; * = $p \leq 0.05$

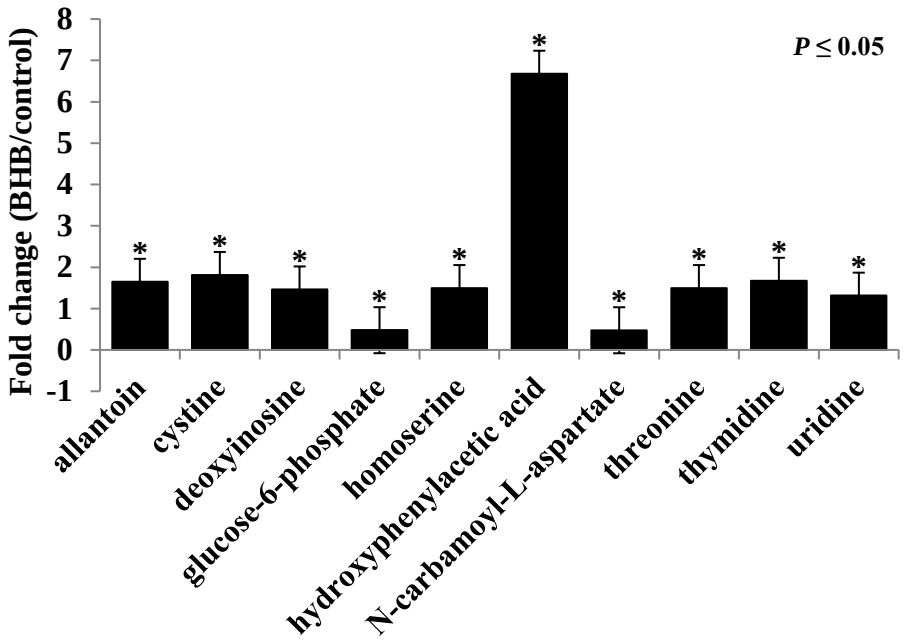

Figure 2. 3: Effects of intracerebroventricular BHB injection on serum metabolites
Serum metabolomes of control (CON; 0.9% NaCl) and BHB (β-hydroxybutyric acid sodium salt solution; 12.8 mM) were profiled using LC-MS, n=5/treatment, using peak area as an index of relative metabolite abundance. Raw peak areas were normalized using median centering, log-transformed, and scaled (using Pareto scaling). Metabolites significantly affected by BHB (* adj. P-value ≥ 0.05) were identified using T-test. Data are represented as fold change (FC) in BHB vs. CON, calculated from mean metabolite abundance in each treatment group. Data were analyzed using Metaboanalyst (V3.5).

CHAPTER III

ESTRADIOL IMPLANTATION SUPPRESSES EFFECT OF CENTRAL ADMINISTRATION OF BETA-HYDROXYBUTYRATE IN WETHERS ON LUTEINIZING HORMONE CONCENTRATION BUT NOT LUTEINIZNG HORMONE AMPLITUDE

A version of this chapter is being prepared for publication by: Emily R. Cope, Victoria J. Morris, Brian K. Whitlock, Brynn H. Voy, Ky G. Pohler, and J.Travis Mulliniks.

ABSTRACT

Metabolic dysfunctions in ruminants manifest in several ways including decreased reproductive efficiency and extended postpartum anestrus. Metabolite and hormonal changes altered by states of metabolic stress can inhibit reproduction through effects to pulsatile luteinizing hormone (LH) secretion suppression. The study objective was to evaluate the effect of a central administration of exogenous β-hydroxybutyrate (BHB) into the lateral ventricle of the brain on LH secretion and circulating blood metabolites. Wethers (n = 12) were individually housed and fed daily at a rate of 1.1 kg/d of a 13.5% crude protein and 72.5% total digestible nutrients ration. Before study initiation, wethers were fitted with lateral ventricle intracerebroventricular (ICV) brain cannulas and implanted with estradiol-containing implants (15 mm packed column of 17β-estradiol) to establish physiological concentrations of LH. Wethers were injected with 1 mL into the ICV with one of 4 treatments: 0, 400, 800, or 1,600 μmol/L of β-hydroxybutyric acid sodium salt (BHB) solution in saline (0.9% NaCl). Blood samples were collected every 10 min for 60 min before treatment injection and every 10 min for 120 min after injection. Serum glucose concentrations tended ($P = 0.08$) to decrease linearly with increasing concentrations of BHB. Insulin concentrations exhibited a linear decrease ($P < 0.01$) with increasing concentrations of BHB injection. Nonesterified fatty acids (NEFA) concentration was lower (0 vs. all BHB response; $P = 0.02$) for wethers

injected with 0 μmol/L compared with BHB-injected wethers. Circulating serum BHB and urea N were unaffected ($P \geq 0.46$) by concentrations of exogenous BHB injection. There was no effect of treatment on mean serum LH concentrations ($P = 0.27$) or number of LH peaks ($P = 0.66$). In contrast, amplitude of LH peaks decreased linearly ($P = 0.03$) with increasing concentrations of BHB injection. These results indicate elevated BHB in the brain influences the circulating peripheral metabolic status and may influence LH concentrations in wethers. Amplitude of LH peaks were suppressed with increasing concentrations of BHB and estradiol implants, indicating LH amplitude was inhibited with increasing concentrations of BHB and estradiol, which may alter reproductive efficiency. Additionally, increasing concentrations of BHB may have initiated a glucose-sparing effect.

INTRODUCTION

Reproduction in livestock is adversely affected by metabolic dysfunctions and nutrient imbalances. Livestock experiencing negative energy balance (NEB) can have a reduced reproductive performance (Beam and Butler, 1999), which may be mediated through endogenous metabolic signals (Mulliniks et al., 2013). Manifestation of metabolic dysfunctions occurring during NEB can be presented as ketosis, delayed resumption of estrous, suppressed LH secretion, and delayed conception (Randel, 1990; Butler, 2000; Mulliniks et al., 2013). During periods of metabolic imbalances, low circulating concentrations of blood glucose and insulin are observed concomitantly with elevated nonesterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) concentrations (Zarrin et al., 2013), which may indicate a poor adaptation to NEB (Herdt, 2000).

During the early postpartum period, dairy cattle display depressed LH pulse frequency that corresponded with elevated BHB (Walsh et al., 2007). Tatman et al. (1990) suggested the pituitary gland may be highly influenced by changes in nutritional status. Metabolite changes have direct influences on reproduction; however, the mechanisms regulating these processes are not fully elucidated. Therefore, the hypothesis for our research was that exogenous administration of BHB in the lateral ventricle of the brain would decrease LH concentration in castrated sheep. The objective of this study was to evaluate the effect of a central injection BHB into the lateral ventricle on circulating blood metabolites and LH concentration.

MATERIALS AND METHODS

All animal handling and experimental procedures were in accordance with guidelines set and approved by the University of Tennessee's Institution of Animal Care and Use Committee (IACUC #2148).

Animals

Twelve 10-mo-old Suffolk and Suffolk-cross wethers (49 ± 2 kg) were housed individually in a temperature controlled environment (~21°C) for a 12 h light: 12 h dark photoperiod cycle at the University of Tennessee Johnson Animal Research and Teaching Unit, Knoxville, TN. Wethers were utilized for this study to minimize sex steroid variation associated with the use of cycling females (Kesner et al., 1981; Miller et al., 2002; Kadokawa et al., 2009). Additionally, orchiectomized males have been used to reduce confounding effects of endogenous LH surges as seen in female animals (Kesner

et al., 1981). Wethers were fed once a day at a rate of 1.1 kg/d of a 13.5% crude protein and 72.5% total digestible nutrients complete feed ration (Table 1) to maintain body weight with ad libitum access to water. Two weeks before experimental treatments, wethers were fitted with lateral ventricle intracerebroventricular (ICV) brain cannulas as previously described (Whitlock et al., 2010).

To establish physiological concentrations of LH (Beckett et al., 1997), wethers were implanted with two subcutaneous estradiol-containing implants near the scapular area during the ICV surgeries. Implants were made from 4.8 mm Silastic tubing (Osteotec Ltd, Christchurch, Dorset, UK) containing 15 mm packed column of 17β-estradiol (Sigma). Estradiol-containing implants have been shown to elevate circulating estradiol concentrations to 2 to 4 pg/ml (Adam and Findlay, 1998). Though estradiol was not measured in this study for these wethers, the authors expect similar concentrations as previously reported (Mann et al., 1995).

Treatments

Wethers were centrally injected with 1 mL into the lateral ventricle with one of 4 treatments that were randomly selected: 0, 400, 800, or 1,600 μmol/L of β-hydroxybutyric acid sodium salt; solution (BHB). Preparation of BHB solution was made according to previously defined methods by Zarrin et al. (2013). The BHB solution was prepared using a DL-β-hydroxybutyric acid sodium salt (BHB salt) (H6501, Sigma-Aldrich, St. Louis, MO). Once the BHB solution reached treatment concentrations, it was stored at 4°C until injection. Following aseptic preparation, treatments were administered through the ICV cannula with a 25 gauge Huber Point needle followed by 250 μl of

saline (0.9% NaCl). Two hours post experimental treatment administration, all wethers received an intravenous (i.v.) injection 100 mL of 50% dextrose to wash out the effect of BHB. To increase the experimental units, wethers were re-randomized to different experimental treatment following a 72-hr washout period. Application of treatments in period 2 was exactly the same as mentioned above in the first period. A carryover effect was not found in the second period.

Sampling and analyses

Wethers were fit individually with indwelling jugular catheters approximately 24 h before serial blood collection. Before injection of experimental treatments, jugular blood samples were collected at approximately 10-min intervals for 60-min for a baseline. After the 60-min baseline collection, assigned treatments were injected and blood was collected at approximately 10-min intervals for an additional 120-min. Blood samples were collected, cooled, and centrifuged at 2,000 × *g* at 4°C for 20-min. Serum was harvested, poured into conical tubes, and stored at -20°C for later analysis.

Serum samples were analyzed in duplicate for NEFA, urea N (SUN), glucose, BHB, insulin, and LH. Serum samples were analyzed using a 96-well microplate reader spectrophotometer with commercial kits for NEFA (Wako Chemicals, Richmond, VA), glucose (Thermo Electron Corp., Waltham, MA), and SUN (Thermo Electron Corp., Waltham, MA). Serum samples of BHB were analyzed using a of DL-β-Hydoroxybutyric acid sodium salt, Tris Buffer (10 ml of Tris hydrochloric acid + 40 ml of deionized water, pH 9) with 30 mg of β-Nicotinamide adenine dinucleotide (β-NAD), and an enzyme of 3-hydroxybutyrate dehydrogenase (Sigma-Aldrich, St. Louis, MO).

Concentrations of serum insulin were determined by RIA (EMD Millipore's Porcine Insulin RIA) using Wizard2 Gamma Counter (Perkin Elmer, Waltham, MA). Analysis for LH was conducted by solid-phase radioimmunoassay (Lee et al., 1976) with NIH-bLH-B9 for standards. The intra- and interassay CV were, respectively, 1.8% and 2.2% for serum NEFA, 3.3% and 3.8% for serum glucose, 3.2% and 4.6% for SUN, 4.0% and 4.2% for serum BHB, and 5.1% and 4.2% for serum insulin.

Determination of LH profiles utilized serum samples that were harvested during the 3-h collection period for each treatment group by previously established methods (Clarke, 1993). The number of LH peaks during the 2-h sampling period post injection of treatments was determined by taking the overall LH mean concentration and then adding 1 standard deviation. In addition, LH amplitude was determined by taking the LH concentration at the baseline (60-min before treatment administration) to the top of the peak as previously described methods by Foster and Olster, (1985).

Statistical analyses

Repeated measures in the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, version 9.4) were used to analyze serum metabolite data. The repeated measure was time and the model was tested using sheep as the subject term. Data were analyzed using the Kenward-Roger degrees of freedom method. The model included fixed effects of period (Treatment 1, Treatment 2), sheep, time, and treatment. The compounded symmetry structure was determined to be most desirable covariance structure according the Akaike's information criterion. The MIXED procedure of SAS was used to test all the main effects of overall LH mean, number of pulses, and LH amplitude. The model

included fixed effects of period, sheep, time, and treatment. Preplanned contrasts were used to test for linear, quadratic, and control vs. all doses of BHB. Significance was determined at $P \leq 0.05$.

RESULTS

Serum metabolites

Serum glucose concentrations tended ($P = 0.08$; Table 2) to decrease in a linear fashion with increasing BHB dose. Insulin concentrations decreased linearly ($P < 0.01$) with increasing doses of BHB. Serum NEFA concentrations were greater in wethers receiving BHB compared with the 0 µmol/L (0 vs. BHB response; $P = 0.02$). Serum NEFA concentration was greatest after treatment with 1,600 µmol/ml concentration of BHB. Endogenous circulating serum BHB was unaffected ($P \geq 0.66$) by increasing doses of exogenous BHB. Circulating SUN ($P = 0.46$) was unaffected by dose of exogenous BHB.

Luteinizing hormone

Mean LH concentration ($P = 0.27$; Table 3) and number of LH peaks ($P = 0.66$) were unaffected by increasing doses of BHB injection. However, amplitude of LH peaks decreased linearly ($P = 0.03$) with increasing doses of BHB injection.

DISCUSSION

The tendency of the linear decrease in glucose may be attributed to a glucose-sparing effect (Moore et al., 1976; Zarrin et al., 2013). Central ketone bodies may affect glucose and energy balance through the hypothalamus, because the hypothalamus is the

primary regulatory organ for energy homeostasis (Park et al., 2011). However, circulating glucose concentrations are tightly regulated in ruminants (Kaneko, 2008). In a study by Park et al. (2011), short-term ICV infusion of BHB in rats decreased hepatic glucose output. In contrast, Mulliniks et al. (2013) reported decreased serum glucose concentrations with decreased endogenous concentrations of BHB in lactating beef cows. An explanation for the results reported by Mulliniks et al. (2013) is that the potential serum glucose tissue rate was faster allowing for better acetate oxidation, subsequently preventing an increase in endogenous BHB concentration. In support of this, Mulliniks et al. (2011) reported that with improved utilization of metabolizable acetate, ketone body concentrations were reduced. In contrast, other studies involving dogs, pigs, and ewes have reported decreased serum glucose concentrations with intravenous injection of BHB, suggesting an inhibition of gluconeogenesis or decrease in glucose utilization (Madison et al., 1964, Müller et al., 1984; Schlumbohm and Harmeyer, 2003).

Serum insulin concentrations decreased linearly with increasing does of BHB. In contrast to our insulin results, Mulliniks et al. (2013) reported decreased insulin concentrations with decreased serum BHB concentrations. Studies involving monogastrics have reported increased insulin concentrations with elevated circulating BHB concentrations (Madison et al., 1964). Madison et al. (1964) demonstrated that pancreatic β-cells responded to elevated BHB concentrations. In dairy cattle chronically intravenously infused with BHB, insulin concentration was unaffected (Zarrin et al, .2013). In the current study, circulating glucose concentration tended to decrease linearly, which may have resulted in the decrease in insulin (Chan and Sherwin, 2012). In

addition, the increase in injected-BHB may have directly decreased insulin because elevated BHB concentrations inhibit insulin secretion (Zhou and Grill, 1995; Takehiro et al., 2005).

Exogenous administration of BHB increased NEFA concentrations. The NEFA concentration results indicate that the BHB injection may be acting as an energy status signal; thus, indicating incomplete oxidation of fatty acids. Chronic intravenous infusion of BHB in dairy cows did not affect circulating NEFA concentrations (Zarrin et al., 2013). Similarly, NEFA concentrations were not different between cows with low or elevated BHB concentrations (Mulliniks et al., 2013). In agreement with our results, low insulin concentrations have been reported with elevated NEFA concentrations resulting from increased lipid mobilization (Fiore et al., 2014).

Endogenous BHB concentration was unaffected by exogenous BHB administration. Ketone bodies are used as an alternate fuel source during times of physiological stress or starvation, allowing for a glucose-sparing effect to take place in certain tissue in which ketones can be used as an energy source (Zarrin et al., 2013). While ketone bodies are always present in the blood, increases in ketone bodies are frequently observed concurrently with a NEB and lack of glucose (Laffel, 1999; Zarrin et al., 2013). In mice intracarotidly infused with BHB to mimic BHB transport across the blood brain barrier, an increase in BHB was reported in the periphery (Carneiro et al., 2016). In contrast to our results, Park et al. (2011) and Iwata et al. (2011a) reported an increase in serum BHB concentrations in rats infused with exogenous BHB into the lateral ventricle of the brain.

Serum urea N was unaffected by exogenous BHB infusion. Commonly, SUN and BHB concentrations are often evaluated together to determine the energy status of the animal necessary to meet production needs (Nozard et al, 2012). Similarly, SUN was not different between lactating beef cows with low or elevated BHB concentrations (Mulliniks et al. 2013). In contrast, Zarrin et al. (2013) reported a decrease in SUN concentrations in dairy cows intravenously infused with BHB.

Though LH mean concentration and LH pulse were unaffected by BHB administration, LH amplitude decreased linearly with increasing BHB administration. DiVall et al. (2015) suggested in diet-induced obese mice increases in gonadotropin-releasing hormone (GnRH) secretion may be due to an increase in to LH amplitude; thus suggesting the importance of LH amplitude in regulation of reproductive function. Jain et al. (2007) suggested suppressed amplitude of LH pulsatility results in inadequate luteal stimulation. Also, inadequate luteal stimulation is further depressed by deficient LH secretion caused by impaired LH pulse amplitude (Jain et al., 2007). Furthermore, ovariectomized heifers with elevated serum BHB experienced reduced LH amplitude (DiCostanzo et al., 1999). Similarly, Iwata et al. (2011b) reported decreased amplitude of LH with increased concentrations of exogenous BHB injection in ovariectomized rats. This decrease in LH amplitude may be caused by a decreased responsiveness by the pituitary (Fitzgerald et al., 1987), potentially resulting from the exogenous BHB. Wagenmaker et al. (2009) reported LH pulse amplitude suppression is due in part to a reduction in pituitary responsiveness to GnRH. Central effects to decrease GnRH pulse amplitude could potentially reduce LH amplitude or the LH reduction may be the result

of a direct pituitary effect on the gonadotrope (Tilbrook et al., 2000). Thus, findings from the current study and others (DiCostanzo et al., 1999; Iwata et al., 2011b) may indicate LH amplitude may be sensitive to elevated concentrations of BHB both centrally and peripherally.

During metabolic dysfunctions, such as undernutrition, BHB may be elevated such that reproductive function is comprised by enhancing the negative feedback potency of gonadal steroids (Beckett et al., 1997). Previous studies have indicated suppression of LH secretion with exogenous administration of estradiol (Riggs and Malven, 1974). Though peak signals were present, LH amplitude and overall mean were further inhibited by the potential negative energy signal from BHB and estradiol implants. Foster and Olster (1985) reported that *ad libitum* fed estradiol-treated ovariectomized ewes had a reduced rise in LH amplitude; thus further supporting estradiols negative feedback ability. The potential role of BHB to act as negative central energy signal highlights the enhancement of negative feedback potency of estradiol to further suppress LH. Beckett et al. (1997) offers a possible explanation for the central sensitivity to estradiol by suggesting the chronic nutrient restriction, or in this study elevated BHB, may alter the estrogen receptor isoform distribution in the hypothalamus. During times of elevated BHB concentrations, such as periods of metabolic dysfunctions or nutrient restriction, estradiol may act locally to further inhibit LH secretion (McManus et al, 2005).

CONCLUSIONS

These results indicate that elevated BHB in the brain may potentially influence the peripheral metabolic status and may influence LH profile in wethers. The decrease in

serum glucose concentrations may suggest a glucose sparing effect was initiated by the increasing concentrations of BHB. The amplitude of LH peaks was suppressed with increasing concentrations of BHB, which may alter reproductive efficiency by altering the timing ovulation.

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APPENDIX

Table 3. 1: Feed ration composition (DM) of wethers

Item	
Ingredient, %	
Cotton seed hulls	25.00
Molasses	7.00
Cracked corn	56.00
Soybean meal	10.00
Calcium phosphate	0.20
Limestone calcium	1.00
Ade premix	0.10
TM salt	0.50
Dynamate	0.20
Nutrient composition	
Dry matter, %	88.33
CP, %	13.47
ADF, %	16.37
aNDF, %	24.7
TDN, %	72.52
Calcium, %	0.74
Phosphorus, %	0.37
Potassium, %	1.08
Magnesium, %	0.23
Sodium, %	0.26
Sulfur, %	0.25
Aluminum, mg/kg	132.00
Cobalt, mg/kg	<0.20
Copper, mg/kg	5.06
Iron, mg/kg	157.00
Magnesium, mg/kg	18.70
Molybdenum, mg/kg	1.22
Zinc, mg/kg	30.70

*DM reported on an as fed basis

Table 3. 2: Effect of central injection of exogenous beta-hydroxybutyrate (BHB) into the lateral ventricle on circulating serum metabolites in wethers

	Treatment[1]					Contrast[2] *P*-value		
Measurement	0	400	800	1600	SEM	L	Q	0 vs BHB
Metabolites								
Glucose, mg/dL	93.61	97.24	91.31	83.11	9.47	0.08	0.19	0.58
Insulin, ng/mL	0.20	0.15	0.13	0.15	0.05	<0.01	<0.01	<0.01
NEFA, µmol/L	490.34	582.25	547.62	611.47	42.41	0.04	0.66	0.02
BHB, µmol/L	117.65	116.93	123.47	109.67	22.31	0.80	0.66	0.96
SUN[3], mg/dL	12.22	9.08	13.40	11.50	23.90	0.65	0.51	0.46

[1]Treatment: A single central injection into the lateral ventricle with **1 mL** of one of four treatments: 0, 400, 800, or 1,600 µmol/L of β-hydroxybutyric acid sodium salt solution.
[2]Contrast: L = Linear, Q = Quadratic; 0 vs BHB = 0 µmol/L vs 400, 800, and 1,600 µmol/L of β-hydroxybutyric acid sodium salt solution.
[3]SUN: serum urea N.

Table 3. 3: Effect of a central injection of exogenous β-hydroxybutyrate (BHBA) into the lateral ventricle on LH parameters in wethers

	Treatment[1]					Contrast[2] *P*-value		
Measurement	0	400	800	1,600	SEM	Lin	Q	0 vs BHB
LH mean, ng/mL	0.57	0.53	0.56	0.52	0.05	0.27	0.94	0.27
LH peaks, no.	1.77	1.57	1.57	1.83	0.57	0.93	0.66	0.85
LH amplitude, ng/mL	0.64	0.54	0.41	0.25	0.13	0.03	0.81	0.11

[1]Treatment: A single central injection into the lateral ventricle with 1 mL of one of four treatments: 0, 400, 800, or 1,600 μmol/L of β-hydroxybutyric acid sodium salt solution

[2]Contrast: Lin = Linear, Q = Quadratic; 0 vs BHB = 0 μmol/L vs 400, 800, and 1,600 μmol/L of β-hydroxybutyric acid sodium salt solution.

CHAPTER IV

LUTEINIZING HORMONE MEAN CONCENTRATION AND PULSE AMPLITUDE REDUCED IN WETHERS WITH CENTRAL ADMINISTRATION OF BETA-HYDROXYBUTYRATE

A version of this chapter is being prepared for publication by: Emily R. Cope, Victoria J. Morris, Brian K. Whitlock, Brynn H. Voy, Ky G. Pohler, and J.Travis Mulliniks.

ABSTRACT

Metabolic dysfunctions are known to have negative impacts on reproduction in several mammalian species. Changes in blood metabolites and metabolic hormones in response to metabolic dysfunctions can inhibit reproduction in response to the suppression of pulsatile luteinizing hormone (LH) secretion. Therefore, the objective of this study was to evaluate the effect of a central injection of exogenous β-hydroxybutyrate (BHB) into the lateral ventricle of the brain on serum metabolites and LH secretion patterns in wethers. Ten wethers (n=10) were housed individually and fed once a day at a rate of 1.1 kg/d of a 13.5% CP and 72.5% TDN complete feed ration. Before experimental treatments, wethers (n=10) were fitted with lateral ventricle intracerebroventricular brain cannulas and randomly assigned to be centrally injected with 1 mL into the lateral ventricle with one of two treatments (n=5): (1) β-hydroxybutyric acid sodium salt solution (**BHB**; 12,800 µmol/L) or (2) saline solution (**CON;** 0.9% NaCl). Blood samples were collected every 10-min for 60-min prior to treatment injection and every 10 min for 120 min after injections. Serum glucose and insulin concentrations increased ($P < 0.01$) with BHB injection. However, injection of BHB decreased ($P < 0.01$) circulating serum NEFA concentrations. In addition, circulating endogenous serum BHB concentrations increased ($P < 0.01$) in wethers injected with exogenous BHB. There was a tendency ($P = 0.10$) for serum urea N to be

greater in the CON wethers compared to BHB-injected wethers. Injection of BHB did decrease ($P < 0.01$) overall mean LH. In addition, wethers injected with BHB had decreased ($P < 0.01$) amplitudes of LH peaks. In contrast, number of LH peaks during the 2 h sampling period after injection of treatments did not differ ($P = 0.18$) between wethers injected with BHB or CON. The results of this study indicate that elevated BHB in the brain mimics a negative energy signal leading to an increase in the mobilization of glucose, while reducing the amplitude of LH peaks.

INTRODUCTION

Metabolic dysfunctions and nutrient imbalances are known to have adverse impacts on reproduction (Mulliniks et al., 2013). Effects of metabolic dysfunction are characterized by ketosis, fatty liver, delayed resumption of estrous, reduced LH secretion, and delayed conception (Randel 1990; Herdt, 2000; Mulliniks et al., 2013). Metabolic imbalances can be further defined by low serum glucose and insulin concentrations detected concomitantly with elevated NEFA and β-hydroxybutyrate (BHB) concentrations (Butler, 2003; Zarrin et al., 2013). With these metabolic changes and decreases in energy status, increases in body tissue mobilization occur, resulting in increased NEFA concentrations. Increases in NEFA concentrations may exceed the ability of the liver to appropriately oxidize fatty acids to acetyl CoA to and subsequently lead to elevated circulating ketones, especially BHB.

Manifestation of metabolic dysfunction through elevated BHB concentrations could be an indication of poor adaptation to negative energy balance (NEB; Herdt, 2000) and modulate reproductive incompetence in domestic ruminants (Mulliniks et al., 2013;

Hobbs, 2016). Suppression of episodic releases of LH are detected in cattle and sheep during NEB (Imakawa et al., 1987; Thomas et al., 1990). Therefore, BHB concentration may be an indicator of metabolic status (Pushpakumara et al., 2003) and reproductive performance (Hobbs, 2016). Dairy cattle, during the early postpartum period, display a depressed LH pulse frequency coupled with elevated BHB (Walsh et al., 2007). Intraruminal infusion of acetate has been shown to increase circulating BHB concentrations while depressing mean LH and pulse amplitude (DiCostanzo et al., 1999). Therefore, the hypothesis for our research was that exogenous injection of BHB in the lateral ventricle of the brain will decrease LH secretion patterns in wethers. The objective of this study was to evaluate the effect of a central injection of exogenous BHB into the lateral ventricle on serum metabolites and LH concentrations in wethers.

MATERIALS AND METHODS

All experimental procedures and animal handling agreed with guidelines established and approved by the University of Tennessee's Institution of Animal Care and Use Committee (IACUC #2148).

Animals

Ten Suffolk and Suffolk-cross castrated male lambs [n=10; 49 ± 2 kg] were housed individually at the University of Tennessee Johnson Animal Research and Teaching Unit, Knoxville, TN with a 12 h light:12 h dark photoperiod cycle in a climate-controlled environment (~21°C). Wethers were utilized for the evaluation of LH profiles as previously indicated for the study of the reproductive axis (Beckett, et al., 1997; McManus et al., 2005; Renquist et al., 2007). Gonadectomized males can be used to

reduce confounding effects of endogenous LH surges as seen in female animals (Kesner et al., 1981). Additionally, wethers were utilized because castrated wethers can be used to study regulatory mechanisms for tonic and pulsatile release (Riggs and Malven, 1974). Wethers were offered a complete feed ration once daily at a rate of 1.1 kg/d of a 13.5% CP and 72.5% TDN (Table 1). Random samples of the ration were collected at the beginning and end of the study, composited into a single sample, and analyzed for nutrient content using wet chemistry procedures at a commercial laboratory (SDK Laboratories, Hutchinson, KS). Ad libitum water access was offered to the wethers throughout the duration of this study.

Treatments

Wethers were fit individually with intracerebroventricular (ICV) cannulas into the lateral ventricle of the brain as previously described (Whitlock et al., 2010) at least 2 wk before experimental treatments. Wethers were randomly assigned to receive a single injection into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or saline solution (CON; 0.9% NaCl). The dosage of BHB injection was determined based on previous research by Mulliniks et al. (2013) and Hobbs et al. (2016) to increase endogenous BHB by ~ 200 µmol/L. The pH of both BHB and CON injection treatments were measured at 7.4. Following aseptic preparation, treatments were administered through the ICV cannula with a 25 gauge Huber Point needle followed by 250 µl of saline. Beta-hydroxybutyrate solution was prepared according to a previously defined method by Zarrin et al. (2013). A DL-β-hydroxybutyric acid sodium salt (H6501, Sigma-Aldrich, St. Louis, MO) was used for the BHB solution

to reach a 12,800 μmol/L BHB solution. Once the solution reached 12,800 μmol/L of BHB, it was stored at 4°C until injection.

Sampling and analyses

In the morning of the serial blood collections, wethers were fit individually with indwelling jugular catheters. To establish baseline parameters prior to injection of experimental treatments, fasted (24 h) blood samples were collected for 60-min at 10-min intervals. After the 60-min baseline period, treatments were injected by the ICV cannulas and blood samples were collected for an additional 120-min at 10-min intervals. Ten-mL blood samples were collected at each collection time and placed in Corvac serum separator tubes (Corvac, Sherwood Medical, St. Louis, MO). Blood samples were collected, cooled, and centrifuged at 2,000 × *g* at 4°C for 20-min. Serum was collected, transferred to conical tubes, and stored at -20°C for subsequent analyses.

Serum samples were analyzed in duplicate for BHB, glucose, NEFA, urea N (SUN), insulin, and LH. Serum analysis for NEFA and SUN were performed using commercial kits (NEFA, Wako Chemicals, Richmond, VA) (SUN, Thermo Scientific, Middletown, VA). A glucose commercial kit was utilized for analysis (enzymatic endpoint, Thermo Scientific, Middletown, VA). Serum BHB concentrations were determined with the use of DL-β-hydoroxybutyric acid sodium salt, β-Nicotinamide adenine dinucleotide hydrate, and 3-hydroxybutyrate dehydrogenase (Sigma-Aldrich, St. Louis, MO) as described by McCarthy et al. (2015). Serum insulin concentrations were determined by RIA (EMD Millipore's Porcine Insulin RIA) using Wizard2 Gamma Counter (Perkin Elmer, Waltham, MA). Serum concentrations of LH were quantified by

RIA (Bishop and Wettemann, 1993) with NIH-bLH-B9 for standards. The intra- and inter-assay CV were, respectively, 1.8% and 2.2% for serum NEFA, 3.3% and 3.8% for serum glucose, 3.2% and 4.6% for SUN, 4.0% and 4.2% for serum BHB, and 5.1% and 4.2% for serum insulin.

Serum samples harvested during the 3 h collection period were utilized for the determination of LH pulse profiles for each treatment group (n = 5 per treatment) by previously established method (Schiewe et al., 1991; Clarke, 1993; Battaglia et al., 2000). LH pulsatility was determined from time -60 to 120 min relative to treatment administration using Pulse XP Software (Version 20090124; Johnson et al., 2008). Baseline LH concentration (-60 to -10 min immediately preceding treatment administration) were excluded from the determination of LH pulse. In addition, LH amplitude was determined by calculating the difference in the LH concentration between the baseline and that of the pulse peak as previously described methods by Kletter et al. (1997) and Foster and Olster (1985).

Statistical analyses

Data were analyzed using PROC MIXED in SAS (SAS Institute Inc., Cary, NC, version 9.4). Repeated measures in the MIXED procedure were used to analyze serum metabolite data. The repeated measure was time and the model was tested using sheep as the subject term. The model included fixed effects of sheep, time, treatment, and treatment by time interaction. Variance Components structure was determined to be most desirable covariance structure according the Akaike's information criterion. The data was analyzed using the Kenward-Roger degrees of freedom method. The MIXED procedure

of SAS was used to test all the main effects of the LH measurements. The main effects included treatment, time, and treatment by time interaction. Separation of Least Squares Means was performed using the PDIFF option in SAS when a significant ($P \leq 0.05$) effect by treatment was detected.

RESULTS

Serum metabolites

Serum glucose concentrations increased ($P < 0.01$; Table 2) with injection of BHB. Administration of BHB increased ($P < 0.01$) serum insulin concentrations, which is expected in response to an increase in serum glucose concentrations. Administration of BHB decreased ($P < 0.01$) serum NEFA concentrations. Endogenous serum BHB concentrations increased ($P = 0.002$) in BHB-injected wethers compared to their control counterparts. Serum urea N tended ($P = 0.10$) to be greater in the CON-injected wethers compared to BHB-injected wethers.

Luteinizing hormone

Administration of BHB did decrease ($P< 0.01$); Table 3; Figure 1) mean LH secretion. In addition, wethers administered BHB had lower ($P < 0.01$; Table 3) LH amplitudes of the pulses. In contrast, number of LH peaks during the 2 h sampling period after injection of treatments did not differ ($P = 0.58$; Table 3) between wethers administered BHB or CON.

DISCUSSION

The increase in serum glucose following BHB injection may indicate a stimulation of gluconeogenesis by the increase in BHB. Furthermore, the exogenous BHB may have indicated to the brain a state of low glucose availability, thus initiating gluconeogenesis. Contrarily, other studies have reported decreased concentration of glucose with intravenous infusion of BHB (Schlumbohm and Harmeyer 2003; Zarrin et al., 2013). In contrast, Rossi et al. (2000) reported circulating serum glucose concentrations were unaffected by the infusion of BHB. In agreement with Rossi et al. (2000), Arase et al. (1988) reported no differences in glucose concentrations with chronic infusion of BHB or saline into the third ventricle of rats. Unlike the present study, these studies did not infuse directly into the brain, but rather intravenously or intraperitoneally. The decrease in circulating glucose concentrations in the above-mentioned studies may have been related to a decrease in glucose production (Shaw and Wolfe 1984) rather than the utilization rate of the glucose. Furthermore, Park et al. (2011) reported glucose tended to be lower in rats after ICV infusion of BHB rather than infusion of artificial cerebrospinal fluid. However, Park et al. (2011) reported an increase of GLUT2 and glucokinase expression in the hypothalamus after BHB infusion into the lateral ventricle, indicating that an increase in BHB may enhance glucose sensing in the liver. Expression of GLUT2 in the nervous systems regulates glucose-sensing autonomic nervous activity, which could influence the liver (Thorens, 2015). Therefore, the enhanced glucose sensing in the brain post BHB injection may explain the contradiction in glucose results. In agreement with our study, Iwata et al. (2011a) reported an increase in serum glucose after

ventricular infusion of BHB in rats. Likewise, Iwata et al. (2011b) reported an increase in plasma glucose concentrations after injection of BHB into the fourth ventricle in male rats. Iwata et al. (2011a) suggested that ependymocyte glucose metabolism may be inhibited through an association with BHB, in response to an inhibition of pyruvate oxidation by 3-hydroxybutyrate in cerebrocortical mitochondria (Lai et al., 1987). Furthermore, the increase in glucose concomitantly with elevated BHB may be simulating an insulin resistant state (Tardiff et al., 2001). Insulin resistance increase gluconeogenesis and further decreases glucose uptake in insulin sensitive tissues (Ferris and Kahn, 2016). With that, accelerated gluconeogenesis is often observed along with elevated ketones (Kaneko).

Central administration of exogenous BHB increased insulin concentrations. Contrary to our results, Park et al. (2011) reported no differences in insulin concentrations after ICV BHB infusion in rats. In beef cows, a decrease in BHB concentrations has been reported to be associated with increased insulin concentrations (Mulliniks et al., 2013). By intravenously infusing BHB in pigs, Müller et al. (1984) also reported an increase in circulating insulin concentration. Furthermore, Madison et al. (1964) suggested that the increase in insulin to elevated BHB concentrations could potentially be attributed to the pancreatic beta cells response to the elevated ketone bodies. The increase in both glucose and insulin concentrations after BHB injection may be indicative a presumed insulin resistant state. In addition, elevated BHB has been reported to impair insulin action in rat cardiomyocytes (Tardif et al., 2001). Beta-hydroxybutyrate further exerts inhibitory effects on insulin by altering mitochondrial

metabolism (Yamada et al., 2010). Additionally, BHB may be inhibiting glucose uptake by peripheral tissues (Yamada et al., 2010).

Serum NEFA concentrations decreased with BHB injection. In agreement with our results, intravenously infusing BHB in pregnant ewes decreased plasma NEFA concentration (Harmeyer and Schlumbohm, 2006), which may be attributed to the inhibition of lipolysis by elevated circulating BHB (Lemosquet et al., 1997; Schiffelers et al., 1998; Dolnikoff et al., 2001). Contrary to our results, Mulliniks et al. (2013) did not report a decrease in NEFA concentration in cows with elevated endogenous BHB concentration. Iwata et al. (2011b) reported that an increase in NEFA concentration with ventricle injection of BHB in male rats. During periods of increased lipid mobilization NEFA enter the liver and potentially exceed the capacity of the liver to oxidize triglycerides appropriately (Ospina et al., 2010); thus, a feedback mechanism may inhibit lipolysis through the regulation of fat mobilization (Metz et al., 1974).

Endogenous BHB concentration increased with exogenous BHB injection. In a review, Laeger et al. (2010) suggested several possibilities for the effect BHB has on the periphery: (1) BHB acts as a cellular signal, (2) BHB provides necessary energy, or (3) BHB reflects fluctuations in liver fatty acid oxidation. Because ketone diffusion across the blood-brain-barrier is unidirectional (Hasselbalch et al., 1995), it was unexpected to see an increase in serum BHB after ICV injection of BHB. Additionally, Iwata et al. (2011b) reported an increase in plasma BHB levels after ventricle injection of BHB in male rats. Likewise, Park et al. (2011) reported an increase in circulating endogenous BHB concentrations in rats infused with exogenous BHB into the lateral ventricle of the

brain. These authors suggested that with the infusion of BHB, ketone body synthesis may be initiated within the liver (Park et al., 2011).

Central of infusion of BHB did not affect SUN concentration. In contrast to our results, SUN was not different between beef cows with high and low BHB concentrations (Mulliniks et al. 2013). Contrarily, SUN concentrations were decreased in dairy cows intravenously infused with BHB (Zarrin et al. 2013). Elevated BHB coincided with elevated SUN in dairy cows experiencing induced severe negative energy balance (Fenwick et al., 2008), which is contradictory to our results.

Exogenous administration of BHB decreased LH mean and pulsatility. Similarly to our results, elevated BHB in female ovariectomized rats suppressed LH mean and pulsatility suggesting that the increased concentration of BHB may function as a negative energy signal to inhibit gonadotropin secretion (Iwata, 2011a), thus inhibiting gonadal function. In a previous study in our lab, estradiol implanted wethers centrally injected with BHB had decreased LH amplitude; however, LH mean concentration was unaffected. The contradicting results are likely because of the enhanced negative potency of estradiol during energy deficit (Beckett et al., 1997), or BHB in this instance. Deficient LH secretion in response to impaired LH amplitude results in inadequate luteal stimulation in women (Jain et al., 2007). Therefore, the hindbrain may possess a sensor to detect the energy status in the cerebral spinal fluid (CSF), relaying information of nutrient status to regulate gonadotropin release (Iwata et al. 2011a). Fitzgerald et al. (1987) suggested that a decrease in LH amplitude may be caused by decreased responsiveness by the pituitary; thus, potentially explain LH amplitude sensitivity to energy status.

Additionally, axons of GnRH extend through the median eminence to release GnRH into the capillary bed of the hypophyseal portal blood system (Constantin, 2011). This connection through the median eminence to the anterior pituitary may offer an explanation for elevated BHB concentrations to alter GnRH release, subsequently influencing LH release from the anterior pituitary. Another possible regulatory site may have been altered expression of neuropeptide Y (NPY) in the hypothalamus to affect LH secretion. This neuropeptide is involved in energy availability and reproduction (Hess et al., 2005). Decreased energy availability increases NPY, subsequently resulting in an inhibition of GnRH and LH release (Daniel et al., 2013; Waterman and Butler, 2010). The interaction of NPY and reproductive hormones suggests a mechanism by which the interaction of peripheral signals, through energy availability, controls reproduction (Daniel et al., 2013). Reproductive activity through neuroendocrine control by energy status and nutritive state may be influencing the release of LH (Schillo, 1992). Therefore, energy substrate availability may mediate signals necessary to elicit hormonal responses necessary for reproduction (Chilliard et al., 1998).

Regarding the effect of BHB on reproduction, previous studies have linked associations with elevated endogenous BHB with reduced LH secretion (Randel, 1990) and delayed conceptions (Mulliniks et al., 2013). In dairy cows, increasing concentrations of serum BHB linearly decreases the likelihood of conceiving after first AI (Walsh et al., 2007). In a study with multiparous dairy cows, elevated BHB coincided with irregular or delayed estrus cycles (Pushpakumara et al., 2003). It has been established that with a lower amplitude of LH, follicular development is affected, and resumption of estrus is

delayed (Rawlings et al, 1984). The delay in luteal activity and resumption of estrus are attributed to the interface between the hypothalamus-pituitary-ovarian axis (Reist et al., 2000; Butler, 2003). However, it is unclear if BHB effects on the hypothalamus-pituitary axis are direct affects or the BHB action is through intermediate mechanisms (DiCostanzo et al., 1999).

Metabolic homeostasis is an orchestration of communication between serum metabolites and such orchestration is especially important during periods of metabolite alternations. Overall, the injection of exogenous BHB into the lateral ventricle may have influenced peripheral metabolites via direct action in the brain. This is further supported by the increase in insulin after BHB injection. Concurrently, the decrease in NEFA and increase in insulin may be indicative of decreased lipolysis because of the signaling abilities of β-hydroxybutyrate. During times of insulin resistance, when BHB is elevated circulating glucose increases along with insulin, resulting in a decrease in the adipose tissue mobilization. Insulin resistance is further defined by elevated BHB concentrations and hyperglycemia (Veech, 2004) and low free fatty acids (Brockman and Larvel, 1986).

CONCLUSIONS

The results of this study indicate that central injection of exogenous BHB effects the serum metabolite profile of wethers by increasing serum glucose and BHB concentrations, as well as decreasing circulating NEFA concentrations. Therefore, these results suggest that elevated BHB in the brain may mimic a negative energy signal leading to an increase in the mobilization of glucose and decrease circulating NEFA concentrations, which may indicate an increase in fatty acid oxidation. In addition, central injection of BHB reduced mean LH and amplitude of LH pulses. Thus, without sufficient LH amplitude, timing of estrus may be affected, which may be attributable to increased concentrations of endogenous BHB. Thus, BHB may act as a negative energy signal in the brain, which may lead to altered estrous cyclicity in response to reduction of LH.

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APPENDIX

Table 4. 1: Composition (DM basis) of wether feed ration

Item	
Ingredient, %	
Cotton seed hulls	25.00
Molasses	7.00
Cracked corn	56.00
Soybean meal	10.00
Calcium phosphate	0.20
Limestone calcium	1.00
Ade premix	0.10
TM salt	0.50
Dynamate	0.20
Nutrient composition	
Dry matter, %	88.33*
CP, %	13.47
ADF, %	16.37
aNDF, %	24.7
TDN, %	72.52
Calcium, %	0.74
Phosphorus, %	0.37
Potassium, %	1.08
Magnesium, %	0.23
Sodium, %	0.26
Sulfur, %	0.25
Aluminum, mg/kg	132.00
Cobalt, mg/kg	<0.20
Copper, mg/kg	5.06
Iron, mg/kg	157.00
Magnesium, mg/kg	18.70
Molybdenum, mg/kg	1.22
Zinc, mg/kg	30.70

*DM reported on an as fed basis

Table 4. 2: Effect of central injection of exogenous beta-hydroxybutyrate (BHB) into the lateral ventricle on circulating serum metabolites

	Treatments[1]			
Measurement	BHB	CON	SEM	P-value
Metabolites				
Glucose, mg/dL	68.79	59.34	2.27	< 0.01
Insulin, ng/mL	0.48	0.33	0.02	< 0.01
NEFA, µmol/L	229.56	322.74	18.90	< 0.01
BHB, µmol/L	90.64	47.29	9.81	< 0.01
SUN[2], mg/dL	18.07	24.39	2.70	0.10

[1]Treatment: A single centrally injection into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or saline solution (CON; 0.9% NaCl).

[2]SUN: serum urea N.

Table 4. 3: Effect of central injection of exogenous beta-hydroxybutyrate (BHB) into the lateral ventricle on LH parameters in wethers

	Treatments[1]			
Measurement	BHB	CON	SEM	*P*-value
LH mean, ng/mL	4.02	5.38	0.2	< 0.01
LH amplitude, ng/mL	1.61	3.97	0.3	< 0.01
LH peaks, no.	1.80	2.60	0.4	0.58

[1]Treatment: A single centrally injection into the lateral ventricle with 1 mL of β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or saline solution (CON; 0.9% NaCl).

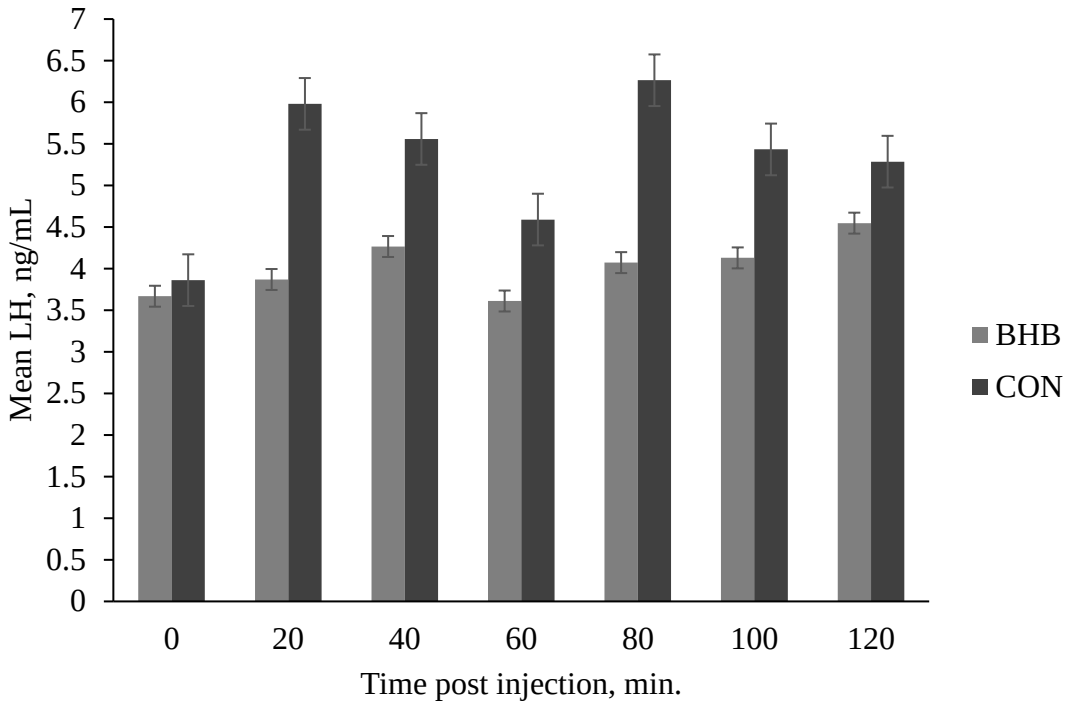

Figure 4. 1: Effect of central injection of 1 mL of β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or saline solution (CON; 0.9% NaCl) on mean LH concentration in wethers ($p < 0.01$)

CHAPTER V

EFFECT OF NUTRIENT RESTRICTION AND CENTRAL ADMINISTRATION OF BETA-HYDROXYBUTYRATE ON SERUM METABOLITES AND LUTEINIZING HORMONE IN OVARIEXCTOMIZED EWES

A version of this chapter is being prepared for publication by: Emily R. Cope, Jeremy D. Hobbs, Zachary D. MacFarlane, Brian K. Whitlock, Brynn H. Voy, Ky G. Pohler, and J.Travis Mulliniks.

ABSTRACT

The objective of this study was to evaluate the effect of nutrient restriction and lateral ventricle administration of exogenous β-hydroxybutyrate (BHB) on serum metabolites and LH concentration in ovariectomized ewes. Twenty-one ovariectomized Suffolk-crossed ewes were individual fed once daily of a 13.5% CP and 72.5% TDN diet. Ewes were stratified by BW and randomly assigned to be fed either at BW maintenance (MAINT) or fed at a 30% feed reduction (RES) for 79 d. On 64 d, ewes were fit with intracerebroventricular (ICV) cannulas. On 70 d, ewes were randomly assigned to be centrally injected for 10 d with 300 µl into the lateral ventricle twice daily with one of two treatments: (1) β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or (2) saline solution (SAL 0.9% NaCl). On d 57, 61, 70, 73, 77, 78 jugular blood samples were collected for analysis of serum glucose, insulin, BHB, NEFA, and urea N concentrations. On d 77, blood samples were collected every 15 min for 3 h for LH response. As designed, BW and BCS decreased ($P < 0.02$) in the RES ewes. Serum glucose concentrations decreased ($P = 0.02$) with infusion of BHB; however, glucose concentration was unaffected ($P = 0.88$) by feed restriction. Serum insulin concentrations were unaffected ($P = 0.81$) by central infusion of BHB; however, insulin concentrations did decrease ($P < 0.01$) in RES ewes. Endogenous BHB concentration was unaffected ($P > 0.73$) by exogenous BHB infusion or dietary treatments. Serum NEFA and urea N

concentrations increased ($P < 0.01$) in RES ewes; however, NEFA and SUN concentrations were unaffected ($P > 0.74$) by BHB infusion. Luteinizing hormone exhibited a tendency ($P = 0.06$) for a diet × treatment interaction. Exogenous administration of BHB did not affect LH pulse ($P = 0.69$); however, there was a tendency ($P = 0.09$) for BHB administration to increase LH amplitude. Additionally, LH pulse was decreased ($P = 0.05$) in feed restricted ewes. This study indicates that central infusion of exogenous BHB may be a metabolic signal to alter metabolic homeostasis through regulation of glucose. However, central infusion of BHB did not exacerbate the effects of negative energy balance or deleteriously affect LH parameters.

INTRODUCTION

Glucose metabolism is one of the most important aspects for metabolic homeostasis in ruminants. If glucose availability is decreased or glucose metabolism is impaired, then the depletion of oxaloacetate as a key intermediate for gluconeogenesis can occur (Hawkins et al., 2000). With these impaired metabolic processes and decreases in energy status, increases in body tissue mobilization occurs, resulting in increased circulating peripheral NEFA concentrations. Increases in NEFA concentrations may exceed the ability of the liver to appropriately oxidize fatty acids to acetyl CoA and subsequently lead to elevated circulating ketones, especially beta-hydroxybutyrate (BHB). Elevated concentrations of BHB have been associated with poor adaptation to negative energy balance (NEB: Herdt, 2000) and reproductive incompetence in domestic ruminants (Mulliniks et al., 2013; Hobbs, 2016). During NEB, suppression of episodic release of LH has been detected in cattle and sheep (Imakawa et al., 1987, Thomas et al.,

1990). Aside from functioning as a metabolic intermediate during periods of metabolic dysfunction, BHB can influence processes related to nutrient utilization both centrally and peripherally (Rojas-Morales et al., 2016). The hypothalamus and pituitary are known for playing central roles in the integration of information for energy balance (St-Amand et al., 2011). However, it is unclear if BHB effects on the hypothalamus-pituitary axis are direct affects or the BHB action is through intermediate mechanisms (DiCostanzo et al., 1999). Therefore, BHB may be a mediator of homeorhesis, which leads to altered metabolic and reproductive homeostasis.

The hypothesis for our research was that chronic exogenous infusion of BHB in the lateral ventricle of the brain will alter metabolites and LH in ovariectomized ewes and will be exacerbated by nutrient restriction and negative energy balance. Therefore, the objective of this study was to evaluate the effect of nutrient restriction and lateral ventricle infusions of exogenous BHB on circulating serum metabolites and LH in ovariectomized ewes.

MATERIALS AND METHODS

All animal handling and experimental procedures were in agreement with guidelines established and approved by the University of Tennessee's Institution of Animal Care and Use Committee (IACUC #2445).

Animals and treatments

Yearling Suffolk-crossed ovariectomized ewes [n = 21; 40 ± 2 (SD) kg] were stratified by BW and randomly assigned to be fed to either BW maintenance (MAINT) or

fed at a 30% feed restriction (RES) for a 79 d feeding period. Daily at approximately 0800 h, ewes were individually fed a diet that was 13.5% CP and 72.5% TDN (SDK Laboratories). Feed intake was recorded for each individual ewe by recording feed offered and refused. Throughout the duration of the study, ewes were provided ad libitum access to water.

All ewes were housed under natural light conditions for approximately 49 d at the University of Tennessee East Tennessee Research and Education Center, Knoxville TN. On 49 d of dietary treatments, all ewes were moved indoors and maintained under 12 h light: 12 h dark photoperiod in a temperature-controlled environment (~21°C) at the University of Tennessee Johnson Animal Research and Teaching Unit, Knoxville, TN.

On 64 d of dietary treatments, ewes were fit individually with intracerebroventricular cannulas (ICV) into the lateral ventricle of the brain as previously described (Whitlock et al., 2010). Briefly, ewes were maintained under general anesthesia and restrained in a sheep stereotaxic (David Kopf Instruments, Tujunga, CA, USA). A skull skin flap was created in order to place a reservoir port under the flap to facilitate future treatment infusions. A bone burr was used create a burr hole in the skull and to facilitate advancement of a Touhy needle into the lateral ventricle. Correct ventricular placement was determined when sterile physiological saline demonstrated a significant drop and pulse. On 70 d, ewes were randomly assigned to receive twice daily central infusions for 10 consecutive days with 300 µl into the lateral ventricle (~12 h apart) with one of two treatments: (1) β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or (2) saline solution (SAL; 0.9% NaCl). The dosage of BHB injection was

determined based on previous research by Mulliniks et al. (2013) and Hobbs et al. (2016). The pH of both BHB and CON injection treatments were measured at 7.4. After aseptic preparation, administration of ICV treatments were infused through ICV cannulas with a 25 gauge Huber Point needle followed by 400 µl of sterile saline to ensure the catheters were cleared of treatments. The BHB solution was prepared according to a previously described method by Zarrin et al. (2013). To avoid contamination of the ICV cannula, amounts for daily infusions were aliquoted under a sterile hood into sterile conical tubes. The solution of BHB was stored at 4°C until infusion.

Sampling and analyses

Blood samples (~ 9 ml) via jugular venipuncture were collected from ewes on d 57, 61, 70, 73, 77, and 78 for analysis of metabolic status. On the morning (~ 0800 h) of 77 d, ewes were individually fitted with indwelling jugular catheters prior to intensive blood sampling. Blood samples were collected every 15 min for 3 h for LH concentration. Blood samples were collected in Corvac serum separator tubes (Corvac, Sherwood Medical, St. Louis, MO), cooled, and centrifuged at 2,000 × g at 4°C for 20 min. Serum was harvested, poured into conical tubes, and stored at -20°C for subsequent analyses. On 70 d and 75 d, post morning infusions (~ 12 h), cerebral spinal fluid was successfully collected from approximately 71% and 81% of the ewes, respectively. Before CSF collection, 400 µl was first removed and discarded from the catheter to account for any remaining sterile saline from the previous treatment in the ICV catheter.

At approximately 0000 h on 78 d, all ewes received 100 µg of 17-β estradiol i.m. as to ensure measurable LH secretion baseline (Elsasser et al., 1983; Clark, 1993) and to

elicit an predictable LH surge approximately 12 h post estradiol injection (Malven et al., 1995; Rozell and Keisler, 1990). Estradiol was compounded under a sterile hood using 250 mg with Sesame oil NF (Letco; lot # 1603210062) to make a 2.5 mg/ml solution of β-estradiol (Sigma-Aldrich; lot # SLBP6339V). To achieve 0.05 mg/ml of solution, 2 ml of the previously compounded β-estradiol solution was added to 98 ml of Sesame oil NF. On 78 d, approximately 8 h (0700 h) after estradiol administration, blood samples were collected every 15 min for 8 h (Atkinson et al., 1989; Hileman et al., 1993) for LH concentration and serum metabolite analysis.

Blood samples collected during the serial blood collections were utilized for the determination of LH profiles for each ewe by previously established method (Schiewe et al., 1991; Clarke, 1993; Battaglia et al., 2000). Luteinizing hormone pulsatility was determined from time -60 to 120 min on d 77 and until -60 to 480 min on d 78 using Pulse XP Software (Version 20090124; Johnson et al., 2008). Pulse XP determines at what time point pulses occurred; however, pulse amplitude is not reported from the software. Pulse amplitude for each individual ewe was calculated based upon previously defined methods (Kletter et al, 1997; Foster and Olster 1985). In brief, the mean LH amplitude was calculated by subtracting the leading value nadir from the highest peak. The intra- and interassay CV were, 2.99% and 5.70% for LH.

Serum samples were analyzed for glucose, insulin, NEFA, BHB, urea N (SUN). Serum samples were analyzed using a 96-well microplate reader spectrophotometer with commercial kits for glucose (Thermo Electron Corp., Waltham, MA; sensitivity of 0.3 mg/dL), NEFA (Wako Chemicals, Richmond, VA; sensitivity of 0.01 mmol/L), and SUN

(Thermo Electron Corp., Waltham, MA; sensitivity of 2.0 mg/dL). Endogenous serum BHB concentrations were determined as previously described by McCarthy et al. (2015) using DL-β-hydoroxybutyric acid sodium salt, β-Nicotinamide adenine dinucleotide hydrate, and 3-hydroxybutyrate dehydrogenase (Sigma-Aldrich, St. Louis, MO). Serum insulin concentrations were determined by RIA (EMD Millipore's Porcine Insulin RIA) with a Wizard2 Gamma Counter (Perkin Elmer, Waltham, MA). Through the use of a commercial quantitative sandwich ELISA kit for Beta-Hydroxybutyratic Acid (MyBioSource, San Diego, CA) the CSF concentrations (µmol/L) were determined. The intra- and interassay CV were, respectively, 2.0% and 2.2% for serum NEFA, 3.3% and 1.8% for serum glucose, 2.3% and 2.6% for SUN, 3.0% and 3.6% for serum BHB, and 5.76% and 4.55% for serum insulin.

Ewes BW were recorded twice weekly for a weekly average BW. Individual ewe feed amounts were adjusted using weekly BW. In addition, BCS (1-5; Russel et al., 1969) were recorded throughout study duration by two trained technicians. Briefly, BCS was performed by palpating along the spinal processes of the lumbar vertebrae, between the last rib and the front of the hip bones on each individually ewe.

Statistical analyses

Data were analyzed using PROC MIXED in SAS (SAS Institute Inc., Cary, NC, version 9.4). Serum metabolite data were analyzed using repeated measures in the MIXED procedure. Day was the repeated measure and the models were tested using sheep as the subject term. The models for glucose, insulin, NEFA, and SUN included fixed effects of day, treatment, diet, breed, and treatment by diet interaction. The model

for BHB included fixed effects of treatment, diet, diet by treatment interaction, and day by diet interaction. The models for LH concentration, LH pulse, and LH amplitude included fixed effects of diet, treatment, and diet by treatment interaction using the MIXED procedure in SAS. In addition, LH concentration used a repeated measure of time and the model was tested using sheep as the subject. Interaction statements for LH concentration included diet by treatment, diet by time, and time by treatment. The most desirable covariance structure was Variance Components structure according the Akaike's information criterion. The Kenward-Roger degrees of freedom method was utilized for analyzing the data. Data are presented as least squares means and differences were considered significant at $P \leq 0.05$.

RESULTS

Animal performance

Ewe BW or BCS did not display ($P > 0.64$; Table 2) a diet × ICV infusion interaction. Ewe BW was reduced ($P < 0.01$) in RES ewes; however, BW was unaffected ($P = 0.56$) by BHB infusion. Likewise, BCS was decreased ($P = 0.02$) in RES ewes, but ewe BCS was unaffected ($P = 0.47$) by BHB infusion.

Serum metabolites and cerebral spinal fluid

Serum glucose concentrations decreased ($P = 0.02$; Table 2) with infusion of BHB. However, serum glucose concentrations were not different ($P = 0.88$) between dietary treatments. In contrast, serum insulin concentrations were unaffected ($P = 0.81$; Table 2) by central infusion of BHB; however, serum insulin concentrations were lower

($P < 0.01$) in RES-fed ewes. Endogenous BHB concentration was unaffected ($P \geq 0.73$) by exogenous BHB infusion and dietary treatments. Serum NEFA and SUN concentration were unaffected ($P \geq 0.74$) by BHB infusion. Both serum NEFA and SUN concentrations increased ($P < 0.01$) with RES-fed ewes. Concentration of BHB in the CSF was unaffected ($P = 0.49$; Table 3) by diet. Likewise, CSF BHB concentration was unaffected ($P = 0.86$) by exogenous administration of BHB.

During the intensive serial serum sampling on d 78, serum glucose concentration exhibited ($P = 0.01$; Table 4) diet × treatment interaction, with MAINT-fed, SAL-infused ewes having increased glucose concentrations. Serum NEFA concentration displayed ($P < 0.01$) a diet × treatment interaction such that RES-fed ewes with exogenous BHB infusion had increased concentrations of NEFA. Exogenous BHB infusion decreased endogenous circulating BHB concentration ($P < 0.01$). Likewise, feed restriction decreased ($P = 0.03$) endogenous circulating BHB concentration. Additionally, there was an interaction between diet × treatment such that RES-fed, BHB-infused ewes had increased SUN concentration ($P < 0.01$).

Luteinizing hormone

Luteinizing hormone concentration on d 78 during the 3 h sampling period exhibited a tendency ($P = 0.06$; Table 5) for a diet × treatment interaction with RES-fed ewes receiving either SAL BHB infusion having decreased LH concentration compared to MAINT-fed ewes receiving either BHB or SAL infusion. Exogenous infusion of BHB did not affect LH pulse ($P = 0.69$); however, there was a tendency ($P = 0.09$) for BHB administration to increase LH amplitude. In contrast, number of LH pulses was decreased

($P = 0.05$) in feed restricted ewes; however, LH pulse amplitude was unaffected by diet ($P = 0.29$).

DISCUSSION

As designed, ewe BW and BCS decreased with feed restriction compared to maintenance fed ewes. Previous studies in ruminants have reported nutrient restriction may cause a decrease in circulating metabolites and hormones (Blache et al., 2006: Roberts et al., 2009; Miller et al., 2011). During times of nutrient restriction body tissue mobilization can occur, such that mobilization of lean and adipose tissue is sufficient to meet animal requirements (Fenwick et al., 2008; Bjerre-Harpøth et al., 2012; Fiore et al., 2014).

Pooled serum glucose concentration across the duration of the study decreased with central administration of exogenous BHB. In addition, during intensive bleeding on d 78, circulating glucose concentration decreased for MAINT-fed ewes that were infused with BHB with no difference in RES ewes receiving either infusion. Park et al. (2011) reported male rats centrally infused with BHB had decreased glucose concentrations. The decrease in serum glucose concentration with BHB infusion may be indicative of an increase in glucose utilization in the TCA cycle. Lin et al. (2015) suggested an increase in ketone body metabolism as a compensatory mechanism following caloric restriction and elevated BHB levels in rats. Likewise, carotid infusion of BHB in mice resulted in a decrease in hepatic glucose production (Carneiro et al., 2016). The authors suggest the decreased glucose was the result of a country regulator response resulting in a normalization of metabolic parameters. Therefore, the results in the current study suggest

central infusion of BHB may have elicited a re-establishment of energy homeostasis, likely because glucose was not a limiting factor. As the hypothalamus is the primary regulator of homeostasis, the central response to ketone bodies may be mediated through the hypothalamus (Park et al., 2011). In contrast, previous work in our lab reported an increase in serum glucose in wethers centrally infused with BHB (Cope et al., 2016). The contradictory results may be explained by the duration of infusion or timing of sampling. Additionally, a glucose-sparing effect may be attributed for the decrease in glucose concentration reported in the BHB infused ewes (Moore et al., 1976; Zarrin et al., 2013). Despite feed restriction, glucose remained tightly regulated within both feed restricted and maintenance fed groups (Brockman and Laarveld, 1986). In contrast, glucose concentration decreased in RES with BHB- or SAL-infused ewes compared to MAINT-SAL infused ewes during the intensive serial bleeding. Additionally, the tight regulation of the pooled glucose during feed restriction period across time may be explained by the decrease in insulin concentrations to lower tissue glucose utilization in the RES-fed ewes (Ouellet et al., 2001).

Central exogenous administration of BHB did not affect serum insulin concentrations during the course of the study. In agreement, Zarrin et al. (2013) reported unchanged insulin concentration in cows intravenously infused with BHB. Likewise, serum insulin concentrations were not different between control and centrally infused BHB rats (Park et al., 2011). The increased nutrient intake in MAINT ewes suggests the increased insulin may be attributable to augmentation of insulin mediated peripheral glucose disposal (Schugar et al., 2012). In ruminants, ketone bodies represent a modest

stimulus for insulin secretion, which may explain the unchanged insulin concentration in BHB infused ewes (Jordan and Phillips, 1978). Serum insulin concentrations decreased in RES ewes compared to MAINT ewes. The decrease in insulin concentration in RES ewes is further explained by insulin being directly related to feed and energy intake (Brockman and Laarveld, 1986). Additionally, the elevated NEFA concentrations have been reported to be inversely related with insulin concentration (Snoj et al., 2014); therefore, increases in circulating NEFA concentrations may have inhibited insulin secretion.

Endogenous BHB concentration was unaffected by dietary treatments and exogenous BHB infusion with pooled samples across the study; however, during the 3 h serial sampling endogenous BHB concentration decreased with BHB infusion and feed restriction. According to Carneiro et al. (2016), short-term infusion of BHB into rat brains resulted in dysregulation of metabolic normalization; however long-term infusion resulted in a counter-regulatory response of metabolic normalization. Ketone bodies are utilized as alternate fuel sources during times of physiological stress or starvation, allowing for a glucose-sparing effect in certain tissues (Zarrin et al., 2013). While ketone bodies are always present in circulation, increases in ketone bodies are frequently observed concurrently with a NEB and lack of glucose (Laffel, 1999).

Due to increased BW loss, serum NEFA concentrations increased in RES ewes. Feed restricted and BHB infusion increased NEFA concentrations ewes during the intensive serial bleeds. Elevated NEFA concentrations have been previously reported in feed restricted steers (Hayden et al. 1993; Wertz-Lutz et al., 2008), thus indicating a prolonged catabolic state. The increase in NEFA in RES ewes was expected due to an

expected increase in tissue mobilization to offset the decreased nutrient intake for body maintenance. In agreement with our results, decreased insulin concentrations have been reported with elevated NEFA concentrations as a result of increased lipid mobilization (Fiore et al., 2014). In contrast to feed restriction, NEFA concentration was unaffected by exogenous infusion of BHB. This result may be indicative of increased BHB inhibiting lipolysis, as seen in cattle (Metz et al., 1974) and dogs (Fredholm, 1972). During the intensive bleeds, RES-fed, BHB-infused ewes NEFA concentrations increased; thus indicating an increase in tissue mobilization. Also, the BHB may have acted as negative energy signal to elicit the increase in tissue mobilization.

Serum urea N increased in RES ewes; however, SUN was unaffected by BHB infusion. In agreement, SUN was not different between lactating beef cows with low or elevated BHB concentrations (Mulliniks et al., 2013). Zarrin et al. (2013) reported decreased SUN concentrations in dairy cows intravenously infused with BHB. Fenwick et al. (2008) reported elevated SUN concentrations in dairy cows experiencing severe NEB, which may indicate an increased lean protein mobilization and explain our increase in SUN in RES ewes. Increased catabolism of amino acids from tissue proteins is stimulated by energy deficient states, which can result in elevated SUN concentrations (Bell, 1995). Between high and low milk producing dairy cows, decreased concentrations of circulating SUN and BHB were indicative of optimal production conditions (Nozard et al., 2012).

Luteinizing hormone exhibited a tendency for a diet × treatment interaction with RES-fed SAL-infused ewes having decreased LH concentration. Ewes fed RES and

infused with BHB had lower LH concentration compared to either of the MAINT-fed BHB-infused ewes or MAINT-fed SAL-infused ewes. Likely, the increase in LH concentration in MAINT ewes is an indication that because of adequate energy status from the MAINT diet, BHB infusion to not elicit a negative energy response. Furthermore, the availability of glucose thought to contribute the suppression of LH during NEB (Butler and Smith, 1989; Wade et al., 1996); therefore, with adequate glucose available to completely oxidize BHB, LH suppression may have been inhibited. In contradiction to our results, previous studies have reported decreased LH concentration with increasing BHB concentration (Randel, 1990; Matsuyama et al., 2009; Iwata et al., 2011). Additionally, elevated BHB concentrations have been reported to disrupt estrus cycles (Pushpakumara et al., 2003) and delay conception in beef (Mulliniks et al., 2013) and dairy cows (Walsh et al., 2007). In the current study, RES ewes had a decrease in LH concentration compared to their MAINT counterparts with either BHB or SAL treatment infusion. Ewes fed MAINT had increased LH concentration with either BHB or SAL infusion. In agreement, Schillo et al. (1992) reported heifers fed energy deficient diets had suppression of LH pulse frequencies compared to heifers fed maintenance diets. During acute nutrition deprivation, beef heifers failed to ovulate due to the absence of LH surges (Mackey et al., 1999). Ovariectomized beef cows that were fed a maintenance diet and to achieve a moderate body condition had greater LH concentrations compared to feed restricted cows fed to achieve a thin body condition (Looper et al., 1996). Taken together, the increase in LH concentration with MAINT-fed diets was not likely a biological effect of BHB infusion, but rather a response of adequate energy supply in

MAINT-fed ewes. Furthermore, increases in growth hormone have been reported concomitantly with increases in estradiol (Scanlan and Skinner, 2002); which may explain the inconsistencies between the LH concentrations between treatment groups and metabolites between the pooled and serial samples. In contrast, RES-fed ewes receiving either BHB or SAL infusions had decreased LH concentrations. It has been previous reported altered clearance rates of estradiol during feed restriction in ewes (Adams et al., 1994); which may have contributed the differences in LH concentrations.

On 79 d of serial blood collection, LH concentrations increased with regard to BHB treatment administration; however, LH surges for the ewes were not detected. According to previous literature, ovine LH surges are detected approximately 12 h post estradiol administration (Rozell and Keisler, 1990; Malven et al., 1995). In our study on 79 d of serial blood collection, blood sampling started approximately 8 h (0070 h) after estradiol administration and continued for a total of 15 h (1600 h) post estradiol administration.

Kaneko (1989) reported BHB concentrations accumulate in the blood when acetate oxidation is inhibited when glucose derived oxaloacetate is in inadequate supply. During prolong periods of starvation, ketone uptake by the brain increases (Gjedde and Crone, 1975); thus, indicating the potential for BHB to act as negative energy signal. The detection of BHB in the brain may be sensed by energy sensors, such that energy deficit is perceived (Iwata et al., 2011). Additionally, the rate of ketone body utilization by the brain is a direct reflection of circulating peripheral concentrations of BHB (Hasselbalch, 1995). According to Titgemeyer et al. (2011), a G-protein, GPR109A, may monitor

circulating levels of BHB concentrations when BHB binds to and activates the G-protein in the hypothalamus. Therefore, BHB may potentially affect intracellular signaling through transport or by binding to the membrane of GPR109A (Laeger et al., 2012). Specifically, endogenous BHB concentration decreased with endogenous BHB infusion and feed restriction. The BHB response may further support the idea that glucose was not limiting and was perhaps being spared for use in glucose specific tissues (Moore et al., 1976; Zarrin et al., 2013). With that, ruminants consuming a low-quality nutrient restricted diet experienced more deleterious effects than animals consuming a feed restricted, high-quality diet (Muna and Ammar, 2001). Therefore, our study design may not have been appropriate for a model of livestock experiencing diet-induced insulin resistance.

CONCLUSIONS

Due to the decrease in circulating glucose concentrations exhibited in beta-hydroxybutyrate infused ewes in this study, beta-hydroxybutyrate may serve as a potential energy signal to modulate peripheral metabolic status through a glucose-sparing effect. Whole animal metabolism is coordinated by glucose through the orchestration of alterations both centrally and peripherally. Additionally, chronic feed restriction may further comprise overall metabolic state; however, central infusion of beta-hydroxybutyrate in the lateral ventricle did not further exacerbate the effect of negative energy balance. The increase in LH concentration in this study was being driven by quality and nutrient supply of the diet, which did not inhibit oxidation. Though restricted-fed diets decreased ewe BW, glucose supply was likely not a limiting factor.

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APPENDIX

Table 5. 1: Composition (DM basis) of ewe feed ration

Item	
Ingredient, %	
Cottonseed hulls	25.00
Molasses	7.00
Cracked corn	56.00
Soybean meal	10.00
Calcium phosphate	0.20
Limestone calcium	1.00
Ade premix	0.10
TM salt	0.50
Dynamate	0.20
Nutrient composition	
Dry matter, %	88.33*
CP, %	13.47
ADF, %	16.37
aNDF, %	24.7
TDN, %	72.52
Calcium, %	0.74
Phosphorus, %	0.37
Potassium, %	1.08
Magnesium, %	0.23
Sodium, %	0.26
Sulfur, %	0.25
Aluminum, mg/kg	132.00
Cobalt, mg/kg	<0.20
Copper, mg/kg	5.06
Iron, mg/kg	157.00
Magnesium, mg/kg	18.70
Molybdenum, mg/kg	1.22
Zinc, mg/kg	30.70

*DM reported on an as fed basis

Table 5. 2: Effect of feed restriction and of a central injection of exogenous β-hydroxybutyrate (BHB) into the lateral ventricle on circulating serum metabolites

	Diet[1]							
	MAINT		RES			*P*-value		
Measurement	BHB[2]	SAL[3]	BHB	SAL	SEM	Diet	TRT[6]	Interaction[7]
Metabolites								
BW, kg	40.59	40.47	36.85	36.29	0.60	< 0.01	0.55	0.70
BCS	2.50	2.50	2.24	2.34	2.52	0.02	0.47	0.64
Glucose, mg/dL	76.31	90.14	80.89	84.48	4.82	0.88	0.02	0.18
Insulin, ng/mL	0.47	0.50	0.31	0.31	0.07	< 0.01	0.80	0.80
BHB, µmol/L	337.41	332.43	336.7	324.42	27.22	0.86	0.73	0.88
NEFA, µmol/L	80.52	86.67	131.98	134.39	16.82	< 0.01	0.74	0.88
SUN[8], mg/dL	12.44	14.57	19.78	18.12	2.06	< 0.01	0.88	0.24

[1]Diet: Ewes were assigned to one of two diets: maintenance of BW (MAINT) or 30% feed reduction (RES)

[2,3]Infusion treatment: A single centrally infusion into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB[2]; 12,800 µmol/L) or saline solution (SAL[3]; 0.9% NaCl)

[6]TRT: Treatment

[7]Interaction: Diet × Treatment

[8]SUN: Serum urea N

Table 5. 3 Effect feed restriction and of a central injection of exogenous β-hydroxybutyrate (BHB) into the lateral ventricle during intensive bleeding on cerebral spinal fluid (CSF)

	Diet[1]							
	MAINT		RES			*P*-value		
Measurement	BHB[2]	SAL[3]	BHB	SAL	SEM	Diet	TRT[5]	Interaction[6]
CSF	256.48	244.00	238.46	247.08	12.06	0.49	0.86	0.33

[1]Diet: Ewes were assigned to one of two diets: maintenance of BW (MAINT) or 30% feed reduction (RES)

[2,3]Infusion treatment: A single centrally infusion into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB[2]; 12,800 µmol/L) or saline solution (SAL[3]; 0.9% NaCl)

[4]CSF: Cerebral Spinal Fluid

[5]TRT: Treatment

[6]Interaction: Diet × Treatment

Table 5. 4 Effect feed restriction and of a central injection of exogenous β-hydroxybutyrate (BHB) into the lateral ventricle during intensive bleeding on circulating serum metabolites

	Diet[1]							
	MAINT		RES			*P*-value		
Measurement	BHB[2]	SAL[3]	BHB	SAL	SEM	Diet	TRT[4]	Interaction[5]
Metabolites								
Glucose, mg/dL	72.45^{c}	83.72^{a}	77.91^{bc}	78.48^{ab}	2.13	0.95	< 0.01	0.01
BHB, µmol/L	296.91^{bc}	339.13^{a}	276.55^{c}	311.79^{ab}	11.36	0.03	< 0.01	0.75
NEFA, µmol/L	85.27^{c}	90.74^{c}	130.72^{a}	107.65^{b}	5.54	0.11	< 0.01	< 0.01
SUN[6], mg/dL	10.24^{c}	12.03^{b}	13.89^{a}	10.58^{bc}	0.58	0.04	0.16	< 0.01

[1]Diet: Ewes were assigned to one of two diets: maintenance of BW (MAINT) or 30% feed reduction (RES)

[2,3]Infusion treatment: A single centrally infusion into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB[2]; 12,800 µmol/L) or saline solution (SAL[3]; 0.9% NaCl)

[4]TRT: Treatment

[5]Interaction: Diet × Treatment

[6]SUN: Serum urea N

Table 5. 5: Effect feed restriction and of a central injection of exogenous β-hydroxybutyrate (BHB) into the lateral ventricle on LH parameters pre-estradiol treatment

	Diet[1]							
	MAINT		RES			*P*-value		
Measurement	BHB[2]	SAL[3]	BHB	SAL	SEM	Diet	TRT[4]	Interaction[5]
LH mean, ng/mL	1.43[a]	1.16[b]	0.98[c]	0.90[c]	0.05	< 0.01	< 0.01	0.06
LH peaks, no.	4.40	4.80	3.30	3.40	0.59	0.05	0.69	0.77
LH amplitude, ng/mL	0.96	0.65	1.16	0.83	1.80	0.29	0.09	0.95

[1]Diet: Ewes were assigned to one of two diets: maintenance of BW (MAINT[2]) or 30% feed reduction (RES[3])

[2,3]Infusion treatment: A single centrally infusion into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB[2]; 12,800 µmol/L) or saline solution (SAL[3]; 0.9% NaCl)

[4]TRT: Treatment

[5]Interaction: Diet × Treatment

[a, b, c] Within diet, means with different superscripts differ ($P < 0.05$).

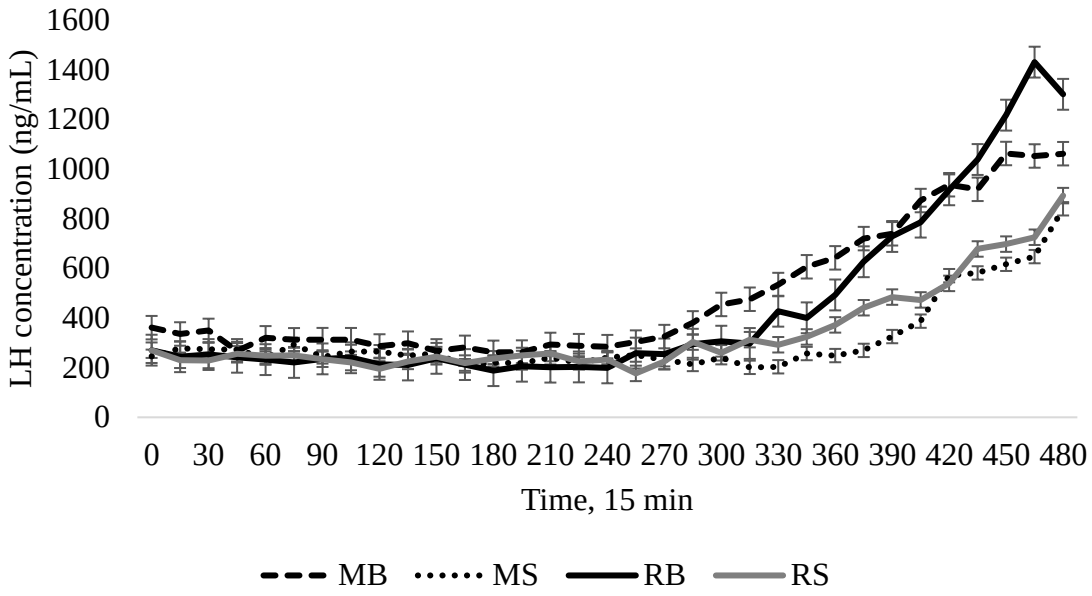

Figure 5. 1 Effect of feed restriction (RES) or maintenance (MAINT) diets and central injection of exogenous beta-hydroxybutyrate (BHB) or saline (SAL) into the lateral ventricle on LH estradiol-induced surge concentration in ewes
Infusion treatment: A single centrally infusion into the lateral ventricle with 1 mL of either β-hydroxybutyric acid sodium salt solution (BHB; 12,800 µmol/L) or saline solution (SAL; 0.9% NaCl)

CHAPTER VI

CONCLUSIONS

The inability to adapt to negative energy balance can have costly consequences on metabolic homeostasis and reproduction in ruminants. Central concentrations of BHB, which is both an energy source and a signaling metabolite that may connect peripheral energy status with reproduction and metabolism in ruminants. Elevated concentrations of BHB may act as a negative energy balance signal leading to alterations in serum metabolites and LH parameters in sheep. In ruminants depending on available intercellular glucose, BHB may control peripheral metabolic status through a glucose-sparing effect. Additionally, increased concentrations of BHB does alter gene expression in both the hypothalamus and pituitary. Specifically, exogenous BHB administration upregulated genes related to nutrient stress sensing; therefore potentially establishing a foundation to identify pathways that mediate the effects of BHB metabolically and reproductively. In 2 of the 3 studies, exogenous administration of BHB decreased LH pulse amplitude and mean LH concentrations. Suppression of LH amplitude can alter timing estrus and may be attributed to elevated endogenous BHB concentrations. The inconsistencies reported across studies may likely be due duration of treatment administration, as well as timing of sample collection. Additionally, the estradiol administration may have confounded some of the results through an enhanced effect of negative feedback potency, altered estradiol clearance rate due to feed restriction, or a concomitant increase in growth hormone secretion with increased estradiol. Metabolism is a coordinated process that is regulated by alterations both centrally and peripherally. During times of nutrient deficits metabolic state may be comprised; however, the quality of the diet may be more important than quantity for sustaining metabolic and

reproductive homeostasis. Specifically, if glucose is in adequate supply then oxaloacetate will be sufficient to appropriately oxidize accumulated BHB that may arise during NEB. Concentrations of BHB may be used as marker for identifying reproductive and metabolic efficient ruminants.

VITA

Emily Rebecca Cope was born in Raleigh, NC on November 3, 1986. She attended Athens Drive High School in Raleigh, NC where she graduated in May, 2005. She then attended North Carolina State University, majoring in Animal Science. She graduated from North Carolina State University with a Bachelor of Science degree in Animal Science in May, 2009. On May 29, 2010 she married Douglas Andrew Cope in Morehead City, NC. She earned her non-thesis Masters of Science degree in Animal Science from North Carolina State University in December, 2014. She continued her graduate work at the University of Tennessee, Knoxville, under the guidance of Drs. J. Travis Mulliniks and Brynn H. Voy. She received her Doctor of Philosophy degree, with a major in Animal Science in May, 2018.