AN ABSTRACT OF THE DISSERTATION OF

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In the first set of studies, 2 experiments evaluated the influence of supplement composition on ruminal forage disappearance, performance, and physiological responses of Angus × Hereford cattle consuming a low-quality, cool-season forage (8.7 % CP and 57 % TDN). In Exp. 1, 6 rumen-fistulated steers housed in individual pens were assigned to an incomplete 3 x 2 Latin square design containing 2 periods of 11 d each and the following treatments: 1) supplementation with soybean meal (PROT), 2) supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis; ENER), or 3) no supplementation (CON). Steers were offered meadow foxtail (*Alopecurus pratensis* L.) hay for ad libitum consumption. Treatments were provided daily at 0.50 and 0.54 % of shrunk BW/steer for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. No treatment effects were detected on rumen disappearance parameters of forage DM ($P \geq 0.33$) and NDF ($P \geq 0.66$). In Exp. 2, 35 pregnant heifers were ranked by initial BW on d -7 of the study, allocated into 12 feedlot pens (4 pens/treatment), and assigned to the same treatments and forage intake regimen as in Exp. 1 for 19 d. Treatments were fed once daily at 1.77 and 1.92 kg of DM/heifer for PROT and ENER, respectively, to achieve the

same treatment intake as % of initial BW used in Exp. 1 (0.50 and 0.54 % for PROT and ENER, respectively). No treatment effects ($P = 0.17$) were detected on forage DMI. Total DMI was greater ($P < 0.01$) for PROT and ENER compared with CON, and similar between PROT and ENER ($P = 0.36$). Accordingly, ADG was greater ($P = 0.01$) for PROT compared with CON, tended to be greater for ENER compared with CON ($P = 0.08$), and was similar between ENER and PROT ($P = 0.28$). Heifers receiving PROT and ENER had greater mean concentrations of plasma glucose ($P = 0.03$), insulin ($P \leq 0.09$), IGF-I ($P \leq 0.04$), and progesterone (P_4; $P = 0.01$) compared to CON, whereas ENER and PROT had similar concentrations of these variables ($P \geq 0.15$). A treatment × hour interaction was detected ($P < 0.01$) for plasma urea N (PUN), given that PUN concentrations increased after supplementation for ENER and PROT (time effect, $P < 0.01$), but did not change for CON (time effect; $P = 0.62$). In conclusion, beef cattle consuming low-quality cool-season forages had similar ruminal forage disappearance and intake, performance, and physiological status if offered supplements based on soybean meal or corn at approximately 0.5 % of BW (DM basis).

The following experiment evaluated the influence of supplement composition on performance, reproductive, and metabolic responses of Angus × Hereford heifers consuming a low-quality cool-season forage (8.7 % CP and 57 % TDN). Sixty heifers (initial age = 226 ± 3 d) were allocated into 15 drylot pens (4 heifers/pen; 5 pens/treatment), and assigned to the same treatments as reported above. Heifers were offered meadow foxtail (*Alopecurus pratensis L.*) hay for ad libitum consumption during the experiment (d -10 to 160). Beginning on d 0, PROT and ENER were provided daily at a rate of 1.30 and 1.40 kg of DM/heifer to ensure that PROT and ENER intakes were

isocaloric and isonitrogenous. Hay and total DMI were recorded for 5 consecutive days during each month of the experiment. Blood was collected every 10 d for analysis of plasma P_4 to evaluate puberty attainment. Blood samples collected on d -10, 60, 120, and 150 were also analyzed for PUN, glucose, insulin, IGF-I, NEFA, and leptin. Liver samples were collected on d 100 from 2 heifers/pen, and analyzed for mRNA expression of genes associated with nutritional metabolism. No treatment effect was detected (P = 0.33) on forage DMI. Total DMI, ADG, mean concentrations of glucose, insulin, and IGF-I, as well as hepatic mRNA expression of IGF-I and IGFBP-3 were greater ($P \leq$ 0.02) for PROT and ENER compared with CON, and similar between PROT and ENER ($P \geq 0.13$). Mean PUN concentrations were also greater ($P < 0.01$) for PROT and ENER compared with CON, whereas PROT heifers had greater ($P < 0.01$) PUN compared with ENER. Plasma leptin concentrations were similar between ENER and PROT ($P \geq 0.19$), and greater ($P \leq 0.03$) for ENER and PROT compared with CON on d 120 and 150 (treatment × day interaction; P = 0.03). Hepatic mRNA expression of mitochondrial phosphoenolpyruvate carboxykinase was greater (P = 0.05) in PROT compared with CON and ENER, and similar between CON and ENER (P = 0.98). The proportion of heifers pubertal on d 160 was greater ($P < 0.01$) in ENER compared with PROT and CON, and similar between PROT and CON (P = 0.38). In conclusion, beef heifers consuming a low-quality cool-season forage had a similar increase in DMI, growth, and overall metabolic status if offered supplements based on soybean meal or corn at 0.5 % of BW.

The last experiment was designed to determine if frequency of protein supplementation impacts physiological responses associated with reproduction in beef

cows. Fourteen non-pregnant, non-lactating beef cows were ranked by age and BW, and allocated to 3 groups. Groups were assigned to a 3 × 3 Latin square design, containing 3 periods of 21 d and the following treatments: 1) soybean meal (SB) supplementation daily (D), 2) SB supplementation 3 times/wk (3WK), and 3) SB supplementation once/wk (1WK). Within each period, cows were assigned to an estrus synchronization protocol; 100 µg of GnRH + controlled internal drug release (CIDR) containing 1.38 g of P_4 on d 1, 25 mg of $PGF_{2\alpha}$ on d 8, and CIDR removal + 100 µg of GnRH on d 11. Grass-seed straw was offered for ad libitum consumption. Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Moreover, 3WK were supplemented on d 0, 2, 4, 7, 9, 11, 14, 16, and 18, whereas 1WK were supplemented on d 4, 11, and 18. Blood samples were collected from 0 (prior to) to 72 h after supplementation on d 11 and 18, and analyzed for PUN. Samples collected from 0 to 12 h were also analyzed for plasma glucose, insulin, and P_4 (d 18 only). Uterine flushing fluid was collected concurrently with blood sampling at 28 h for pH evaluation. Liver biopsies were performed concurrently with blood sampling at 0, 4, and 28 h, and analyzed for mRNA expression of carbamoylphosphate synthetase I (CPS-I; h 28), and CYP2C19 and CYP3A4 (h 0 and 4 on d 18). Plasma urea-N concentrations were greater ($P < 0.01$) for 1WK vs. 3WK from 20 to 72 h, and greater ($P < 0.01$) for 1WK vs. D from 16 to 48 h and at 72 h after supplementation (treatment × hour interaction; $P < 0.01$). Moreover, PUN concentrations peaked at 28 h after supplementation for 3WK and 1WK ($P < 0.01$), and were greater ($P < 0.01$) at this time for 1WK vs. 3WK and D and for 3WK vs. D. Expression of CPS-I was greater ($P < 0.01$) for 1WK vs. D and 3WK. Uterine flushing pH tended ($P \leq 0.10$) to be greater for 1WK vs. 3WK and D. No treatment effects were

detected ($P \geq 0.15$) on expression of CYP2C19 and CYP3A4, plasma glucose and P_4 concentrations, whereas plasma insulin concentrations were greater ($P \leq 0.03$) in D and 3WK vs. 1WK. Hence, decreasing frequency of protein supplementation did not reduce uterine flushing pH or plasma P_4 concentrations, which are known to impact reproduction in beef cows.

In summary for all the experiments presented herein: (1) pregnant and developing replacement beef heifers consuming a low-quality, cool-season forage equally utilize and benefit, in terms of growth and metabolic parameters, from supplements based on protein or energy ingredients provided at approximately 0.5 % of heifer BW/d, (2) energetic supplementation at approximately 0.5 % BW/d did not impair forage disappearance parameters in rumen-fistulated steers, and (3) decreasing soybean meal supplementation frequency to once a week did not increase uterine pH, plasma P_4, and expression of hepatic enzymes associated with steroid catabolism in ruminants.

NUTRITIONAL STRATEGIES TO IMPROVE THE REPRODUCTIVE PERFORMANCE OF BEEF FEMALES

by

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I understand that my dissertation will become part of the collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Bruno Ieda Cappellozza, Author

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ABBREVIATION LIST

ADF = Acid Detergent Fiber

ADG = Average Daily Gain

AI = Artificial Insemination

ATP = Adenosine triphosphate

bST = Bovine Somatotropin

BW = Body Weight

CL = Corpus Luteum

CNS = Central Nervous System

CP = Crude Protein

CPS-I = Carbamoyl-phosphate synthetase-I

C_T = Threshold Cycle

CYP_{450} = Cytochrome P_{450} enzymes

d = Day

DM = Dry Matter

DMI = Dry Matter Intake

GH = Growth Hormone

GHR-1A = Growth Hormone Receptor-1A

GnRH = Gonadotropin-releasing Hormone

h = Hour

HS-60 = High-starch diet offered for 60 days

IGF-I = Insulin-like Growth Factor-I

IGFBP = Insulin-like Growth Factor-Binding Protein

IL-6 = Interleukin-6

IP_3 = Inositol Triphosphate

JAK = Janus-kinase Pathway

LH = Luteinizing Hormone

LS-30 = low-starch diet offered for 30 days

MCP = Microbial Crude Protein

min = Minutes

mRNA = Messenger RNA

NADPH = Nicotinamide adenine dinucleotide phosphate

NDF = Neutral Detergent Fiber

NEB = Negative Energy Balance

NEFA = Non-esterified Fatty Acids

NH_4^+ = ammonium

NPN = Non-protein Nitrogen

PC = Pyruvate Carboxylase

PEPCK-C = Cytosolic Phosphoenolpyruvate Carboxykinase

PEPCK-M = Mitochondrial Phosphoenolpyruvate Carboxykinase

P_4 = Progesterone

PI3-K = Phosphatidylinositol-3-kinase

PUN = Plasma urea-N

RPS-9 = Ribosomal Protein S-9

RDP = Rumen Degradable Protein

RUP = Rumen Undegradable Protein

TDN = Total Digestible Nutrients

VFA = Volatile Fatty Acids

wk = Week

CHAPTER 1

INTRODUCTION

Beef cattle production in Oregon is mainly constituted by forage-based cow-calf operations, representing the largest agricultural commodity in the state (OASS, 2014). In these operations, supplementation is often required to meet cattle nutritional requirements (Schillo et al., 1992), independent of the animal category (e.g., developing heifers and mature cows). In the Pacific Northwest, grasses are generally low-quality, cool-season forages (temperate or C_3), presenting a greater nutritional content when compared with warm-season roughages (Bohnert et al., 2011), but often not enough to fulfill the nutrient requirements of the cowherd. Historically, protein has been considered the most limiting nutrient when the herd consumes low-quality forages (DelCurto et al., 2000; Bodine and Purvis, 2003) and protein supplementation usually increases forage DMI and nutrient utilization when cattle consume low-quality, warm-season forages (Lintzenich et al., 1995; Koster et al., 1996; Kunkle et al., 2000). Conversely, Bohnert et al. (2011) demonstrated that protein supplementation to cattle consuming low-quality, cool-season forages often does not increase forage digestibility and DMI. Hence, inclusion of energy ingredients into supplements may be beneficial for growth and reproduction of ruminants consuming such forages.

Even though supplementation of the herd is important and paramount for adequate performance and reproductive responses, supplementation programs significantly increase production costs, including expenses associated with feed purchase and labor required for supplement feeding (Miller et al., 2001). One strategy that reduces the aforementioned expenses while maintaining adequate performance of the herd is to decrease the frequency in which the supplements are offered, from daily to once a week

(Huston et al., 199a,b). However, no research has directly evaluated the effects of this strategy on the reproductive responses of beef females.

CHAPTER 2

LITERATURE REVIEW

Reproductive maturation and resumption of estrous cycles

The reproductive development of ruminants starts during gestation, in a manner that severe nutritional challenges during fetal development permanently affects the future reproductive function of these animals (Caton and Hess, 2010; Ford and Long, 2012). The early stages of reproductive development through puberty attainment, and mechanisms associated with reproductive function of mature cattle are presented as follows:

Onset of Puberty in Heifers

The inclusion of replacement heifers into the cowherd is one of the most important factors affecting the overall efficiency of cow-calf production systems (Bagley, 1993). For optimal economic return and lifetime productivity, replacement heifers should attain puberty by 12 months of age, conceive by 15 months of age, and calve for the first time as 2-year olds (Lesmeister et al., 1973). Nevertheless, in this optimal scenario, replacement heifers will only provide initial economical returns to beef producers when they wean their first calf at 30 months of age.

Puberty in heifers is defined as the first estrous behavior, and ovulation of a fertile oocyte (which may or may not be associated with estrous behavior), followed by a luteal phase of normal duration (Larson, 2007). In other words, puberty is the manifestation of reproductive competence, which includes the ability to ovulate a viable oocyte,

demonstrate estrous behavior, and develop and maintain a functional corpus luteum (**CL**; Day and Anderson, 1998). The post-natal development and function of the reproductive tract starts as early as 1 month of age. During this period, the responsiveness of pituitary gonadotropin releasing hormone (**GnRH**) receptors begin to develop, and the rising luteinizing hormone (**LH**) concentrations at 3 months of age are coincident with the increased number of small- and medium-sized ovarian follicles, resulting in alterations in the population of vesicular follicles on the ovary (Day and Anderson, 1998). Consequently, there are alterations in the circulating concentrations of estradiol of ovarian origin, starting to rise at 2 months of age, but peaking only at 6 months of age, when the number of antral follicles is maximum. The increased levels of estradiol will, in turn, result in a negative feedback on the hypothalamic-pituitary axis (GnRH and LH, respectively), inhibiting the release of the GnRH and LH, thus preventing the pre-ovulatory surge of LH and consequent ovulation. The significance of this early rise in gonadotropin secretion, before 6 months of age in beef heifers, could be a critical early step in postnatal sexual development, stimulating an increase in follicular activity, ovarian cyclicity that persists throughout reproductive life, and triggering the reproductive tract development (Madgwick et al., 2005).

From 6 months of age until the peri-pubertal period (50 days before and after puberty), the main endocrine factor that the ruminant female must overcome in order to reach sexual maturation and become pubertal is to reduce the sensitivity of the hypothalamus to the negative feedback of estradiol, which in turn, will increase the pulsatile release of the GnRH from the hypothalamus and stimulate LH secretion (Day and Anderson, 1998). The mechanism by which this decreased feedback occurs likely

involves the reduction in the number of estradiol receptors in the tonic center of the hypothalamus as the heifer ages, increasing the pulsatile release of GnRH and the responsiveness of the pituitary gland to this hormone, allowing an increase in the pulsatile release of LH (roughly 1 pulse/h). Luteinizing hormone is responsible for the maturation of the follicle, which in turn will increase the synthesis and release of estradiol, leading to the pre-ovulatory surge of LH, consequent ovulation, and formation of the corpus luteum (Kinder et al., 1995; Williams and Amstalden, 2010). Furthermore, it has been demonstrated in female rabbits that as puberty approaches, the characteristics of GnRH neurons located in the arcuate and ventromedial hypothalamus change from a relatively smooth surface to cells with irregular, spiny surfaces (Foster et al., 1993). These authors reported that factors that hasten puberty accelerate these changes, whereas factors that delay puberty postpone these morphological transitions of the GnRH neurons.

Progesterone (**P_4**) also seems to be required for proper establishment of puberty in heifers (Gonzalez-Padilla et al., 1975). Although the major sources of P_4 in the ruminant are the CL and the placenta (Hoffmann and Schuler, 2002), significant increases in blood concentrations of P_4 are observed in heifers up to 2 wk prior to the onset of puberty (Gonzales-Padilla et al., 1975). It seems that the adrenal gland and luteal structures found within the ovary are the prepubertal sources for this hormone (Gonzales-Padilla et al., 1975; Berardinelli et al., 1979). Progesterone stimulates the onset of puberty by reducing the numbers of estradiol receptors in the hypothalamus, resulting in enhanced LH secretion (Anderson et al., 1996) and priming the hypothalamic-pituitary-ovarian axis to respond to the pre-ovulatory surge of estradiol (Looper et al., 2003).

In cattle, the puberal process is influenced by several factors, including nutrition, environment (season of the year, date of birth), genetics (breed), and management. Among these, nutrition, more specifically energy intake, is the most important environmental factor impacting the reproductive development and efficiency of beef herds (Maas, 1987). As an example, energy restriction during the pre-pubertal period delayed puberty attainment (Day et al., 1986) and ovulation (Lents et al., 2013) in beef heifers, up-regulating the numbers of estradiol receptors in the hypothalamus, and consequently delaying the decrease of the negative feedback of estradiol required to increase the responsiveness of the pituitary to GnRH (Day et al., 1986). Conversely, a negative relationship between pre- (Gasser et al., 2006a,b,c) and post-weaning (Arije and Wiltbank, 1974) body weight (**BW**) gains exist with age and BW at which beef heifers attain puberty. Furthermore, these associations seem to be regulated by hormones and metabolites that significantly impact the reproductive function of beef females, including glucose, insulin, insulin-like growth factor-I (**IGF-I**), and leptin (Spicer, 2001; Wettemann et al., 2003; Hess et al., 2005; Cooke et al., 2007).

Reproductive Function of Mature Cows

The main goal of cow-calf operations is to produce one calf/cow every year, whereas the major factor affecting the attainment of this goal is the post-partum period length (or calving-to-conception interval). Gestation length in *Bos taurus* beef cows is approximately 285 d; therefore, cows must be able to conceive within 80 d after calving to optimize reproductive efficiency of the herd.

Unlike early and mid-pregnancy, follicular waves are not detectable during the last wk of pregnancy (Ginther et al., 1996) and the first dominant follicle emerges only

10 to 12 d after calving (Savio et al., 1990). These outcomes indicate that other factors besides regular follicular growth are the limiting factor for estrus resumption in post-partum cows. Secretion of gonadotropins is a rate-limiting step for the initiation of follicular growth and estrus after calving. During late pregnancy, circulating concentrations of gonadotropins are reduced in cows because the constant production of estradiol and P_4 by maternal tissues impairs the synthesis of GnRH by the hypothalamus (Short et al., 1990; Lucy, 2003), and thus depletes pituitary reserves of LH and FSH (Wettemann et al., 2003). However, LH reserves and follicular growth are re-established wk prior to the resumption of cyclicity in most beef cows (Yavas and Walton, 2000; Day, 2004), but postpartum anestrous is sustained because estradiol still exerts a negative feedback on gonadotropin synthesis (Nett et al., 1987; Short et al., 1990), whereas estradiol is not produced by the dominant follicle in sufficient amounts to trigger the LH surge required for ovulation (Day, 1994). Reduced pulsatile secretion of LH during the early post-partum period is likely associated with decreased GnRH secretion because the number and affinity of GnRH binding sites on the pituitary do not change during the post-partum period (Moss et al., 1985). Moreover, pulsatile secretion of LH is associated with secretion of GnRH in cows (Yoshioka et al., 2001) and increased frequency of exogenous GnRH pulses increased the frequency and mean concentrations of LH in anovulatory post-partum cows (Vizcarra et al., 1997).

Progesterone also appears to be required for proper resumption of estrous cycles in post-partum cows. Plasma concentrations of P_4 are minimal at parturition (Smith et al., 1973) and transient increases prior to first ovulation seems to influence the endocrine changes required by the reproductive system of post-partum cows to attain normal and

fertile estrous cycles (Kinder et al., 1987). The first increase in plasma P_4 in beef cows after calving usually persists for 3 to 9 d (Looper et al., 1999), originating from luteinized follicles (Rawlings et al., 1980) or short-lived CL tissues from undetected ovulations (Perry et al., 1991), though it is not preceded by behavioral estrus. Looper et al. (2003) reported that 81 % of cows that had transient increases in concentrations of P_4 before first estrus had normal luteal activity compared to 36 % of cows that did not present an increase in P_4.

The effects of P_4 in ruminants are not limited to the attainment of puberty and resumption of estrous cycles, being also required for proper establishment and maintenance of pregnancy (Spencer and Bazer, 2002). Progesterone, produced by the CL originating from the ovulated dominant follicle, suppresses gonadotropin synthesis, release, and consequent ovulation during gestation (Hess et al., 2005). Furthermore, P_4 prepares the uterine environment for the growth and development of the conceptus, and modulates the release of hormones that may regress the CL and disrupt pregnancy (Bazer et al., 1998). Therefore, it is paramount to increase the lifespan of the CL, whereas nutritional and/or management strategies that increase the plasma concentration of P_4 before and after breeding/AI increase conception rates in cattle. As an example, low systemic P_4 concentrations on d 5 post-ovulation, or a delay in the normal rise in P_4 between d 4 and 5 post-ovulation, has been associated with reduced pregnancy rates in cattle (Larson et al., 1997; Starbuck et al., 2001). Fonseca et al. (1983) indicated that conception rates to AI in dairy cows increased by 13 % for each ng/mL increase in average blood P_4 concentration during the 12-d period preceding AI. Similarly, Folman et al. (1990) reported that plasma P_4 concentrations prior to AI were highly correlated (r

= 0.87) with conception rates in dairy cows. Robinson et al. (1989) observed an increase in the proportion of pregnant cows that received a progesterone-releasing intravaginal device from d 5 to 17 after breeding. Demetrio et al. (2007) reported a positive relationship between conception rates and serum P_4 concentrations on d 7 following AI in dairy cows. Stronge et al. (2005) reported that both sub- and supra-optimal concentrations of P_4 from d 4 to 7 after AI or a sub-optimal rate of increase in the concentration of P_4 during this interval were negatively associated with embryo survival rate in dairy cows. Circulating concentrations of P_4 may also be modulated by diet, more specifically high-starch or propiogenic diets (Lemley et al., 2008; Lemley et al., 2010), in a manner that increased concentrations of glucose, that subsequently will increase circulating concentrations of insulin and regulate the activity and expression of enzymes associated with hepatic steroid catabolism (Vieira et al., 2013). More information regarding these mechanisms will be presented in a later section.

Post-partum anestrus is primarily caused by nutritional stress and/or calf suckling. However, due to the subject of the studies presented herein, we will focus on the effects of nutrition in the anestrus occurrence, and for additional information on suckling, the reader is referred to other research papers (Stevenson et al., 1997; Lamb et al., 1999). Briefly, calf suckling stimulates the release of endogenous opioids, which in turn will inhibit the pulsatile release of LH by the animal, consequently inhibiting ovulation to occur. The anestrus condition in post-partum cows is caused by reduced ovarian follicular growth and the absence of luteal activity (Wettemann et al., 1980). The mean concentration and frequency of LH pulses increase with time before the first post-partum ovulation, while inadequate pulses of LH due to poor nutrition may cause recurring

follicular waves and atresia of the dominant follicle (Wettemann et al., 2003). However, it is still unknown if these mechanisms are associated with an increased sensitivity to the negative effects of estradiol on LH concentrations and pulsatility.

Supplementation strategies for forage-based cow-calf systems

The vast majority of cow-calf operations in the Pacific Northwest rely on cool-season forages as the main feed source for beef cattle. Also, ruminants often consume low-quality (< 7 % CP) forages for extended periods during a year (DelCurto et al., 2000), given that these forages vary significantly with respect to quality parameters such as DM digestibility and CP content, mainly due to differences in plant variety, stage of maturity, and management practices. Ganskopp and Bohnert (2001) demonstrated that forages sampled from July to December in Southern Eastern Oregon are unable to provide the minimum amount of CP needed for proper function of the rumen, suggesting that CP supplementation is required.

As previously mentioned, protein has been considered the limiting nutrient in forage-based cow-calf systems in the Pacific Northwest (DelCurto et al., 2000), and supplemental CP is often provided to increase forage intake (Lintzenich et al., 1995), DM digestibility (DelCurto et al., 1990), and BW gain (Bodine et al., 2001). However, it is important to note that all these research studies used low-quality warm-season forages, whereas Bohnert et al. (2011) reported that the same outcome often does not occur in cattle consuming low-quality, cool-season forages. In fact, Bohnert et al. (2011) demonstrated that forage intake and nutrient digestibility increased only when animals were fed a low-quality warm-season forage (tallgrass prairie), indicating that CP

supplementation may not be the best option to enhance nutrient intake with C_3 forage consumption. Given that the hormones and metabolites associated with energy intake and metabolism are important for proper growth and reproductive function in beef females, it might be speculated that inclusion of energy ingredients into diets of ruminants consuming low-quality cool-season forages might be beneficial to growth and reproductive performance of ruminants.

Energy metabolism

As aforementioned, a balanced ration is the primary nutritional consideration for optimal reproductive performance of beef females, accounting for most of the costs associated with the beef production system (Miller et al., 2001) and that the producers are able to control at some point (Dunn and Moss, 1992). Among these, energy is the primary nutrient involved in the process described above (Maas, 1987). Puberty can be hastened by increasing energy intake and the consequent BW gain (Schillo et al., 1992). Ciccioli et al. (2005) evaluated the effects of energy supplementation programs and the amount of starch in the diet on incidence of puberty in spring-born heifers. Heifers fed a high-starch (**HS-60**) diet (73 % corn; 53 % starch) in a drylot for 60 d before breeding season had similar BW at breeding season compared with heifers self-fed a low-starch (**LS-30**) diet (49 % corn; 37 % starch) on pasture for 30 d; however, the HS-60 treatment had 31 % more heifers reaching puberty than LS-30. Those authors associated these results with a shift in ruminal fermentation towards propionate production. Greater ruminal concentrations of propionate have increased blood glucose concentrations, the secretion of LH after a GnRH challenge (Rutter et al., 1983), and decreased age at puberty of

prepubertal heifers (Moseley et al., 1977; McCartor et al., 1979). These beneficial effects associated with energy feeding can be attributed to plasma hormones and metabolites, such as glucose, insulin, leptin, and IGF-I (Spicer, 2001; Hess et al., 2005), that are influenced positively by energy intake and by levels of body energy reserves. Cooke et al. (2012) reported an increase in glucose and insulin following exogenous administration of both glucose and insulin, which in turn, stimulated a positive response on circulating concentrations of P_4.

Glucose

Glucose is a small, polar, and water-soluble monosaccharide (Nussey and Whitehead, 2001), essential for maintenance and productive functions of ruminants (Huntington, 1997; Reynolds, 2005). Several body cells and tissues, such as red blood cells and the central nervous system (**CNS**), are predominantly dependent on glucose in order to maintain their normal function. Previous research has indicated that blood glucose concentrations in beef cattle are positively associated with feed intake and rates of BW gain (Vizcarra et al., 1998; Hersom et al., 2004). Glucose is the major energy source required by the ruminant and they meet their requirements primarily through the process of gluconeogenesis, the synthesis of glucose from non-carbohydrate sources (Randel, 1990). Metabolic fates of glucose include generation of ATP via glycolysis and the Krebs cycle (or TCA cycle), as well as generation of NADPH through the hexose monophosphate shunt (Huntington, 1997).

The process of gluconeogenesis occurs predominantly in the liver (90 %), whereas the remaining occurs in the kidneys (Huntington, 1997). The major substrate used for gluconeogenesis in ruminants is propionate, a volatile fatty acid (**VFA**)

originating from ruminal fermentation (Reynolds et al., 1994; Reynolds et al., 2005), followed by other substrates originating from the digestive process, such as glycerol, lactate, and gluconeogenic amino acids (Huntington, 1997). After ruminal metabolism, these substrates are removed by the liver from the portal circulation, where they go through further metabolism. Propionate is oxidized into succinyl-CoA, a Krebs cycle intermediate, and then converted to oxaloacetate, which in turn can be converted into phosphoenolpyruvate (Nelson and Cox, 2005) and follow the gluconeogenesis pathway. Lactate is primarily converted into pyruvate as part of the Cori cycle, whereas amino acids can be either degraded into pyruvate, oxaloacetate, or several Krebs cycle intermediates (Nelson and Cox, 2005). As aforementioned, the steps of the gluconeogenesis pathway are the conversion of pyruvate into oxaloacetate by the enzyme pyruvate carboxylase (**PC**), and oxaloacetate into phosphoenolpyruvate catalyzed by the phosphoenolpyruvate carboxykinase (**PEPCK**; Nelson and Cox, 2005). Following these steps, the gluconeogenesis process uses the majority of the same enzymes and substrates seen in the glycolysis pathway. Drackley et al. (2006) reported that these enzymes, PC and PEPCK, regulate the rate of gluconeogenesis in mammals, including cattle. Furthermore, PEPCK has two isoforms of similar activities and kinetic properties, subdivided into the cell mitochondria (**PEPCK-M**) and cytosol (**PEPCK-C**; Agca et al., 2002).

Previous studies have demonstrated that the expression of the gluconeogenic enzymes PC and PEPCK-C reflect the differences in nutrient intake pattern observed in ruminants (Cooke et al., 2008), and the expression of these enzymes is positively associated with enzymatic activity and consequent glucose synthesis in cattle (Greenfield

et al., 2000; Agca et al., 2002; Bradford and Allen, 2005). Furthermore, the availability of gluconeogenic precursors is positively correlated with the mRNA expression of PC and PEPCK-C in the liver of dairy cattle (Hammon et al., 2003; Karcher et al., 2007). On the other hand, other authors demonstrated that the expression of PC and PEPCK-C were inhibited directly by elevated plasma concentrations of glucose and insulin (Agca et al., 2002; Hammon et al., 2003), corroborating the statement that nutrients and hormones are able to regulate PC and PEPCK-C expression. Nutritional deficiencies also have been shown to regulate the expression of the gluconeogenic enzymes. Velez and Donkin (2005) reported an increase in PC in lactating cows fed 50 % of maintenance requirements for 5 d compared to ad libitum-fed cows, while PEPCK was unaffected by feed restriction. White et al. (2011) demonstrated that exposure of rat hepatocyte cells to serum from feed-restricted and serum from normal-fed cows plus fatty acids increased the expression of PC, indicating that NEFA may signal inadequate availability of nutrients and stimulate the gluconeogenesis process. Conversely, PEPCK-M is considered constitutive and not responsive to hormones and nutritional state (Greenfield et al., 2000; Agca et al., 2002), although it may be responsible for as much as 61 % of glucose synthesis in ruminant hepatocytes (Aiello and Armentano, 1987).

Given the importance that glucose has in maintaining proper functioning of the CNS and red blood cells, it is not surprising that the lack of this nutrient significantly impairs several body functions. Inadequate glucose availability reduces synthesis and release of GnRH and gonadotropins by the brain, impairing reproductive function of the herd (Bucholtz et al., 1996). In fact, previous studies in the literature reported that monensin supplementation, an additive that increases propionate and consequently

glucose concentrations, decreased age at puberty in beef heifers (Purvis and Whittier, 1996) and shortened the postpartum interval in beef cows (Hixon et al., 1982). Previous studies have shown that ruminants administered glucose antagonists experienced induced anestrus, anovulation (McClure et al., 1978), and impaired LH secretion (Funston et al., 1995; Bucholtz et al., 1996). In cattle, the hypothalamus may recognize low blood glucose concentrations in a threshold-dependent fashion, given that GnRH secretion is impaired when glucose availability is inadequate, but resumed when glucose levels are adequate (Hess et al., 2005). On the other hand, Wettemann and Bossis (2000) indicated that glucose concentrations in blood are probably not a major factor controlling follicular growth and ovulation because glucose concentrations in anestrous heifers were similar to those observed in cycling heifers at least two follicular waves before resumption of cyclicity. Blood glucose concentration in cattle is fairly stable due to the role of insulin, which may prevent proper assessment of glucose on reproductive function and nutrient flux often observed (Marston et al., 1995; Hess et al., 2005).

Insulin

Bovine insulin is a small peptide hormone containing 51 amino acid residues and a molecular weight of 5.7 kDa (Smith, 1966). Insulin is synthesized within the pancreas and initially stored as a pro-hormone in secretory granules in the pancreatic beta-cells (Nelson and Cox, 2005). Several stimuli trigger the conversion of pro-insulin into active insulin, which is consequently released into the bloodstream, whereas the other side chain that was cleaved has almost no action in the body (Nelson and Cox, 2005). The main effects of insulin are on liver, muscle, and adipose tissues, where it increases anabolic processes and consequently decreases tissue catabolism (Nussey and Whitehead, 2001).

Pancreatic secretions differ in ruminants compared to non-ruminants because ruminant species lack a measurable, postprandial, absorptive and post-absorptive state (Harmon, 1992), due in part to ruminal fermentation that leads to a continuous absorption of fermentation end products and a semi-constant flow of digesta. In ruminants, VFAs have a dramatic influence on insulin secretion. Bassett (1972) reported an increase in insulin and glucagon secretion after equal molar infusions of propionate, butyrate, and valerate by itself compared to infusions of acetate or a mixture of VFAs.

Although insulin is secreted in pulses approximately every 10 min (Nussey and Whitehead, 2001), it follows a biphasic release pattern by the pancreatic β-cells: (1) quick release in response to increased blood glucose concentrations, therefore maintaining metabolic homeostasis because blood glucose is maintained within constant concentrations, and (2) a sustained, slow release of newly-formed vesicles independent of sugar stimulus. Additionally, insulin secretion is also stimulated by gastrointestinal hormones and neural/paracrine mechanisms associated with feed intake (Nussey and Whitehead, 2001). Glucose, in turn, enters the muscle and fat tissues via intracellular GLUT-4 transporters that are recruited and increase their expression as a result of insulin stimulation, enhancing cell ATP production, glycogen synthesis in muscle, and lipogenesis in fat tissues (Nelson and Cox, 2005). In the liver, insulin promotes glycogenesis, but does not modulate glucose uptake by the hepatocytes via GLUT-2 transporter. Insulin also modulates cellular uptake of amino acids and some electrolytes (Austgen et al., 2003). Insulin has been shown to be highly correlated with nutrient intake and ADG in cattle (Vizcarra et al., 1998; Cooke et al., 2007a; Cooke et al., 2008). Cooke et al., (2007a) reported greater mean insulin concentrations (0.89 vs. 0.75 ng/mL,

respectively) and ADG (0.40 vs. 0.30 kg/d, respectively) for grazing heifers consuming citrus pulp-based supplements instead of isocaloric and isonitrogenous supplements based on sugarcane molasses. Frequency of supplementation has also been shown to alter the circulating concentrations of insulin. Cooke et al. (2007b) reported that forage-fed steers supplemented daily with a citrus pulp-based supplement had greater ADG (0.30 vs. 0.18 kg/d, respectively), but inferior mean insulin concentrations (0.46 vs. 0.60 ng/mL, respectively) compared to steers offered supplements three times a wk.

Furthermore, insulin has been recognized as an important metabolic signal affecting the reproductive function of ruminant females, by stimulating ovarian steroidogenesis and mitosis (Spicer and Echternkamp, 1995). Hence, cows with low insulin concentrations have impaired LH surge, and/or reduced numbers of LH receptors in the dominant follicle, which fails to ovulate even if the LH surge is present (Diskin et al., 2003). In a review on the control of follicular growth, Webb et al. (2004) reported that insulin infusion in beef heifers increased dominant follicle diameter, as well as ovulation rate in energy-deprived heifers, suggesting that management practices designed to increase circulating insulin concentrations may help to improve heifer reproductive performance. Gong et al. (2002) fed lactating dairy cows with diets to stimulate (propiogenic ingredients) or maintain insulin concentrations at normal levels (acetogenic ingredients) from d 0 to 50 post-calving. These authors reported similar circulating concentrations of gonadotropin, milk yield, as well as BW and BCS changes, whereas cows fed insulin-stimulating diets had greater plasma insulin concentrations and reduced postpartum interval (34 vs. 48 d) compared to cows offered the acetogenic diet.

Besides a direct effect on steroidogenesis, insulin also can regulate the expression and/or activity of hepatic cytochrome P_{450} enzymes involved in steroid catabolism. Cooke et al. (2012) demonstrated that non-lactating and non-pregnant cows receiving exogenous glucose and insulin had greater concentrations of P_4 compared with cohorts receiving saline. Arias et al. (1992) reported that GnRH release from perifused hypothalamic fragments of female rats was dramatically increased when glucose and insulin were added simultaneously to the perifusion medium compared to those containing no supplemental glucose, insulin, or both. Conversely, insulin administration by itself failed to increase LH secretion in lambs (Hileman et al., 1993) and to inhibit hepatic steroid catabolism in beef females (Cooke et al., 2012), indicating that glucose might be needed so that insulin mat affect the reproductive function of ruminants.

Insulin-like Growth Factor-I (IGF-I)

The somatotropic axis is an essential constituent of multiple systems controlling growth (Le Roith et al., 2001) and reproduction (Hess et al., 2005). The key components of the somatotropic axis include growth hormone (**GH**) and its receptors, IGF-I, IGF-receptors, and the IGF-binding proteins (**IGFBPs**; Lucy et al., 2001). The bovine IGF-I is a single-chain, polypeptide hormone with a molecular weight of roughly 7.6 kDa (Etherton, 2004) that is involved in carbohydrate, protein, and fat metabolism, as well as cell proliferation and differentiation (Jones and Clemmons, 1995). The liver is the primary source of circulating IGF-I, with hepatic IGF-I responsible primarily for its systemic effects (Yakar et al., 1999), whereas IGF-I synthesized by non-hepatic tissues mainly exert autocrine and paracrine effects (McGuire et al., 1992). Growth hormone is the primary regulator of IGF-I synthesis (McGuire et al., 1992), and its secretion into the

circulation occurs in a constant pattern (Thissen et al., 1994). The activity of IGF-I within target tissues is modulated by the binding of IGF-I to the IGFBPs and IGF-I receptors (Thissen et al., 1994; Le Roith et al., 2001). In fact, the majority of IGF-I (~ 90 %) found in the blood and other body fluids is bound with high affinity to one of six different IGFBPs, which vary in length from 201 to 289 amino acid residues, and molecular weight from 24 to 44 kDa (Thissen et al., 1994; Beattie et al., 2006). The IGFBPs bind to IGF-I with high affinity and are responsible for transporting IGF-I among body tissues, extending its half-life and enhancing or blocking its binding to IGF-I receptor (Le Roith et al., 2001). Approximately 90 % of circulating IGF-I is bound to IGFPB-3 (Martin and Baxter, 1992), whereas the remainder is bound to IGFBP-1, IGFBP-2, or IGFBP-4 (Clemmons, 1991). The binding of IGF-I to IGFBP-3 generates a complex that is incapable of crossing the capillary endothelium (Binoux and Hossenlopp, 1988). Hence, IGF-I must be released from IGFBP-3, and associate with 4 to 5-fold smaller complexes (IGFBP-1 and IGFBP-2) that are capable of crossing the capillary endothelium and reaching the target cells (Thissen et al., 1994). The presence of IGFBP-5 and IGFBP-6 is not abundant in the blood; however, IGFBP-5 has been associated with bone and muscle development because of its role in cell survival, differentiation, and apoptosis (Beattie et al., 2006; Dayton and White, 2008). The IGF-I receptor is widely expressed in the body tissues, including muscle, adipose tissue, hypothalamus, pituitary, gonads, and reproductive tract (Codner and Cassorla, 2002; Vestergaard et al., 2003), and shares similarity with the insulin receptor of approximately 60 and 85 % at the amino acid level and tyrosine kinase domain, respectively (Ullrich et al., 1986; Thissen et al., 1994). The intracellular domain of the IGF-I receptor contains the tyrosine kinase activity that

becomes phosphorylated upon binding of IGF-I, which triggers a cascade of intracellular events beginning with IRS-I phosphorylation (Thissen et al., 1994) Furthermore, anabolic effects are stimulated via pathways such as phosphatidylinositol-3-kinase (**PI3K**) and MAPK kinase, whereas catabolism is inhibited via BAD phosphorylation (Le Roith et al., 2001). Other processes, such as cell function, immune response, and also cell secretory activity are positively influenced by IGF-I (Jones and Clemmons, 1995). To fuel all these activities, IGF-I has insulin-like stimulatory effects on most cells with IGF-I receptors, including enhancing glucose and amino acid uptake, and glycogen synthesis (Dimitriadis et al., 1992).

Similarly to what was described for glucose and insulin, feed intake and BW gain have been positively associated with circulating concentrations of IGF-I (Bossis et al., 2000; Armstrong et al., 2001), but negatively with GH concentrations (Ellenberger et al., 1989; Lapierre et al., 2000). Therefore, IGF-I has been used as an indicator of the nutritional status and nutrient intake of ruminants, whereas its synthesis at adequate levels of nutrition relies on additional mechanisms besides direct GH stimuli. Insulin facilitates IGF-I synthesis in the liver by enhancing the binding of GH to hepatic GH receptors (McGuire et al., 1995; Molento et al., 2002; Butler et al., 2003), whereas concentrations of insulin are positively correlated with IGF-I concentrations in cattle (Keisler and Lucy, 1996; Webb et al., 2004; Cooke et al., 2007a). Binding of GH to hepatic membranes is highly correlated with growth hormone receptor-1A (**GHR-1A**) mRNA expression (Radcliff et al., 2003), which is also highly correlated with hepatic expression of IGF-I mRNA (Lucy et al., 2001). Increased expression of GHR-1A mRNA has been observed in lactating dairy cows following chronic infusions of insulin (Butler

et al., 2003). Thus, increased hepatic expression of GHR-1A enhances the capacity for GH binding (Lapierre et al., 1992), and consequently, IGF-I synthesis (Radcliff et al., 2004). Nutrient restriction, such as amino acids (Philips et al., 1991) and hypothyroidism (Burnstein et al., 1979), have been shown to impair IGF-I synthesis, most likely at a pre-translational stage because IGF-I concentrations are preceded by changes in hepatic IGF-I mRNA expression (Thissen et al., 1994).

Previous researchers suggested that IGF-I is a major metabolic signal regulating reproductive function in cattle (Wettemann and Bossis, 2000; Hess et al., 2005), being positively associated with ADG and reproductive performance (Cooke et al., 2007a,b; Cooke et al., 2008) and negatively associated with age at puberty (Granger et al., 1989; Cooke et al., 2013). Cooke et al. (2007a) demonstrated that beef heifers with greater concentrations of IGF-I achieved puberty earlier (120 vs. 99 ng/mL) and had greater pregnancy rates (123 vs. 11 ng/mL) in their first breeding season when compared with cohorts having reduced IGF-I concentrations. Cooke et al. (2013) administered bovine somatotropin (**bST**) every 2 wk to developing beef heifers from weaning until the beginning of the breeding season. These authors reported that heifers receiving bST had greater circulating concentrations of IGF-I and attained puberty earlier compared with heifers receiving saline in the same time-frame. The circulating concentrations of IGF-I are positively associated with the body condition score (**BCS**) and nutrient intake (Yelich et al., 1996), whereas reduced IGF-I concentrations are associated with a longer post-partum interval in beef cows (Roberts et al., 1997). Concentrations of IGF-I decline at calving and gradually increase during the post-partum period (Vicini et al., 1991). Roche et al. (2000) stated that depending on the intensity of the post-partum negative energy

balance (**NEB**), there are significant decreases in the circulating concentration and follicular availability of IGF-I, reduced LH pulse frequency, as well as decreased diameter of the dominant follicle, resulting in an extended post-partum period.

Because of their close association with IGF-I, the IGFBPs have also been shown to influence performance and reproduction of cattle. Blood concentrations and mRNA expression of IGFBP-3 are associated positively with nutrient intake and rates of BW gain in cattle (Thissen et al., 1994; Vestergaard et al., 1995). In turn, availability of energy substrates and circulating insulin concentrations positively modulate the expression of liver IGF-I and IGFBP-3 mRNA and the consequent hepatic synthesis of these proteins (McGuire et al., 1992; Thissen et al., 1994; Cooke et al., 2008). On the other hand, IGFBP-2 is believed to decrease IGF-I bioavailability and consequently conserve IGF-I when the hormone synthesis is reduced (Jones and Clemmons, 1995; Armstrong et al., 2001). Ruminants in a poor nutritional status have increased blood concentrations and mRNA expression of IGFBP-2 in several tissues (Vandehaar et al., 1995; Armstrong and Benoit, 1996; Armstrong et al., 2001). Roberts et al. (1997) reported that serum concentrations of IGFBP-2 were decreased whereas serum concentrations of IGFBP-3 were increased in beef cows that resumed estrus by 20 wk post-partum compared with anestrus cows. Hence, IGF-I and IGFBP-3 have been recognized as indicators of nutritional status in cattle (Yelich et al., 1995; Wettemann and Bossis, 2000; Hess et al., 2005).

Yelich et al. (1996) reported increasing circulating concentrations of IGF-I in heifers as puberty approached, as well as an increase in LH. Also, an advancement of puberty in pre-pubertal rats was observed when IGF-I was infused into the third ventricle

(Hiney et al., 1996), suggesting that IGF-I may act centrally in the hypothalamus and/or pituitary to modulate gonadotropin secretion (Amstalden et al., 2000). In fact, IGF-I receptors have been detected in the hypothalamus and pituitary gland, indicating that GnRH and gonadotropin secretion are potentially modulated by IGF-I. Conversely, Rutter et al. (1989) reported that increases in IGF-I via glucose supplementation to beef cows at adequate planes of nutrition did not influence LH pulsatile pattern. Consequently, a large portion of IGF-I effects on reproduction of cattle may rely on its effects within the ovary. Receptors for IGF-I and also IGF-I mRNA were detected in several ovarian cells, such as granulosa, thecal, and luteal cells (Spicer and Echternkamp, 1995; Armstrong and Benoit, 1996; Bao and Garverick, 1998). Previous studies demonstrated that IGF-I stimulates mitogenic growth and ovarian steroidogenesis (Spicer and Echternkamp, 1995), and increases the number of LH receptors on follicles (Stewart et al., 1995). The ovulation process also appears to be dependent on IGF-I stimulus. Roche (2006) indicated that follicle size, LH pulsatility, and systemic concentrations of IGF-I are the factors responsible for the successful ovulation of the dominant follicle.

Non-esterified fatty acids (NEFA)

During the post-partum period, fasting, and/or nutrient restriction, ruminant females go through a period of NEB, where nutrient intake is less than their requirements, resulting in reduced concentrations of glucose, insulin, and increased circulating concentrations of NEFA (Bossis et al., 1999). Non-esterified fatty acids and glycerol are mobilized from adipose tissues upon action by glucocorticoids, catecholamines, and cytokines in order to supply energy to the animal during a stressful or nutritional challenge. After entering the circulation, NEFAs are taken up by the liver where they are

converted into acetyl-CoA, which in turn is used for energy production (Nussey and Whitehead, 2001). Partial oxidation of NEFA in the liver results in ketosis, whereas triglyceride formation can lead to fatty liver (Brown et al., 2012), conditions often associated with delayed ovulation, delayed resumption of estrous, and a longer post-partum interval (Jorritsma et al., 2000; Brown et al., 2012).

The effect of NEFAs in the reproductive performance of ruminants is controversial. Vanholder et al. (2004) reported that NEFA likely impairs the proliferation of granulosa cells, suggesting a possible mechanism by which NEB affects folliculogenesis during the post-partum period. Moreover, Lents et al. (2013) indicated that nutrient restriction and circulating concentrations of NEFA are negatively correlated with the frequency of LH pulses. On the other hand, previous studies demonstrated that LH secretion is not influenced by circulating concentrations of NEFA (Estienne et al., 1990; DiConstanzo et al., 1999). Similarly, the effects, if any, of NEFA on puberty attainment have not been demonstrated and deserve further investigation.

Leptin

Leptin, a 16 kDa hormone, has a gene that is highly conserved across species and has a half-life in humans of about 30 min (Trayhurn et al., 1999), with the kidneys being responsible for approximately 80 % of the clearance from the peripheral circulation (Meyer et al., 1997). In addition, leptin secretion follows a circadian rhythm, with a nadir early in the morning, an increase during the day, and a peak between midnight and 2:00 am; however, this pattern has not been observed in ruminants (Daniel et al., 2002).

In ruminants, similar to other mammals, leptin is mainly produced by the adipocytes and circulating blood levels are positively correlated with the body fat content

(Houseknecht et al., 1998). Other sites of leptin production in the body include the placenta, ovaries, skeletal muscle, stomach, macrophages, and liver. The leptin receptor was first identified by expression cloning (Tartaglia et al., 1995) and has been classified as a member of the class-I cytokine receptor due to its structural homology to interleukin-6 (**IL-6**) and GH receptors and common downstream signaling pathways. At least 6 receptor isoforms arise due to alternative splicing (Ob-Ra through Ob-Rf), including a functional long form containing a long intracellular domain (Ob-Rb), as well as several short and one soluble form. Distribution of the Ob-Rb varies among species, but mRNA abundance has been localized in the ventromedial, median eminence, medial preoptic area, and arcuate nucleus of the hypothalamus (Zieba et al., 2004), as well as the anterior pituitary of the ruminants (Dyer et al., 1997). Hence, leptin could act at the brain and/or pituitary to regulate gonadotropin secretion and modulate reproduction in cattle.

Due to the importance that has been given to the effects of leptin on cattle reproductive function, most of the leptin data available regarding beef cattle production pertains to this topic. Nonetheless, other tissues that possess leptin receptors and deserve further investigation include the adipose tissue, macrophages, liver, pancreas, heart, and skeletal muscle (Keisler et al., 1999). Moreover, since its discovery in 1994 (Zhang et al., 1994), leptin is considered a pleiotropic hormone, working in different systems, including the control of feed intake, energy expenditure, immune system (i.e., maturation of T-lymphocytes, up-regulation of pro-inflammatory cytokine secretion), regulation of reproductive function, and endocrinology (Marie et al., 2012).

Leptin exerts its actions centrally (hypothalamus and pituitary), but also in peripheral tissues. Briefly, circulating leptin is transported into the brain via a saturable

system (Banks et al., 1996), where it causes the release or inhibition of factors that ultimately impact feed intake, energy expenditure, reproductive function, and physical activity. Additionally, leptin acts in a negative feedback loop to inhibit further expression of its own gene. Nonetheless, besides these central actions, leptin may also act peripherally, regulating pancreatic synthesis and release of insulin (Muñoz-Gutiérrez et al., 2005). Intracerebroventricular administration of leptin into mature beef cows fasted for 60 h resulted in concentrations of insulin similar to cows with ad libitum feed intake, with no changes in circulating glucose (Amstalden et al., 2002). Conversely, other studies reported that leptin can regulate the pancreatic secretion of insulin in a manner that causes insulin resistance by attenuating its action and signaling in various insulin-responsive cell types, including white adipose tissue (Kieffer et al., 1997). These differential results may relate to the dose-dependent influence of leptin on pancreatic β-cells, with lower doses stimulating insulin secretion and higher doses attenuating this pathway (Zieba et al., 2003).

The majority of the studies evaluating the effects of leptin on animal reproductive function were conducted in *ob/ob* mice, and the importance of leptin was recognized because: (1) this particular strain lacks a functional leptin gene, is infertile, and has atrophic reproductive organs, (2) gonadotropin secretion is impaired and the central reproductive axis is very sensitive to negative feedback by gonadal steroids, and (3) treatment with leptin rejuvenates the reproductive system in *ob/ob* mice, leading to growth and function of the reproductive organs and fertility, via secretion of gonadotropins. Additionally, several species have observed a positive effect of leptin on puberty attainment (Ahima et al., 1997; Garcia et al., 2002). The positive influence of

leptin on sexual maturation appears to be driven by its action within the hypothalamic-pituitary axis (Garcia et al., 2002), likely signaling energy status to the central reproductive axis. One of the potential signaling pathways through which leptin could interact with GnRH in the exocytosis of secretory granules of LH involves inositol triphosphate (**IP_3**), which is a second messenger for GnRH action at the level of gonadotrophs, activating the janus-kinase (**JAK**) pathway (Kellerer et al., 1997).

The exact mechanisms by which leptin affects the puberty process in ruminants have not been fully elucidated, but may be related to the presence of leptin receptors within the arcuate nucleus of the hypothalamus and on the pituitary gland, regulating the release of GnRH and LH, respectively (Carvalho et al., 2013; Amstalden et al., 2014). Nutrition, more specifically the energy status of the animal, likely plays a role in mediating the effects of leptin on the reproductive function and puberty attainment in beef animals. Fasted beef heifers (48 h) have reduced leptin, insulin, and IGF-I concentrations compared to full-fed cohorts (Amstalden et al., 2000). Moreover, frequency of LH pulses was also reduced in the fasted group, indicating that leptin synthesis/secretion is acutely responsive to changes in nutritional status. In fact, Maciel et al. (2004a) reported that beef heifers nutrient-restricted for 72 hours and receiving subcutaneous injections of recombinant ovine leptin (38.4 μg/kg BW daily) for 5 days had greater mean concentrations of GH and frequency of LH pulses compared with nutrient-restricted heifers injected with saline. Following the leptin injections, GnRH was administered to all heifers, and leptin-treated heifers had greater release of LH compared with cohorts receiving saline. Due to the importance of estradiol in the puberty process and its effects on the negative feedback of LH, it may be speculated the ability of

exogenous leptin to prevent fasting-mediated reductions in LH pulse frequency involves interactions among the leptin receptor, estradiol, and nutritional status (Maciel et al., 2004a). On the other hand, other studies demonstrated no effect of leptin administration to ruminants in adequate nutritional status (Maciel et al., 2004b; Carvalho et al., 2013). Maciel et al. (2004b) reported that pre-pubertal beef heifers maintained in a positive energy balance (ADG = 0.32 kg/d) and infused with 38.4 µg/kg BW daily of recombinant ovine leptin for 40 days failed to reach puberty earlier when compared with cohorts administered saline for the same period. Furthermore, no differences were observed on the frequency of pulses of LH, mean serum LH, and the concentrations of the metabolic hormones, insulin, GH, and IGF-I (Maciel et al., 2004b) following the administration of GnRH. Overall, these results suggest that the mechanism(s) by which leptin affect reproductive function in cattle might involve intermediary pathways.

Another proposed mechanism by which leptin affects puberty and reproductive function in ruminants may be associated with the presence of leptin receptors (Ob-Rb) in pre-ovulatory follicles and in mature oocytes, indicating that this hormone may have a local role in the ovary (Sarkar et al., 2009). Leptin inhibits insulin- and IGF-I-stimulated estradiol production from cultured granulosa cells (Spicer and Francisco, 1997), suggesting that leptin has a negative interaction with the intra-follicular insulin-IGF system. In fact, leptin infusion (1 µg/h) for 72 h stimulated folliculogenesis by increasing the number of large follicles and the presence of leptin receptor in ovine granulosa cells, whereas the number of IGF-I receptors were reduced (Muñoz-Gutiérrez et al., 2005).

In ruminants, it is theorized that females should achieve a certain percentage of body fat to attain puberty (Frisch and McArthur, 1974). This relationship supports the use

of circulating leptin as indicator of puberty attainment based on the positive correlation among body fat and leptin. In fact, plasma leptin concentrations increase as puberty approaches in beef heifers (Garcia et al., 2002). However, a recent work from our research group (Cooke et al., 2013) demonstrated that puberty in heifers is not entirely dependent on circulating leptin concentrations, whereas leptin appears to play a permissive role during the puberty process (Barb and Kraeling, 2004). More specifically, Cooke et al. (2013) reported that beef heifers that had reduced backfat thickness and plasma concentrations of leptin due to bST administration attained puberty earlier compared with non-treated heifers.

Protein metabolism

Upon intake, dietary CP reaches the rumen, where the portion that is degradable in the rumen will be progressively colonized and digested by the microorganisms, resulting in ammonia release (Bach et al., 2005). This ammonia can be used by rumen microorganisms to produce microbial crude protein (**MCP**), which will be absorbed in the small intestine of the animal, significantly accounting for the metabolizable protein (**MP**) pool of the animal (NRC, 1996). According to the NRC (1996), the MCP contains 80 % of true protein and a digestibility ratio of 80 %, resulting in a coefficient of amino acid absorption of 64 %. The other constituents of this pool include endogenous protein and rumen undegradable protein (**RUP**). In the rumen, ammonia can also be used as a growth factor for rumen bacteria, mainly the ones that are responsible for cellulose digestion (cellulolytic; Bach et al., 2005).

At this point, two routes of escape of the ammonia produced in the rumen have been identified: (1) MCP and absorption in the small intestine, and (2) utilization by rumen microorganisms. Another important route is the absorption of ammonia through the rumen wall and transport via the portal blood to the liver for further metabolism. In the liver, ammonia is converted into urea via the urea (or ornithine) cycle (Lobley et al., 1995). The following steps of this cycle are: (1) ammonia + HCO_3 are converted into carbamoyl phosphate by the mitochondrial enzyme carbamoyl-phosphate synthetase (**CPS-I**). This reaction uses 2 moles of ATP to be completed, (2) carbamoyl phosphate and ornithine are converted into citrulline by the action of the ornithine transcarbamoylase (also mitochondrial), (3) in turn citrulline is converted into argininosuccinate in a reaction that uses aspartate, the cytosolic enzyme argininosuccinate synthetase, and 1 molecule of ATP, (4) argininosuccinate is degraded by argininosuccinase (or argininosuccinate lyase) into fumarate and arginine. Fumarate, in turn, can be used into the Krebs cycle as a way to at least try to recover some of the energy invested into this cycle, and (5) in the final step of this cycle, arginine is converted into ornithine and urea by arginase. Ornithine can be shuttle down to reaction #2 to maintain the cycle running. The other end product of the metabolism (urea) can have 3 fates: (1) excretion in the urine, (2) incorporated into the saliva, and (3) directly absorbed by the rumen. These possible fates of the ammonia metabolism and "N recycling" make the ruminant more efficient in CP utilization when the same is unavailable in adequate amounts (Farmer et al., 2004), given that they can maintain their homeostasis and digestive function when consuming diets with reduced CP content or amount (Wickersham et al., 2008).

Urea is a source of non-protein N (**NPN**) that can be used in livestock operations and its utilization must be done with caution, given that urea toxicity occurs when the ammonia-urea conversion capacity of the liver is exceeded, resulting in increased blood ammonia levels (Chalupa, 1968; Essig et al., 1988). Consequently, it is suggested that urea intake not exceed 0.3 g/kg BW (Helmer and Bartley, 1971). Before being excreted, urea has another chance to be kept in the peripheral blood supply: renal reabsorption cycle. Moreover, if urea is to be incorporated in the saliva, the urea molecule will eventually reach the rumen and similar to the molecule that was absorbed directly into the rumen, these molecules will be converted into ammonia and used to support microbial growth. The MCP produced in the rumen, endogenous protein, and RUP also might have different fates: (1) enterocytes (cells of the small intestine) use amino acids as energy sources, such as glutamine. Hence, they can deaminate and convert some amino acids into this molecule to be used, (2) the remaining protein (endogenous, MCP, and RUP) that was broken down in the intestine into smaller tri-, di, or peptides can also be transported to the liver for further metabolism. In the liver, these molecules can be used to supply compounds to the urea cycle (i.e., aspartate and arginine), deaminated to glutamate and/or glutamine, and if not utilized in the liver, can be exported for extrahepatic tissues, and (3) in extrahepatic tissues, amino acids can be used for anabolic reactions, such as muscle deposition. In lactating animals, amino acids can also be used by the mammary gland for milk protein synthesis.

The gluconeogenic amino acids reaching the liver can also be used in the gluconeogenesis process, supplying the body with glucose. Other process in which amino acids help metabolism is the Krebs Cycle, by providing substrates and maintaining the

proper functioning of this cycle for ATP production. Insulin synthesis in the pancreas has been shown to be positively associated with the CP content and intake of the diet (Caton et al., 1988; Cheema et al., 1991). Reed et al. (2007) reported that diets containing greater amounts of RUP (20 and 41 %) resulted in greater elevation of plasma insulin concentrations compared with diets containing low RUP (1 %). Similarly, Sletmoen-Olson et al. (2000) reported an increase in plasma insulin concentration in response to protein supplementation of beef cows compared with cohorts fed reduced amounts of CP. However, not all the protein available in the diet is absorbed in the small intestine. A portion can be excreted in the feces of the animals, however, the quantity of protein digested in the small intestine may be the causal factor in determining plasma insulin concentrations (Bassett et al., 1971).

Frequency of Supplementation to Ruminants

Even though supplementation might be required when they are fed low-quality cool-season forages throughout the year (Ganskopp and Bohnert, 2001) to maintain adequate performance, consequently affecting the productivity and profitability of the beef operations, producers often are discouraged to adopt such practice because of the costs associated with feed purchase and labor-associated costs, such as fuel and labor expenses (Miller et al., 2001). Therefore, developing a supplementation alternative that reduces the costs associated with feed and labor, while maintaining adequate growth and reproductive function, is imperative to beef operations. One such alternative might be to reduce the frequency by which supplements are offered to ruminants. This strategy consists of offering the same amount of supplement on a weekly basis, but in different frequencies (i.e., once a wk instead of daily or 3 times per wk). However, it is important

to keep in mind that the supplement type offered to the herd may significantly impact the usefulness of this strategy in beef cattle operations.

Decreasing the supplementation frequency of energy ingredients may not be a feasible alternative to reduce supplementation costs while maintaining and/or increasing the performance of the herd (Cooke et al., 2008; Moriel et al., 2012). Cooke et al. (2008) demonstrated that replacement heifers consuming low-quality warm-season forages and receiving a low-starch energy supplement daily had greater forage intake, growth rates, hastened puberty attainment, and improved pregnancy rates compared with cohorts supplemented 3 times weekly. Moreover, Moriel et al. (2012) corroborated these results, independently of the forage quality (low- or medium-quality warm-season forages) offered to the herd. These results can be primarily attributed to the improved ruminal function and energy metabolism in daily-fed cattle when compared with cohorts fed 3 times weekly, indicating that decreasing the supplementation frequency of energy-based ingredients impairs the performance of the herd. Conversely, Drewnoski et al. (2014) reported no negative effects of infrequent energy supplementation when animals were offered low-quality, cool-season forages.

On the other hand, several research studies indicated that the same nutritional strategy is feasible when protein supplements are offered, helping to reduce the labor and fuel costs associated with supplementation, while maintaining performance comparable to daily supplementation (Schauer et al., 2005). Supporting this statement, Bohnert et al. (2002) demonstrated that performance is not altered when animals are fed soybean meal daily or once every 6-d. Huston et al. (1999b) reported that pregnant cows consuming low-quality forages (warm-season perennial grasses) and receiving protein

supplementation (cottonseed meal; 41 % CP) as infrequent as once a wk had similar BW and BCS compared with cohorts supplemented daily. Farmer et al. (2004) suggested a few mechanisms that may play a role in buffering the impact of infrequent supplementation and minimize differences in performance of cattle. These include N recycling, a lag in peak ruminal ammonia concentration, and prolonged elevation of ammonia (Farmer et al., 2004). As previously explained, excess N leaving the rumen as ammonia is taken up by the liver, where it is converted into urea. Urea, in turn, can return to the circulation via the saliva, be reabsorbed by the rumen, or excreted in the urine (Bach et al., 2005). An attenuated peak and prolonged maintenance of elevated ruminal ammonia would facilitate the maintenance of the activity of cellulolytic microbes and the conservation of N, thus reducing potential negative effects as a result of the infrequent supplementation.

Frequency of Supplementation and Reproductive Function

Although decreased supplementation frequency may reduce the costs associated with feeding and labor, the same must be assessed when evaluating the reproductive performance of the herd. Decreasing the frequency at which supplements are offered may impair reproductive function of ruminants primarily by 2 mechanisms: hepatic steroid catabolism and excessive protein intake.

Progesterone Catabolism. Cooke et al. (2007) observed that decreasing the frequency of supplementation to 3 times a wk decreased the circulating concentrations of P_4 in beef females. These results are likely explained by the increased blood flow to the liver, which in turn, increases the catabolism of this hormone (Sangsritavong et al., 2002). Vasconcelos et al. (2003) demonstrated that dairy cows offered the ration in a single meal

had decreased concentrations of P_4 when compared to cohorts offered the ration in several, smaller meals. Taking into account that the liver is the primary site of P_4 catabolism, several researchers have correlated liver blood flow, through the hepatic portal vein, and the metabolic clearance rate of P_4. Parr et al. (1993) showed that a 40 % increase in liver blood flow in sheep would double the metabolic clearance rate of P_4. Studies in the dairy cow by Sangsritavong et al. (2002) determined a positive regression between liver blood flow and metabolic clearance rate of P_4. These strong correlations between P_4 clearance and liver blood flow strengthen the notion that the primary site of P_4 catabolism is the liver, and that altering liver physiology may turn out to be a rational approach in order to raise the circulating P_4 concentrations during early pregnancy, when the same is essential.

Cytochrome P_{450s} are present in most tissues, but most highly expressed in hepatocytes (P_{450} enzymes account for 1 to 2 % of hepatocytes by weight) and are typically embedded in the smooth endoplasmic reticulum (Ruckpaul and Rein, 1984). Cytochrome P_{450s} function as monooxygenases and are involved in the metabolism of a number of important endogenous compounds including vitamin D_3 activation, catabolism of cholesterol to bile acids, and metabolism of all major classes of steroid hormones (Waxman et al., 1991). Moreover, cytochrome P_{450} reductase serves as an electron carrier by transferring electrons from NADPH to cytochrome P_{450s} (Yamazaki et al., 1999). In humans, the most abundant cytochrome P_{450} belongs to the 3 family, accounting for approximately 30 to 40 % of total P_{450} protein in human liver. Of particular interest is the hydroxylation of testosterone at the 6β position, which is accomplished by CYP3A4, CYP3A5, CYP3A7, CYP2C9 and CYP2C19; however, CYP3A4 accounts for 90 % of

6β-hydroxytestosterone formation (Yuan et al., 2002), allowing for a fairly selective measurement of CYP3A4 activity with very low reactivity with the other members of the cytochrome P_{450} superfamily. Studies by Li et al. (2005) showed that CYP2C19 and CYP3A4 both catabolize omeprazole and that specific inhibitors of CYP2C19 or CYP3A4 are needed to determine the activities for either one of the isozymes individually. Using antibodies raised against different isozymes of rat cytochrome P_{450s}, Murray (1991; 1992) demonstrated that the principal isozymes involved in P_4 catabolism in sheep belonged to the cytochrome P_{450} 2C and 3A subfamilies and the major metabolites were found to be 21-hydroxyprogesterone and 6β-hydroxyprogesterone, respectively (Murray, 1991; Murray, 1992). These enzymes were also shown to catabolize testosterone in a manner similar to that demonstrated by Waxman et al. (1991) adding substantial evidence that cytochrome P_{450} isozymes are conserved not only amongst monogastrics, but in ruminant species as well.

In a series of studies, Lemley et al. (2008; 2010) demonstrated that cows fed high-starch supplements and consequently with greater circulating concentrations of glucose and insulin, had greater concentrations of P_4. Recently, Vieira et al. (2010) demonstrated that non-lactating, non-pregnant ovariectomized cows in positive energy balance had greater P_4 and insulin concentrations after an exogenous administration of glucose compared with cows that were in NEB and received an exogenous P_4-releasing device (CIDR), and attributed these results to the insulin-effect on inhibiting the steroid catabolism by the CYP_{450} enzymes. Subsequently, Vieira et al. (2013) demonstrated that the expression of the CYP3A4 and CYP2C19 were reduced in animals that had greater circulating concentrations of insulin, indicating that insulin may reduce the metabolic

clearance of P_4, by regulating the activity and/or expression of the enzymes associated with hepatic steroid catabolism in ruminants.

Excessive protein intake. In dairy cattle, diets containing high levels of protein have been associated with impaired reproductive performance (Canfield et al., 1990; Elrod and Butler, 1993; Elrod et al., 1993; Butler et al., 1996). Jordan and Swanson (1979) reported that dairy cows fed a 19.3 % CP diet exhibited an increased number of days open, services per conception, and calving intervals compared with cohorts fed a diet containing 12.7 % CP. Canfield et al. (1990) demonstrated that feeding high-protein (19 % CP) diets to dairy heifers and cows resulted in reduced conception rates and greater PUN compared with cohorts fed a control diet (16 % CP). Interestingly, these authors reported that animals that conceived to first service had lower plateau PUN than those that did not conceive, indicating that urea might play a role in the reproductive function of ruminant females. Elrod and Butler (1993) offered Holstein heifers diets that were 100 (control) and 150 (excess) % of their RDP requirements during the estrus synchronization protocol. Feeding 150 % of RDP requirements did not improve growth rates, but reduced first-service pregnancy rates to 61 % compared with 82 % on the control group. Plasma urea-N concentrations were greater in heifers that were not pregnant compared with cohorts that did conceive and the uterine pH was reduced in heifers that were fed protein in excess on d 7 of the estrous cycle (Elrod and Butler, 1993). High-protein diet consumption blocked the luteal phase rise in uterine pH that occurs between estrus and d 7 (Elrod et al., 1993), whereas no differences were observed between treatments at estrus (Elrod and Butler, 1993). Conversely, Grant et al. (2013) demonstrated that beef cows offered diets high in CP content (16.3 % CP) had greater uterine pH values during the

estrous cycle compared with cohorts fed diets lower in CP (11 % CP). It is also important to note that for both high- and low-CP diets in Grant et al. (2013), the uterine pH values were within the recommended range for semen maintenance and embryo development.

The pH of ejaculated bull semen is approximately 6.8 (Mann and Lutwak-Mann, 1981); hence, changes in uterine pH that may subsequently affect the viability and survival of the semen. Moreover, the regulation of intracellular uterine pH is important for proper embryonic growth and development (Bavister, 2000). Alteration of intracellular uterine pH greater than (Zhao et al., 1995) or less than (Leclerc et al., 1996; Edwards et al., 1998; Lane et al., 1998) normal physiological values can result in loss of embryonic developmental competence. This happens because most cellular processes are pH sensitive and deregulation of intracellular pH can result in loss of normal cell function (FitzHarris and Baltz, 2009), impaired cell growth and proliferation (Grinstein et al., 1989; Kapus et al., 1994), as well as decreased cell survival (Pouysségur et al., 1984).

The exact mechanisms by which ruminants consuming high protein diets had decreased uterine pH remain unknown at this point, but Elrod and Butler (1993) suggested that the alteration of uterine pH in response to high protein intake was unique to this organ. Two mechanisms might be involved in controlling the pH in the uterus of the ruminants, including PUN and/or ammonia levels circulating in the body. Hammon et al. (2005) reported that cows having greater PUN also had greater uterine fluid urea-N and ammonia concentrations compared with cows with reduced PUN levels, suggesting that ammonia could also be a factor affecting reproductive performance of cattle consuming high protein diets (Hammon et al., 2005). As previously mentioned, after absorption through the rumen wall into the circulation, ammonia can be transported to the

liver and converted into urea (Bach et al., 2005). However, the rate of ammonia escaping this hepatic conversion and flowing directly into the reproductive tract has not been shown and warrants further investigation. Glutamine, which is a non-toxic transport mechanism, carries ammonia to the liver for urea synthesis and to other tissues for biosynthesis of amino acids (Dimski, 1994), and upon its catabolism, ammonium ($\mathbf{NH_4^+}$) is generated (Dimski, 1994). Thus, glutamine may carry ammonia into the reproductive tract and its utilization by the uterine tissue would result in greater NH_4^+ levels in the uterine fluid, which could account for the changes in pH. However, more studies should be designed to evaluate these facts.

Development of nutritional strategies to improve the reproductive function of beef females

Beef cattle production is the second largest commodity in the state of Oregon, with a beef cow herd of approximately 518,000 cows and it is estimated that 100,000 replacement heifers are introduced into the Oregon cow-calf industry every year (OASS, 2014). Hence, the development of nutritional strategies that improve the growth and reproductive performance of the beef cattle herd are warranted.

Beef producers are always challenged to increase the profitability of the system, either by increasing the number of animals becoming pregnant during the breeding season or reducing the costs associated with the production system. To evaluate the first challenge, it is imperative to develop new strategic nutritional and/or management alternatives that can be used into beef cattle operations, aiming to improve conception and pregnancy rates at the end of the breeding season. In order to evaluate the latter,

decreasing the frequency by which the supplements are offered might significantly reduce the costs associated with feed and labor, while maintaining the reproductive function of the cowherd. Therefore, to address the aforementioned issues, 4 experiments were conducted to evaluate the effects of different supplementation strategies on ruminal forage disappearance, growth and reproductive performance, as well as metabolic responses of beef females. Methods and results from these experiments are reported and discussed in the following chapters.

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CHAPTER 3

SUPPLEMENTATION BASED ON PROTEIN OR ENERGY INGREDIENTS TO BEEF CATTLE CONSUMING LOW-QUALITY COOL-SEASON FORAGES: I. FORAGE DISAPPEARANCE PARAMETERS IN RUMEN-FISTULATED STEERS AND PHYSIOLOGICAL RESPONSES IN PREGNANT BEEF HEIFERS

Introduction

Supplementation is often required in heifer development programs based on low-quality forages (Schillo et al., 1992). Protein is traditionally considered the limiting nutrient in Western U.S. cow-calf systems (DelCurto et al., 2000), although energy is the primary dietary consideration for female development (Maas, 1987) and forages typically represent the main energy source for forage-fed cattle. Indeed, supplemental protein has been shown to improve digestibility and DMI of low-quality warm-season forages, resulting in increased energy utilization from the forage and cattle performance (DelCurto et al., 1990; Lintzenich et al., 1995). However, supplemental protein did not increase forage digestibility and DMI of low-quality cool-season forages (Bohnert et al., 2011a). Hence, inclusion of energy ingredients into supplements may be beneficial for growth and reproduction of heifers consuming such forages.

After the first breeding season, pregnant heifers still need to grow while maintaining the pregnancy. Energy intake modulates BW gain and circulating concentration of progesterone (**P_4**); a steroid required for pregnancy establishment and maintenance (Spencer and Bazer, 2002). The hormones associated with the metabolism of energy substrates, particularly starch, increase P_4 concentration by reducing hepatic P_4 catabolism (Cooke et al., 2012) and stimulating ovarian steroidogenesis (Spicer and Echternkamp, 1995). Hence, inclusion of energy ingredients into supplements may further benefit reproductive performance of pregnant heifers consuming low-quality cool-

season forages by increasing circulating P_4 concentration. However, supplements based on energy ingredients often impair forage digestibility and DMI in cattle (DelCurto et al., 2000). Therefore, 2 experiments compared the effects of supplements based on protein or energy ingredients on ruminal forage disappearance in steers (Exp. 1), and performance and physiological parameters of pregnant beef heifers (Exp. 2).

Materials and Methods

Both experiments were conducted at the Oregon State University – Eastern Oregon Agricultural Research Center (Burns) from August to September 2012 (43°31’06” N, 119°01’21” W, and 1,370 m elevation). All cattle utilized were cared for in accordance with acceptable practices and experimental protocols reviewed and approved by the Oregon State University, Institutional Animal Care and Use Committee.

Supplement ingredients provided during Exp. 1 and 2 were from the same batch, whereas the hay (meadow foxtail; *Alopecurus pratensis* L.) provided during both experiments was harvested from the same field in June 2012. A sample of hay (according to Bohnert et al., 2011b) and each supplement ingredient was collected prior to the beginning of both experiments, and analyzed for nutrient content by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY). Samples were analyzed in triplicates by wet chemistry procedures for concentrations of CP (method 984.13; AOAC, 2006), RDP (Roe et al., 1990 for supplement ingredients; Coblentz et al., 1999 for hay), ADF (method 973.18 modified for use in an Ankom 200 fiber analyzer, Ankom Technology Corp., Fairport, NY; AOAC, 2006), and NDF (Van Soest et al., 1991; modified for use in an Ankom 200 fiber analyzer, Ankom Technology Corp.).

Calculations for TDN used the equations proposed by Weiss et al. (1992), whereas NE_m and NE_g were calculated with the equations proposed by the NRC (1996). Hay nutritive value was (DM basis) 57 % TDN, 58 % NDF, 37 % ADF, 1.12 Mcal/kg of NE_m, 0.57 Mcal/kg of NE_g, 8.7 % CP, 6.0 % RDP, and 2.1 % ether extract.

Experiment 1

Steers and diets. Six Angus × Hereford steers (initial shrunk BW 494 ± 11 kg), housed in individual pens (8 × 20 m) and fitted with a ruminal cannula, were assigned to an incomplete 3 x 2 Latin square design containing 2 periods of 11 d each (2 steers/treatment in each period) and the following treatments: 1) supplementation with soybean [*Glycine max* (L.) *Merr*.] meal (**PROT**), 2) supplementation with a mixture of cracked corn (*Zea mays* L.), soybean meal, and urea (68:22:10 ratio, DM basis; **ENER**), or 3) no supplementation (**CON**). Steers were offered meadow foxtail hay for ad libitum consumption during the entire experiment. The PROT and ENER treatments were provided daily (0800 h) at 0.50 and 0.54 % of steer shrunk BW recorded at the beginning of each period, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous (Table 1). Urea was included into ENER to result in isocaloric and isonitrogenous intakes of PROT and ENER. Treatment intake during the experiment averaged at 2.20 and 2.37 kg of DM/steer for PROT and ENER, respectively. Treatments were inserted directly into the ruminal cannula of each steer to ensure readily supplement consumption. All steers had ad libitum access to water and a mineral and vitamin mix (Cattleman's Choice, Performix Nutrition Systems, Nampa, ID) containing 14 % Ca, 10 % P, 16 % NaCl, 1.5 % Mg, 3,200 mg/kg of Cu, 65 mg/kg of I, 900 mg/kg of Mn, 140

mg/kg of Se, 6,000 mg/kg of Zn, 136,000 IU/kg of vitamin A, 13,000 IU/kg of vitamin D3, and 50 IU/kg of vitamin E throughout the experimental period.

Sampling. Within each period (d 0 to 11), steer shrunk BW was recorded on d 0 after 16 h of feed and water restriction to determine steer initial BW. From d 1 to 7 of each period, voluntary forage DMI was recorded daily by collecting and weighing refusals. Samples of the offered and non-consumed forage were collected daily from each pen and dried for 96 h at 50°C in forced-air ovens for DM calculation. From d 8 to 11 of each period, steers were offered 90 % of their voluntary forage DMI determined from d 1 to 7. Immediately before treatments were provided on d 8, Dacron bags (50 ± 10 µm pore size and 10 × 20 cm bag size; Ankom Technology Corp.) containing 4 g (DM basis) of ground dietary hay (2-mm screen; Wiley Mill, Model 4; Arthur H. Thomas, Philadelphia, PA) were suspended into the ruminal ventral sac of each steer, and incubated in triplicates for 0, 1, 3, 5, 8, 12, 24, 36, 48, 72, and 96 h. Before ruminal incubation, all bags were soaked in warm water (39°C) for 15 min. After ruminal incubation, bags were washed repeatedly with running water until the rinse water was colorless, and subsequently dried for 96 h at 50°C in a forced-air oven. The 0-h bags were not incubated in the rumen, but were subjected to the same soaking, rinsing, and drying procedures applied to the ruminally incubated bags. Dried samples were weighed for residual DM determination, and triplicates were combined and analyzed for NDF (Robertson and Van Soest, 1981) using procedures modified for use in an Ankom 200 Fiber Analyzer (Ankom Technology Corp.).

Statistical Analysis. All data were analyzed using steer as the experimental unit and Satterthwaite approximation to determine the denominator degrees of freedom for the

tests of fixed effects. Kinetic parameters of forage DM and NDF disappearance were estimated using nonlinear regression procedures of SAS (SAS Inst., Cary, NC), as described by Vendramini et al. (2008). Effective degradability of forage DM and NDF were calculated by fixing ruminal passage rate at 0.046/h (Poore et al., 1990) and using the model proposed by Ørskov and McDonald (1979), whereas treatment effects on these parameters were analyzed using the MIXED procedure of SAS (SAS Inst.). The model statement contained the effects of treatment and period as independent variables. Data were analyzed using steer(treatment × period) as the random variable. Results are reported as least square means and separated using PDIFF. Significance was set at $P \leq 0.05$ and tendencies were denoted if $P > 0.05$ and ≤ 0.10.

Experiment 2

Heifers and diets. Thirty-five pregnant Angus x Hereford heifers (initial shrunk BW 354 ± 4 kg, initial age = 508 ± 4 d) were utilized in the study. Heifers were concurrently exposed and became pregnant to a fixed-time AI protocol (CO-Synch + controlled internal progesterone-release device; Larson et al., 2006) 90 d prior to the beginning of the experiment. Pregnancy status to AI was verified by detecting a fetus via transrectal ultrasonography (5.0-MHz transducer; 500V, Aloka, Wallingford, CT) 80 d after AI (d -10 of the experiment). On d -7, all heifers were ranked by initial shrunk (after 16 h of feed and water restriction) BW, and allocated to 12 feedlot pens (4 pens/treatment; 11 pens with 3 heifers and 1 pen with 2 heifers; 8 × 20 m) in a manner which all pens had equivalent initial average shrunk BW. Pens were randomly assigned to receive the same treatments described in Exp. 1. Heifers were offered meadow foxtail

hay for ad libitum consumption during the entire experiment (d -7 to 19). Beginning on d 1, PROT and ENER treatments were fed once daily (0700 h) at a rate of 1.77 and 1.92 kg of DM/heifer, respectively, to achieve the same treatment intake as % of initial shrunk BW used in Exp. 1 (0.50 and 0.54 % of initial BW for PROT and ENER, respectively), and to ensure isocaloric and isonitrogenous intakes (Table 1). The ENER and PROT treatments were not mixed with hay, and were readily consumed by heifers. Water availability, and mineral and vitamin mix supplementation were the same as in Exp. 1.

Sampling. Heifer shrunk BW was collected prior to the beginning (d -7) and at the end of the study (d 20; also after 16 h of feed and water restriction) for ADG calculation. Hay DMI was evaluated daily from each pen from d 1 to 19 by collecting and weighing refusals daily. Samples of the offered and non-consumed feed were collected daily from each pen and dried for 96 h at 50°C in forced-air ovens for DM calculation. Hay, concentrate, and total daily DMI of each pen were divided by the number of heifers within each pen, and expressed as kg per heifer/d. In addition, daily intake/heifer of NE_m, NE_g, CP, RDP, and starch were estimated based on total DMI of each pen, and nutritive value of hay and treatments (Table 1).

Blood samples were collected immediately prior to and 2, 4, 6, and 8 h after treatment feeding (h 0) on d 13, 15, 17, and 19 of the experiment and analyzed for plasma concentrations of glucose, urea N (**PUN**), insulin, IGF-I, and P_4. Blood samples were also collected on d 0 of the experiment, immediately prior to and 4 and 8 h after hay feeding (h 0) to determine if ENER, PROT, and CON heifers had similar P_4 concentrations prior to the beginning of treatment administration (d 1 to 19). All blood samples were collected via jugular venipuncture into commercial blood collection tubes

(Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) containing 158 USP units of freeze-dried sodium heparin. After collection, blood samples were placed immediately on ice, subsequently centrifuged (2,500 × *g* for 30 min; 4°C) for plasma harvest, and stored at -80°C on the same day of collection. Plasma concentrations of P_4 and insulin were determined using Coat-A-Count solid phase ^{125}I RIA kits (Siemens Healthcare Diagnostics, Los Angeles, CA) previously used for bovine samples (Moriel et al., 2008). Plasma glucose and PUN concentrations were determined using quantitative colorimetric kits (#G7521 and B7551, respectively; Pointe Scientific, Inc., Canton, MI). Concentration of IGF-I was determined in samples collected at 0 and 4 h after feeding, using a human-specific commercial ELISA kit (SG100; R&D Systems, Inc., Minneapolis, MN) with 100 % cross-reactivity with bovine IGF-I and previously validated for bovine samples (Cooke et al., 2012). The intra- and inter-assay CV were, respectively, 1.94 and 3.30 % for glucose, 8.55 and 8.64 % for PUN, 2.34 and 4.74 % for IGF-I, 2.98 and 3.29 % for insulin, and 6.87 and 7.19 % for P_4. The minimum detectable concentrations were 0.02 μIU/mL for insulin, and 0.056, and 0.07 ng/mL for IGF-I and P_4, respectively.

Statistical Analysis. All data were analyzed using the MIXED procedure of SAS (SAS Inst.), using pen as experimental unit, and Satterthwaite approximation to determine the denominator df for the tests of fixed effects. The model statement used for ADG contained only the effect of treatment. Data were analyzed using pen(treatment) and heifer(pen) as random variables. The model statement used for feed and nutrient intake contained the effects of treatment, day, and the treatment × day interaction. Data were analyzed using pen(treatment) as the random variable, given that DMI was recorded from each pen. The specified term for the repeated statement was day and subject was

pen(treatment). The model statement used for plasma variables contained the effects of treatment, hour, day, and all the resultant interactions. The model statement for P_4 also contained the average P_4 concentration on d 0 as covariate. Data were analyzed using pen(treatment) and heifer(pen) as random variables. The specified term for the repeated statement was hour(day), whereas heifer(treatment × day) was the subject. For both intake and plasma variables, the covariance structure used was first-order autoregressive, which provided the smallest Akaike Information Criterion and hence the best fit for all variables analyzed. Results are reported as least square means, or covariately adjusted means for plasma P_4 concentration, and separated using PDIFF. Significance was set at $P \leq 0.05$ and tendencies were denoted if $P > 0.05$ and ≤ 0.10. Results are reported according to main effects if no interactions were significant, or according to highest-order interaction detected.

Results and Discussion

As previously stated, inclusion of energy ingredients into supplements may benefit growth and reproductive performance of beef heifers consuming low-quality cool-season forages (Bohnert et al., 2011a; Cooke et al., 2012), although supplemental energy ingredients may impair forage digestibility and intake (DelCurto et al., 2000). To address these theories, the experiments reported herein evaluated performance and physiological responses in pregnant heifers provided PROT and ENER at 0.50 and 0.54 % of BW, as well as in situ forage disappearance in rumen-fistulated steers to estimate supplementation effects on ruminal forage degradability parameters. Similar treatments were applied to replacement heifers following weaning, and these results are being

reported in the next chapter. These supplementation rates were adopted to yield adequate ADG of beef heifers, either non-pregnant or pregnant, consuming low-quality cool-season forages (NRC, 1996).

Experiment 1

No treatment effects were detected for ruminal disappearance rate or effective ruminal degradability of hay DM ($P \geq 0.33$) and NDF ($P \geq 0.66$; Table 2), indicating that PROT and ENER did not impact rumen in situ disappearance parameters of a low-quality cool-season forage. Supporting these results, Caton and Dhuyvetter (1997) suggested that ruminal disappearance rate of low-quality forages is not impacted by energy or protein-based supplementation. Nevertheless, supplements based on protein and energy ingredients are often associated, respectively, with improved and decreased ruminal forage digestibility in beef cattle (DelCurto et al., 2000). However, protein supplementation is generally beneficial to forage digestibility when the CP content of the basal forage is less than 8 % (DelCurto et al., 2000), whereas the forage utilized herein had 8.7 % CP (DM basis). Supplements based on energy ingredients can be provided to forage-fed cattle at 0.5 % of BW without major impacts on forage digestibility and intake (Bowman and Sanson, 1996), whereas the ENER treatment was provided at 0.54 % of steer BW.

Corn intake above 0.25 % of BW has been shown to impair forage utilization in cattle (Bowman and Sanson, 1996) by reducing ruminal pH, shifting rumen microbes from a cellulolytic population towards an amylolytic population, and decreasing ruminal NH_3 concentration (Chase and Hibberd 1987; Sanson et al., 1990; Caton and Dhuyvetter, 1997). In the present experiment, ENER steers consumed corn at 0.37 % of their BW.

However, inclusion of a RDP source into corn-based supplements may offset the negative impacts of corn-based supplements on rumen function and digestibility (Olson et al., 1999). Hence, the inclusion of soybean meal and urea into the ENER treatment, as well as the equivalent intake of CP and RDP by ENER and PROT steers, may also have contributed to the similar ruminal forage digestibility among treatments.

In summary, results from this experiment suggest that ruminal in situ disappearance and estimated degradability parameters of a low-quality cool-season forage in beef steers is not impacted by supplements based on protein or energy ingredients provided as 0.5 % of steer BW/d at isocaloric and isonitrogenous rates.

Experiment 2

No treatment effects (P = 0.17) were detected on forage DMI (Table 3). This outcome agrees with the lack of treatment effects on ruminal degradability parameters of the forage utilized herein reported in Exp. 1, given that ruminal forage digestibility is positively associated with intake (Allen, 1996). Bohnert et al. (2011a) also reported that protein supplementation did not impact DMI of a low-quality cool-season forage, whereas Bowman and Sanson (1996) suggested that supplements based on energy ingredients may be fed at 0.5 % of BW without impacting forage intake. In the following experiment from Chapter 4, hay intake was also similar among growing replacement heifers receiving CON, ENER, and PROT. As expected due to the lack of treatment effects on forage intake, as well as treatment design and intake rate, total daily DMI, NE_m, NE_g, CP, and RDP intake were greater ($P < 0.01$) for PROT and ENER compared with CON heifers, and similar ($P \geq 0.18$) between PROT and ENER heifers (treatment effects, $P < 0.01$; Table 3). In addition, estimated mean daily intake of starch was greater

($P < 0.01$) for ENER compared with PROT and CON, and similar ($P = 0.40$) between PROT and CON (Table 3). Hence, PROT and ENER had a similar increase in energy and protein intake compared with CON heifers, although starch was the main energy source provided by ENER.

A treatment effect ($P = 0.03$) was detected for ADG (Table 3). In agreement with the treatment effects observed for DMI and nutrient intake, ADG was greater ($P = 0.01$) for PROT compared with CON, tended to be greater for ENER compared with CON ($P = 0.08$), and was similar between ENER and PROT ($P = 0.28$). Cappellozza et al. (2013) also reported that growing replacement heifers receiving ENER and PROT had similar ADG, which were greater compared with CON cohorts. These results provide evidence that beef heifers consuming low-quality cool-season forages can equally utilize nutrients provided by supplements based on protein or energy ingredients to support BW gain. Supporting this rationale, similar treatment effects were detected for plasma concentrations of PUN ($P < 0.01$), glucose ($P = 0.04$), insulin ($P < 0.01$), and IGF-I ($P = 0.03$) in the present study (Table 4), which are hormones and metabolites associated with dietary protein and energy metabolism in cattle (Hammond, 1997; Huntington, 1997; Wettemann et al., 2003).

A treatment × hour interaction was detected ($P < 0.01$) for PUN (Figure 1), given that PUN concentrations increased after supplementation for ENER and PROT heifers (time effect, $P < 0.01$), but did not change for CON (time effect; $P = 0.62$). In addition, mean PUN concentrations were greater ($P < 0.01$) for ENER and PROT heifers compared with CON, and were similar ($P = 0.44$) between ENER and PROT heifers (Table 4). Concentration of PUN is positively associated with intake of CP, RDP, and concentration

of ruminal ammonia (Broderick and Clayton, 1997). Therefore, treatment effects detected for PUN can be attributed to the equivalent treatment effects detected for CP and RDP intake (Table 3). In addition, PUN concentration has been shown to promptly increase after consumption of supplements containing RDP sources such as the ENER and PROT utilized herein (Cooke et al., 2007a; Cooke et al., 2007b), likely due to prompt degradation of soluble protein by rumen microbes and subsequent absorption of ammonia by ruminal tissues (Broderick and Clayton, 1997). Optimal PUN concentration in growing beef heifers range from 15 to 19 mg/dL (Hammond, 1997), which suggests that CON heifers in present study required supplemental CP and RDP. Conversely, PUN concentrations were similar between ENER and PROT heifers and within the optimal level proposed by Hammond (1997), suggesting that these heifers had adequate and equivalent protein intake, utilization, and metabolism despite differences in CP and RDP sources between treatments.

Mean plasma glucose concentration was greater (P = 0.03) for ENER and PROT compared with CON heifers, and were similar (P = 0.96) between ENER and PROT heifers (Table 4). A similar outcome was detected in our other study with replacement beef heifers, which was unexpected given the difference in starch intake between ENER and PROT heifers (Table 3). Glucose concentration in beef cattle was positively associated with feed intake and rate of BW gain (Vizcarra et al., 1998; Hersom et al., 2004), as observed herein based on the greater nutrient intake and ADG of PROT and ENER compared with CON heifers (Table 3). However, starch is the major dietary precursor for glucose in ruminants (Huntington, 1997); hence, it would be expected that ENER heifers had greater plasma glucose concentration compared to PROT.

Nevertheless, Huntington (1997) indicated that growing cattle are highly capable of synthesizing glucose from amino acids, such as those provided in the PROT treatment or produced by rumen microbes. In addition, blood glucose concentration in cattle are fairly stable due to the role of insulin, which may have prevented proper assessment of treatment effects on glucose flux herein (Marston et al., 1995).

Mean plasma insulin concentration was greater ($P < 0.01$) for PROT compared with CON heifers, tended ($P = 0.08$) to be greater for ENER compared with CON heifers, and did not differ ($P = 0.15$) between PROT and ENER heifers (Table 4). Mean plasma IGF-I concentration was greater ($P \leq 0.04$) for PROT and ENER compared with CON heifers, and did not differ ($P = 0.55$) between PROT and ENER heifers (Table 4). In cattle, circulating insulin is directly influenced by nutrient intake and blood glucose concentration (Vizcarra et al., 1998; Nussey and Whitehead, 2001), and is known to stimulate hepatic IGF-I synthesis (Molento et al., 2002). Hence, plasma insulin and IGF-I concentrations have been recognized as indicators of nutrient intake and nutritional status of cattle (Yelich et al., 1995; Wettemann and Bossis, 2000; Hess et al., 2005). Similar treatment effects were detected for plasma insulin and IGF-I in results reported later, which supports the results detected herein for plasma glucose concentration, and suggests that ENER and PROT heifers had equivalent intake, utilization, and metabolism of dietary substrates despite differences in ingredients between treatments.

A treatment effect was also detected ($P = 0.01$) for plasma P_4 concentration. Progesterone concentrations on d 0 were significant covariates ($P < 0.01$) but did not differ ($P = 0.98$) among treatments (6.84, 6.84, and 6.99 ng/mL for CON, ENER, and PROT, respectively; SEM = 0.71), indicating that heifers from all treatment groups had

similar plasma P_4 concentration prior to the beginning of treatment administration. Within samples collected on d 13, 15, 17, and 19, mean plasma P_4 concentrations were greater ($P \leq 0.01$) for PROT and ENER compared with CON heifers, and did not differ ($P = 0.93$) between PROT and ENER heifers (Table 4). The main hypothesis of this experiment was that beef heifers consuming a low-quality cool-season forage and receiving a supplement containing an energy ingredient would have greater plasma P_4 compared with unsupplemented or cohorts receiving a supplement based on a protein ingredient. This hypothesis was developed based on the premise that energy ingredients such as corn favor circulating concentrations of glucose, insulin, and IGF-I (Huntington, 1990; Nussey and Whitehead, 2001; Molento et al., 2002), whereas insulin and IGF-I have been positively associated with circulating P_4 concentration. More specifically, IGF-I is known to stimulate luteal P_4 synthesis (Spicer and Echternkamp, 1995). Insulin also stimulates luteal P_4 synthesis (Spicer and Echternkamp, 1995), and alleviates hepatic P_4 catabolism by CYP2C and CYP3A enzymes (Murray, 1991; Cooke et al., 2012; Vieira et al., 2013). In the present experiment, the lack of differences in plasma P_4 concentrations between ENER and PROT heifers, which were greater compared with CON heifers, can be directly attributed to the equivalent treatment effects detected for insulin and IGF-I. Hence, the ENER and PROT treatments utilized herein equally increased plasma P_4 concentrations in pregnant beef heifers consuming a low-quality cool-season forage.

In summary, heifers offered PROT and ENER had a similar increase in nutrient intake, ADG, plasma concentrations of hormones and metabolites associated with dietary protein and energy metabolism, as well as plasma P_4 concentration compared with CON

heifers, despite differences in ingredients between supplement treatments. Hence, pregnant beef heifers consuming a low-quality cool-season forage equally utilize and benefit, in terms of performance and physiological parameters, from supplements based on protein or energy ingredients provided as 0.5 % of heifer BW/d at isocaloric and isonitrogenous rates.

Table 1. Ingredient composition and nutrient profile of treatments offered during Exp. 1 and Exp. 2.

Item	Exp. 1		Exp. 2	
	PROT	ENER	PROT	ENER
Ingredients, % DM				
Cracked corn	--	68	--	68
Soybean meal	100	22	100	22
Urea	--	10	--	10
Nutrient profile,[1] DM basis				
TDN, [2] %	85.4	77.0	85.4	77.0
NE_m,[3] Mcal/kg	2.02	1.91	2.02	1.91
NE_g,[3] Mcal/kg	1.37	1.31	1.37	1.31
CP, %	50.1	45.0	50.1	45.0
RDP, %	28.3	36.0	28.3	36.0
NFC, %	33.5	59.0	33.5	59.0
NDF, %	8.6	9.0	8.6	9.0
Starch, %	5.4	48.4	5.4	48.4
Ether extract, %	1.5	2.9	1.5	2.9
Daily intake[4]				
DM, kg	2.20	2.37	1.77	1.92
TDN,[2] kg	1.88	1.82	1.51	1.48
NE_m,[3] Mcal	4.44	4.53	3.58	3.67
NE_g,[3] Mcal	3.01	3.10	2.42	2.52
CP, kg	1.10	1.08	0.89	0.86
RDP, kg	0.62	0.85	0.50	0.69
NFC, kg	0.74	1.40	0.59	1.13
NDF, kg	0.19	0.21	0.15	0.17
Starch, kg	0.12	1.15	0.10	0.93
Ether extract, kg	0.03	0.07	0.03	0.06

[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea. Values obtained from a commercial laboratory wet chemistry analysis (Dairy One Forage Laboratory, Ithaca, NY).
[2]Calculated according to the equations described by Weiss et al. (1992).
[3]Calculated with the following equations (NRC, 1996): $NE_m = 1.37\ ME - 0.138\ ME^2 + 0.0105\ ME^3 - 1.12$; $NE_g = 1.42\ ME - 0.174\ ME^2 + 0.0122\ ME^3 - 0.165$. Given that ME = DE × 0.82, and 1 kg of TDN = 4.4 Mcal of DE.
[4] Estimated from the concentrate consumption of individual experimental unit.

Table 2. Ruminal in situ disappearance parameters of meadow foxtail (*Alopecurus pratensis* L.) hay incubated in forage-fed steers receiving no supplementation (**CON**; n = 4), or supplements based on a protein (**PROT**; n = 4) or energy ingredient (**ENER**; n = 4).[1]

	Treatments				
Item	CON	PROT	ENER	SEM	*P*-Value
Ruminal disappearance rate, %/h					
DM	2.88	3.36	3.67	0.35	0.33
NDF	3.64	4.24	4.06	0.51	0.71
Effective degradability,[2] %					
DM	60.7	60.8	60.3	1.1	0.95
NDF	55.4	55.5	53.7	1.5	0.66

[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis). All steers were offered meadow foxtail hay for ad libitum consumption. Treatments were provided daily at 0.50 and 0.54 % of BW/steer for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous.

[2]Calculated by fixing ruminal passage rate at 0.046/h (Poore et al., 1990) and using the model proposed by Ørskov and McDonald (1979).

Table 3. Performance parameters of pregnant beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis* L.) and receiving no supplementation (**CON**; n = 4), or supplements based on a protein (**PROT**; n = 4) or energy ingredient (**ENER**; n = 4).[1]

	Treatments				
Item	CON	PROT	ENER	SEM	*P-Value*
ADG,[2] kg/d	0.49[a]	0.89[b]	0.75[b]	0.09	0.03
DMI, [3] kg/d					
Hay	8.60	8.42	8.84	0.14	0.17
Total	8.60[a]	10.19[b]	10.50[b]	0.22	< 0.01
Daily nutrient intake[4]					
NE_m, Mcal	9.46[a]	12.84[b]	12.89[b]	0.35	< 0.01
NE_g, Mcal/d	4.73[a]	7.06[b]	7.03[b]	0.22	< 0.01
CP, kg	0.74[a]	1.62[b]	1.51[b]	0.07	< 0.01
RDP, kg	0.51[a]	1.00[b]	1.12[b]	0.06	< 0.01
Starch, kg	0.146[a]	0.239[a]	0.950[b]	0.075	< 0.01

[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis). All heifers were offered meadow foxtail hay for ad libitum consumption. Treatments were offered and consumed (d 1 to 19) daily at 1.77 and 1.92 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Within rows, values with different superscripts differ ($P \leq 0.05$).

[2]Calculated using initial and final shrunk BW (after 16 h of feed and water restriction) obtained on d -7 and 20 of the experiment.

[3]Recorded from each pen from d 1 to 19 of the experiment, divided by the number of heifers within each pen, and expressed as kg per heifer/d.

[4]Estimated based on total DMI of each pen, and nutritive value of hay and treatments.

Table 4. Plasma concentrations of urea N (**PUN**), glucose, insulin, IGF-I, and P_4 of pregnant beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis* L.) and receiving no supplementation (**CON**; n = 4), or supplements based on a protein (**PROT**; n = 4) or energy ingredient (**ENER**; n = 4).[1,2]

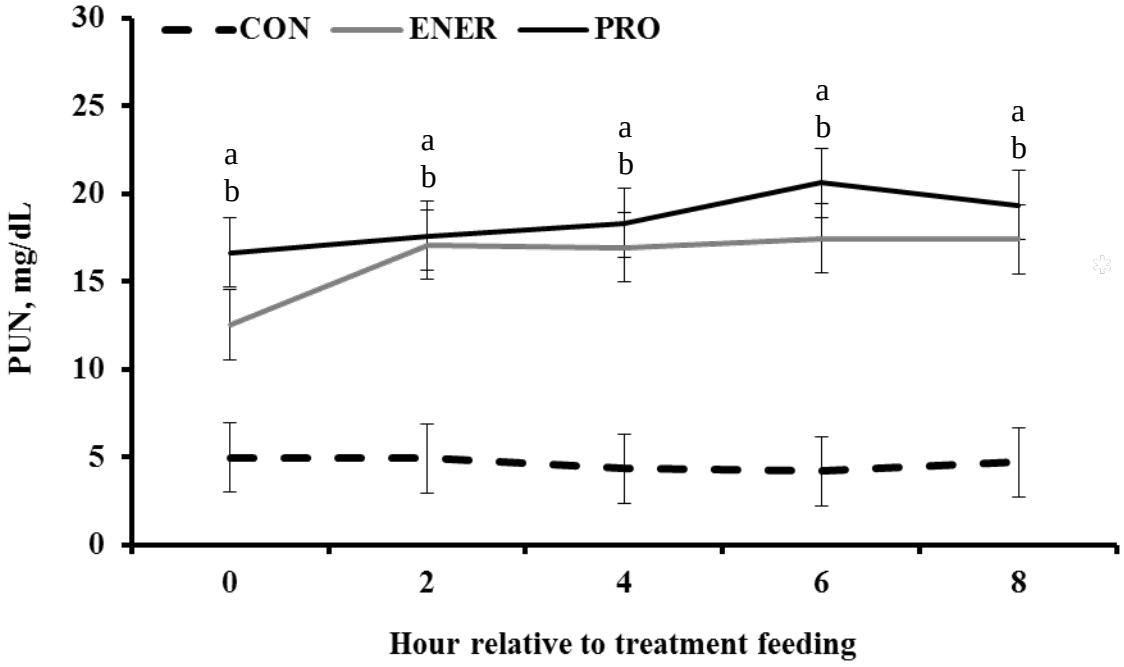
	Treatments				
Item	CON	PROT	ENER	SEM	*P-Value*
PUN, mg/dL	4.6[a]	16.3[b]	18.5[b]	1.9	< 0.01
Glucose, mg/dL	62.2[a]	66.5[b]	66.6[b]	1.3	0.04
Insulin, µIU/mL	2.48[a]	3.65[b]	3.09[ab]	0.25	< 0.01
IGF-I, ng/mL	112.9[a]	143.6[b]	137.3[b]	7.3	0.03
Progesterone,[3] ng/mL	6.38[a]	7.79[b]	7.75[b]	0.36	0.01

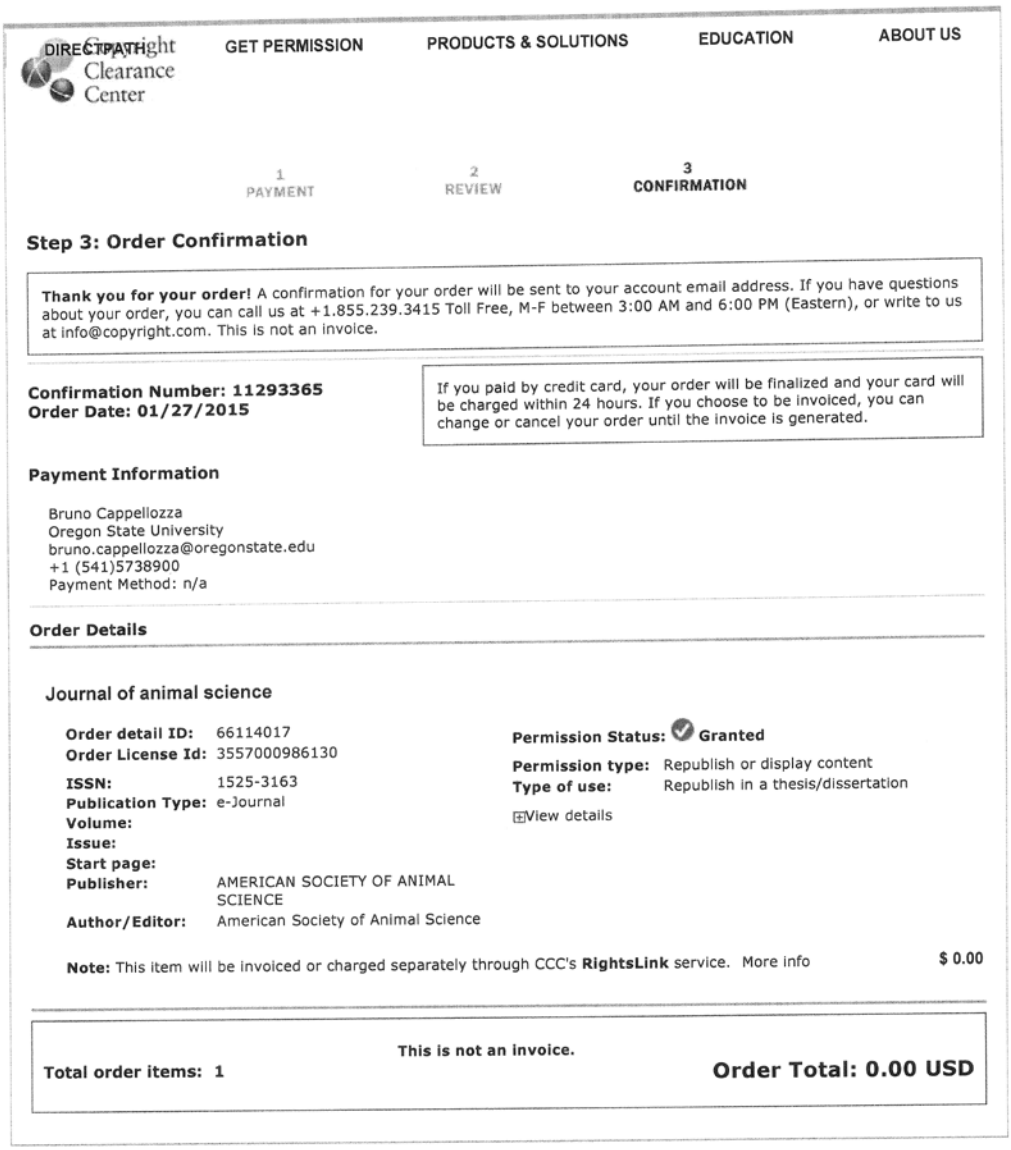
[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis). All heifers were offered meadow foxtail hay for ad libitum consumption. Treatments were offered and consumed (d 1 to 19) daily at 1.77 and 1.92 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Within rows, values with different superscripts differ ($P \leq 0.05$).

[2]Blood samples were collected on d 13, 15, 17, and 19 of the study immediately prior to, and 2, 4, 6, and 8 h relative to supplement feeding (h 0).

[3] Covariately adjusted to samples collected on d 0, immediately prior to and 4 and 8 h relative to hay feeding (h 0).

Figure 1. Plasma concentration of urea N (PUN) in pregnant beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis* L.) and receiving no supplementation (**CON**; n = 4), or supplements based on a protein (**PROT**; n = 4; 100 % soybean meal on DM basis) or energy ingredient (**ENER**; n = 4; 68 % cracked corn, 22 % soybean meal, and 10 % urea on DM basis). Treatments were offered and consumed at 1.77 and 1.92 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Blood samples were collected on d 13, 15, 17, and 19 of the experiment immediately prior to, and at 2, 4, 6, and 8 h relative to treatment feeding (h 0). A treatment × hour interaction was detected ($P < 0.01$) for PUN (Figure 1), given that PUN concentrations increased after supplementation for ENER and PROT heifers (time effect, $P < 0.01$), but did not change for CON (time effect; $P = 0.62$).Within hour, letters indicate the following treatment differences; a = PROT vs. CON ($P < 0.01$), b = ENER vs. CON ($P < 0.02$).





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CHAPTER 4

SUPPLEMENTATION BASED ON PROTEIN OR ENERGY INGREDIENTS TO BEEF CATTLE CONSUMING LOW-QUALITY COOL-SEASON FORAGES: II. PERFORMANCE, REPRODUCTIVE, AND METABOLIC RESPONSES OF REPLACEMENT HEIFERS.

Introduction

Supplementation is often required in heifer development programs based on low-quality forages (Schillo et al., 1992). Although forages typically represent the main energy source for forage-fed cattle, and energy is the primary dietary consideration for heifer development (Mass, 1987), protein is traditionally considered the limiting nutrient in Western U.S. cow-calf operations (DelCurto et al., 2000). Indeed, protein supplementation generally improves digestibility and DMI of low-quality warm-season forages, resulting in increased energy utilization from the forage and cattle BW gain (DelCurto et al., 1990; Lintzenich et al., 1995). However, Bohnert et al. (2011a) reported that protein supplementation did not increase digestibility and DMI of low-quality cool-season forages. Hence, inclusion of energy ingredients into supplements may be beneficial for growth and reproductive development of heifers consuming such forages.

Beef heifers, particularly *Bos taurus*, should attain puberty by 12 mo of age to maximize lifetime productivity (Lesmeister et al., 1973). Energy intake influences puberty attainment in heifers by other mechanisms besides BW gain, including modulation of hormones known to mediate the puberty process such as insulin and IGF-I (Schillo et al., 1992). Accordingly, Ciccioli et al. (2005) reported that feeding starch-based supplements hastened puberty attainment in beef heifers independently of BW gain. Hence, inclusion of energy ingredients, such as starch, into supplements may further

benefit reproductive development of heifers consuming low-quality cool-season forages by favoring circulating concentrations of nutritional mediators of puberty. To test this hypothesis, this experiment compared the effects of supplements based on protein or energy ingredients on performance, plasma metabolites and hormones, expression of hepatic genes associated with nutritional metabolism, and puberty attainment of beef heifers consuming a low-quality cool-season forage.

Materials and Methods

This experiment was conducted at the Oregon State University – Eastern Oregon Agricultural Research Center (Burns; 43°29’31” N, 119°42’40” W, and 1,425 m elevation) from November 2012 to April 2013 (d -10 to 160). All heifers utilized were cared for in accordance with acceptable practices and experimental protocols reviewed and approved by the Oregon State University, Institutional Animal Care and Use Committee.

Hay (meadow foxtail; *Alopecurus pratensis* L.) and supplement ingredients utilized in this experiment originated from the same field and batch, respectively, as the dietary ingredients utilized in the previously reported experiments (Chapter 3). A sample of hay (according to Bohnert et al., 2011b) and each supplement ingredient was collected prior to the beginning of the experiment reported herein and those previously described (Chapter 3), and analyzed by nutrient content by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY) also as described previously (Chapter 3). Hay nutritive value was (DM basis) 57 % TDN, 58 % NDF, 37 % ADF, 1.12 Mcal/kg of NE_m, 0.57 Mcal/kg of NE_g, 8.7 % CP, 6.0 % RDP, and 2.1 % ether extract.

Heifers and diets

Sixty Angus × Hereford weaned heifers (initial age 226 ± 3 d; initial BW 200 ± 2 kg) were utilized in this experiment. On d -10 of the study, heifers were ranked by initial BW and age and allocated to 15 drylot pens (7 × 15 m; 5 pens/treatment; 4 heifers/pen), in a manner which all pens had equivalent initial average BW and age. Pens were randomly assigned to receive 1 of 3 treatments: 1) supplementation with soybean [*Glycine max* (L.) *Merr*.] meal (**PROT**), 2) supplementation with a mixture of cracked corn (*Zea mays* L.), soybean meal, and urea (68:22:10 ratio, DM basis; **ENER**), or 3) no supplementation (**CON**). Heifers were offered meadow foxtail hay for ad libitum consumption during the entire experiment (d -10 to 160). Beginning on d 0, PROT and ENER treatments were fed once daily (0800 h) at a rate of 1.30 and 1.40 kg of DM/heifer, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous (Table 1). Urea was included into ENER to result in isocaloric and isonitrogenous intakes of PROT and ENER. Further, treatment intakes were formulated at 0.50 and 0.54 % of the expected average heifer shrunk BW during the experiment for PROT and ENER, respectively, to achieve the same treatment intake as % of BW used in our other experiments (Chapter 3). Average heifer shrunk BW during the experiment was estimated based on initial shrunk BW (d -9) and expected final shrunk BW (d 161). Expected final shrunk BW was projected based on previous research from our group (Cooke et al., 2012; Cooke et al., 2013), which was conducted at the same research station and using the same cowherd as the experiment described herein.

The ENER and PROT treatments were not mixed with hay, and were readily consumed by heifers. All heifers had ad libitum access to water and the same mineral and vitamin mix previously described throughout the experimental period.

Sampling

Heifers were weighed on 2 consecutive days to determine both full and shrunk (after 16 h of feed and water restriction) BW at the beginning (d -10 and d -9) and end of the experiment (d 160 and 161). Shrunk BW was used to determine heifer ADG during the study. Blood samples were collected at 10-d intervals throughout the entire experiment (d -10 to 160), starting 4 h after the ENER and PROT treatments were offered, to determine onset of puberty according to plasma progesterone concentration. Heifers were considered pubertal when plasma progesterone concentration was equal or greater than 1.0 ng/mL for 2 consecutive samplings (Perry et al., 1991), and puberty attainment was declared at the second sampling of elevated progesterone. In addition, blood samples collected on d -10, 60, 120, and 150 were also analyzed for plasma urea N (**PUN**), glucose, insulin, NEFA, IGF-I, and leptin concentrations.

Hay and total DMI were evaluated from each pen by collecting and weighing refusals from d 12 to 16, d 53 to 57, d 71 to 75, d 93 to 97, d 112 to 116, and d 143 to 147 of the experiment, which were classified as periods (periods 1 to 6, respectively). Samples of the offered and non-consumed hay were collected daily from each pen and dried for 96 h at 50°C in forced-air ovens for DM calculation. Hay, concentrate, and total daily DMI of each pen were divided by the number of heifers within each pen and expressed as kg per heifer/d. In addition, daily intake/heifer of NE_m, NE_g, CP, RDP, and

starch were estimated based on DMI of each pen, and nutritive value of hay and treatments (Table 1).

On d 100 of the experiment, 2 heifers/pen were randomly assigned for liver sample collection via needle biopsy (Arthington and Corah, 1995), which began 4 h after supplements were offered. Immediately after collection, liver samples (average 100 mg of tissue, wet weight) were placed in 1 mL of RNA stabilization solution (RNAlater, Ambion Inc., Austin, TX), maintained at 4°C for 24 h, and stored at -80°C. Samples were analyzed via real-time quantitative reverse transcription (**RT**)-PCR for IGF-I, IGFBP-3, pyruvate carboxylase (**PC**), cytosolic phosphoenolpyruvate carboxykinase (**PEPCK-C**), mitochondrial PEPCK (**PEPCK-M**), and cyclophilin mRNA expression.

Laboratory Analysis

Blood samples. Blood samples were collected via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) containing 158 USP units of freeze-dried sodium heparin for plasma collection. All blood samples were placed immediately on ice, subsequently centrifuged (2,500 × *g* for 30 min; 4°C) for plasma harvest, and stored at -80°C on the same day of collection. Plasma concentrations of glucose, PUN, insulin, progesterone, and IGF-I were determined as described in our previous studies (Chapter 3). Plasma concentration of NEFA was determined using a colorimetric commercial kit (HR Series NEFA-2; Wako Pure Chemical Industries Ltd. USA, Richmond, VA) with the modifications described by Pescara et al. (2010). Plasma concentration of leptin was determined according to procedures described by Delavaud et al. (2000). The intra- and inter-assay CV were, respectively, 4.82 and 3.53 % for NEFA, 0.93 and 5.69 % for glucose, 10.31 and 6.54 %

for PUN, 6.17 and 3.37 % for IGF-I, 7.92 and 4.27 % for insulin, and 5.01 and 4.97 % for progesterone. All samples were analyzed for leptin concentration within a single assay, and the intra-assay CV was 4.40 %. The minimum detectable concentrations were 0.02 μIU/mL for insulin, and 0.056, 0.10, and 0.10 ng/mL for IGF-I, leptin, and progesterone, respectively.

Tissue Samples. Total RNA was extracted from tissue samples using TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). Quantity and quality of isolated RNA were assessed via UV absorbance (NanoDrop 2000; Thermo Fisher Scientific, Minneapolis, MN) at 260 nm and 260/280 nm ratio, respectively (Fleige and Pfaffl, 2006). Extracted RNA was stored at -80°C until further processing.

Extracted hepatic RNA (2.5 μg) was incubated at 37°C for 30 min in the presence of RNase free DNase (New England Biolabs Inc., Ipswich, MA) to remove contaminant genomic DNA. After inactivating the DNase (75°C for 15 min), samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random hexamers (Applied Biosystems, Foster City, CA). Quantity and quality of cDNA were again assessed via UV absorbance at 260 nm and 260/280 nm ratio, respectively (NanoDrop 2000; Thermo Fisher Scientific). Real-time RT-PCR was completed using the Rotor-Gene SYBR Green PCR Kit (Qiagen Inc., Valencia, CA) and specific primer sets (25 ng/mL; Table 3), with a Rotor-Gene Q real-time PCR cycler (Qiagen Inc.) according to procedures described by Yoganathan et al. (2012). At the end of each RT-PCR, amplified products were subjected to a dissociation gradient (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) to verify the amplification of a single product by denaturation at the anticipated temperature. Responses were quantified based on the threshold cycle ($\mathbf{C_T}$),

the number of PCR cycles required for target amplification to reach a predetermined threshold. All C_T responses from genes of interest were normalized to cyclophilin C_T examined in the same sample and assessed at the same time as the targets. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$), as described by Ocón-Grove et al. (2008).

Statistical Analysis. All data were analyzed using pen as experimental unit, and Satterthwaite approximation to determine the denominator df for the tests of fixed effects. Performance, plasma variables, and gene expression data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The model statement used for BW, ADG, and gene expression contained only the effects of treatment. Data were analyzed using heifer(pen) and pen(treatment) as the random variables. The model statement used for plasma variables contained the effects of treatment, day, the treatment × day interaction, and values obtained on d -10 as covariate. Data were analyzed using heifer(pen) and pen(treatment) as random variables, with day as the specified term for the repeated statement and heifer(pen) as subject. The model statement used for feed and nutrient intake contained the effects of treatment, day, period, and all the resultant interactions. Data were analyzed using pen(treatment) as the random variable, given that DMI was recorded daily from each pen, as well as day(period) as the specified term for the repeated statement and pen(treatment) as subject. For both intake and plasma variables, the covariance structure used was first-order autoregressive, which provided the smallest Akaike Information Criterion and hence the best fit for all variables analyzed. Puberty data were analyzed using the GLIMMIX procedure of SAS (SAS Inst. Inc.). The model statement used contained the effects of treatment, day, and the resultant interaction. Data were analyzed using heifer(pen) and pen(treatment) as the random

variables. Results are reported as least square means, or covariately adjusted means for plasma variables, and separated using PDIFF. Significance was set at $P \leq 0.05$ and tendencies were denoted if $P > 0.05$ and ≤ 0.10. Results are reported according to main effects if no interactions were significant, or according to highest-order interaction detected.

Results and Discussion

As previously stated, inclusion of energy ingredients into supplements may benefit growth and reproductive performance of replacement heifers consuming low-quality cool-season forages (Schillo et al., 1992; Ciccioli et al., 2005; Bohnert et al., 2011a). To test this theory, a series of experiments evaluated productive and biological responses in beef cattle consuming a low-quality cool-season forage and receiving CON, PROT, or ENER. The experiments reported in Chapter 3 evaluated forage disappearance parameters in rumen-fistulated steers, as well as performance and physiological responses in pregnant heifers provided PROT and ENER at 0.50 and 0.54 % of shrunk BW, respectively. The experiment reported herein compared growth, puberty attainment, and metabolic responses of beef heifers assigned to CON, or PROT and ENER after weaning. It is important to note that average shrunk BW during the present experiment was 227, 257, and 264 kg for CON, ENER, and PROT, respectively (SEM = 3.3), which resulted in an average treatment intake of 0.54 and 0.49 % of shrunk BW for ENER and PROT, respectively. Hence, average intake of ENER and PROT during the present experiment as % of shrunk BW was similar to our previous studies reported in Chapter 3. These

supplementation rates were adopted to yield adequate ADG of beef heifers, either non-pregnant or pregnant, consuming low-quality cool-season forages (NRC, 1996).

No treatment effects were detected (P = 0.33) on forage DMI (Table 3). Accordingly, rumen-fistulated steers receiving CON, ENER, or PROT had similar ruminal disappearance and estimated degradability of the same forage utilized herein, whereas ruminal forage digestibility is positively associated with intake (Allen, 1996). In addition, results from studies reported herein (Chapter 3) also demonstrated similar hay intake among pregnant replacement heifers receiving CON, ENER, and PROT. These results support that protein supplementation does not impact DMI of a low-quality cool-season forage (Bohnert et al., 2011a), and that supplements based on energy ingredients can be fed at approximately 0.5 % of BW without impacting forage intake (Bowman and Sanson, 1996). Total daily DMI, and estimated daily intake of NE_m, and NE_g, were greater (P < 0.01) for PROT and ENER compared with CON heifers, and similar ($P \geq$ 0.41) between PROT and ENER heifers (Table 3). Estimated daily intake of CP, RDP, and starch were greater (P < 0.01) for PROT and ENER compared with CON heifers, whereas ENER had greater (P < 0.01) RDP and starch intake, and tended (P = 0.09) to have less CP intake compared to PROT heifers (Table 3). Hence, PROT and ENER had greater overall nutrient intake compared with CON heifers, although starch was the main energy source provided by ENER. The greater RDP intake of ENER compared with PROT heifers can be attributed to the inclusion of urea into the ENER treatment (Horn and McCollum, 1987), and consequent RDP content of treatments (Table 1). In addition, the slightly greater CP intake of PROT compared with ENER heifers, despite similar CP content of treatments (Table 1) can be attributed to the numerical difference in hay intake

between PROT and ENER heifers. However, CP and RDP intakes were not limited in ENER or PROT heifers, based on supplement formulation and intake rate (NRC, 1996).

A treatment effect ($P < 0.01$) was detected for ADG (Table 3), which was greater ($P < 0.01$) for PROT and ENER compared with CON heifers, and similar between ENER and PROT ($P = 0.52$). Our previous studies (Chapter 3) also reported that pregnant heifers receiving ENER and PROT had similar ADG, which were greater compared with CON cohorts. Collectively, these results provide evidence that beef heifers consuming low-quality cool-season forages can equally utilize nutrients provided by supplements based on protein or energy ingredients to support BW gain. These results also indicate that differences in CP and RDP intakes between ENER and PROT in the present experiment were minimal and not sufficient to impact heifer ADG. Supporting this rationale, equivalent treatment effects were detected ($P \leq 0.05$) for the plasma variables associated with dietary energy and protein metabolism evaluated herein (Table 4 and Figure 1; Hammond, 1997; Huntington, 1997; Hess et al., 2005).

A treatment effect was detected ($P < 0.01$) for plasma NEFA (Table 4). Values obtained on d -10 were significant covariates ($P < 0.01$), but did not differ ($P = 0.93$) among treatments (0.121, 0.124, and 0.119 μEq/L for CON, PROT, and ENER, respectively; SEM = 0.01). During the experiment, mean NEFA concentration was greater ($P < 0.01$) for CON compared with PROT and ENER heifers, and similar ($P = 0.13$) between PROT and ENER heifers (Table 4). Accordingly, circulating NEFA concentration in cattle was negatively associated with nutrient intake and ADG, whereas elevated NEFA is often associated with negative energy balance (Lucy et al., 1991; Peters, 1986). Nevertheless, it is important to note that heifers from all treatments were in

a positive nutritional status based on their ADG (Table 3). Hence, the elevated NEFA concentration in CON heifers were somewhat unexpected, given that Bossis et al. (2000) and Ellenberger et al. (1989) reported similar NEFA concentrations in beef cattle being managed to achieve different but positive rates of ADG.

A treatment effect was detected ($P < 0.01$) for PUN (Table 4). Values obtained on d -10 were not significant covariates ($P = 0.40$), and did not differ ($P = 0.22$) among treatments (22.74, 20.26, and 22.28 mg/dL for CON, PROT, and ENER, respectively; SEM = 1.06). During the experiment, mean PUN concentrations were greater ($P < 0.01$) for PROT and ENER compared with CON, whereas PROT also had greater ($P < 0.01$) PUN concentration compared with ENER heifers (Table 4). Concentration of PUN is positively associated with intake of CP, RDP, and ruminal ammonia concentration (Broderick and Clayton, 1997). In addition, optimal PUN concentrations in growing beef heifers range from 15 to 19 mg/dL (Hammond, 1997). Hence, the greater PUN concentrations of PROT and ENER compared with CON heifers can be directly attributed to their greater CP and RDP intake, and suggest that CON heifers required supplemental CP and RDP. Differences in PUN concentrations between ENER and PROT heifers can also be attributed to the slightly greater CP intake of PROT heifers, as well as improved N utilization by ruminal microbes in ENER heifers (Hall and Huntington, 2008). Although RDP intake was greater in ENER compared with PROT heifers, the ENER treatment also contained a greater proportion of starch and NFC, which are known to optimize the synchrony in energy and protein utilization by rumen microbes and reduce the amount of ammonia and subsequent PUN in the circulation (Hammond, 1997; Hall and Huntington, 2008). Moreover, PUN concentrations in ENER

and PROT heifers further corroborates that CP and RDP intakes were not limiting in ENER or PROT heifers (Hammond, 1997). Hence, differences between ENER and PROT heifers on the parameters evaluate herein, besides PUN concentrations, should not be associated with CP and RDP intake.

A treatment effect was detected ($P < 0.01$) for plasma glucose (Table 4). Values obtained on d -10 tended to be significant covariates ($P = 0.08$), but did not differ ($P = 0.52$) among treatments (58.3, 61.4, and 58.1 mg/dL for CON, PROT, and ENER, respectively; SEM = 2.2). During the experiment, mean glucose concentrations were greater ($P < 0.01$) for PROT and ENER compared with CON heifers, and similar ($P = 0.91$) between PROT and ENER heifers (Table 4). A similar treatment effect was also detected in plasma glucose concentrations of pregnant heifers, as previously described herein. Supporting these results, glucose concentration was positively associated with feed intake and rates of BW gain (Vizcarra et al., 1998; Hersom et al., 2004), as observed herein based on the greater nutrient intake and ADG of PROT and ENER compared with CON heifers (Table 3). However, starch is the major dietary precursor for glucose in ruminants (Huntington, 1997); hence, it would be expected that ENER heifers had greater plasma glucose concentration compared to PROT. Nevertheless, blood glucose concentrations in cattle are fairly stable due to the role of insulin, which may have prevented proper assessment of treatment effects on glucose flux herein (Marston et al., 1995). In addition, Huntington (1997) reported that growing cattle are highly capable of synthesizing glucose from amino acids, such as those provided in the PROT treatment or produced by rumen microbes.

Supporting this latter rationale, PROT heifers had greater (P = 0.05) mRNA expression of liver PEPCK-M compared with ENER and CON, which was similar (P = 0.98) between ENER and CON (treatment effect, P = 0.08; Table 5). Although liver PEPCK-M is considered constitutive and not highly responsive to hormones and nutritional state (Agca et al., 2002), it may account for up to 61 % of glucose synthesis in ruminant hepatocytes (Aiello and Armentano, 1987). Moreover, Cooke et al. (2008) also reported that PEPCK-M mRNA expression was influenced by supplementation and reflective of overall nutritional status of beef heifers. No treatment effects were detected ($P \geq 0.28$; Table 5) for mRNA expression of PC and PEPCK-C, although mRNA expression of these enzymes are modulated by nutrient intake (Cooke et al., 2008) and are positively associated with glucose synthesis in cattle (Greenfield et al., 2000; Bradford and Allen, 2005). Nevertheless, circulating NEFA are known to stimulate mRNA expression of hepatic PC and PEPCK-C, but not PEPCK-M, to preserve gluconeogenesis in cattle with insufficient nutrient intake (Agca et al., 2002; White et al., 2011). Hence, the greater NEFA concentration in CON heifers may have maintained mRNA expression of hepatic PC and PEPCK-C similar to that of ENER and PROT heifers. In addition, it may be speculated that a greater gluconeogenesis through hepatic PEPCK-M in PROT heifers contributed to their greater glucose concentration compared with CON, and to the similar glucose concentration compared with ENER heifers despite treatment differences in starch intake.

Treatment effects were detected ($P \leq 0.05$) for plasma insulin and IGF-I (Table 4), as well as mRNA expression of liver IGF-I and IGFBP-3 (Table 5). Values obtained on d -10 were significant covariates for plasma insulin and IGF-I analyses ($P < 0.01$), but did

not differ ($P \geq 0.66$) among treatments (5.77, 5.52, and 5.68 μIU/mL of insulin, SEM = 0.57, and 92.3, 85.8, and 85.8 ng/mL of IGF-I, SEM = 7.6; for CON, PROT, and ENER, respectively). During the experiment, mean insulin and IGF-I concentrations were greater ($P < 0.01$) for PROT and ENER compared with CON heifers, and similar ($P \geq 0.21$) between PROT and ENER heifers (Table 4). In our previous studies (Chapter 3), ENER and PROT also increased plasma concentrations of insulin and IGF-I compared to CON in pregnant beef heifers. Expression of liver IGF-I and IGFBP-3 mRNA were also greater ($P \leq 0.05$) in PROT and ENER compared with CON, and similar ($P \geq 0.29$) between PROT and ENER (Table 5). Collectively, these results corroborate with treatment effects detected for DMI, nutrient intake, and plasma glucose, given that circulating concentration of insulin is positively regulated by nutrient intake and blood glucose (Vizcarra et al., 1998; Nussey and Whitehead, 2001). In turn, availability of energy substrates and circulating insulin positively modulate the expression of liver IGF-I and IGFBP-3 mRNA, and consequent hepatic synthesis of these proteins (McGuire et al., 1992; Thissen et al., 1994; Cooke et al., 2008). For these reasons, plasma insulin and IGF-I have been recognized as indicators of nutritional status of cattle (Yelich et al., 1995; Wettemann and Bossis, 2000; Hess et al., 2005), suggesting that ENER and PROT heifers in the present experiment had equivalent intake, utilization, and metabolism of dietary substrates despite differences in ingredients between treatments.

A treatment × day interaction was detected ($P = 0.03$) for plasma leptin (Figure 1). Values obtained on d -10 were significant covariates ($P = 0.03$), but did not differ ($P = 0.19$) among treatments (4.34, 4.87, and 4.49 ng/mL for CON, PROT, and ENER, respectively; SEM = 0.20). Plasma leptin concentrations were similar between ENER and

PROT throughout the experiment ($P \geq 0.19$), and greater for ENER and PROT compared with CON on d 120 ($P \leq 0.01$) and 150 ($P \leq 0.03$; Figure 1). Circulating leptin concentration is regulated by body fat content, nutrient intake, and circulating insulin (Houseknecht et al., 1998). Hence, the similar plasma leptin concentrations between PROT and ENER corroborate with the similar nutrient intake, growth rates, and plasma insulin concentrations between treatments (Tables 3 and 4). Nevertheless, the greater ADG, nutrient intake, and plasma insulin concentrations of PROT and ENER heifers compared with CON only resulted in a similar effect on plasma leptin beginning on d 120 of the experiment. The reason for this delay is unknown and deserves further investigation, but may be associated with heifer age and rate of body fat accretion (Houseknecht et al., 1998).

No overall treatment effects were detected ($P = 0.25$) on puberty attainment (data not shown). However, a greater ($P < 0.01$) proportion of ENER heifers were pubertal at the end of the experiment (d 160) compared with CON and PROT cohorts, whereas no differences were detected ($P = 0.38$) between CON and PROT heifers (Table 3). The main hypothesis of the experiment was that replacement beef heifers consuming a low-quality cool-season forage and receiving a supplement based on an energy ingredient would have enhanced ADG and hastened puberty attainment compared with heifers receiving no supplementation or supplemented with a protein ingredient. This hypothesis was developed based on the premise that energy ingredients such as corn favor circulating concentrations of insulin, IGF-I, and leptin (Huntington, 1990; Molento et al., 2002; Lents et al., 2005), and these hormones are known to impact the puberty process by mediating synthesis and activity of GnRH and gonadotropin (Butler and Smith, 1989;

Schillo et al., 1992; Maciel et al., 2004). Indeed, a greater proportion of ENER heifers were pubertal at the end of experiment compared with PROT and CON, but this outcome disagrees with the similar ADG and metabolic status between PROT and ENER heifers. Supporting our findings, Ciccioli et al. (2005) also reported that heifers receiving a high-starch supplement had hastened puberty attainment but similar ADG compared with cohorts receiving an isocaloric and isonitrogenous low-starch supplement.

Nevertheless, puberty results reported herein should be adopted with caution, given that overall puberty attainment was lower than expected according to previous work from our research group (Cooke et al., 2012: Cooke et al., 2013). Based on the mature BW of the cowherd utilized herein (535 kg; Bohnert et al. 2013), mean full BW and % of mature BW at the end of the experiment (d 160) were greater ($P < 0.01$) for ENER and PROT compared to CON, and similar ($P = 0.13$) between ENER and PROT (271, 335, and 348 kg of BW, SEM = 7, and 50.7, 62.6, and 65.1 % of mature BW, SEM = 1.2, for CON, ENER, and PROT, respectively). Heifer age at the end of the experiment was also similar among treatments ($P = 0.97$) and averaged 396 ± 6 d. Hence, ENER and PROT heifers achieved the BW recommended for puberty achievement at 13 mo of age (Patterson et al., 2000). It is important to note that heifers utilized herein were reared in a 7 × 15 m drylot pens, whereas heifers utilized by Cooke et al. (2012) and Cooke et al. (2013) were reared on 6 ha pastures. Exercise may be required for adequate reproductive function in cattle (Lamb et al., 1979; Lamb et al., 1981; Cooke et al., 2012) via endogenous opioids known to modulate gonadotropin secretion and consequent onset of puberty, cyclicity, and fertility (Harber and Sutton, 1984; Mahmoud et al., 1989). Accordingly, Mulliniks et al. (2013) reported that heifers reared in drylots had greater

ADG, but reduced pregnancy rates compared with cohorts reared on range pastures. Therefore, it may be speculated that the lack of exercise haltered puberty attainment in the present experiment, despite adequate growth rates and final BW of ENER and PROT heifers.

In summary, replacement beef heifers offered PROT and ENER had a similar increase in nutrient intake, ADG, and overall metabolic status compared with CON heifers, despite differences in ingredients between supplement treatments. Puberty attainment was enhanced in ENER heifers only, although this outcome should be interpreted with caution due to the reduced number of pubertal heifers across all treatments. Hence, replacement beef heifers consuming a low-quality cool-season forage equally utilize and benefit, in terms of growth and metabolic parameters, from supplements based on protein or energy ingredients provided as 0.5 % of heifer BW/d at isocaloric and isonitrogenous rates.

Table 1. Ingredient composition and nutrient profile of treatments offered during the experiment.

	Treatments	
Item	PROT	ENER
Ingredients, % DM		
Cracked corn	--	68
Soybean meal	100	22
Urea	--	10
Nutrient profile[1], DM basis		
TDN, [2] %	85.4	77.0
NE_m, [3] Mcal/kg	2.02	1.91
NE_g, [3] Mcal/kg	1.37	1.31
CP, %	50.1	45.0
RDP, %	28.3	36.0
NFC, %	33.5	59.0
NDF, %	8.6	9.0
Starch, %	5.4	48.4
Ether extract, %	1.5	2.9
Daily intake[4]		
DM, kg	1.30	1.40
TDN, [2] kg	1.11	1.08
NE_m, [3] Mcal	2.63	2.67
NE_g, [3] Mcal	1.78	1.83
CP, kg	0.65	0.63
RDP, kg	0.37	0.50
NFC, kg	0.44	0.83
NDF, kg	0.11	0.13
Starch, kg	0.07	0.68
Ether extract, kg	0.02	0.04

[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea. Values obtained from a commercial laboratory wet chemistry analysis (Dairy One Forage Laboratory, Ithaca, NY).
[2]Calculated according to the equations described by Weiss et al. (1992).
[3]Calculated with the following equations (NRC, 1996): $NE_m = 1.37\ ME - 0.138\ ME^2 + 0.0105\ ME^3 - 1.12$; $NE_g = 1.42\ ME - 0.174\ ME^2 + 0.0122\ ME^3 - 0.165$. Given that ME = DE × 0.82, and 1 kg of TDN = 4.4 Mcal of DE.
[4] Estimated from the concentrate consumption of individual experimental unit.

Table 2. Primer sequences and accession number for all gene transcripts analyzed by quantitative real-time RT-PCR.

Target gene	Primer sequence[1]	Accession no.
IGF-I		
Forward	CTC CTC GCA TCT CTT CTA TCT	NM_001077828
Reverse	ACT CAT CCA CGA TTC CTG TCT	
IGFBP-3		
Forward	AAT GGC AGT GAG TCG GAA GA	NM_174556.1
Reverse	AAG TTC TGG GTG TCT GTG CT	
Pyruvate carboxylase		
Forward	CCA ACG GGT TTC AGA GAC AT	NM_177946.3
Reverse	TGA AGC TGT GGG CAA CAT AG	
Cytosolic phosphoenolpyruvate carboxykinase		
Forward	CAA CTA CTC AGC CAA AAT CG	NM_174737.2
Reverse	ATC GCA GAT GTG GAC TTG	
Mitochondrial phosphoenolpyruvate carboxykinase		
Forward	GCT ACA ACT TTG GGC GCT AC	XM_583200
Reverse	GTC GGC AGA TCC AGT CTA GC	
Cyclophilin		
Forward	GGT ACT GGT GGC AAG TCC AT	NM_178320.2
Reverse	GCC ATC CAA CCA CTC AGT CT	

[1]All primer sequences were obtained from Cooke et al. (2008).

Table 3. Performance and puberty parameters of replacement beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis L.*) and receiving no supplementation (**CON**; n = 5), or supplements based on a protein (**PROT**; n = 5) or energy ingredient (**ENER**; n = 5).

	Treatments				
Item	CON	PROT	ENER	SEM	*P-Value*
ADG, [2] kg/d	0.36[a]	0.76[b]	0.72[b]	0.04	< 0.01
DMI, [3] kg/d					
Hay	5.94	5.79	5.51	0.20	0.33
Total	5.92[a]	7.10[b]	6.91[b]	0.19	< 0.01
Daily nutrient intake [4]					
NE_m, Mcal	6.54[a]	9.00[b]	8.74[b]	0.22	< 0.01
NE_g, Mcal/d	3.27[a]	4.97[b]	4.87[b]	0.11	< 0.01
CP, kg	0.51[a]	1.15[b]	1.11[b]	0.02	< 0.01
RDP, kg	0.35[a]	0.71[b]	0.83[c]	0.01	< 0.01
Starch, kg	0.10[a]	0.17[b]	0.77[c]	0.003	< 0.01
Pubertal heifers on d 160,[5] %	10 (2/20)[a]	5 (1/20)[a]	25 (5/20)[b]	4	< 0.01

[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis). All heifers were offered meadow foxtail hay for ad libitum consumption. Treatments were offered and consumed daily (d 0 to 160) at 1.30 and 1.40 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Within rows, values with different superscript differ ($P \leq 0.05$).

[2]Calculated using initial and final shrunk BW (after 16 h of feed and water restriction) obtained on d -9 and 161 of the experiment.

[3]Recorded monthly from each pen during 5 consecutive days, but divided by the number of heifers within each pen and expressed as kg per heifer/d.

[4]Estimated based on total DMI of each pen, and nutritive value of hay and treatments.

[5]Estimated based from blood samples collected every 10 d during the experimental period (d -10 to 160). Heifers were considered pubertal once plasma progesterone concentration was equal or greater than 1.0 ng/mL for 2 consecutive wk (Perry et al., 1991), and puberty attainment was declared at the second wk of elevated progesterone. Values within parenthesis represent pubertal heifers/total heifers.

Table 4. Plasma concentrations of urea N (**PUN**), glucose, insulin, IGF-I, leptin, and NEFA of replacement beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis L.*) and receiving no supplementation (**CON**; n = 5), or supplements based on a protein (**PROT**; n = 5) or energy ingredient (**ENER**; n = 5). [1,2]

	Treatments				
Item[3]	CON	PROT	ENER	SEM	*P-Value*
NEFA, µEq/L	0.412[a]	0.194[b]	0.241[b]	0.022	< 0.01
PUN, mg/dL	3.57[a]	20.07[b]	17.87[c]	0.54	< 0.01
Glucose, mg/dL	59.3[a]	65.1[b]	65.0[b]	1.1	< 0.01
Insulin, µIU/mL	5.20[a]	6.72[b]	6.69[b]	0.35	0.02
IGF-I, ng/mL	79.5[a]	159.4[b]	149.5[b]	5.5	< 0.01

[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis). All heifers were offered meadow foxtail hay for ad libitum consumption. Treatments were offered and consumed daily (d 0 to 160) at 1.30 and 1.40 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Within rows, values with different superscript differ ($P \leq 0.05$).

[2]Blood samples were on d -10, 60, 120 and 150, starting at 4 h after supplements were offered.

[3]Results covariately adjusted to samples collected on d -11 of the study.

Table 5. Expression of hepatic genes associated with nutritional metabolism and growth of replacement beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis L.*) and receiving no supplementation (**CON**; n = 5), or supplements based on a protein (**PROT**; n = 5) or energy ingredient (**ENER**; n = 5). [1,2]

	Treatments				
Item [3,4]	CON	PROT	ENER	SEM	*P-Value*
PC	3.64	2.77	2.66	0.45	0.28
PEPCK-C	5.00	4.68	3.92	0.68	0.52
PEPCK-M	2.92[a]	4.19[b]	2.90[a]	0.42	0.08
IGF-I	3.71[a]	8.31[b]	6.75[b]	1.00	0.02
IGFBP-3	1.62[a]	2.46[b]	2.38[b]	0.21	0.03

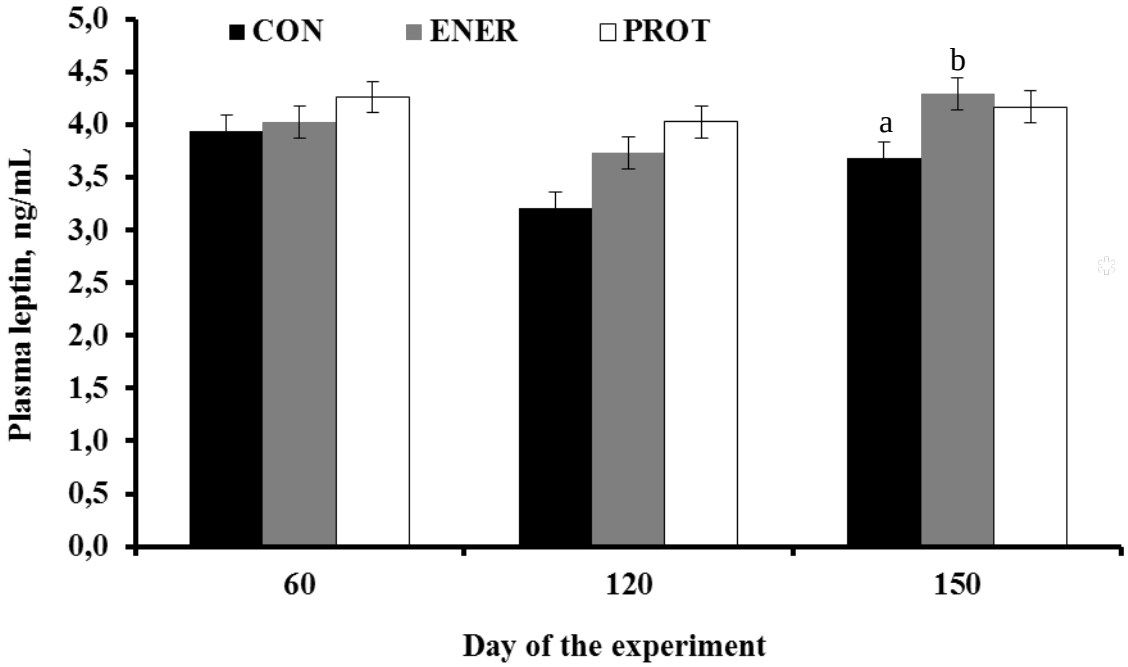
[1]PROT = supplementation with soybean meal; ENER = supplementation with a mixture of cracked corn, soybean meal, and urea (68:22:10 ratio, DM basis). All heifers were offered meadow foxtail hay for ad libitum consumption. Treatments were offered and consumed daily (d 0 to 160) at 1.30 and 1.40 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Within rows, values with different superscript differ ($P \leq 0.10$).

[2]Liver samples collected on d 100 of the experiment from 2 heifers per pen. Values are expressed as relative fold change (Ocón-Grove et al., 2008; Cooke et al., 2008).

[3]PC = Pyruvate carboxylase; PEPCK-C = cytosolic phosphoenolpyruvate carboxykinase; PEPCK-M = mitochondrial PEPCK.

[4] All data were normalized to the threshold-cycle value ($\mathbf{C_T}$) obtained for the housekeeping gene (**cyclophilin**) and calculated as relative fold change using the $2^{-\Delta\Delta CT}$ (Ocon-Grove et al., 2008).

Figure 1. Plasma concentration of leptin in replacement beef heifers consuming a low-quality cool-season forage (meadow foxtail; *Alopecurus pratensis L.*) and receiving no supplementation (**CON**; n = 5), or supplements based on a protein (**PROT**; n = 5; 100 % soybean meal on DM basis) or energy ingredient (**ENER**; n = 5; 68 % cracked corn, 22 % soybean meal, and 10 % urea on DM basis). Treatments were fed and consumed daily (d 0 to 160) at 1.30 and 1.40 kg of DM for PROT and ENER, respectively, to ensure that PROT and ENER intakes were isocaloric and isonitrogenous. Blood samples were collected on d -10, 60, 120, and 150, starting at 4 h after supplements were offered. Results are covariately adjusted to values obtained on d -10. A treatment × hour interaction was detected ($P < 0.01$). Within day, letters indicate differences between treatments ($P \leq 0.03$).



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CHAPTER 5

EFFECTS OF PROTEIN SUPPLEMENTATION FREQUENCY ON PHYSIOLOGICAL RESPONSES ASSOCIATED WITH REPRODUCTION IN BEEF COWS

Introduction

Fall-calving herds often require protein supplementation during the winter breeding season, particularly due to the limited availability and nutritional content of forages available (DelCurto et al., 2000). Nevertheless, supplementation programs substantially increase production costs in cattle systems, including expenses associated with feed purchase and labor required for supplement feeding (Miller et al., 2001). Hence, offering supplements 3 times or once weekly instead of daily are common strategies to lessen supplementation and overall production costs in cow-calf operations.

Research has shown that pregnant cattle consuming low-quality forages and receiving protein supplementation as infrequent as once a week had similar BW and BCS compared with cohorts supplemented daily (Huston et al., 1999a). However, no research has yet evaluated the effects of infrequent protein supplementation to beef cows during the breeding season. Decreasing protein supplementation frequency results in increased protein intake per meal, as well as size of each meal. Excessive protein intake increases PUN concentrations to levels that may decrease uterine pH (Elrod and Butler, 1993), which impairs reproductive function of beef females by reducing sperm motility and embryo survival (Acott and Carr, 1984; Butler, 1998). Large meal size may reduce circulating P_4 concentrations, the hormone required for establishment and maintenance of pregnancy (Spencer and Bazer, 2002), by stimulating hepatic blood flow and subsequent hepatic P_4 catabolism (Sangsritavong et al., 2002). Based on this rationale, we

hypothesized that beef cows supplemented infrequently would have reduced uterine pH and circulating P_4 concentrations following a supplementation event. Hence, our objective was to determine the effects of protein supplementation frequency on physiological responses and hepatic expression of genes associated with metabolism and reproductive function of beef cows.

Materials and Methods

This experiment was conducted at the Oregon State University – Eastern Oregon Agricultural Research Center (Burns Station) from October 2013 to January 2014. All animals utilized herein were cared for in accordance with acceptable practices and experimental protocols reviewed and approved by the Oregon State University, Institutional Animal Care and Use Committee.

Animals and diets

Fourteen non-pregnant, non-lactating mature Angus × Hereford cows (initial BW = 471 ± 13 kg, age 4.1 ± 0.4 yr) were ranked by initial BW and age, and allocated to 3 groups (2 groups containing 5 cows, and 1 group containing 4 cows). Groups were assigned to a 3 × 3 Latin square design, containing 3 periods of 21 d each, and the following treatments: 1) daily supplementation of soybean meal (**D**), 2) soybean meal supplementation 3 times/wk (**3WK**), and 3) soybean meal supplementation once/wk (**1WK**). Within each period (d 1 to 21), cows were assigned to the following estrus synchronization protocol: 100 µg of GnRH (Factrel; Zoetis, Florham Park, NJ) + controlled internal drug release (**CIDR**) containing 1.38 g of P_4 (Zoetis) on d 1, 25 mg of $PGF_{2\alpha}$ (Lutalyse, Zoetis) on d 8, followed by CIDR removal + 100 µg of GnRH on d 11

of each period. The CIDR was maintained in cows for an additional 72 h compared with the original Co-Synch + CIDR protocol (Lamb et al., 2001) to prevent cows from entering estrus before the beginning of sample collection, given that estrus alters uterine pH in beef females (Perry and Perry, 2008a,b).

All animals had ad libitum access to grass-seed straw during the entire experiment. Soybean meal was individually supplemented to cows (0700 h) at a daily rate of 1 kg/cow (as-fed basis). Cows receiving 3WK were supplemented on Mondays, Wednesdays, and Fridays (d 0, 2, 4, 7, 9, 11, 14, 16, and 18 of each period; 2.33 kg of soybean meal/feeding), whereas 1WK were supplemented on Fridays (d 4, 11, and 18 of each period; 7 kg of soybean meal/feeding), in a manner that all cows received the same amount of supplement on a weekly basis. Cows from all treatments consumed their entire supplement allocation within 30 min after feeding. Water and a complete commercial mineral-vitamin mix (Cattleman's Choice, Performix Nutrition Systems, Nampa, ID), containing 14% Ca, 10% P, 16% NaCl, 1.5% Mg, 3,200 mg/kg of Cu, 65 mg/kg of I, 900 mg/kg of Mn, 140 mg/kg of Se, 6,000 mg/kg of Zn, 136,000 IU/kg of vitamin A, 13,000 IU/kg of vitamin D_3, and 50 IU/kg of vitamin E, were offered for ad libitum consumption throughout the experiment.

A sample of straw and soybean meal was collected prior to the beginning of the experiment, and analyzed for nutrient content by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY). Samples were analyzed in triplicates by wet chemistry procedures for concentrations of CP (method 984.13; AOAC, 2006), ADF (method 973.18 modified for use in an Ankom 200 fiber analyzer, Ankom Technology Corp., Fairport, NY; AOAC, 2006), and NDF (Van Soest et al., 1991; modified for use in an

Ankom 200 fiber analyzer, Ankom Technology Corp.). Calculations for TDN used the equations proposed by Weiss et al. (1992), whereas NE_m and NE_g were calculated with the equations proposed by the NRC (1996). Nutritive values for straw and soybean meal were, respectively (DM basis), 54 and 80% TDN, 73 and 11% NDF, 50 and 8% ADF, 0.97 and 1.93 Mcal/kg of NE_m, 0.42 and 1.27 Mcal/kg of NE_g, and 4.7 and 54.1% CP.

Sampling

Individual full BW was recorded at the beginning (d 1) and at the end (d 21) of each experimental period to evaluate if cows were in adequate nutritional status during the experiment, which is known to impact the reproductive function in beef females (Randel, 1990; Bossis et al., 2000; Hess et al., 2005).

Blood samples. Blood samples were collected from 0 (immediately prior to) to 72 h after supplements were offered on d 11 and 18 of each period. More specifically, samples were collected every 2 h from 0 to 12 h, every 4 h from 16 to 28 h, and every 12 h from 36 to 72 h after supplementation. This sampling schedule was adopted to assess the impacts of infrequent protein supplementation on: a) d 11 – when cows would be inseminated following the estrus synchronization protocol, given that uterine pH impacts sperm motility and viability (Acott and Carr, 1984), and b) d 18 – corresponding to d 7 of gestation, when uterine pH and circulating P_4 concentrations are known to modulate embryonic development and survival (Mann and Lamming, 2001; Ocón and Hansen, 2003).

Blood samples were collected via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) containing 158 USP units of freeze-dried sodium heparin. After collection, blood samples were

placed immediately on ice, centrifuged (2,500 × *g* for 30 min; 4°C) for plasma harvest, and plasma was stored at -80°C on the same day of collection. Blood samples collected from 0 to 72 h were analyzed for plasma urea-N (**PUN**) concentrations, while samples collected from 0 to 12 h were analyzed for plasma glucose, insulin, and P_4 concentrations (d 18 only). Additionally, blood samples collected 24 h after supplement feeding on d 11 were also analyzed for plasma P_4 concentrations, in order to assess estrus synchronization rate. Cow was considered responsive to the estrus synchronization protocol if plasma P_4 concentration was < 1.0 ng/mL on the aforementioned sample, and > 1.0 ng/mL on the first sample collected on d 18. Plasma PUN and glucose concentrations were determined using quantitative colorimetric kits (#B7551 and G7521, respectively; Pointe Scientific, Inc., Canton, MI). Plasma insulin and P_4 concentrations were analyzed using a chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). Only cows that responded to the estrus synchronization protocol had all samples from d 18 analyzed for plasma P_4 concentrations. The intra- and inter-assay CV were, respectively, 3.78 and 11.05% for PUN and 3.78 and 6.44% for glucose. All samples were analyzed for insulin and P_4 concentrations within a single assay, and the intra-assay CV were 2.3 and 3.8%, respectively.

Uterine flushing. One month before the beginning of the experiment, a pre-trial with 9 non-lactating and non-pregnant Angus × Hereford cows assigned to the same treatments (3 cows/treatment) and blood sampling schedule reported herein was conducted in order to evaluate when PUN concentrations would peak following supplementation. These results determined the appropriate sampling time for uterine

flushing, more specifically when PUN peaked due to the negative correlation among PUN and uterine pH reported by Hammon et al. (2005). In this pre-trial, PUN concentrations were numerically stable for D and peaked at 28 h after supplementation for 3WK and 1WK (20.26, 28.68, and 32.72 mg/dL, respectively, at 28 h relative to supplementation; SEM = 1.93). Hence, in the present experiment, uterine flushing fluid was collected 28 h after supplementation on d 11 and 18 for pH measurement according to procedures previously described by Hersom et al. (2010). More specifically, uterine flush samples were collected by passing a sterile Foley 2-way, 16-French catheter (C. R. Bard, Covington, GA) into the uterus. Thirty-five milliliters of sterile saline (0.9%; pH = 7.0) was gently infused through the catheter. Saline was allowed to equilibrate for 90 s and then flushed from the uterus through the catheter into a 50-mL Falcon tube (BD Biosciences, Bedford, MA). Flushing fluid was measured for pH immediately after collection (Orion SA 520, American Instrument Exchange Inc., Haverhill, MA). The pH of sterile saline was used to standardize the pH calibration before each cow was flushed.

Liver samples. Liver sampling was performed via needle (Tru-Cut biopsy needle; CareFusion Corporation, San Diego, CA) biopsy 28 h after supplement feeding on d 11, and at 0, 4, and 28 h relative to supplement feeding on d 18 according to the procedures described by Arthington and Corah (1995). During biopsies, incisions were made between the 11^{th} and 12^{th} ribs for collection of samples from the right hepatic lobe (Miranda et al., 2010), and at least 2 cm from previous incision(s) of the same period to prevent collection of damaged hepatic tissue. Immediately after collection, liver samples (average 100 mg of tissue; wet weight) were placed in 1 mL of RNA stabilization solution (RNAlater, Ambion Inc., Austin, TX), maintained at 4°C for 24 h, and stored at -

80°C. Samples were analyzed via real-time quantitative reverse transcription (**RT**)-PCR for carbamoylphosphate synthetase I (**CPS-I**; rate-limiting enzyme in the urea cycle; Takagi et al., 2008), CYP2C19 and CYP3A4 (enzymes that regulate P_4 steroid catabolism; Lemley et al., 2008), and ribosomal protein S-9 (**RPS-9;** hepatic housekeeping gene; Janovick-Guretzky et al., 2007) mRNA expression. Expression of CPS-I was assessed only on samples collected 28 h relative to supplementation based on the results from our pre-trial. Expression of CYP2C19 and CYP3A4 was assessed in samples collected at 0 and 4 h relative to supplementation on d 18, from cows that responded to the estrus synchronization protocol, based on previous research from our group (Vieira et al., 2013).

Total RNA was extracted from liver tissue samples using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) and stored at -80°C until further processing. Quantity and quality of isolated RNA were assessed via UV absorbance (NanoPhotometer Version 2.1, Implen, Munich, Germany) at 260 nm and 260/280 nm ratio, respectively (Fleige and Pfaffl, 2006). Extracted RNA (2.5 µg) was incubated at 37°C for 30 min in the presence of RNase-free DNase (New England Biolabs Inc., Ipswich, MA) to remove contaminant genomic DNA. After inactivating the DNase (75°C for 15 min), samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random hexamers (Applied Biosystems, Foster City, CA). Real-time RT-PCR was completed using the SYBR Green PCR Master Mix (Applied Biosystems) and specific primer sets (20 pM; Table 1) with a 7900HT Fast Real-time PCR cycler (Applied Biosystems) according to procedures described by Cooke et al. (2008). At the end of each RT-PCR, amplified products were subjected to a dissociation

gradient (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) to verify the amplification of a single product by denaturation at the anticipated temperature. Responses were quantified based on the threshold cycle ($\mathbf{C_T}$), the number of PCR cycles required for target amplification to reach a predetermined threshold. All C_T responses from genes of interest were normalized to RPS-9 C_T examined in the same sample and assessed at the same time as the targets. Results are expressed as relative fold change ($2^{-\Delta\Delta CT}$), as described by Ocón-Grove et al. (2008).

Statistical analysis

All data were analyzed using cow as the experimental unit, cow and group as random variables, and using the Satterthwaite approximation to determine the denominator df for the tests of fixed effects. Response to estrus synchronization was analyzed using the GLIMMIX procedure of SAS (version 9.3; SAS Inst., Cary, NC), with a model statement containing the effects of treatment and period as independent variables. All other data were analyzed using the MIXED procedure of SAS (version 9.3; SAS Inst.). The model statement for BW change contained the effects of treatment and period as independent variables. The model statement for plasma variables contained the effects of treatment, day (for PUN, glucose, and insulin), hour, all the resultant interactions, and period as an independent variable. The specified term for the repeated statement was hour, subject was cow(treatment × day × period) or cow(treatment × period) for P4 analysis only, and the covariance structure used was compound symmetry based on the Akaike information criterion. The model statement used for uterine flushing pH contained the effects of treatment, day, synchronization status, all interactions, and period as an independent variable. The model statement for hepatic gene expression

contained the effects of treatment, day (for CPS-I) or hour (for CYP2C19 and CYP3A4), all resultant interactions, and period as an independent variable. For uterine flushing pH and CPS-I expression, the specified term for the repeated statement was day, subject was cow(treatment × period), and the covariance structure used was first-order autoregressive based on the Akaike information criterion. For CYP2C19 and CYP3A4 expression, the specified term for the repeated statement was hour, subject was cow(treatment × period), and the covariance structure used was also first-order autoregressive. Results are reported as least-squares means, which were separated using the PDIFF option in SAS (version 9.3; SAS Inst.). Significance was set at $P \leq 0.05$ and tendencies were denoted if $P > 0.05$ and ≤ 0.10. Results are reported according to main effects if no interactions were significant, or according to highest-order interaction detected.

Results and Discussion

No treatment ($P = 0.97$) effects were observed on estrus synchronization rate (79, 80, and 78 % for D, 3WK, and 1WK, respectively; SEM = 1.2). Furthermore, BW change did not differ ($P = 0.35$) between treatments (13.4, 18.1, and 13.6 kg of BW change for D, 3WK, and 1WK, respectively; SEM = 3.0). Although the present experiment was not designed to evaluate these parameters, it is important to note that cows used herein were in similar and positive nutritional status, and responsiveness to the estrus synchronization protocol was adequate to properly test our hypothesis.

Parameters associated with protein intake

A treatment × hour interaction was detected ($P < 0.01$) for PUN concentrations, which were greater ($P < 0.01$) for 1WK compared with 3WK from 20 to 72 h, and greater

($P < 0.01$) for 1WK compared with D from 16 to 48 h and at 72 h after supplementation (Figure 1). Conversely, D and 3WK had greater ($P < 0.01$) PUN concentrations compared with 1WK from 0 to 6 h relative to supplementation. Concentrations of PUN in 3WK were also greater ($P < 0.01$) from 12 to 28 h and reduced ($P < 0.01$) from 48 to 72 h after supplementation compared with D (Figure 1). Moreover, PUN concentrations peaked at 28 h after supplementation for 3WK and 1WK ($P < 0.01$), and were greater ($P < 0.01$) at this time for 1WK and 3WK compared with D, as well as for 1WK compared with 3WK (Figure 1). Similarly, others have also reported that PUN peaked 1 d after supplementation in ruminants consuming low-quality forage and receiving a protein supplement as infrequent as once/week (Krehbiel et al., 1998; Huston et al., 1999b; Bohnert et al., 2002). Treatment effects detected for PUN herein reflect the designed differences in protein intake across treatments for each supplementation event, given that PUN concentrations are positively correlated with the amount of protein and RDP consumed by ruminants (Broderick and Clayton, 1997). Moreover, Hammond (1997) suggested that PUN concentrations should range from 7 to 8 mg/dL for mature beef cows. Therefore, cows from all treatments had PUN concentrations, as well as N supply (Bach et al., 2005), beyond the adequate range throughout the sampling period.

A treatment effect was detected ($P < 0.01$) for the hepatic mRNA expression of CPS-I on samples collected 28 h after supplements were offered. Cows fed 1WK had greater ($P < 0.01$) hepatic CPS-I mRNA expression compared with D and 3WK, while CPS-I expression was similar ($P = 0.67$) between D and 3WK (Table 2). Carbamoylphosphate synthetase-I is expressed in hepatocytes and epithelial cells of the intestinal mucosa (Tillman et al., 1996), and is considered a rate-limiting step within the

urea cycle (Takagi et al., 2008) by converting ammonia into carbamoylphosphate (Visek, 1979). In addition, expression and activity of urea cycle enzymes are affected by hormones and nutrients (Takiguchi and Mori, 1995). Hence, the greater mRNA expression of CPS-I in 1WK compared with 3WK and D corroborate treatment differences in protein intake, as well as PUN concentrations 28 h after supplementation (Ryall et al., 1984; Hayden and Straus, 1995; Takagi et al., 2008). Nevertheless, the similar CPS-I mRNA expression between 3WK and D was unexpected, although others have also reported that enzyme activity can be increased without changes in its expression (Banu et al., 2009; Lebovic et al., 2013). Moreover, mRNA translation into the active enzyme requires time (Clancy and Brown, 2008); hence, one can speculate that CPS-I expression assessed 28 h after supplementation reflected the ureagenesis rate at h 36 after supplementation, when PUN concentrations were similar between 3WK and D, and reduced for both treatments compared with 1WK (Figure 1).

A tendency for a treatment effect was detected ($P = 0.10$) for uterine flushing pH, given that 1WK tended to have greater ($P \leq 0.10$) flushing pH compared with D and 3WK, whereas flushing pH was similar ($P = 0.79$) between D and 3WK (Table 2). Previous research reported that uterine pH was negatively associated with protein intake and PUN concentrations in cattle (Elrod et al., 1993; Elrod and Butler, 1993; Hammon et al., 2005). Hence, excessive protein intake has been associated with impaired reproductive performance in females (Butler et al., 1996), given that decreased uterine pH can result in loss of sperm viability and embryonic competence (Pouysségur et al., 1984; FitzHarris and Baltz, 2009). Conversely, Grant et al. (2013) fed diets containing different levels of protein (ranging from 10 to 16% CP) to mature beef cows, and

reported that PUN concentrations and uterine pH were greater for cows fed the 16% CP diet compared with cohorts receiving the 10% CP diet. These latter results support differences detected herein on protein intake during the day of supplementation, and subsequent PUN concentrations and uterine flushing pH in 1WK compared with 3WK and D. In fact, urea and ammonia have positive charges and alkaline properties when in solution (Haynes, 2014). Thus, neither of these compounds are expected to reduce pH of biological neutral environments. Nevertheless, the exact mechanism(s) by which excessive protein intake modulates uterine pH in cattle remains unknown and deserves further investigation, particularly because alteration of uterine pH in response to protein intake may be unique to this organ (Elrod and Butler (1993).

Parameters associated with meal size

No treatment effects were detected (P = 0.97) on plasma glucose (Table 3). Although plasma glucose concentrations are directly associated with nutrient intake (Vizcarra et al., 1998), the supplement utilized herein was based on soybean meal, which contains limited amounts of carbohydrates and is not considered a glucogenic precursor to cattle (NRC, 1996). Hence, the greater soybean meal intake of 1WK and 3WK following a supplementation event was likely not sufficient to impact plasma glucose concentrations, at least within 12 h after supplementation, compared with D. Accordingly, previous research from our (Cooke et al., 2008; Moriel et al., 2012) and other research groups (Drewnoski et al., 2014) also reported similar plasma glucose in beef cattle assigned to different supplementation frequencies during the days in which all cattle were supplemented.

A treatment effect was detected ($P = 0.01$) for plasma insulin, given that plasma insulin concentrations were greater ($P \leq 0.03$) for D and 3WK compared with 1WK, but similar ($P = 0.58$) between D and 3WK (Table 3). These results were unexpected because circulating insulin concentrations are also associated with nutrient intake, and regulated by blood glucose concentrations (Vizcarra et al., 1998). Nevertheless, cattle consuming excessive protein may shift amino acids required for synthesis of insulin (Reed et al., 2007), such as aspartate and arginine, to support the urea cycle (Lobley et al., 1995). Therefore, the greater ureagenesis rate of 1WK compared with 3WK and D, based on the rapid increase in PUN concentrations after supplement feeding and past research documenting increased ureagenesis with infrequent supplementation (Wickersham et al., 2008), may have impaired pancreatic insulin synthesis and subsequent circulating concentrations of this hormone. In addition, insulin has been shown to inhibit the expression of urea cycle enzymes, including CPS-I (Kitagawa et al., 1985; Morris Jr., 1992). Therefore, the greater mRNA expression of CPS-I in 1WK compared with 3WK and D (Table 2) may also be attributed to treatment effects detected for plasma insulin concentrations (Table 3).

No treatment effects were detected ($P = 0.65$) on plasma P_4 concentrations (Table 3). Similarly, no treatment effects were detected on hepatic mRNA expression of CYP2C19 ($P = 0.25$) and CYP3A4 ($P = 0.15$; Table 2). These results indicate that infrequent protein supplementation did not impact plasma P_4 concentrations, the hormone required for pregnancy establishment and maintenance (Spencer and Bazer, 2002), within 12 h after supplements were offered. One of the hypotheses of this experiment was that beef cows supplemented infrequently would have reduced plasma P_4 concentrations

following a supplementation event, which could negatively impact cattle reproductive efficiency given that circulating P_4 concentration 7 d after AI impacts embryonic viability and subsequent pregnancy rates (Mann and Lamming, 2001; Demetrio et al., 2007). This hypothesis was developed based on the premise that large meal size reduces circulating P_4 concentrations by increasing hepatic blood flow and subsequent hepatic P_4 catabolism by CYP2C19 and CYP3A4 (Sangsritavong et al., 2002). Supporting our rationale, Vasconcelos et al. (2003) demonstrated that dairy cows receiving 100% of their diet in a single meal had reduced P_4 concentrations compared with cohorts fed their diets in multiple, but smaller meals. Similarly, Cooke et al. (2007) reported that beef females receiving energy-based supplements 3 times weekly had reduced circulating P_4 concentrations after supplementation. In the present experiment, the lack of differences in plasma P_4 concentrations among treatments suggest that the increased meal size resultant from reduced supplementation frequency, at the rate utilized herein, was not sufficient to impact hepatic steroid catabolism and subsequent plasma P_4 concentrations. Perhaps a greater increase in meal size (Vasconcelos et al., 2003), or inclusion of highly-fermentable substrates such as energy ingredients (Cooke et al., 2007), are required to impact these parameters. Last but not least, insulin impacts plasma P_4 concentrations by stimulating luteal P_4 synthesis (Spicer and Echternkamp, 1995) and reducing hepatic P_4 catabolism by CYP2C19 and CYP3A4 (Cooke et al., 2012; Vieira et al., 2013). Therefore, the difference in plasma insulin concentrations of D and 3WK compared with 1WK following a supplementation event was not sufficient to elicit a similar effect on plasma P_4 concentrations. Accordingly, research from Lemley et al. (2008) suggested that there might be a threshold in circulating insulin concentrations that must be reached

in order to alleviate expression of CYP2C19 and CYP3A4, and consequently increase circulating P_4 concentrations.

Overall conclusion

Supplementing protein to beef cows as infrequent as once weekly increased PUN concentrations and hepatic mRNA expression of the rate-limiting enzyme of the urea cycle, but did not reduce uterine flushing pH or circulating P4 concentrations after supplements were offered. Nevertheless, additional research within this subject is warranted, including experiments designed to determine whether protein supplementation frequency to lactating beef cows during the breeding season can be reduced, such as 3 times or once weekly, without impairing pregnancy rates.

Overall conclusion for all the experiments reported herein

In summary for all the experiments presented herein: (1) pregnant and developing replacement beef heifers consuming a low-quality, cool-season forage equally utilize and benefit, in terms of growth and metabolic parameters, from supplements based on protein or energy ingredients provided at approximately 0.5 % of heifer BW/d, (2) energetic supplementation at approximately 0.5 % BW/d did not impair forage disappearance parameters in rumen-fistulated steers, and (3) decreasing soybean meal supplementation frequency to once a week did not increase uterine pH, plasma P_4, and expression of hepatic enzymes associated with steroid catabolism in ruminants.

Table 1. Primer sequences and accession number for all gene transcripts analyzed by quantitative real-time RT-PCR.

Target gene	Primer sequence[1]	Accession no.
Carbamoyl phosphate synthetase-I		
Forward	5'-ACACTGGCTGCAGAATACCC-3'	XM_587645
Reverse	5'-TTCTTGCCAAGCTGACGCAA-3'	
CYP2C19		
Forward	5'-TATGGACTCCTGCTCCTGCT-3'	NM_001109792
Reverse	5'-CATCTGTGTAGGGCATGCAG-3'	
CYP3A4		
Forward	5'-GTGCCAATCTCTGTGCTTCA-3'	BT030557
Reverse	5'-CCAGTTCCAAAAGGCAGGTA-3'	
Ribosomal protein S-9		
Forward	5'-CCTCGACCAAGAGCTGAAG -3'	DT860044
Reverse	5'-CCTCCAGACCTCACGTTTGTTC-3'	

[1] Primer sequences obtained from: carbamoyl phosphate synthetase, Takagi et al. (2008); CYP2C19 and CYP3A4, Lemley et al. (2008); ribosomal protein S-9, Janovick-Guretzky et al. (2007).

Table 2. Uterine flushing pH and expression of hepatic genes associated with ureagenesis (carbamoyl phosphate synthetase; **CPS**) and steroid catabolism (**CYP2C19** and **CYP3A4**) in beef cows receiving protein supplementation daily (**D**; n = 14), 3 times per week (**3WK**; n = 14), or once a week (**1WK**; n = 14).[1]

	Treatments				
Item	D	3WK	1WK	SEM	*P-Value*
Uterine flushing pH [2]	6.14	6.13	6.20	0.03	0.10
mRNA expression, relative fold change [3,4]					
CPS	4.22[a]	4.59[a]	7.21[b]	0.69	< 0.01
CYP2C19	10.32	10.67	8.24	1.16	0.25
CYP3A4	13.82	5.65	9.82	3.24	0.15

[1] Cows were assigned to a 3 × 3 Latin square design, containing 3 periods of 21 d. Within each period, cows were assigned to the following estrus synchronization protocol: 100 µg of GnRH + controlled internal drug release (**CIDR**) containing 1.38 g of progesterone (**P_4**) on d 1, 25 mg of $PGF_{2\alpha}$ on d 8, and CIDR removal + 100 µg of GnRH on d 11. Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Moreover, 3WK were supplemented on d 0, 2, 4, 7, 9, 11, 14, 16, and 18, whereas 1WK were supplemented on d 4, 11, and 18. Within rows, values with different superscript differ ($P \leq 0.05$).

[2] Uterine flushing fluid pH was collected 28 h after supplements were offered on d 11 and 18, according to Hersom et al. (2010).

[3] Liver samples were collected via needle biopsy (Arthington and Corah, 1995) 28 h after supplement feeding on d 11, and at 0, 4, and 28 h relative to supplement feeding on d 18 according to the procedures described by Arthington and Corah (1995). Samples were analyzed for mRNA expression of CPS-I (h 28), and CYP2C19 and CYP3A4 (h 0 and 4 on d 18).

[4] All data were normalized to the threshold-cycle value (**C_T**) obtained for the housekeeping gene (**RPS-9**) and calculated as relative fold change using the $2^{-\Delta\Delta CT}$ (Ocon-Grove et al., 2008).

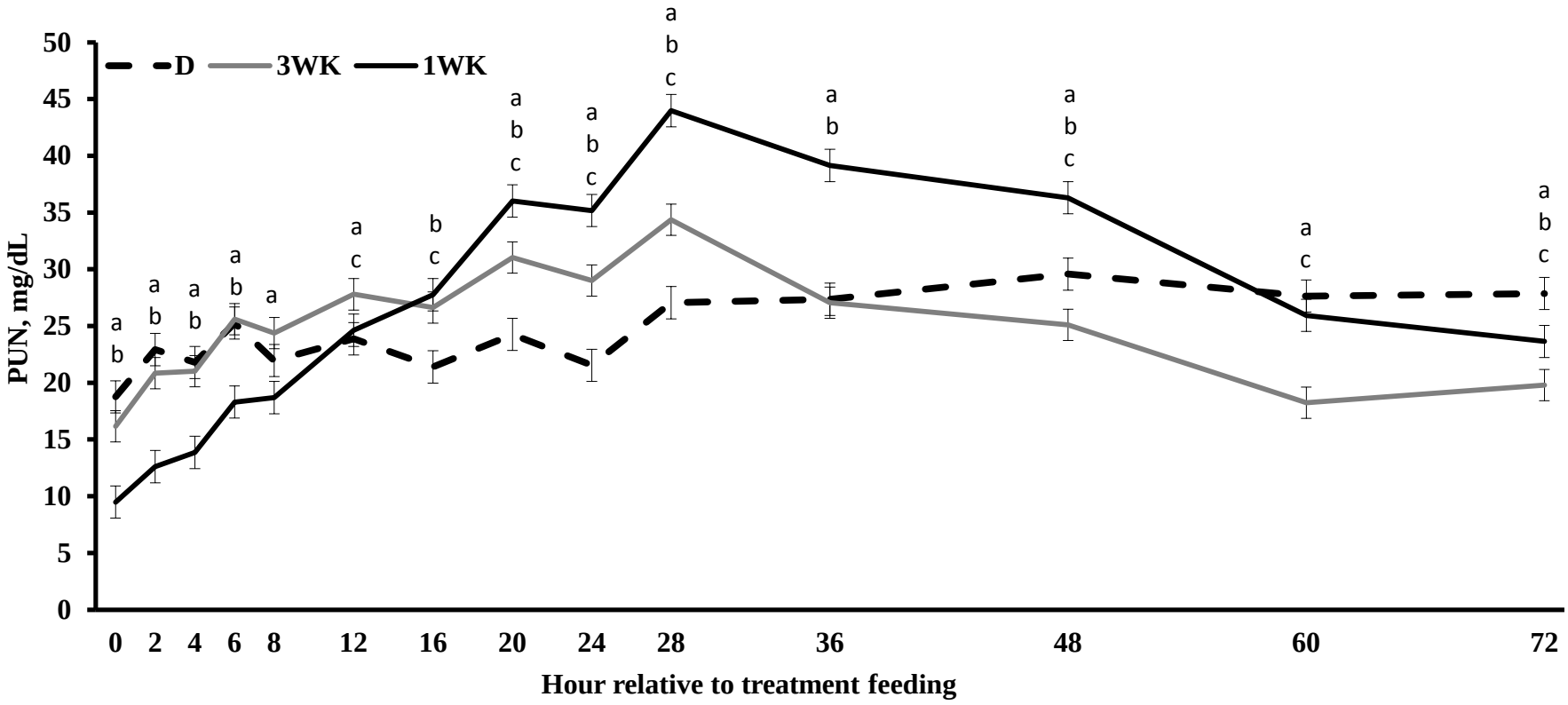
Table 3. Mean plasma concentration glucose, insulin, and progesterone (**P_4**) of beef cows receiving protein supplementation daily (**D**; n = 14), 3 times per week (**3WK**; n = 14), or once a week (**1WK**; n = 14).[1,2]

	Treatments				
Item	D	3WK	1WK	SEM	*P-Value*
Glucose, mg/dL	60.35	60.38	60.20	1.06	0.97
Insulin, µIU/mL	4.57[a]	4.76[a]	3.80[b]	0.44	0.01
Progesterone, ng/mL	2.78	2.53	2.84	0.32	0.65

[1] Cows were assigned to a 3 × 3 Latin square design, containing 3 periods of 21 d. Within each period, cows were assigned to the following estrus synchronization protocol: 100 µg of GnRH + controlled internal drug release (**CIDR**) containing 1.38 g of progesterone (**P_4**) on d 1, 25 mg of $PGF_{2\alpha}$ on d 8, and CIDR removal + 100 µg of GnRH on d 11. Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Moreover, 3WK were supplemented on d 0, 2, 4, 7, 9, 11, 14, 16, and 18, whereas 1WK were supplemented on d 4, 11, and 18. Within rows, values with different superscript differ ($P \leq 0.10$).

[2] Blood samples were collected from 0 (prior to) to 72 h after supplementation on d 11 and 18, and analyzed for PUN. Samples collected from 0 to 12 h were also analyzed for plasma glucose, insulin, and P_4 (d 18 only).**Figure 1.** Plasma urea-N (**PUN**) concentrations in beef cows receiving protein supplementation daily (**D**; n = 14), 3 times per week (**3WK**; n = 14), or once a week (**1WK**; n = 14). Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Blood samples were collected from 0 (prior to) to 72 h after all treatments received supplementation. A treatment × hour interaction was detected ($P < 0.01$). Within hours, letters indicate the following treatment differences; a = 3WK vs. 1WK ($P \leq 0.04$), b = D vs. 1WK ($P < 0.01$), c = D vs. 3WK ($P \leq 0.01$).

Figure 1. Plasma urea-N (**PUN**) concentrations in beef cows receiving protein supplementation daily (**D**; n = 14), 3 times per week (**3WK**; n = 14), or once a week (**1WK**; n = 14). Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Blood samples were collected from 0 (prior to) to 72 h after all treatments received supplementation. A treatment × hour interaction was detected ($P < 0.01$). Within hours, letters indicate the following treatment differences; a = 3WK vs. 1WK ($P \leq 0.04$), b = D vs. 1WK ($P < 0.01$), c = D vs. 3WK ($P \leq 0.01$).



CHAPTER 6

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