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EFFECT OF ESTRADIOL SUPPLEMENTATION ON BLOOD ESTRADIOL AND METABOLITE LEVELS, AND HEPATIC PROTEIN EXPRESSION, IN GROWING, MATURE, AND SENESCENT BEEF CATTLE

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By
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Lexington, Kentucky

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2013

ABSTRACT OF DISSERTATION

EFFECT OF ESTRADIOL SUPPLEMENTATION ON BLOOD ESTRADIOL AND METABOLITE LEVELS, AND HEPATIC PROTEIN EXPRESSION, IN GROWING, MATURE, AND SENESCENT BEEF CATTLE

Estradiol (Compudose®, COM) implants are extensively used in beef cattle production systems to alter body composition and feed efficiency. Little information exists about the physiological mechanisms affected by COM treatment in growing, mature, and senescent female cattle. Moreover, no reports describe the level of blood estradiol resulting from COM treatment. The effect of COM on levels of plasma estradiol and blood metabolites and proteins, and relative content of glutamine synthetase (GS) and other amino acid nitrogen-metabolizing enzymes in liver tissue, was studied using three experimental models relevant to cow-calf production regimens: senescent cows (Trial 1), young mature (young) versus senescent (old) cows (Trial 2), and growing heifers (Trial 3). In Trial 1, plasma estradiol concentrations were 222 % more after 14 and 28 d in COM-implanted than sham implanted (Control) cows. COM treatment did not affect measured blood metabolites and enzymes, but increased hepatic GS protein expression by 350% after 14 d and 200% after 28 d of implantation. In contrast, protein expression of alanine transaminase, aspartate transaminase, glutamate dehydrogenase, and two glutamate transporters was not affected by COM. In Trial 2, plasma estradiol concentrations of COM implanted young and old cows were 48% higher than Control groups, whereas blood metabolites were not affected. COM implantation did not affect GS protein expression in young cows, but tended to increase GS expression in the old cows by 283% after 14 d and 41% after 28 d. GS mRNA content was increased about 38% in both young and old COM-treated cows. Hepatic content of beta-catenin and G protein-coupled receptor 30 (GPR30) content was not affected by COM treatment, indicating that estradiol-mediated GS expression was not regulated by beta-catenin- or GPR30-controlled pathways. In Trial 3, plasma estradiol levels in COM-treated heifers were 70% higher in COM heifers, concomitant with increased levels of total bilirubin and creatine kinase, and decreased creatinine. Correlation analysis of plasma estradiol levels and blood constituents only identified a positive correlation between plasma estradiol and potassium. Collectively, these data describe positive estradiol-mediated effects on hepatic metabolism and blood parameters in female cattle.

Keywords: Aging, Cattle, Estradiol, Glutamine Synthetase, Alanine Transaminase

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Chapter 1

Introduction

The age of a beef cow has a major impact on many economically important production traits of beef cattle, including milk yield and calf weaning weight (BIF, 1996). The physiological and molecular mechanisms responsible for these age-related effects are not well understood. It is known that aging can have detrimental effects on the function of many organs including the small intestine, muscle, and liver. The liver is an important central organ because it is responsible for coordinating whole-body energy and nitrogen metabolism. Two metabolically important enzymes involved with these processes are glutamine synthetase (GS) and alanine transaminase (ALT). Initial studies from this laboratory have found that hepatic glutamine synthetase and alanine transaminase protein expression is decreased in aged cows (Matthews and Sipe, 2006).

Given the current international, and growing national, resistance to consumable beef products from cattle raised using exogenous agents, there is a need in the beef cattle industry to understand the underlying cellular mechanisms and processes by which the administration of exogenous estradiol and other anabolic steroids promote the efficient growth of developing cattle (Chung and Johnson, 2008). Much research has been performed regarding the effects of anabolic implants on growth rate and carcass traits of growing steers and heifers (Heitzman, 1976). However, given the length of time that estradiol supplements have been used in cattle, little is known regarding the relationship between time of estradiol implantation, blood estradiol levels, and their correlation to clinical blood parameters. Moreover, little is known about the effect of estradiol supplementation on hepatic cellular mechanisms. Recently, research from this laboratory

found that administration of exogenous estradiol plus progesterone (Synovex-S® ear implants) to growing steers increased hepatic glutamine synthetase protein expression (Sipe, 2004).

The purpose of this dissertational research was to determine if estradiol administration positively affects the expression of two important nitrogen-metabolizing enzymes, in old cows (greater than 10 years of age), young cows, and growing heifers. The specific objectives of this dissertational research were:

1. To determine the effects of supplemental exogenous estradiol to old beef cows on (1) plasma estradiol and progesterone concentrations, (2) hepatic expression of GS and ALT, and other proteins involved in the hepatic glutamate/glutamine/alanine metabolism, and (3) blood biochemical and clinical indicators of whole body metabolism. (Chapter 3).
2. To test the hypotheses that (1) glutamine synthetase expression will be up-regulated by estrogen in old and young mature cows and (2) that this regulation is affected by the β-catenin/Wnt pathway and/or GPR30. (Chapter 4)
3. To evaluate the effects of estrogen on the clinical blood metabolites of growing heifers (Chapter 5).

Chapter 2

Literature Review

Amino Acid Metabolism

In ruminants versus non-ruminants, glutamate has a heightened role in nitrogen and carbon metabolism given the importance of glutamate for amino acid-derived gluconeogenesis (Heitmann and Bergman, 1981).

From a whole-body view, glutamate is important for neurotransmission, protein synthesis, nitrogen turnover, and energy metabolism, but its use by specific tissues in the body differ. In the mammalian CNS, glutamate is considered to be the major excitatory neurotransmitter and an important component of many processes by the brain (Fonnum, 1984; Collingridge and Lester, 1989).

In peripheral tissues, glutamate serves as a source of oxidizable fuel and plays an antecedent role in the synthesis of new amino acids. Also, glutamate can be utilized to support whole-animal energy and nitrogen metabolism (Figure 2.1) as well as tissue- and cellular specific functions within the peripheral tissues (Heitmann and Bergman, 1981; Wu, 1998; Matthews, 2005). In renal tissue, reabsorbed glutamate acts as an ammoniagenesis regulator and acts as a precursor for glutathione and extracellular matrix protein (Welbourne and Matthews, 1999). In the placenta, primarily fetal-derived glutamate pools serve as sources of oxidizable fuel, with a limited addition of maternal-derived glutamate into the pool (Moores et al., 1994; Vaughn et al., 1995). Also, adipose subcutaneous fat is another peripheral tissue thought to be involved in ammonia detoxification (Kowalski and Watford, 1994). In the gastrointestinal tract, glutamate metabolized from glutamine (a major fuel for intestinal tissue) by phosphate-dependent

glutaminase, undergoes transamination with pyruvate (by alanine transaminase or aspartate transaminase) generating L-alanine and α-ketoglutarate (Heidger and Welbourne, 1999).

In ruminants, glutamate is an important precursor of metabolic energy in certain peripheral tissues. In sheep, the small intestinal mucosa extensively oxidized glutamate as indicated by the recovery of only 4% of gut-infused [^{14}C]glutamate appears in the form of [^{14}C]-labeled glutamate and/or glutamine in the portal blood (Tagari and Bergman, 1978). Furthermore, the kidney provides reserved glutamate-derived gluconeogenic capacity, accounting for 33 to 50% of glutamate-derived glucose in fasted and starved sheep, respectively (Heitmann and Bergman, 1981).

Muscle Glutamate and Alanine Metabolism. Glutamate taken from the blood as a main anaplerotic precursor for the tricarboxylic acid cycle (Bowtell and Bruce, 2002) is utilized by the muscle to synthesize glutamine and alanine (Holecek, 2002). Delivered from the blood, branched-chain amino acids (valine, isoleucine, and leucine) can be also utilized by the muscle to make new proteins or to produce glutamine and alanine (Holecek, 2002). Muscle-derived glutamine and alanine are released in large amounts in the postabsorptive state and during the absorption of a protein-containing meal in humans (Wagenmakers, 1998).

Muscle Glutamate Metabolism. The skeletal muscular uptake of plasma glutamate, its conversion into glutamine, and the ensuing release of glutamine into the blood allows the skeletal muscle to play an important role in ammonia recovery and detoxification of the body (Biolo et al., 1995; Rennie et al., 1996; Vesli et al., 2002). Glutamate levels in skeletal muscle can decrease quickly during vigorous exercising, even though plasma

glutamate levels are constant (Rennie et al., 1996). For example, human muscle and the canine gastrocnemius exhibit an efflux of ammonia and glutamine during extended and intense exercises, whereas intramuscular glutamate level quickly decrease by more than 50% and remain steady during the exercising period (Graham and MacLean, 1998). Glutamate seems to be an important substrate for the rapid increase in muscle tricarboxylic acid cycle intermediates that arises at the beginning of moderate to intense exercise because alanine transaminase is converting glutamate and pyruvate to alanine and α-ketoglutarate (Gibala, 2001).

After ingestion of a protein-inclusive meal, branched chain amino acid and glutamate are taken up by the muscle, in which their carbon atoms are used for the synthesis of glutamine (Wagenmaker, 1998). As a net exporter of glutamine, skeletal muscle branched-chain transaminase transfers α-amino groups from branched-chain amino acids to α-ketoglutarate to produce glutamate, which is then aminated with ammonia by glutamine synthetase activity (Rennie et al., 1996).

Muscle Alanine Metabolism. Muscle-type alanine transaminase converts glutamate into pyruvate, alanine and α-ketoglutarate (Palmer et al., 1985; Wagenmakers, 1998; Gibala, 2001; Holecek, 2002). In the skeletal muscle of humans, alanine transaminase helps to build and maintain high levels of tricarboxylic acid cycle intermediates during the first ten minutes of exercise (Wagenmakers, 1998). The carbon skeleton of the exported alanine is derived predominantly from breakdown of blood glucose and from muscle glycogen (Wagenmakers, 1998). The released alanine is transported into the blood and is utilized by the liver to synthesized glucose via gluconeogenesis (Palmer et al., 1985; Holecek, 2002). For instance, human muscle and

the canine gastrocnemius have been reported to produce alanine during intense and extended exercises, concurrently with the export of muscle-derived glutamine and ammonia (Graham and MacLean, 1998).

Hepatic Glutamate and Alanine Metabolism. In the liver, hepatic transport and intermediary metabolism of glutamate and alanine are necessary for nitrogen metabolism and whole-body energy regulation (Figure 2.2).

Hepatic Glutamate Metabolism. Glutamate plays an important role in the intercellular glutamate/glutamine cycle in the liver, specifically acting as the main substrate for hepatic ureagenesis, gluconeogenesis, de novo protein synthesis, and nitrogen shuttling via glutamine (Matthews, 2005). Hepatic entry of glutamine from the portal vein is thoroughly uptaken by the periportal heptacytes and deaminated by liver-specific glutaminase to release ammonia and glutamate (Kilberg et al., 1980). The liberated ammonia is incorporated into carbamoyl phosphate by carbamoyl phosphate synthetase for ureagenesis, whereas glutamate is available for conversion to α-ketoglutarate to be used for gluconeogenesis, protein synthesis, or transported back into hepatic sinusoids. When α-ketoglutarate is oxidized in the tricarboxylic acid cycle, it generates malate, which, by the action of $NADP^+$-dependent malic enzyme, generates pyruvate. The NADH and FADH2 generated via this pathway are used for electron donation to the electron transporting chain in the mitochondria and thus promote ATP synthesis.

Once glutamate is released into the sinusoidal blood, it is available for absorption by the pericentral hepatocytes and is incorporated with free sinusoidal ammonia having escaped conversion into urea by the periportal hepacytes for the synthesis of glutamine.

In the pericentral hepatocytes, glutamine synthetase catalyzes the ATP-dependent conversion of glutamate and ammonia into glutamine (Cadoret et al., 2002; Gebhardt andn Mecke, 1983).

Hepatic Alanine Metabolism. In the periportal hepatocytes, alanine transaminase converts alanine into pyrvate, which is utilized in hepatic gluconeogenesis to produce glucose for whole-body energy metabolism (Schutz, 2011). Sakagishi (1995) has suggested that cytosol alanine transaminase is connected to the utilization of pyruvate in glycolysis, whereas mitochondrial alanine transaminase is involved in the production of alanine-derived pyruvate for gluconeogenesis in humans.

Molecular Components of Alanine Transaminase and Glutamine Synthetase.

Alanine Transaminase (ALT). Alanine transaminase is found in the blood, muscle, liver, and kidney (Baudhuin et al., 1984; DeRosa and Swick, 1975; Philippson, 1979). Two genes account for ALT activities (Sookoian and Pirola, 2012). GPT1 (glutamate-pyruvate transaminase 1) encodes for the protein that accounts for cytosolic ALT activity (ALT1) whereas GPT2 encodes for mitochondrial ALT activity (ALT2). GPT1 has been reported to be located to chromosomes 8 and 16 in humans whereas GPT2 is located on chromosome 16 (Yang et al., 2002). Also, GPT1 and GPT2 differ in mRNA expression in that GPT1 is mainly expressed in kidney, liver, and heart whereas GPT2 is expressed highly in muscle, fat, and kidney (Yang et al., 2002).

In rats and pigs, glycolytic tissues (ex. skeletal muscle), cytosolic alanine transaminase is the predominant form and is responsible for the conversion of pyruvate into alanine (DeRosa and Swick, 1975). In gluconeogenic tissues (ex. liver and kidney), mitochondrial alanine transaminase has been found in cattle, pig, and rat; where it

catalyzes alanine into pyruvate (DeRosa and Swick, 1975; Sipe et al., 2004). In rats, there is increased expression of ALT mRNA in periportal hepatocytes versus the ALT mRNA expression in pericentral hepatocytes (Boon et al., 1999). Also, rat ALT mRNA expression is affected by the presence of protein in the diet in which mRNA content was lowest at 0% protein diet and highest at 60% protein diet in the periportal hepatocyte, whereas there was no change in ALT mRNA content of pericentral hepatocytes in the presence of 0-60% protein diet (Boon et al., 1999). The specific activity of ALT1 is found to be 15 fold higher than ALT2 during purification from crude hepatic lysate (Liu et al., 2008). In terms of serum ALT activity, ALT1 is mainly responsible for basal ALT activity (Lindblom et al., 2007).

Glutamine synthetase (GS). Whereas prokaryotes express glutamine synthetase I, eukaryotes express glutamine synthetase II (GS; Eisenberg et al., 2000). In a report by Eisenberg et al. (2000), the structure of glutamine synthetase was characterized by X-ray crystallography, using *S. typhimurium* bacteria as a source sample, which has a molecular mass of 620 kDa. It was determined that *Mycobacterium tuberculosis* GS has a molecular weight of 640 kDa. In bacteria, GS form dodecamers, containing 12 active sites (Almassy, et al., 1986, Valentine et al., 1968). The GS dodecamer is held together mainly by hydrophobic and hydrogen bonding between two hexameric rings (Almassy et al., 1986). Both the N- and C-terminus of each subunit form a helical structure. The N-terminal helix is exposed to solvent whereas the C-terminal helix is covered by a hydrophobic subunit on the opposite hexameric ring. At each active site, bidirectional binding in which glutamate and ATP bind at opposite ends. Also, the main channel of the

dodecamer contained six four-stranded beta-sheets. In this study, non-bacteria GS was not examined for structural analysis via X-ray crystallography.

The gene GLUL (glutamate-ammonia ligase) encodes mammalian glutamine synthetase (GS; Kung et al., 2011). GLUL is expressed by pericentral hepatocytes (hepatocytes surrounding the central lobular vein) (Brosnan and Brosnan, 2009). It is known that part of the 5'-upstream enhancer region of the GLUL gene is responsible for GS localization around the pericentral vein (Lie-Venema et al., 1995). The enhancer region contains a single T-cell factor/lymphoid enhancer that is required for responsiveness to β-catenin (Clinkenbeard et al., 2012). It is not known if estrogen receptors directly activate the promoter region for glutamine synthetase. Proximal to the transcription-start site, TATA, CCAAT, and GC elements are present in the first 117 base pairs of rat GS gene. These elements constitute the functional promoter region of GLUL (Fahrner et al., 2005). The addition of an upstream sequence of AP2-binding site triggers a four-fold increase of the GS gene promoter activity (Fahrner et al., 1993). The cloning and characterization of the GS gene has led to the identification of several *cis*-acting elements that enhanced reporter gene expression in transient transfections (Fahrner et al., 1993). The rat GS gene consists of 7 exons separated by 6 introns, which spans 10 kb and containing two consensus sequences for the binding site of the transcriptional regulatory protein SP1 in the first exon, playing a part in the regulation of GS expression (Mill et al., 1991).

Eukaryotic glutamine synthetase has been reported to form an eight-subunit oligomer (Eisenberg et al., 2000). All identified active residues of GS II structure are invariant among all species and are similar to the active sites of bacterial GS I, indicating

that the mechanism of action can be predicted to be similar (Meister, 1974). For activation, eukaryotic GS II can be highly regulated by nitrogen starvation in *E. coli* strains (Eisenberg et al., 2000). It has been reported that insulin and hydrocortisone can trigger changes in the rate of eukaryotic GS II biosynthesis (Meister, 1984; Berl and Clark, 1983; Cooper et al., 1983). Also, GS II can be regulated in vitro by assosication and dissociation of subunits in the presence of manganese and magnesium, presence of substrates, and enzyme concentrations (Denman and Wedler, 1984).

Eukaryotic GS acts in the presence of L- and D-glutamate and its analog (e.g. beta-glutamate, cis-cycloglutamate, and alpha-methly-L-glutamate) but is inhibited by MetSox and carbamoyl phosphate in the presence of manganese ion only (Tate et al., 1972). Furthermore, there are tissue-specific differences in the regulation of eukaryotic GS. Specifically, liver GS isoform reacts differently than brain GS isoform to feedback inhibition by L-glutamate-derived metabolites (ex. glycine, L-alanine, L-serine, L-glutamine, L-histidine, and carbamoyl phosphate in the presence of manganese ion only) and is activated by alpha-ketoglutarate and citrate (Meister, 1974; Meister; 1984). Non-brain GS type II responds to end-product feedback inhibition in which low concentrations of glutamate-dependent metabolites stimulate GS activity and high concentrations of the end products will inhibit it, whereas brain GS type II is not affected by end-product feedback inhibition (Tate et al., 1972). Rat GS mRNA expression is affected by the presence of protein in the diet in which mRNA content was lowest at 0% protein diet and 1.5 to 2.0-fold higher at 20% and 60% protein diets in the periportal hepatocytes (Boon et al., 1999).

Amino Acid Transport Systems

In mammals, System X^-_{AG} is a high affinity glutamate transport system (K_m = 4-40 μ*M*), predominantly transport glutamate and L/D-aspartate with an obligatory counter-exchange of K^+, and plays a major role in the central nervous system (Danbolt, 2001). System ASC is another high affinity Na^+-dependent amino acid transport system which mainly transports L-alanine, L-serine, and L-cysteine (Matthews, 2005). Systems ASC and B_o also transport L-glutamate and L-aspartate, but only in acidic (pH < 5.5) environments (Utsunomiya-Tate et al., 1996).

System X^-_{AG} Transport Proteins. Five mammalian proteins have been cloned that are capable of System X^-_{AG} transport activity. They are known as EAAC1 (SLC1A1), GLT-1 (SLC1A2), GLAST1 (SLC1A3), EAAT4 (SLC1A6), and EAAT5 (SLC1A7) (Kanai and Hediger, 2004). Functionally, the transport process by all SLC1A proteins involves the extracellular binding and translocation of three Na^+ ions and one amino acid (L-glutamate, D-aspartate, or L-aspartate); with reorientation of the transporter to the extracellular face of the membrane being driven by the intracellular-to-extracellular counter-transport of one K^+ ion. Although System X^-_{AG} transporters usually transport anionic amino acids from the extracellular fluid into the cytosol, they are capable of reverse transport when the intracellular K^+ levels are too low (Zerangue and Kavanaugh, 1996; Levy et al., 1998).

The System X^-_{AG} proteins have been reported to be expressed in many tissue types. For instance, EAAC1 mRNA and/or protein have been detected in the heart (Nakayama et al., 1996), small intestine (Kanai and Hediger, 1992; Howell et al., 2001), kidney (Kanai and Hediger, 1992; Gissendanner, 2003; Sipe, 2004), skeletal muscle

(Velaz-Faircloth et al., 1996) and liver (McGivan and Nicholson, 1999; Howell et al., 2001, 2003; Gissendanner, 2004; Sipe, 2004).

Using isolated membrane vesicles, system X^-_{AG} activity has been demonstrated in canalicular-enriched fractions and a Na^+-independent activity in the sinusoidal membrane domain of the liver (Ballatori et al., 1986; Cariappa and Kilberg, 1992). In contrast, others have measured both system X^-_{AG} activity and Na^+-independent (exchanger) glutamate transport activity, in sinusoidal membranes of rat hepatocytes (Low et al., 1992). System X^-_{AG} is known to be extremely high in cells that also express high activities of glutamine synthetase (Hertz, 1976), as is the case for pericentral hepatocytes. Glutmate transporters EAAC1, EAAC2, and GLT-1 are reported to be expressed in the liver of rodents (Fremeaux et al., 2002). By Northern blot analysis, EAAT5 appears to be expressed at low levels in liver. In cattle, both of the protein and mRNA expressions of EAAC1 and GLT-1 were found in the liver, whereas other transporters from system X^-_{AG} were not detected (Howell et al., 2001).

Moreover, EAAC1 protein has been observed on the apical membrane of rat renal proximal tubule brush borders (Shayakul et al., 1997), nephrons isolated from rats (Verrey et al., 2005), and neonatal porcine epithelial cells (Fan et al., 2004). GLT-1 mRNA has been detected in the kidney (Welbourne and Matthews, 1999) and liver (Howell et al., 2001, 2003; Kim et al., 2003). In sheep and cattle, our group found that both proteins and mRNA of EAAC1 and GLT-1 were expressed in small intestinal epithelia isolated from the rumen, omasum, duodenum, jejunum, ileum, cecum, and colon (Howell et al., 2001). Also, GLAST1 mRNA has been detected in the heart (Nakayama et al., 1996). In placental tissue, EAAC1, GLAST1, and GLT-1 mRNA and protein are

expressed (Matthews et al., 1998a; 1998b; Danbolt et al., 2001). In the brain, only GLAST1 and GLT-1 proteins (Torp et al., 1994; Schmitt et al., 1996; Torp et al., 1997; Berger and Hediger, 1998) are expressed in astrocytes, whereas EAAC1 and EAAT4 are the predominant transporters in neurons (Rothstein et al., 1994; Kanai et al., 1995; Berger and Hediger, 1998). High concentration of GLT-1 and GLAST1 proteins has been identified in astroglial membranes facing the nerve terminals (synaptic cleft), axons and/or dendrites, whereas GLT-1 and GLAST-1 levels were lower in astroglial membranes facing other astrocytes, cell bodies, or the basement membrane of the endothelium (Chaudhry et al., 1995). Furthermore, EAAT4 and EAAT5 are predominantly expressed in the cerebellum (Fairman et al., 1995; Nagao et al., 1997) and in the retina (Arriza et al., 1997), respectively.

Glutamate absorption by mammalian skeletal muscle initially was thought to occur by a Na^+-independent process, based on conclusions drawn from a rat hindlimb perfusion study (Hundal et al, 1989). Subsequently, and in accordance with that observed in the muscle of barnacles (Revest and Baker, 1988), the expression of both Na^+-dependent and –independent glutamate uptake activities was identified in skeletal muscle using primary cultures of rat myotubules (Low et al, 1994). The Na^+-dependent uptake of glutamate activity was inhibited by both L- and D-aspartate and displayed an affinity constant for glutamate of 0.7 m*M*. Therefore, the affinity is too low, although the profile of substrates that inhibit Na^+-dependent uptake of glutamate by myotubules is consistent with system X^-_{AG} transporters. Though specific information about the molecular identity of potential glutamate transporters expressed by muscle tissue is very limited, EAACl mRNA has been identified in skeletal muscle (Kanai and Hediger, 1992), which

confirmed the possibility that EAACl could account for the system X^-_{AG} activity found in skeletal (Revest and Barker, 1988; Low et al., 1994; Frank et al, 2002) myocytes. In cattle, both mRNA and protein for two system X^-_{AG} transporters (EAACl and GLT-1) are expressed in the longissimus dorsi muscle (Gissendanner et al, 2003).

Also, the activities of some SLC1A transporters can be regulated by protein-protein interaction. For example, EAAC1 function is regulated by glutamate transport associated protein 3-18 (GTRAP3-18). GTRAP3-18 is a protein that reduces the affinity of EAAC1 for glutamate by interacting with the carboxy-terminal end of EAAC1, as shown with yeast two-hybrid analysis (Lin et al., 2001).

System X^-_c Transport Proteins. System X_c^- is the most widely expressed Na^+-independent transport activity for anionic amino acids, mediating the influx of L-cystine into the cell coupled to the efflux of glutamate (predominant anionic amino acid) or aspartate at a 1:1 ratio (Bannai and Kitamura, 1980).

Administration of Exogenous Sex Steroids and its Application in the Beef Cattle Industry

In the United States feedlot industry, the administration of exogenous sex steroids (e.g.-androgens, estrogens, and progestogens) and their derivatives are given to improve production efficiency of the cattle while reducing the production cost. Specifically, these steroidal implants influence growth rates and body composition of growing and mature cattle (Kahl, 1978). In the United States feedlots, the common estrogenic hormones utilized are estradiol 17β, zeranol and estradiol benzoate, whereas trenbolone acetate and testosterone are common androgenic compounds given to cattle through ear implants

(Reinhardt, 2007).

After implantation, exogenous estrogen and other steroidal compounds are transferred from the ear implant into the blood when it dissociates from the delivery capsule. The length of time in which the implant can trigger growth depends on the release rate of the sex steroids from the implant which usually occurs in a biphasic pattern in which there is a higher rate of release earlier in the growth-promoting period than later in the time period (Reinhardt, 2007). Compudose® (25.7 mg estradiol 17beta) has been indicated to be effective for 200 days, whereas Synovex-S (200 mg progesterone plus 20 mg estradiol benzoate) is effective for 70 days (per manufacturers' directions). During the release of these hormones, a concentration threshold needs to be reached before these compounds can improve growth rates (Preston, 1999). After 60 days of Synovex-S implantation and then its removal, 24% of the initial estradiol and 27% of the initial progesterone dosage remained in the implant, with a linear reduction of 0.16 and 0.15% of the initial dosage per day for estrogen and progesterone, respectively (Rumsey et al., 1992). However, surprisingly, there are no published reports describing the concentration of estradiol in blood in response to Compudose® implantation.

Reproductive Physiology of Endogenous Estrogen and Progesterone in Cattle

The estrous cycle in cattle lasts for 18 to 24 days, with an average of 21 days, consisting of a luteal phase (14-18 days) and a follicular phase (4-6 days) (Hadley and Levine, 2007; Forde et al., 2011). During the beginning stage of estrus, the hypothalamic emergence of gonadotropin-releasing hormone (GnRH) causes the anterior pituitary release of the follicle stimulating hormone (FSH), which initiates follicle maturation, and

thus increasing secretion of estradiol to induce estrous behavior in cattle (Allrich, 1994). After ovulation, in the luteal stage, estrogen-induced circulation of the luteinizing hormone (LH) causes the formation of the corpus luteum, and thus the production of progesterone, an inhibitor of the gonadotropins (LH and FSH) to prevent ovulation (Hadley and Levine, 2007).

Whole-Animal Parameters Affected by Steroidal Implantation

Exogenous estrogen and its combination with progesterone or with androgenic derivatives have been well characterized to affect certain steer carcass parameters including improved muscle composition and fat beds (Heitzman, 1976; Duckett et al., 1996; 1999). In feedlot heifers, the implant combination of estrogen and trenbolone acetate at various concentrations positively affected carcass traits including increased longissimus muscle area (Heitzman, 1976; Schneider et al., 2007; Boles et al., 2009). Increased weight gain of steer and heifer calves occurs when these animals are treated with either estradiol 17 beta or zeranol, a non-steroidal estrogen agonist (Sawyer, 1987). However, there are conflicting reports about the effectiveness of implants containing both estrogen and testosterone in improving growth rates and carcass parameters. For instance, the carcass composition of mature cows has been shown to be affected by 200 mg testosterone proprionate plus 20 mg estradiol benzoate (Cranwell et al., 1996; Neill et al., 2009). In contrast, carcass traits of culled cows were not affected by a combination of 200 mg testosterone and 20 mg estradiol benozoate (Jones, 1982; Matulis et al., 1987). In pigs, nitrogen retention was improved by the treatment of 20 mg 17beta-estradiol + 140 mg trenbolone acetate (van Weerden and Grandadam, 1976).

Mechanistic Regulation of Steroidal Implants

The effect of steroidal implants on whole-animal parameters has been well elucidated. However, little information is known regarding how sex steroid administration affects specific mechanisms (genes, proteins, biochemical pathways) responsible for alteration of muscle and adipose tissue composition. It has been reported that estrogenic implants affect muscle protein accumulation by increasing the concentration of hepatic somatotropin and insulin-like growth factor 1 (Reinhardt, 2007).

Even less is known about whether/how sex steroid administration alters specific mechanisms of the liver, the tissue responsible for coordinating alteration of whole-body nutrient fluxes. For example, in the liver, glutamate serves as precursor for glutamine (which is exported to the plasma as a nitrogen carrier), gluconeogenic carbons, and incorporation into de novo protein synthesis (Haussinger and Gerok, 1983). Prior findings from our lab demonstrated that liver glutamine synthetase is up-regulated by Synovex-S® (20 mg 17β-estradiol benzoate + 200 mg progesterone) in finishing steers (Sipe et al., 2004). Also, glutamine synthetase activity and protein expression in rat C6-glioma cells is increased by estrogen (Haghighat, 2005). In the hypothalamus and hippocampus of humans, estradiol up-regulates glutamine synthetase mRNA and protein content (Blutstein et al., 2006). In addition, the activity of ALT and aspartate transaminase (AST) can be down-regulated by estradiol, and by the combination of estradiol, and by the progesterone, in aged cyclical rats (Moorthy et al., 2005).

Recently, the detection of mRNA for estrogen receptors α and β in cattle liver (Pfaffl et al., 2001) suggests that sex steroid administration to cattle has a direct effect on

hepatic mechanisms through specific sex steroid receptor-activated mechanisms, and not only through IGF-mediated effects.

Estrogen and Progesterone Receptor Cell Signaling

Estrogen and progesterone receptors belong to the nuclear receptor superfamily and have also been expressed on the cell surface membranes. To date, the expression of progesterone receptors has not been reported in the liver of cattle. It has been reported that estrogen α and β receptors are expressed in the liver of cattle and that the mRNA content of these receptors is not affected by administration of zeranol, a strong estrogenic and anabolic compound (Pfaffl et al., 2001). In contrast, mRNA expression of estrogen receptor α appeared to increase with increasing concentrations of melengestrol acetate, a potent synthetic progestagen (Pfaffl et al., 2002). Furthermore, estrogen receptor β has been shown to inhibit estrogen α receptor-dependent transcription of target genes when co-expressed together in murine and breast cancer cells (Matthews and Gustafssoni, 2006). It has been suggested that estrogen receptor β can inhibit estrogen receptor α by altering binding of co-transcriptional factors to estrogen receptor α and by causing enhanced the degradation of estrogen receptor α in T47D cell lines (Heldring et al., 2007).

Even though estrogen receptor isoforms result from the transcription of different genes, these estrogen receptors can mediate the activation of target proteins through multiple cell signaling pathways when they form homeodimers (Matthews and Gustafsson, 2003). The classical pathway involves the transport of the ligand-bound estrogen receptors from the plasma membrane into the cell to promote transcription and/or mediate cell signaling cascades (Figure 2.3). In the genomic pathway, estrogen-

bound estrogen receptors can directly bind to the estrogen response element located on the promoter region of target genes or interact with other transcription factors (e.g. β-catenin) to initiate the transcription of target genes with no estrogen response elements present on the promoter region (Heldring et al., 2007).

In the non-genomic pathway, estrogen has been reported to be able to trigger rapid events that occur within seconds or minutes after exposure to estrogen through certain cell signaling cascades. Specifically, these prompt effects involve the activation of protein kinases and phosphatases and increased movement of ions across the plasma membrane and other membranes possibly mediated by GPR30, a newly discovered membrane bound plasma receptor (Heldring et al., 2007).

Moreover, ligand-independent estrogen receptor activation can occur when growth factor cell signaling cascade activates certain protein kinases to phosphorylate/activate estrogen receptors (Kato et al., 1995). In humans, this type of estrogen signaling has been indicated to be a factor in the growth of certain breast cancers in the absence of estrogen (Coutts and Murphy, 1998; Shim et al., 2000).

The cell signaling pathway(s) involved in estrogen up-regulation of glutamine synthetase expression in the liver has not been elucidated. However, two pathways that are good candidates for the regulation of hepatic glutamine synthetase expression by estrogen are those mediated by GPR30 and β-catenin. In the non-classical pathway, GPR30 is thought to be a prime target in the activation of non-genomic regulation of target genes by estrogen. Currently, the non-genomic estrogen pathway is not fully understood. However, in the human brain, estrogen can activate certain cell signaling pathways through GPR30, a plasma membrane-bound receptor. In the human liver,

estrogen has been indicated to enhance liver function after a trauma-induced hepatic hemorrhage via GPR30-mediated protein kinase A activation (Hsieh et al., 2007).

In the canonical Wnt pathway, Wnt signals by activating β-catenin for translocation into the nucleus (Figure 2.4). As lipoglycoproteins, Wnts are secreted from neigboring cells to interact with two plasma-membrane bound receptors, frizzled and low-denisity-lipoprotein-related protein (LRP) receptors (Daugherty and Gottardi, 2007). Wnt triggers the activation of β-catenin by inhibiting the β-catenin degradation complex through the transducer protein, disheveled (Dvl). This complex contains two scaffolding proteins, axin and adenomatous polyposis coli (APC), and two serine/threonine protein kinases, glycogen synthase kinase 3β (GSK3β) and casein kinase I. The regulatory actions of Wnt allows for the non-phosphorylated form of β-catenin to dissociate from the degradation complex and bind to T cell factor (TCF) and other transcription factors for translocation into the nucleus to activate target genes. In the absence of Wnt activation, β-catenin is phosphorylated by GSK3β or casein kinase I and is targeted for ubiquitin-mediated degradation by the APC and axin proteins. Estrogen is thought to be a major modulator in the Wnt pathway. In the uterine epithelium, estradiol can up-regulate the expression of two Wnt proteins (Wnt4 and Wnt5a) to trigger nuclear translocation of β-catenin in an estrogen receptor-independent manner (Hou et al., 2004). In past studies, it has been demonstrated that estrogen receptor alpha can interact with beta catenin in osteoblasts (Foo et al., 2007), *Drosophila* and certain human cancer cell lines (Kouzmenko et al., 2004). Furthermore, in the rat hippocampus, estradiol can inhibit GSK3β and bind to β-catenin for translocation into the nucleus (Cardonna-Gomez et al., 2004).

Certain proteins involved in glutamate metabolism have been shown to be regulated by β-catenin. Ornithine aminotransferase is up-regulated in transgenic mice overexpressing hepatic β-catenin, but is not regulated by β-catenin in human hepatocellular carcinomas, whereas GLT-1 and glutamine synthetase mRNA expression is up-regulated by the overexpression of β-catenin (Cadoret et al., 2002; Zucman et al., 2007). However, the interaction of β-catenin and estrogen on glutamine synthetase expression is unknown.

Age-associated Reduction of Amino Acid N Metabolism

Aging (senescence) can be defined as the "progressive, time dependent deterioration in the ability of an organism to respond adaptively to environmental changes" (Balin and Allen, 1986). Aging influences numerous metabolic and physical mechanisms. Age of a cow has a vital relationship to various cost-effective parameters of beef cattle production including fecundity, milk yield, and weaning weight. For instance, the increase of milk yields and weaning weights has been positively correlated for Angus cows from three to six years of age (Baker and Boyd, 2003). Additionally, increased birth and weaning weights have been shown to be positively associated to multiparous British crossbred beef cows with ages ranging from three to eight years of age (Renquist et al., 2006). However, in senescent cows (beyond 8 yrs of age), age has been negatively correlated with reproductive capacity and weaning weights of numerous beef cattle breeds (BIF, 1996; Renquist et al., 2006).

At the biochemical level, the physiological mechanisms responsible for age-associated changes are poorly understood in cattle and other ruminants. However, age-

related changes in hematologic and metabolic profiles, and amino acid concentrations for non-ruminants, have been reported (Lowseth et al., 1990; Militante and Lombardini, 2004). Consistently, decrease in protein synthesis and putative loss of muscle protein in which amino acid availability is known to be a limiting factor in protein synthesis in older animals has been observed (Volpi et al., 1998; Pitkanen et al., 2003). Nonetheless, it is evident that the degree to which age affects amino acid metabolism is highly dependent upon the species and the sex of the animal (Militante and Lombardini, 2004). In addition to changes in blood amino acid levels, the hematologic and metabolic profiles, including enzymes associated with organ function and blood mineral content also are affected by age, and are differentially altered across species. For example, as dogs age, serum aspartate transferase levels decrease whereas ALT increase (Lowseth et al., 1990; Swanson et al., 2000). Furthermore, ornithine aminotransferase and glutamate dehydrogenase are increased, whereas alanine transaminase is decreased in the liver of senescence accelerated mice via 2-DE proteomic analysis (Cho et al., 2003). Moreover, metabolic syndrome- and sex-independent serum alanine transaminase levels decrease with age in both men and women, after adjustments for alcohol use, sex, metabolic syndrome constituents, and adiposity biomarkers were made (Dong et al., 2010).

The process of senescence is known to be associated by a reported decrease in whole-body protein synthesis (Karakelides and Nair, 2005). In different tissues, the protein synthetic rates may decrease, not change, or increase, depending on the organism (Ward, 2000). In the muscle, aging has been known to affect the nitrogen-metabolizing enzymes involved with ammonia detoxification (Dhahbi, et al., 1999). Also, in humans, there is a reduced capacity for nitrogen retention, which can be up-regulated by growth

hormone therapy (Corpas et al., 1993). Also, in senescent humans, there is a decrease in urinary nitrogen excretion and muscle mass, affecting nitrogen metabolism (Evans, 2004). In the liver, aging has been associated with decreased metabolic functions and decreased gene expression of proteins responsible for intermediary metabolism in the liver (Sersté and Bourgeois, 2006). Moreover, the mRNA content and activity of murine hepatic glutamine synthetase has been reported to decrease (Dhahbi et al., 1999).

In cattle, little information is known about the relationship between hepatic function and aging. However, previous research in our lab has shown that old beef cows vs. young mature beef cows have reduced hepatic expression of glutamine synthetase and alanine transaminase, two enzymes critical for N recycling. Specifically, glutamine synthetase protein content decreased by 46 to 71% in old, mature cows compared to the young mature cows (Matthews et al., 2006). This finding suggests that glutamine synthetase catalyzed conversion of L-glutamate to L-glutamine in pericentral hepatocytes may be reduced in old cows. Old cows also had a 61 to 73% reduction in ALT content, indicating that ALT-mediated conversion of α-ketoglutarate and alanine into glutamate and pyruvate is impaired in periportal hepatocytes (Matthews and Sipe., 2006). GTRAP3-18 content, an inhibitory regulator of EAAC1 glutamate transporter, was increased 132 to 192% in old cows suggesting that the decrease in glutamine synthetase content was related to an inhibition glutamate uptake capacity from plasma (Matthews and Sipe, 2006)

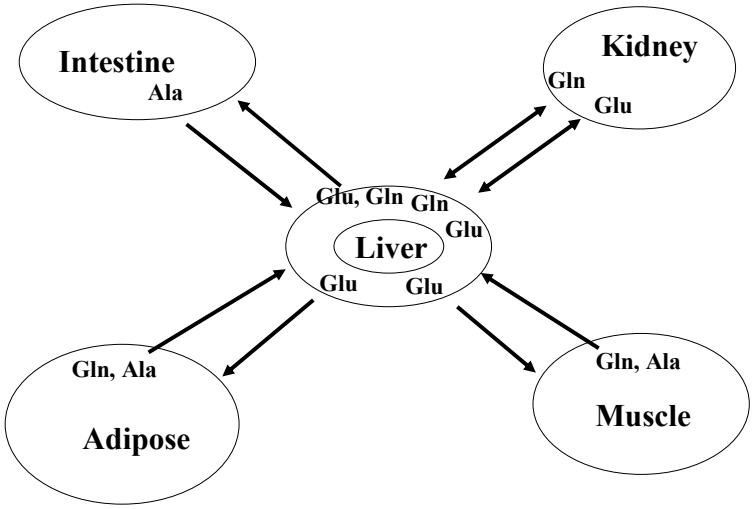
Dissertation Objectives

Because we were interested in understanding the physiological mechanisms involved with steroidal regulation, three animal models (old cows; young mature versus old cows;

developing heifers). The overall goal of the dissertation was to answer the following questions:

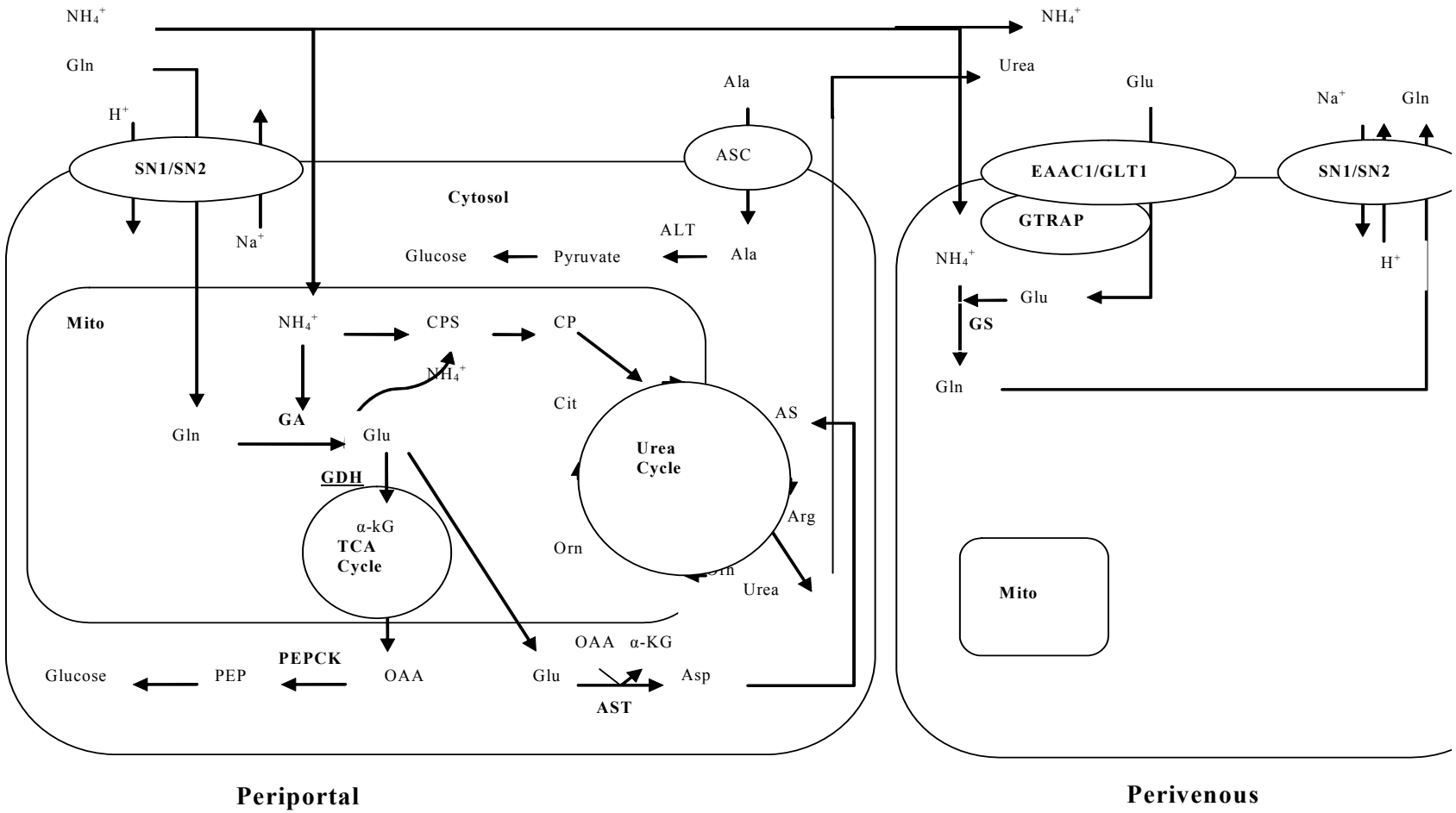
1) Can estrogenic implants increase the expression of glutamine synthetase and other critical liver proteins in old, mature cows? (Trial 1; Chapter 3)
2) Will exogenous estrogen increase the expression of glutamine synthetase and other proteins in the liver of young, mature cows versus old mature cows (Trial 2; Chapter 4)?
3) What are the potential hepatic mechanisms (i.e., estrogen receptors, transcription factors) affected by exogenous estrogen in the liver (Trial 2; Chapter 4)?
4) What are the potential effects of exogenous estrogen on blood parameters of developing heifers (Trial 3; Chapter 5)?

Figure 2.1: Movement and utilization of glutamine, glutamate, and alanine between liver and other peripheral tissues



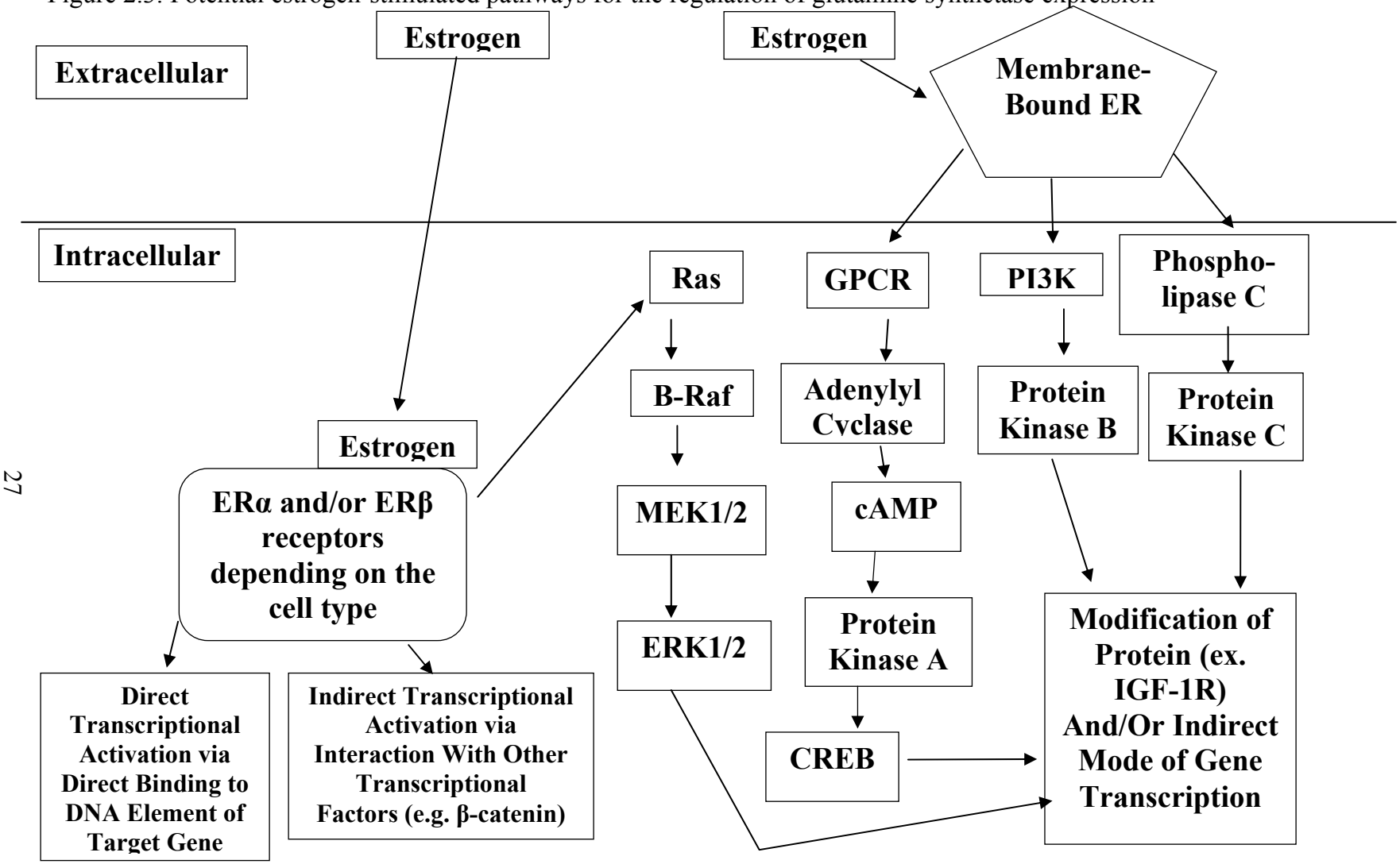
Revised from Heidger and Welbourne(1999).

Figure 2.2: Liver glutamine/glutamate metabolism



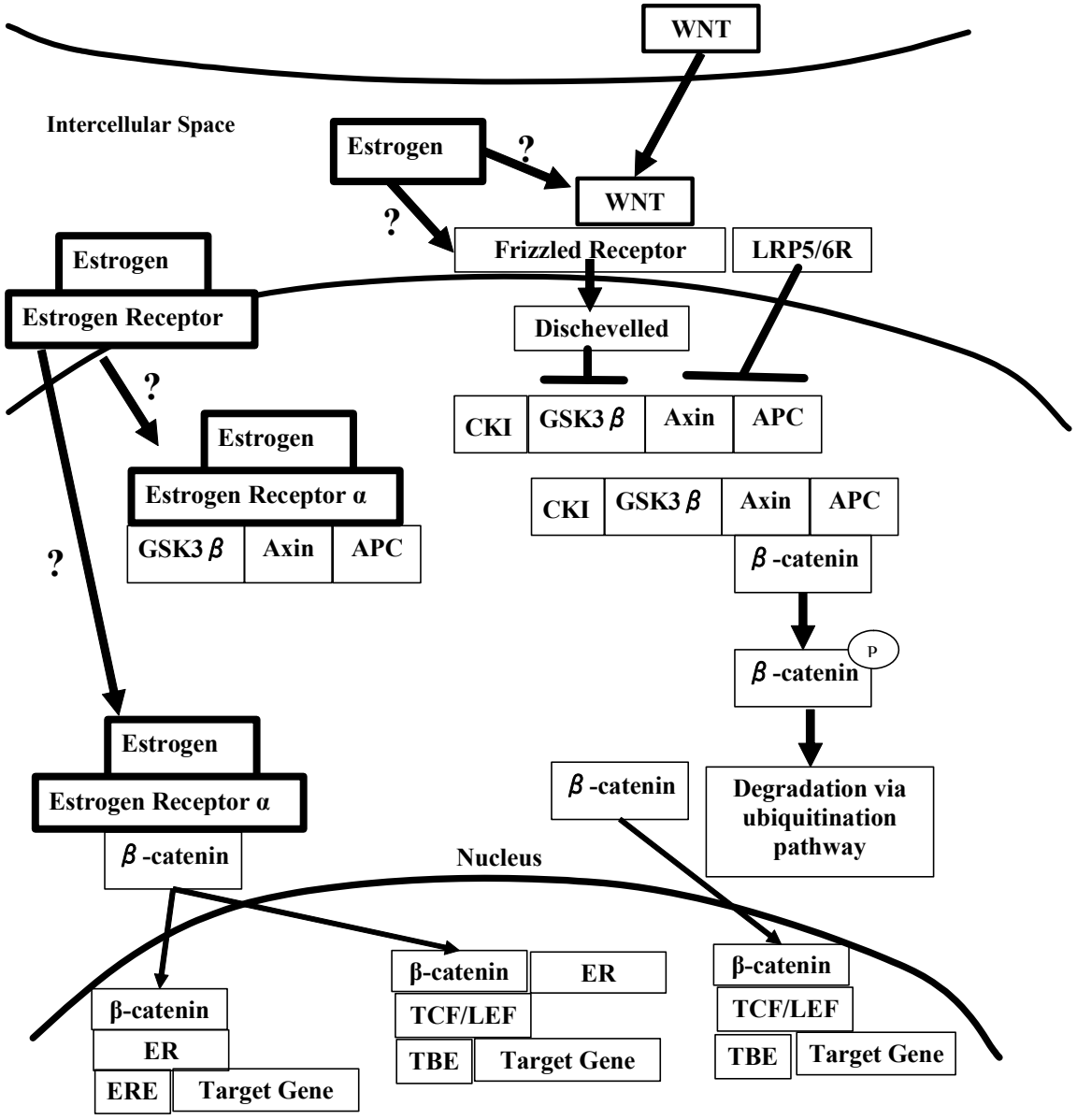
[1]SN1 (SLC38A3), Na^+ and H^+-coupled glutamine transporter; EAAC1, high-affinity glutamate transporter; GTRAP3-18, regulator of EAAC1 function that binds to EAAC1 on cytosolic side of membrane; GLT-1, high-affinity glutamate transporter; ASC, system ASC, originally named for three of its preferred substrates alanine, serine, cysteine; ATA2, SLC38A2; ATA3, SLC38A4; GA, glutaminase;
GDH, glutamate dehydrogenase; ALT, alanine transaminase; AST, aspartate transaminase; GS, glutamine synthetase; PEPCK, Phosphoenolpyruvate carboxykinase, cytosolic; CPS, carbamoylphosphate synthethase; CP, carbamoyl phosphate; Cit, citrulline;
Orn, ornithine; AS, arginiosuccinate;

Figure 2.3: Potential estrogen-stimulated pathways for the regulation of glutamine synthetase expression



Adapted from Behl, 2002; Green et al., 1986; Honda et al., 2000; Kelly et al., 2005; Kuiper et al., 1996; Sing et al., 1999

Figure 2.4: Potential WNT/beta catenin pathways for the regulation of glutamine synthetase expression



Adapted from Cardona-Gomez et al., 2004; Hou et al., 2004; Kouzmenko et al., 2004; Eisenmann, 2005.

Chapter 3

Glutamine synthetase is up-regulated in the liver of old beef cows by 17β-estradiol implants

INTRODUCTION

The age of a beef cow has a major impact on many economically important production traits of beef cattle, including milk yield and calf weaning weight (BIF, 1996). For instance, increased milk yield and weaning weights have been shown to be positively correlated to the productive age of three to six years for Angus cows (Baker and Boyd, 2003). Conversely, after 8 years of age, these aging cows produce calves with lower birth and weaning weights (BIF, 1996). Moreover, old cows (> 8 y) have daughters with reduced milk yield and productive lifespans (Fuerst-Waltl et al., 2004).

The physiological mechanisms responsible for these senescence-related effects are not well understood. It is known that aging can have detrimental effects on the function of many organs including the small intestine, muscle, and liver. In the small intestine, aging can cause a decline in the ability to absorb glucose (Drozdowski et al., 2003). For the muscle, aging can trigger sarcopenia, a loss of muscle mass and strength (Attaix et al., 2005). Furthermore, aging is associated with decreased metabolic functions and decreased gene expression of proteins responsible for intermediary metabolism in the liver (Serstè and Bourgeois, 2006).

The liver is an important central organ because it is responsible for coordinating whole-body energy and nitrogen metabolism. For example, glutamate serves as precursor for glutamine (which is exported to the plasma as a nitrogen carrier), gluconeogenic

carbons, and incorporation into de novo protein synthesis (Haussinger and Gerok, 1983). In cattle, little information is known about the relationship between hepatic function and aging. However, previous research in our lab has shown that old beef cows vs young mature beef cows have reduced hepatic expression of glutamine synthetase (GS) and alanine transaminase (ALT), two enzymes in the liver that are critical for optimal N recycling. Specifically, the content of GS protein in the liver of old (≥ 10 years old), mature cows is decreased by 46 to 71% compared that of young (3-5 years old) adult cows (Matthews et al., 2006). This reduction is important because GS catalyzes the conversion of L-glutamate to L-glutamine in pericentral hepatocytes. The livers of old cows also express 61 to 73% less ALT than young cows (Matthews and Sipe, 2006). In the periportal hepatocytes, ALT is responsible for the conversion of α-ketoglutarate (α-KG) and alanine into glutamate and pyruvate. Furthermore, it was observed that the content of GTRAP3-18, an inhibitory protein regulator of pericentral hepatocyte-localized glutamate transporter (EAAC1) activity, is increased 132 to 192% in old cows (Matthews and Sipe, 2006). Thus, the capacity for plasma glutamate uptake, intracellular glutamate production, and glutamine synthesis all are reduced in liver tissue of old cows.

Prior findings from our lab demonstrated that liver GS mRNA and protein content is up-regulated in finishing steers by implantation of Synovex-S® (20 mg 17β-estradiol benzoate + 200 mg progesterone) into the ear (Sipe et al., 2004). In rat C6-glioma cells, GS activity and protein content is increased by 17β-estradiol (Haghighat, 2005). In the hypothalamus and hippocampus of humans, estradiol up-regulates GS mRNA and protein content (Blutstein et al., 2006). In addition, the activity of ALT and aspartate transaminase (AST) in the brain, heart, liver, kidney and uterus can be down-regulated by

estradiol and by the combination of estradiol and progesterone, in aged, acyclic rats (Moorthy et al., 2005).

The primary goal of this experimentation was to determine if administration of exogenous 17β-estradiol (estradiol), in the form of Compudose ear implants (25.7 mg estradiol), affects the expression of GS, ALT, AST, and other proteins involved in the hepatic glutamate/glutamine/alanine metabolism by old beef cows. The hypothesis tested in this experiment is that hepatic GS mRNA and protein expression would be up-regulated by exogenous estradiol, whereas ALT mRNA and protein, and AST protein, expression would be down-regulated. The specific experimental objectives were to determine the effects of supplemental exogenous estradiol to old beef cows on (1) plasma estradiol and progesterone concentrations, (2) hepatic expression of GS, ALT, AST, and other proteins involved in the hepatic glutamate/glutamine/alanine metabolism, and (3) blood biochemical and clinical indicators of whole-body metabolism.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Fourteen old (> 10 y) non-pregnant beef cows were obtained from a stockyard and housed in a dry lot at the University of Kentucky Agricultural Research Center, located in Woodford County, KY. Cows had *ad libitum* access to alfalfa hay and water for 28 d. Representative sampling of the alfalfa hay was conducted for proximate and mineral analyses (Figure 3.1) by a commercial laboratory (Dairy One Forage Lab, Ithaca, NY).

COMPUDOSE and Sham Implant Treatments

Cows were judged to be at least 10 years of age based on the amount of wear of their teeth (Johnson, 1959). At the beginning of this trial, cows were weighed and subdivided into 2 groups based on BW and then randomly allotted (n = 7) to either receive a sham (control; 0.5 mg oxytetracycline; 585 kg) using 1 mL sterile corn oil as a vehicle for oxytetracycline (0.5 mg) or estradiol (Compudose Implant; 25.7 mg estradiol; Vetlife, Des Moines, IA; 621 kg) implant treatment. The back of the ear was clipped, cleansed with 70% ethyl alcohol solution, and dried. Seven cows received the controlled-release estrogen implant subcutaneously in the dorsal-medial area of the left ear using the manufacturer's implantation device (Compudose® Implanter; Vetlife, Des Moines, IA) and instructions. In addition, seven cows received a sham implant.

Blood Collection and Fractionation

Jugular venous blood samples were collected by venipuncture on d 14 and d 28. For preparation of plasma, 16 mL of blood was collected in EDTA-containing (0.9375 mg/mL) blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). For serum, 16 mL of blood was collected in serum blood collection tubes without an anticoagulant. For whole blood, 2 mL of blood was collected in EDTA containing (2.7 mg/mL) blood collection tubes(Becton Dickinson). Plasma and serum were recovered by refrigerated centrifugation at 3,000 × *g* for 10 min at 4°C and stored at −80°C.

Analysis of Blood Analytes

Plasma estradiol levels were evaluated using the Ultra-Sensitive Estradiol Radioimmunoassay kit (Diagnostic Systems Labs, Webster, TX). Briefly, 125 µL of $MgCl_2$ and 125 µL Dextran were added to microcentrifuge tubes containing 750 µL of the samples to delipidate the plasma. Samples were incubated for 1 hr at room temperature. Tubes were centrifuged for 15 minutes at 10,000 rpm and the supernatant removed for analysis.

Using the RIA kit, all samples, standards, and controls were assayed in duplicate. Tubes provided from the kit were labeled for total counts (TC), non-specific binding (NSB), standards, controls, and the blood samples. The standards used were 0.5, 0.62, 1, 2.5, 5, 7.5, 10, 15, 20, 35, 50, and 250 pg/ml. The volume for the standards and controls was 200 µL per tube. On the other hand, 266.7 µL of plasma samples were added to the proper tubes. To the NSB tubes, 300 µL of the 0 pg/mL estradiol standard was added. Estradiol antiserum (1^{st} antibody; 100 µL) was added to all tubes except for the NSB and TC tubes. All tubes were vortexed and allowed to incubate overnight at 4°C. After the incubation, ^{125}iodine-labeled estradiol (100 µL) was added to each tube. Then, tubes were vortex, covered, and allowed to incubate at 4°C overnight. On the third day, precipitating reagent (secondary antibody; 1 mL) was added to all tubes except for TC. Tubes were incubated at room temperature for 1 hr. Next, tubes were centrifuged for 30 minutes at 4°C with a speed of 3000 rpm. The tubes were decanted except for the TC tube to remove any excess liquid moisture. Afterwards, tubes were counted in the gamma counter for 1 minute. The intraassay CV was 7.1%, whereas the interassay CV was 11.9%. The detection limit of this assay is 0.67 pg/mL.

Plasma progesterone levels were measured using the Coat-A-Count Progesterone ^{125}I Radioimmunoassay kit per manufacturer's instructions (Siemens Medical Solutions Diagnostics, Los Angeles, CA). The sensitivity of this assay was determined to be 0.038 ng/mL, and an interassay CV of 5% was observed. Also, the intrassay CV was 11%. All other serum analytes, minerals, and blood cell types were analyzed by the University of Kentucky Livestock Disease Diagnostic Center as described by Brown et al. (2009).

Plasma samples were analyzed for ammonia-N by modifications of the L-Glu dehydrogenase assay (Da Fonesca-Wollheim, 1973) using the Konelab 20XTi analyzer (Thermo Electron Corp., Vantaa, Finland). The sensitivity of this assay is 0.010 m*M,* and an interassay CV of 11.0% is typically realized. For this experiment, plasma ammonia concentrations were determined in a single assay event. The intraassay CV was 7.4%.

All other serum analytes, minerals, and blood cell types were analyzed by the University of Kentucky Livestock Disease Diagnostic Center as described by Brown et al. (2009). For serum enzymes, the following specific activities were assayed: ALP, E.C. 3.1.3.1; ALT, E.C. 2.6.1.2; AST, E.C. 2.6.1.1; γ-glutamyltransferase, E.C. 2.3.22; creatine kinase, E.C. 2.7.3.2; LDH, E.C. 1.1.1.27.

Liver Tissue Biopsy

On d 14 and 28, hepatic tissue was collected by a modification of the aspiration technique in cattle (Brown et al., 2009). Briefly, the area from the 10th to the 12th intercostal spaces and 10 to 30 cm from the dorsal median plane on the right side of each animal was clipped free of hair and cleansed with povidine-iodine and two subsequent 70% ethyl alcohol solution washes. The remaining 70% ethyl alcohol solution was dried

with gauze. Lidocaine (Lidocaine 2% Injectible; The Butler Company, Dublin, OH; 1.6 mL per biopsy site) was subcutaneously injected between the 12th and 13th ribs approximately 10 cm from the dorsal medial plane. A topical anesthetic spray (Cetacaine® 300 mg; Cetylite Industries, Pennsauken, NJ) was administered to the skin 20 cm from the dorsal median plane at the 12th intercostal space for 2 seconds and an incision made with a scalpel. A trocar (7 mm diameter) was used to obtain tissue from the liver. The collected tissue was weighed and separated for RNA (400 mg wet tissue) and protein extraction (200 mg wet tissue). Samples were placed in foil packs and snap frozen in liquid nitrogen and stored at -80°C. After completion of biopsy, the incision site was treated with a topical broad spectrum antibacterial spray (Furazolidone® 4%; Veterinary Products Laboratories, Phoenix, AZ).

Immunoblot Analysis

Approximately 200 mg of liver, kidney, and LM were homogenized on ice for 30 s (setting 11, Polytron Model PT10/35, Kinematic Inc., Lucerne, Switzerland) in 7.5 mL of 4°C sample extraction buffer solution [0.25 mM sucrose, 10 mM HEPES-KOH pH 7.5, 1 mM EDTA, and 50 µL of protease inhibitor (Sigma, St. Louis, MO)]. Protein was quantified by a modified Lowry assay, using bovine serum albumin as a standard (Kilberg, 1989). Proteins (60 µg/lane) were separated using 12% SDS-PAGE and electrotransferred to a 0.45-µm nitrocellulose membrane (BioRad, Hercules, CA) as described previously (Howell et al., 2001, 2003), except that proteins were separated using a 12% acrylamide gel instead of 7.5%. Blots were stained with fast-green and the relative amount of stained protein per lane/sample determined by densitometric analysis.

The relative tissue content of specific proteins in liver was evaluated using a standard immunoblot protocol as described previously (Howell et al., 2001, 2003; Brown et al., 2009). Relative contents of GS, ALT, AST, G-protein-coupled receptor 30 (GPR30), glutamate dehydrogenase (GDH), glutamate transporter-1 (GLT-1), excitatory AA carrier 1 (EAAC1), and glutamate transporter-associated protein 3–18 (GTRAP3–18) were evaluated. For the detection of GLT-1, EAAC1, GTRAP3–18, and GDH, blots were hybridized with 5 to 10 μg of IgG anti-rat GLT-1 polyclonal antibody (Affinity BioReagents, Golden, CO), 1 μg of IgG anti-human EAAC1 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), 4 μg of IgG anti-human GTRAP3–18 (Abcam Inc., Cambridge, MA), and 85 μg of IgG anti-bovine GDH (United States Biological, Swampscott, MA), respectively, per mL of blocking solution [1% nonfat dry milk (wt/vol; Carnation, Nestle, Solon, OH) in 30 mM Tris-Cl (pH 7.5), 200 mM NaCl, 0.1% Tween 20 (vol/vol)] for 1.5 h at room temperature with gentle rocking. For AST and ALT detection, blots were hybridized with 20 μg of IgG anti-mouse AST (Fitzgerald Industries International Inc., Concord, MA) or 40 μg of IgG anti-porcine ALT (United States Biological), respectively, per mL of blocking solution [1.5% nonfat dry milk (wt/vol) in 30 mM Tris-Cl (pH 7.5), 200 mM NaCl, 0.1% Tween-20] for 1.5 h at room temperature with gentle rocking. GPR30 was probed using 0.4 μg of IgG anti-human GPR30 polyclonal antibody (Abcam Inc., Cambridge, MA) per mL of blocking solution [1% nonfat dry milk (wt/vol), 30 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (vol/vol)] overnight at 4°C with gentle rocking. Lastly, GS was probed using 1.25 μg of IgG anti-sheep polyclonal antibody (BD Biosciences, San Jose, CA) per mL of blocking

solution [5% nonfat dry milk (wt/vol), 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.1% Tween 20 (vol/vol)] for 1 h at 37°C with gentle rocking.

All protein-primary antibody binding reactions were visualized with a chemiluminescence kit (Pierce, Rockford, IL) after hybridization of primary antibodies with horseradish peroxidase-conjugated donkey antirabbit IgG (Amersham, Arlington Heights, IL; GLT-1 and EAAC1, 1:5,000; and GDH, 1:7,500); horseradish peroxidase-conjugated goat antimouse IgG (BD Biosciences; GS, 1:5,000); horseradish peroxidase-conjugated rabbit anti-sheep IgG (Santa Cruz Biotechnology; ALT and AST, 1:5,000); horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam Inc.; GPR30, 1:5,000); or horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology; GTRAP3–18, 1:5,000).

Densitometric analysis of immunoreactive products was performed as described previously (Howell et al., 2003; Fan et al., 2004). Briefly, after exposure of autoradiographic film (Amersham), digital images of all observed immunoreactive species were recorded and quantified using the BioRad Versadoc imaging system and the Quantity One Program (version 2.3, BioRad). A single immunoreaction product was assessed for treatment effect by densitometric analysis as follows:
AST, 41kDa; ALT, 23 kDa; GDH, 55 kDa; GS, 43 kDa; GLT-1, 74 kDa; EAAC1, 69 kDa; and GTRAP3–18, 41 kDa. The linearity of antibody-ligand immunoreactions and densitometry were validated using immunoblots containing protein gradients (data not shown). Data were collected as arbitrary densitometric units and then were corrected for unequal loading, transfer of proteins, or both by normalization to densitometric values of Fast-Green-stained (Fisher Scientific, Pittsburgh, PA) proteins common to all

immunoblot lanes/samples. For all results, densitometric values were normalized to the average d16 control value for each protein by dividing the actual value for the animal by the average control value. Digital images were prepared with Power-Point (Microsoft PowerPoint 2003, Bellevue, MA).

Extraction of Total RNA and Relative Real-Time Reverse-Transcriptase-Polymerase Chain Reaction Assay

RNA Extraction and Purification. Approximately 200 mg of liver were homogenized on ice for 30 s (setting 11, Polytron Model PT10/35, Kinematic, Inc., Switzerland) in 2 mL of 4°C TRIzol Reagent (Invitrogen, Carlsbad, CA).

Total RNA was obtained by an acidic phenol-chloroform extraction as per instruction of the manufacturer (Invitrogen). Briefly, chloroform (3 mL or volume to volume) was added to each TRIzol® homogenate sample in sterile 7-mL polypropylene tubes (Fisher Scientific) and samples were shaken vigorously. After 3 min incubation in ice, samples were spun at 12,000 x g for 10 min at 4°C. The aqueous phase containing RNA was transferred to a fresh, sterile 7-mL polypropylene tube. After the addition of an equal volume of ice-cold isopropanol, samples were precipitated at -80°C for 10 min, and then at -20°C overnight. Samples were then thawed and spun at 12,000 x g for 10 min at 4°C and the supernatant was removed. Each RNA-containing pellet then was washed gently with 75% ethanol (0.5 mL) and spun at 7,500 x g for 5 min at 4°C. After the supernatant was removed, each tube was allowed to dry for 10 min. Pellets were

resuspended in 100 µL of DNAse/RNAse-free water, transferred to 1.5 mL microcentrifuge tubes, and stored at -80°C.

After the crude RNA was recovered, a purification procedure was performed using RNeasy Mini Kit (Qiagen, Valencia, CA) to minimize genomic DNA contamination (Applied Biosystems, 2004) and enrich all the mRNA longer than 200 nucleotides in molecular size. Purified RNA was then eluted with 60 µL of RNase-free distilled H2O and stored at –80°C.

Purified RNA was then eluted with 60 µL of RNase-free distilled H_2O and stored at –80°C. The integrity of the purified RNA was examined by gel electrophoresis using Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA) at the University of Kentucky Microarray Core Facility. Visualization of the gel images and electropherograms showed that all RNA samples had high quality with RNA integrity number greater than 8.0 and 28S/18S rRNA ratio greater than 1.8. The purity and concentration of the purified RNA samples was analyzed by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), which revealed that all the samples were of high purity with 260/280 absorbance ratios greater than 2.0 and 260/230 absorbance ratios greater than 1.75.

Reverse Transcription (RT). Approximately 3 µg of crude RNA was first treated with DNase I enzyme (amplification grade) in accordance with the manufacturer's instructions (Invitrogen) to ensure that no DNA was present. Briefly, one RNA sample was combined with 1 µL of 10x reaction buffer, 1 µL of DNase I (1 U/µL), and DEPC-treated H2O up to 10 µL, incubated at room temperature for 15 min, and then 1 µL of 25 mM EDTA was added to stop the reaction by incubating at 65°C for 10 min. Then the

DNase-treated RNA samples were reverse transcribed to cDNA by using SuperScript III First-Strand Synthesis System in accordance with the manufacturer's instructions (Invitrogen). Briefly, a solution of hexamers (50 ng/μL) and oligo (dT)20 primer (50 μM) mix (1 μL each) was added to one DNase-treated sample (7 μL in volume), incubated at 70°C for 10 min, and then chilled on ice for 1 min. A solution containing 2 μL of RT buffer (10x), 2μL of dithiothreitol (0.1 M), 4 μL of MgCl2 (25 mM), 1 μL of dNTP (10 mM each), and 1 μL of RNAse Out was then added to the reaction. After incubation at 37°C for 2 min, the reaction was incubated with 1 μL reverse transcriptase at room temperature for 10 min, and then incubated at 50°C for 50 min. To stop the reaction, the reaction mixture was incubated at 70°C for 10 min and then chilled on ice. The resulting reaction products, cDNA, were stored at –20°C until used in real-time PCR.

Real-Time RT-PCR. The relative mRNA quantification methodology for the selected DEG identified from the microarray analysis were developed using 2-step real-time RT-PCR technique following the standard procedure routinely used in our laboratory (Liao et al., 2008, 2010). Before conducting real-time RT-PCR with ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), primer and probe sets for GS, ALT, and 18S cDNA were designed and manufactured using ABI Assays-by-Design Service (Applied Biosystems). Bovine-specific nucleotide sequences were obtained from previously published Genbank (http://www. ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) sequences or from the bovine-specific database (Cattle Gene Index database) of The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/tgi/) by using the Basic Local Alignment Search Tool (BLAST) function and the appropriate human ortholog nucleotide sequence (Genbank). These

specific parameters including the sequences of the forward and reverse primers and probes for relative real-time polymerase chain reaction are provided (Table 3.2). Each Assays-by-Design primer and probe set consists of 2 unlabeled PCR primers and one TaqMan Minor Groove Binding probe with FAM, a reporter dye labeled at 5' end. Components of a 25-µL real-time PCR reaction were an Assays-by-Design Primer and Probe set (1.25 µL), TaqMan Universal PCR Master Mix-No AmpErase UNG (12.5 µL), cDNA template (1.0 to 2.0 µL), and DNase/RNase free H_2O (9.25 to 10.25 µL). The PCR conditions used for the amplification and quantification were an initial denaturing stage (95°C for 10 min), followed by 40 cycles of 2 amplification stages for denaturing (95°C for 15 s) and annealing/extension (60°C for 1 min), with a melting curve program (60 to 95°C), a heating rate of 0.15°C/s, and continuous fluorescence measurements. To establish mRNA relative quantification methodology, the real-time PCR products were validated by DNA sequence verification, as per Liao et al. (2008).

Relative mRNA Quantification Methods. For the relative quantification of GS and ALT mRNA expression levels, real-time quantitative RT-PCR methodology that used 2-step regimen was developed in accordance with ABI guidelines (Applied Biosystems, 2004). In the first step, all the RNA samples were reverse-transcribed to cDNA as described above for RT reaction. In the second, real-time PCR step, relative standard curve methods were established for both GS and ALT cDNA. For each mRNA quantified, ribosmal 18S RNA was selected as an endogenous control to normalize the variations in sample preparation, mRNA inputs, and RT efficiencies (Liao et al., 2008). Bovine liver total RNA was reverse transcribed, the cDNA sample was serially diluted 2.5×, 5×, 25×, 125×, 625×, 3,125×, 15,625×, 78,125×, and 390,625×, and the linear range

for target mRNA quantification was established to ascertain an appropriate amount of cDNA to be used for a standard curve method. For each cDNA sample the real-time PCR reactions (as described above) were conducted in triplicate to average out the potential pipetting, mixing, or plate preparation errors. The minimal threshold (CT) values detected using these dilutions were 35 and 21 for the target and 18S cDNA, respectively. As a result, the optimal detection of GS, ALT and 18S cDNA were achieved by using 1:5, 1:5 and 1:15,625 dilutions of the RT product stocks, respectively. The estrogen treatment effect on the expression of 18S rRNA by the liver was evaluated by comparing the CT values obtained from the real-time PCR reactions (Applied Biosystems, 2004). The relative quantities of GS and ALT mRNA expression were normalized to the relative 18S quantities by calculating the target gene:18S relative quantity ratios, and these 18S-normalized quantity ratios were used for estrogen treatment effect on GS and ALT mRNA expression, the 18S-normalized ratio from the control animals (i.e., sham-implanted) was designated as a calibrator. Then the 18S-normalized ratios for the estrogen-implanted cows, as well as the control animals, were divided by the ratio of the calibrator, respectively. The CT values for 18S rRNA quantities and the calibrated values for estradiol treatment effect were all subjected to statistical analysis.

Statistical Analysis

Data are presented as least square means (± SEM). Experimental parameters were evaluated for treatment differences by ANOVA (version 8.01, SAS Inst. Inc., Cary, NC). For all parameters, the MIXED procedure of SAS with the REPEATED statement was used. Cow was the experimental unit and the effects of estrogen treatment (TRT), time

after implant (DAY), and their interaction (TRT x DAY) were assessed. The statistical model used estrogen treatment as the fixed effect. Class variables were estrogen treatment (TRT) and cow, with cow included in the random statement. Kenward-Roger adjustment was used to calculate the denominator df (Kenward and Roger, 1997).

RESULTS AND DISCUSSION

Plasma Estradiol Concentrations, but not Progesterone, Were Increased in Compudose-implanted Cows

The primary goal of this project was to determine if exogenous estradiol affects the expression of GS, ALT, AST, and other proteins associated with hepatic metabolism of alanine and glutamine in old beef cows. To elaborate these findings, the potential effects of estradiol supplementation on various blood analytes also were determined. However, first, to validate that estradiol implantation actually increased plasma estradiol levels, plasma estrogen levels were compared between control and implanted cows (Table 3.3). Estradiol levels of control cows were typical (personal communication, Keith Schillo, University of Kentucky). The overall experimental mean plasma estradiol concentration of implanted cows (5.07 pg/mL) was 221% more ($P = 0.007$) than for control cows (1.58 pg/mL). In contrast, neither DAY nor TRT × DAY interactions were detected. These results reflect similarly increased levels of estrogen in estradiol-implanted cows at d 14 and 28 d after implantation, vs non-implanted control cows.

Normally in reproductively mature cows, the ovary is the main source of progesterone, estrogen, and androgens (Allen and Doisy, 1923) and the relative amounts

of these steroids vary during the estrous cycle and throughout pregnancy. During the follicular stage of the estrous cycle, estradiol is the predominant steroid hormone secreted by the ovarian follicles. Specifically, in the follicle, thecal cells produce androstenedione that is then delivered to the granulosa cells to be aromatized into estradiol and other estrogens (Hillier et al., 1994). As the follicle matures, estrogen production continually increases until an abrupt release of two anterior pituitary hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), induce ovulation. Generally, after ovulation, progesterone becomes the predominant hormone secreted by the ovary. Specifically, the corpus luteum (CL), formed from the ruptured follicle, is responsible for the production of progesterone during the luteal phase of the estrous cycle and, if applicable, throughout pregnancy.

In our old cow model, plasma progesterone levels were not affected by estradiol TRT (P = 0.229), DAY (P = 0.226), or TRT x DAY (P = 0.337) (Table 3.3). These results indicate that the administration of exogenous estradiol did not affect plasma progesterone concentrations after 14 or 28 d of implantation. Thus, exogenous estradiol did not appear to affect the estrus cycle of implanted cows even though, quantitatively, the mean progesterone concentration of implanted cows was about 50% that of implanted cows on d 14 and non-implanted cows on both d 14 and 28. Comparison of d 14 and d 28 estradiol and progesterone levels within each cow (data not shown) revealed that all cows experienced estrous during the experimental period.

Cow BW were measured at d 0, 14, and 28. By design, initial BW did not differ (P = 0.392) between control (585 kg) and implant (621 kg) cows. By d 14, both groups had lost 13 ± 2 kg (P = 0.019), with no difference in BW (P = 0.407) between control

(573 kg) and implant (607 kg) groups. From d 14 to d 28, control group cows gained 4 ± 3 kg and implanted cows gained 6 ± 4 kg. However, these apparent differences in BW gain were not statistically significant ($P \geq 0.407$). Consistently, there was no differences ($P = 0.407$) in the BW between control and implant animals over the whole 28 d experiment. These results indicate that despite an elevated blood estradiol, BW was not affected after 28 d of cows consuming the alfalfa hay-based diet *ad libitum*.

Estradiol Implantation Increases Hepatic GS mRNA and Protein Content, But has Little Effect on Other Glutamate/Glutamine/Alanine Metabolizing Proteins

The activities of GS, ALT, and other anionic amino acid-metabolizing enzymes are essential to facilitate the metabolism of glutamine and aspartate (Heidger and Welbourne, 1999; Welbourne and Nissim, 2001). In the liver, glutamine arriving from the portal vein is absorbed by the periportal hepatocytes by the hepatic system N activity (Kilberg et al., 1980) and converted to L-glutamate and ammonia by mitochondrial glutaminase. Mitochondrial ALT also can produce glutamate by converting skeletal muscle-derived alanine to glutamate and pyruvate in the periportal hepatocytes. Glutaminase- and ALT-derived glutamate can be metabolized by periportal hepatoycte-localized GDH to ammonia and α-ketoglutarate, or glutamate can be exported into the sinusoid and then absorbed by pericentral hepatocyte-localized EAAC1 or GLT-1 glutamate transporter. Recently, GTRAP3-18 has been shown to be an inhibitory regulator of at least neuronal EAAC1 activity and GTRAP3-18 abundance is reversely proportional to EAAC1 activity (Ruggiero et al., 2008; Watabe et al., 2008). Once absorbed by the periportal hepatocytes, sinusoidal glutamate and ammonia can be

conjugated by GS to glutamine. In cultured rat glial cells (Hagheighat, 2005) and mouse brain tissue (Blutstein et al., 2006), estradiol is known to upregulate GS content and function. In contrast, estrogen did not affect GS activity in cultured primary rat hepatocytes (Sirma et al., 1996).

To determine the effect(s) of implant-administered exogenous estrogen (Table 3.3-3.4) on certain proteins responsible for aspartate, alanine, and glutamine metabolism in the liver, immunoblot analysis was performed to quantify the relative liver content of GS, ALT, AST, GDH, GLT-1, EAAC1, and GTRAP3-18 in estradiol implanted vs non-implanted old cows (Table 3.4). For GS, estradiol TRT ($P = 0.008$), DAY ($P = 0.024$), and TRT x DAY ($P = 0.024$) effects were observed. More specifically, GS content was increased 350% by d 14 and 200% by d 28. However, in contrast to GS, estradiol supplementation did not affect ($P \geq 0.159$) the relative content of any other evaluated protein. Although a DAY effect was observed for ALT ($P = 0.033$), AST ($P = 0.083$), GLT-1 ($P = 0.005$), EAAC1 ($P = 0.083$), and GTRAP3-18 ($P = 0.014$), no TRT x DAY interactions were found for these proteins. Collectively, these findings indicate that hepatic expression of GS in old beef cows is sensitive to stimulation by supplemental estradiol, whereas expression of other proteins that support hepatic glutamate metabolism are not.

To determine if estradiol implantation affected the transcription of ALT and GS, the relative mRNA content of ALT and GS was compared in control vs estradiol-implanted cows (Table 3.5). GS mRNA expression was increased ($P = 0.024$) 34 and 99% at d 14 and 28, respectively, in implanted vs non-implanted cows. These results are consistent with the observed increase in GS protein content in estradiol-implanted cows

and indicate that estradiol-mediated upregulation of GS expression occurs at least at the transcriptional level. For ALT mRNA content, a tendency (P = 0.087) for a TRT x DAY interaction was observed, apparently reflecting a 39% increase in ALT mRNA content at d 28 by old cows receiving the estradiol implants, and suggesting that that any effect of estradiol on ALT mRNA expression is over-ridden by post-transcriptional events.

Estradiol Supplementation Increases Blood Content of Total Protein, Globulin, γ-glutamyltransferase, and Potassium

To gain a greater understanding of the potential effects of increased estradiol blood levels and hepatic GS content on whole-body metabolism of old cows, the profiles of serum enzymes and other blood constituents were compared between control and estradiol-implanted cows (Table 3.6). Plasma ammonia and serum urea N levels were not affected by estradiol ($P \geq 0.348$) or day ($P \geq 0.724$), suggesting that the urea cycle function was not influenced by estrogen supplementation. Similarly, no estradiol TRT ($P \geq 0.635$) or DAY ($P \geq 0.255$) effects were found on the amount of serum alkaline phosphatase (ALP), ALT, or AST activities.

As an indicator of whole-body protein synthetic status, the concentration of total, albumin, and globulin serum proteins was determined. Total serum protein was increased by estradiol TRT (P = 0.021) and DAY (P = 0.001), and no TRT x DAY interaction (P = 0.279) was observed, reflecting 6.5 and 8.6% more total serum protein at d 14 and d 28, respectively, in estradiol- vs non-implanted cows. Two principal proteins measured in the total serum protein levels are albumin and globulin. Albumin is produced predominantly by the liver, whereas globulin is synthesized by the immune system and by the liver,

depending on the globulin type. Serum albumin concentration was not affected (P = 0.827) by estradiol TRT. An observed DAY effect (P = 0.011) without a TRT x DAY interaction (P = 0.240) appears to reflect the 3% greater amount of albumin in both groups of cows by d 28 vs d 14. In contrast, serum globulin levels in estradiol-treated cows were greater (P = 0.035) than for control cows, even though globulin levels for both groups were higher on d 28 than 14. Globulins have various functions, including triggering immune responses to infections and transporting metals and other molecules. Even though the albumin/globulin ratio values were numerically lower for estradiol-treated cows, no estradiol TRT (P = 0.122) or DAY (P = 0.796) effects were observed. However, a tendency for a TRT x DAY interaction was observed, which likely reflects the higher globulin, thus lower albumin/globulin ratio, of the estradiol-treated cows by d 28. The observation that serum albumin concentrations were within reference values for both groups, indicates that the cows were not suffering from dehydration. In contrast, serum globulin levels for both groups were higher than the reference value.

Although not affected by TRT alone (P = 0.271), there was a DAY effect (P = 0.011) and DAY x TRT interaction (P = 0.020) on γ-glutamyltransferase activity, apparently reflecting a 36% increase in on γ-glutamyltransferase from 14 d to 28 d. γ-glutamyltransferase is involved in glutathione metabolism by catalyzing the conversion of glutathione + an amino acid to cystinylglycine + 5-L-glutamyl amino acid (Kaneko et al., 1997). GGT is localized to cell membranes that have high secretory and absorptive capacity such as those in the liver, kidney, pancreas, and most other cells, except skeletal muscle which lacks GGT activity. In the liver, γ-glutamyltransferase is produced by the hepatic microsomes which is ubiquitously distributed in cells involved in bile absorption

and secretion, and is an important indicator of liver cell damage and cholestasis (Cabrera-Abreu and Green, 2002). Hepatic γ-glutamyltransferase activity is primarily utilized to identify hepatobillary insults because it is more sensitive to minimal liver damage than ALP (Nemesanszky and Lott, 1985; Bartholomew et al., 1987) and typically increases with liver damage.

The breakdown of hemoglobin by the reticuloendothelial system results in formation of bilirubin (Giannini et al., 2005), and the hemoglobin for the production of bilirubin comes from the senescent erythrocytes (Dufour et al., 2000). An increase in serum bilirubin suggests liver damage, however, total bilirubin activity was not affected by TRT ($P = 0.904$). Similar to bilirubin levels, changes in blood creatinine levels can be indicative of altered levels of protein catabolism in cattle (Roubicek et al., 1970), or be indicative of altered glomerular filtration rates and, thus, renal dysfunction (Perrone et al., 1992). However, creatinine levels in plasma were not affected by estradiol TRT, DAY or their interaction ($P > 0.128$).

In terms of indicators of energy metabolism, no TRT ($P \geq 0.494$), DAY ($P \geq 0.099$), or TRT x DAY interactions ($P \geq 0.556$), were found for concentrations of creatine kinase, glucose, and cholesterol, or for LDH activity. However, a TRT x DAY interaction on triglyceride concentration appears to reflect a % higher ($P = 0.035$) level of triglycerides on d 14 in estradiol-implanted vs control cows.

To determine if estrogen treatment affect mineral metabolism, serum minerals were measured in control and estradiol implanted cows (Table 3.7). There was no TRT effect ($P > 0.197$) on serum levels of calcium, chloride, magnesium, phosphorus, or sodium. However, there was a DAY effect ($P \leq 0.046$) on calcium, chloride, and

magnesium concentrations. Over time, both calcium and chloride increased by 9% and 2%, respectively, whereas, magnesium decreased by 10% from 14 d to 28 d. However, a DAY effect was not detected ($P = 0.598$) on serum phosphorus concentration. In contrast to the other minerals, there was an estrogen TRT ($P = 0.031$) and DAY ($P = 0.001$) effect on potassium concentrations, but no TRT x DAY interaction ($P = 0.367$). Overall, the potassium serum concentration of implanted cows was 8% higher than for control cows and potassium levels of both control and implanted cows increased 14.5% from 14 d to 28 d.

Because estrogen is known to affect immunological responses after injury (Kovacs et al., 2002; Bird et al., 2008), the effect of estrogen on the presence of blood cell types was evaluated (Table 3.8). Between treatments, no TRT or TRT x DAY interaction effects were detected for hemoglobin ($P > 0.599$) or packed cell volume ($P \geq 0.390$), nor for RBC, WBC, neutrophil, lymphocyte, or eosinophil counts ($P \geq 0.227$). In both control and implanted cows, there was a DAY effect ($P \leq 0.006$) on both hemoglobin concentration and monocyte cell counts. From d 14 to 28, hemoglobin levels increased 3%, whereas monocyte levels decreased 60%. Overall, all values from the complete blood count fall within the normal reference ranges, thus indicating a normal health status for both groups.

Estrogen Receptor-mediated Cell Signaling

In representations of the classical (intracellular) estrogen signaling pathway (Behl, 2002; Kelly et al., 2005), estrogen can transverse across the plasma membrane to bind to the estrogen receptor (ER) α (ERα) or β (ERβ). After binding with an ER, the

estrogen/ER complex migrates into the nucleus and initiates transcription by either directly interacting with the estrogen response element (ERE) on the promoter region of target genes or indirectly by interacting with other transcriptional factors (e.g., . β-catenin, Ras). These effects are considered long-term "genomic" effects.

Besides activating the classical estrogen pathway through binding of ERα and ERβ, estrogen also can bind to described membrane-bound plasma receptors, such as GPR30 (Thomas et al., 2005; Heldring et al., 2007). Although this "non-genomic" estrogen pathway is not fully understood, or even described in cattle, in the human brain, estrogen binding of GRP30 activates the adenylyl cyclase, phosphoinositol 3 kinase (PI3K), and the phospholipase C pathways (Thomas et al., 2005; Heldring et al., 2007, Albanito et al., 2007). Also, in the human liver, estrogen has been indicated to enhance liver function after a trauma-induced hepatic hemorrhage via GPR30-mediated protein kinase A activation (Hsieh et al., 2007). Moreover, evidence for cross-talk between ER and GPR30 has been accumulating (Sheng and Zhu, 2011).

In cattle, ERα and ERβ mRNA have been observed in the liver, uterus, mammary gland, lung, heart, kidney, gastrointestinal tract, and skeletal muscle (Pfaffl et al., 2001). We attempted to measure the relative content of ERα but were unable to because of the failure of commercially-available antibodies to recognize ERα in cow liver homogenates. However, the specificity of the human anti-GPR30 antibody against the putative bovine GPR30 was validated using antibody:antigen peptide prehybridization methods (Xue et al., 2011) and homogenates of liver from control animals (Figure 3.1). Two (40 and 58 kDa) GPR30 immuno-reactive products were observed to be sensitive to increasing amounts of the GPR30 antigen. To determine if the expression of GPR30 played a role in

the estrogen-mediated effect of glutamine synthetase up-regulation, the protein content of GPR30 was evaluated in the liver of old cows given exogenous estrogen (Table 3.4). Although a DAY effect was observed (P = 0.010), neither a TRT (P = 0.968) nor TRT x DAY interaction (P = 0.732) were found. This result indicates that GPR30 protein expression was not affected by increased levels of estradiol supplementation achieved through the use of Compudose.

Overall, there are three salient observations from this study. First, the levels of plasma estradiol resulting from 28 days of Compudose implantation in old cycling beef cows have been characterized. To our knowledge, this is the first report of actual plasma estradiol concentrations resulting from estradiol implantation in cows. Second, the hypothesis that hepatic GS mRNA and protein expression would be up-regulated by exogenous estradiol is accepted as the defined (1- to 2-fold) increase in plasma estradiol of Compudose-implanted old cows was concomitant with an increase in GS protein (3-fold) and mRNA (0.3- to 0.6-fold). Conversely, the hypothesis that ALT and AST expression would be decreased is rejected. Future experimentation that characterizes amino acid concentrations in liver and blood of estradiol-implanted cattle will be required to understand the significance of these finding on whole-body N metabolism. Third, the identification that cattle liver expresses GRP30 protein is a novel observation, and enables subsequent research into GRP30-mediated cellular metabolism. Fourth, elevated plasma estradiol for 28 d had little affect on blood biochemical and clinical indicators of whole-body metabolism.

Table 3.1: Proximate and mineral analysis of alfalfa hay fed to old cows throughout the trial (dry matter basis)[1]

Item	
Proximate analysis	
DM, %	91.4
CP, %	13.4
Available Protein, %	12.0
ADICP, %	1.4
Adjusted CP, %	13.3
Soluble Protein % CP	27.5
Degradable Protein %CP	60.0
NDICP, %	4.9
ADF, %	41.2
NDF, %	62.6
Lignin, %	7.9
NFC, %	18.8
NSC, %	7.0
Starch, %	2.0
Sugar, %	5.1
Crude Fat, %	2.0
TDN, %	54
NEL, Mcal/lb	0.47
NEM, Mcal/lb	0.45
NEG, Mcal/lb	0.20
Mineral analysis	
Ash, %	8.18
Calcium, %	0.92
Phosphorus, %	0.29
Magnesium, %	0.24
Potassium, %	2.09
Sulfur, %	0.19
Chloride Ion, %	0.50

[1]Proximate and mineral values are an average of 15 samples collected throughout the trial and presented on a DM basis.

Table 3.2: Primer and probe sets used for the real-time quantitative PCR analyses of metabolic enzyme mRNA and 18S rRNA

Primer and Probe[a]	Location on template (bp)	Sequence[b]	Amplicon size (bp)
GS (TIGR TC274284)			
Forward	702-720	5'-CACGAATGCCGAGGTCATG-3'	
Probe (fwd.)	726-739	5'-FAM-ACAGTGGGAATTCC-3'	62
Reverse	740-763	5'-CGATTCCTTCACAGGGTCCTATCT-3'	
ALT (TIGR TC310617)			
Forward	975-996	5'-CCTCCTTCCACTCGATCTCCAA-3'	
Probe (rev.)	1002-1017	5'-FAM-CCGCACTCGCCCATGT-3'	79
Reverse	1031-1053	5'-TCCATATTCACCACCTCCACGTA-3'	
18S (GenBank DQ222453)			
Forward	548-572	5'-CCCTGTAATTGGAATGAGTCCACTT-3'	
Probe (rev.)	593-611	5'-FAM-CCAGACTTGCCCTCCAATG-3'	100
Reverse	625-647	5'-ACGCTATTGGAGCTGGAATTACC-3'	

[a]GS = glutamine synthetase; ALT = alanine transaminase; 18S = 18S ribosomal RNA. The contents in the parentheses associated with each protein are the accession numbers for the reported sequences retrieved from the public databases (TIGR or GenBank) and used as templates for designing primers and probes. The custom TaqMan probes were supplied in either the forward (fwd.) or the reverse (rev.) orientation as indicated in the parentheses for each probe.

[b]"FAM" labeled at the 5' end of the TaqMan probe is six-carboxy-fluorescein which is used as a reporter dye in the real-time PCR procedure.

Table 3.3: Plasma estradiol and progesterone concentrations of old cows receiving Control or Estradiol treatment[1]

	Treatment							Overall P-value		
	Control			Estradiol						
Item	**14 d**	**28 d**	**Overall**	**14 d**	**28 d**	**Overall**	**SEM[2]**	**TRT**	**DAY**	**TRT × DAY**
Estradiol, pg/mL	1.65	1.50	1.58	4.14	5.99	5.07	1.04	0.007	0.415	0.339
Progesterone, ng/mL	4.56	4.29	4.43	4.45	2.22	3.34	0.92	0.229	0.226	0.337

[1]Data are presented as least square means (n = 7) ± SEM for hormone concentrations of Control and Estradiol (25.7 mg estradiol) treatment cows collected at 14 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.

Table 3.4: Densitometric analysis of content of AA enzymes and transporters in liver homogenates of old cows receiving Control or Estradiol treatment[1]

	Treatment							Overall P-value		
	Control			Estradiol						
Item[2]	14 d	28 d	Overall	14 d	28 d	Overall	SEM[3]	TRT	DAY	TRT× DAY
GS[4]	1.00[a]	0.97[a]	0.99	4.50[b]	2.90[c]	3.70	0.65	0.008	0.024	0.028
ALT	1.00	0.34	0.67	0.61	0.34	0.48	0.21	0.402	0.033	0.332
AST	1.00	1.48	1.24	0.90	1.39	1.15	0.26	0.713	0.083	0.978
GDH	1.00	0.82	0.91	1.00	1.05	1.03	0.14	0.514	0.514	0.251
GLT-1	1.00	1.95	1.48	0.90	1.56	1.23	0.24	0.329	0.005	0.534
EAAC1	1.00	0.63	0.82	1.19	0.84	1.02	0.16	0.159	0.083	0.980
GTRAP3-18	1.00	0.64	0.82	0.86	0.53	0.70	0.13	0.409	0.014	0.874
GPR30	1.00	2.06	1.53	1.13	1.97	1.55	0.44	0.968	0.010	0.732

[1]Values (normalized arbitrary units) are arithmetic means (n = 7) ± SEM of relative protein content from liver biopsy samples of Control and Implant (25.7 mg estradiol) treatment cows collected at 14 and 28 d after implanting. Densitometric evaluation of all immunoreactive species was performed. Data were analyzed as a repeated measure as described in Materials and Methods.

[2]Values were normalized to the average control value at 16 d for each protein by dividing the actual value for the animal by the average control value. The following is a list of those average control values (in arbitrary units) ± SE used for normalization: ALT, 892 ± 259; AST, 1,542 ± 397; EAAC1, 1,614 ± 155; GDH 2,605 ± 270; GPR30, 1,108 ± 425; GS, 569 ± 245; GLT-1, 830 ± 164; GTRAP3-18, 2,590 ± 448.

[3]Most conservative error of the mean.

[4]Means in a column that lacks a common letter differ ($P \leq 0.056$).

Table 3.5: Relative content of ALT and GS mRNA in liver homogenates of old cows receiving Control or Estradiol treatment[1]

	Treatment							Overall P-value		
	Control			Estradiol						
Item[2]	**14 d**	**28 d**	**Overall**	**14 d**	**28 d**	**Overall**	**SEM[3]**	**TRT**	**DAY**	**TRT × DAY**
GS	1.00	0.79	0.90	1.34	1.57	1.46	0.23	0.024	0.961	0.313
ALT	1.00	1.04	1.02	0.90	1.43	1.17	0.16	0.410	0.054	0.087

[1]Data are the least square means (n = 6-7) ± SEM of the relative mRNA quantities from liver biopsy samples of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implanting.
[2]The normalized quantities of the control and estrogen treatment groups on 14 and 28 d calibrated to the control group on 14 d. Data were analyzed as a repeated measure as described in Materials and Methods.
[3]Most conservative error of the mean.

Table 3.6: Serum and plasma analytes of old cows receiving Control or Estradiol treatment[1]

	Treatment							Overall P-value			
	Control			Estradiol							
Item[2]	14 d	28 d	Overall	14 d	28 d	Overall	SEM[3]	TRT	DAY	TRT × DAY	Reference Range[4]
Ammonia, mM[6]	0.028	0.028	0.028	0.030	0.032	0.031	0.004	0.567	0.724	0.724	-
Urea nitrogen, mg/100 mL	11	12	12	11	11	11	1	0.348	1.000	0.341	5.0-27.0
ALP, U/L	50	46	48	54	45	50	7	0.856	0.255	0.674	100-500
ALT, U/L	22	23	23	23	23	23	2	0.889	0.570	0.400	11-40[5]
AST, U/L	79	69	74	70	71	70.5	5	0.635	0.289	0.223	0-160
Total protein, g/100 mL	7.7	8.1	7.9	8.2	8.8	8.5	0.18	0.021	0.001	0.279	6.50-7.50
Albumin, g/100 mL	3.3	3.4	3.4	3.3	3.4	3.4	0.1	0.827	0.011	0.240	2.3-3.7
Globulin, g/100 mL	4.5	4.7	4.6	4.9	5.4	5.2	0.2	0.035	0.014	0.178	3.0-3.5
Albumin/Globulin ratio	0.71	0.75	0.73	0.69	0.63	0.66	0.04	0.122	0.796	0.089	0.8-1.0
γ-glutamyltransferase, U/L	14	14	14	14	19	17	1	0.271	0.011	0.020	2-20
Total bilirubin, mg/100 mL	0.40	0.43	0.42	0.41	0.43	0.42	0.05	0.904	0.577	0.852	0.0-0.5
Creatinine, mg/100 mL	1.4	1.3	1.4	1.5	1.4	1.5	0.1	0.342	0.128	0.381	1.00-2.00

[1]Data are presented as least square means (n = 6-7) ± SEM for serum enzymes of Control and Implant (25.7 mg estradiol) treatment cows collected at 14 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase.
[3]Most conservative error of the mean.
[4]Taken from the University of Kentucky Livestock Disease Diagnostic Lab unless noted otherwise.
[5]Taken from Kaneko et al., 1997.
[6]Plasma.

Table 3.6: Serum and plasma analytes of old cows receiving Control or Estradiol treatment[1] (con't)

	Treatment							Overall P-value			
	Control			Estradiol							
Item[2]	14 d	28 d	Overall	14 d	28 d	Overall	SEM[3]	TRT	DAY	TRT × DAY	Reference Range[4]
Creatine kinase, U/L	114	143	129	124	161	143	19	0.494	0.099	0.834	100-650
Glucose, g/100 mL	105	101	103	105	101	103	13	0.983	0.627	0.977	40-100
LDH, U/L	997	1037	1017	1008	1020	1014	55	0.960	0.510	0.715	629-1445
Triglycerides, g/100 mL	25	29	27	32	30	31	2	0.153	0.351	0.035	-
Cholesterol, mg/100 mL	98	107	103	99	102	101	5	0.757	0.179	0.556	62-193

[1]Data are presented as least square means (n = 6-7) ± SEM for serum enzymes of Control and Implant (25.7 mg estradiol) treatment cows collected at 14 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase.
[3]Most conservative error of the mean.
[4]Taken from the University of Kentucky Livestock Disease Diagnostic Lab unless noted otherwise.
[5]Taken from Kaneko et al., 1997.
[6]Plasma.

Table 3.7: Serum minerals of old cows receiving Control or Estradiol treatment[1]

Item	Treatment						SEM[2]	Overall P-value			Reference Range[3]
	Control			Estradiol							
	14 d	28 d	Overall	14 d	28 d	Overall		TRT	DAY	TRT × DAY	
Calcium, mg/dL	8.4	9.2	8.8	8.5	9.2	8.9	0.2	0.874	0.001	0.653	9-12
Chloride, mg/dL	107	109	108	107	108	108	1	0.928	0.046	0.664	97-111
Magnesium,	1.6	1.5	1.6	1.7	1.4	1.6	0.1	0.673	0.011	0.261	1.5-2.4
Phosphorus, mg/dL	5.6	5.5	5.6	5.9	5.4	5.7	0.6	0.874	0.598	0.719	4-7
Potassium, mmol/L	3.1	3.7	3.4	3.5	3.9	3.7	0.1	0.031	0.001	0.367	3.9-5.8
Sodium, mmol/L	142	143	143	145	143	144	1	0.197	0.438	0.186	132-152

[1]Data are presented as least square means(n = 6-7) ± SEM for serum minerals of Control and Implant (25.7 mg estradiol) treatment cows collected at 14 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.
[3]Taken from the University of Kentucky Livestock Disease Diagnostic Lab.

Table 3.8: Blood cell types of old cows receiving Control or Estradiol treatment[1]

	Treatment							Overall P-value			
	Control			Estradiol							
Item	**14 d**	**28 d**	**Overall**	**14 d**	**28 d**	**Overall**	**SEM[2]**	**TRT**	**DAY**	**TRT × DAY**	**Reference Range[3]**
RBC, $1\times10^6/\mu L$	7.22	7.30	7.26	6.86	7.01	6.94	0.25	0.369	0.367	0.771	5-10
Hemoglobin, g/dL	12.6	13.0	12.8	12.3	12.7	12.5	0.45	0.599	0.006	0.961	8-15
Packed cell volume, %	38.8	39.3	39.1	36.9	37.7	37.3	1.42	0.390	0.312	0.745	24-46
WBC, $1 \times 10^3/\mu L$	7.19	7.18	7.19	7.72	7.40	7.56	1.10	0.801	0.801	0.806	4-12
Neutrophils, $1 \times 10^3/\mu L$	2.236	2.586	3.529	2.226	2.429	2.328	0.556	0.905	0.556	0.874	0.06-4.00[4]
Lymphocytes, $1 \times 10^3/\mu L$	3.724	3.888	3.806	4.360	4.058	4.209	0.843	0.739	0.763	0.320	2.5-7.5[4]
Monocytes, $1 \times 10^3/\mu L$	0.694	0.280	0.487	0.555	0.215	0.385	0.091	0.227	0.001	0.650	0.0-0.9[4]
Eosinophils, $1 \times 10^3/\mu L$	0.508	0.430	0.469	0.573	0.692	0.633	0.196	0.387	0.900	0.551	0.0-2.4[4]

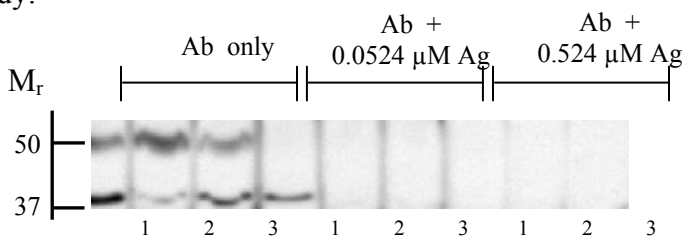
[1]Data are presented as least square means (n = 6-7) ± SEM for serum minerals of Control and Implant (25.7 mg estradiol) treatment cows collected at 14 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.

[2]Most conservative error of the mean.

[3]Taken from the University of Kentucky Livestock Disease Diagnostic Lab unless noted otherwise.

[4]Taken from Duncan et al., 1994.

Figure 3.1: Detection of GPR30 protein in bovine liver, using human anti-GPR30 polyclonal antibody.



The specificity of human anti-GPR30 polyclonal antibody (Ab) to detect bovine GPR30 was tested in homogenates (60 µg) of bovine liver from three cows (lanes 1, 2, 3) by preabsorption of Ab with 0 (Ab only), 0.052 (Ab + 0.0524 µM Ag), or 0.520 (Ab + 0.524 µM Ag) µM of the Ab antigenic polypeptide (Ag) before immunoblotting. The apparent migration weights (M_r) of GPR30 were 40 and 58 kDa.

Chapter 4

17β-estradiol stimulation of glutamine synthetase expression in the liver of old (but not of young) beef cows maybe refractory and independent of β-catenin or GRP30 expression

INTRODUCTION

Aging is associated with decreased metabolic functions and decreased gene expression of proteins responsible for intermediary metabolism in the liver (Serstè and Bourgeois, 2006). In cattle, little information is known about the relationship between hepatic function and aging. However, previous research in our lab has shown that the content of GS protein in the liver of old (≥ 10 years old), mature cows is decreased by 46 to 71% compared that of young (3-5 years old) adult cows, whereas ALT content was 61 to 73% less in old cows (Matthews and Sipe, 2006). In the previous trial (Chapter 3), it was determined that 17β-estradiol (estradiol) supplementation in the form of COMPUDOSE implants for 28 days increased the basal content of GS mRNA and protein, but not ALT, in the liver tissue of old beef cows with unknown and mixed genetic (breed) backgrounds, and having had ad libitum access to alfalfa hay-based diet. However, the role of putative mediators of estradiol stimulation of GS expression were not evaluated.

Estrogen regulated cellular events can be divided into "classical" and "non-classical" pathways. The classical pathway of estrogen regulation of cellular events involves the transport of the ligand-bound α- or β-estrogen receptors (ER) from the plasma membrane into the cell to promote *in nuclear* transcription and/or mediate non-nuclear cell signaling cascades (Figure 4.1). In the genomic pathway, estradiol-bound estrogen receptors bind to the estrogen response element located on the promoter region

of target genes or interact with other transcription factors (e.g. β-catenin) to initiate the transcription of target genes with no estrogen response elements present on the promoter region (Heldring et al., 2007). Estrogen α and β receptor mRNA are expressed by liver tissue of cattle but not affected by zeranol, a strong estrogenic and anabolic compound (Pfaffl et al., 2001). Our attempts to validate commercially-available ERα and ERβ antibodies to detect bovine orthologs failed.

Non-classical estrogen pathways include estrogen activation of the G protein-coupled receptor 30 (GPR30)-dependent and the β-catenin/Wnt pathways (Figure 4.2). G protein-coupled receptor 30, a recently discovered membrane-bound plasma receptor, is thought to be a prime target in the activation of non-genomic regulation of target genes by estrogen (Figures 4.1, 4.2). Although the GPR30 pathway is poorly understood, in the human liver estrogen has been indicated to enhance liver function after a trauma-induced hepatic hemorrhage via GPR30-mediated protein kinase A activation (Hsieh et al., 2007).

In the canonical Wnt pathway, Wnt signals by activating β-catenin for translocation into the nucleus (Figure 4.2). As a lipoglycoprotein, Wnts are secreted from neigboring cells to interact with two plasma-membrane bound receptors, frizzled and low-denisity-lipoprotein-related protein (LRP) receptors (Daugherty and Gottardi, 2007; Sonderegger et al., 2010). Ligand binding (e.g., estradiol) of Wnt and LPR5/6 triggers the activation of β-catenin by inhibiting the β-catenin degradation complex through the transducer protein, disheveled (Dvl). This complex contains two scaffolding proteins, axin and adenomatous polyposis coli (APC), and two serine/threonine protein kinases, glycogen synthase kinase 3β (GSK3β) and casein kinase I. The regulatory actions of Wnt allows for the non-phosphorylated form of β-catenin to dissociate from the degradation

complex and bind to T cell factor (TCF) and other transcription factors for translocation into the nucleus to activate target genes. In the absence of Wnt activation, β-catenin is phosphorylated by GSK3β or casein kinase I and is targeted for ubiquitin-mediated degradation by the APC and axin proteins.

Estrogen is thought to be a major modulator in the Wnt pathway. In the uterine epithelium, estradiol can up-regulate the expression of two Wnt proteins (Wnt4 and Wnt5a) and to trigger nuclear translocation of β-catenin in an estrogen receptor-independent manner (Hou et al., 2004). However, it also has been demonstrated that ERα is known to interact with β-catenin in osteoblasts (Foo et al., 2007), *Drosophila*, and certain human cancer cell lines (Kouzmenko et al., 2004). Furthermore, in the rat hippocampus, estradiol can bind to β-catenin for translocation into the nucleus (Cardonna-Gomez et al., 2004).

β-catenin may be a modulator of estradiol stimulation of GS expression because of its additional reputed role in maintaining GS expression in pericentral hepatocytes (Burke and Tosh, 2006). In addition, other proteins involved in glutamate metabolism are regulated by β-catenin. For example, pericentral hepatocyte-expressed ornithine aminotransferease is up-regulated in transgenic mice overexpressing hepatic β-catenin, as is mRNA for pericentral hepatocyte-expressed high-affinity glutamate transporter GLT-1 and glutamine synthetase is up-regulated by overexpression of β-catenin (Cadoret et al., 2002; Zucman et al., 2007). Although less studied, and not apparently sensitive to estradiol supplementation in old beef cows (Chapter 3), estrogen binding of GPR30 results in activation of the adenylyl cyclase, phosphoinositol 3 kinase (PI3K), and the phospholipase C pathways in several cell types (Thomas et al., 2005; Heldring et al.,

2007, Albanito et al., 2007) and human liver tissue (Hsieh et al., 2007). Moreover, evidence for cross-talk between ER and GPR30 has been accumulating (Sheng and Zhu, 2011).

A direct relationship between estrogen, β-catenin, and GS expression in liver tissue has not been reported. However, mRNA for ERα and ERβ are expressed in the cattle liver (Pfaffl et al., 2001), suggesting that estradiol administration to cattle likely has a direct effect on hepatic mechanisms, and not only through IGF-mediated effects. Moreover, GS activity and protein expression in rat C6-glioma cells is increased by estrogen (Haghighat, 2005), and estradiol up-regulates GS mRNA and protein content in the hypothalamus and hippocampus of humans (Blutstein et al., 2006).

Aging is associated with decreased metabolic functions and decreased gene expression of proteins responsible for intermediary metabolism in the liver (Serstè and Bourgeois, 2006). In cattle, little information is known about the relationship between hepatic function and aging. However, previous research in our lab has shown that the content of GS protein in the liver of old (≥ 10 years old), mature cows is decreased by 46 to 71% compared that of young (3-5 years old) adult cows, whereas ALT content was 61 to 73% less in old cows (Matthews and Sipe, 2006). In the previous trial (Chapter 3), it was determined that 17β-estradiol (estradiol) supplementation (COMPUDOSE implants; Vetlife, Des Moines, IA) for 28 days increased the basal content of GS mRNA and protein, but not ALT, in the liver tissue of old beef cows with unknown and mixed genetic (breed) backgrounds, and having had ad libitum access to an alfalfa hay-based diet.

Our previous finding (Chapter 3) that estradiol supplementation (implants) could increase GS content in the liver of old adult beef cows was exciting because it demonstrated the potential applicability of estradiol supplementation in a typical, commercial beef cattle production regimen. However, not knowing the actual age, feed intake, and breed type, may have confounded or masked treatment effects. In addition, given its potential commercial applicability, it is important to know if estradiol supplementation results in increased GS expression by liver tissue of young adult beef cows. Therefore, to test the effect of estradiol supplementation using more defined cattle phenotypes, the first goal of the present trial was to determine if the same estradiol supplementation regimen equally affected GS expression in old and young adult Angus cows with known ages and pedigrees, receiving a known amount of a corn silage-based diet. The second goal was to gain insight into potential mechanisms affecting these putative changes. The first primary hypothesis tested in this experiment was that COMPUDOSE supplementation of estradiol to young and old adult cows would increase hepatic content of GS. The second primary hypothesis tested was that estradiol supplementation would increase hepatic expression of β-catenin, GRP30, or both, in supplemented young and old adult cows. The third hypothesis tested was that estradiol supplementation would alter blood biochemical and clinical indicators of whole-body physiological status.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Twelve young, mature and twelve old, mature predominately Angus beef cows were housed in individual feeding pens (2.4 × 14.6 m) at the University of Kentucky Agricultural Research Center, located in Woodford County, KY. Beginning 7 d before study initiation, cows were collectively acclimated to a diet consisting of 93.3% corn silage, 5.5% soybean meal, and 1.2% minerals (DM basis). All cows then were fed at 3.37% of BW thereafter. The diet was formulated to contain 10% CP on a DM basis and calculated to provide 1.3 x maintenance NE_m requirements (NRC, 1996). Representative sampling of the experimental diet was conducted for proximate and mineral analyses performed (Table 4.1) by a commercial laboratory (Dairy One Forage Lab, Ithaca, NY). Cows had *ad libitum* access to water for the duration of the trial. Full BW were determined at days 0, 16, and 28 and average daily gain (ADG) calculated from BW difference between d 28 and d 0.

Compudose and Sham Implant Treatments

Before the beginning of this trial, cows were randomly allotted (n = 6) by age and weight into treatment groups to either receive a sham (Control; 0.5 mg oxytetracycline) or COMPUDOSE (estradiol; 25.7 mg estradiol; Vetlife) implant. The back of the ear was clipped, cleansed with 70% ethyl alcohol solution, and dried. Six young and 6 old cows received the controlled-release estradiol implant subcutaneously in the dorsal-medial area of the left ear using the manufacturer's implantation device (COMPUDOSE Implanter;

Vetlife) according to manufacturer's instructions. Furthermore, 6 young and 6 old cows received a sham implant using sterile corn oil as a vehicle for oxytetracycline (0.5 mg).

Blood Collection and Fractionation

Jugular venous blood samples were collected by venipuncture on d 16 and d 28. For preparation of plasma, 16 mL of blood was collected in EDTA-containing (0.9375 mg/mL) vacutainers (Becton Dickinson, Franklin Lakes, NJ). For serum, 16 mL of blood was collected in serum vacutainers without an anticoagulant. For whole blood, 2 mL of blood was collected in EDTA-containing (2.7 mg/mL) blood collection tubes, Becton Dickinson). Plasma and sera were recovered by refrigerated centrifugation at 3,000 × *g* for 10 min at 4°C and stored at −80°C.

Analysis of Blood Analytes

Plasma samples were analyzed for ammonia-N by modifications of the L-Glu dehydrogenase assay (Da Fonesca-Wollheim, 1973) using the Konelab 20XTi analyzer (Thermo Electron Corp., Vantaa, Finland). The sensitivity of this assay is 0.010 m*M,* and an interassay CV of 11.0% is typically realized. For this experiment, plasma ammonia concentrations were determined in a single assay event. The intraassay CV was 7.4%.

Plasma estradiol levels were evaluated using the Ultra-Sensitive Estradiol Radioimmunoassay kit (Diagnostic Systems Labs, Webster, TX) by Dr. Brian McBride (University of Guelph in Ontario, Canada). Briefly, 125 µL of $MgCl_2$ and 125 µL Dextran were added to microcentrifuge tubes containing 750 µL of the samples to duplicate the plasma. Samples were incubated for 1 hr at room temperature. Tubes were

centrifuged for 15 minutes at 10,000 rpm. The resulting spin produced a small pellet and the supernatant removed for further analysis.

Using the RIA kit, all samples, standards, and controls were assayed in duplicate. Tubes provided from the kit were labeled for Total counts (TC), Non-specific binding (NSB), standards, controls, and samples. The estradiol standards used were 0.5, 0.62, 1, 2.5, 5, 7.5, 10, 15, 20, 35, 50, and 250 pg/ml. The volume for the standards and controls was 200 µL per tube. On the other hand, 266.7 µL of plasma samples were added to the proper tubes. To the NSB tubes, 300 µL of the 0 pg/mL estradiol standard was added. Estradiol antiserum (1^{st} antibody; 100 µL) was added to all tubes except for the NSB and TC tubes. All tubes were vortexed and allowed to incubate overnight at 4°C. After the incubation, ^{125}iodine-labeled estradiol (100 µL) was added to each tube. Then, tubes were vortexed, covered, and allowed to incubate at 4°C overnight. On the third day, precipitating reagent (secondary antibody; 1 mL) was added to all tubes except for TC. Tubes were incubated at room temperature for 1 hr. Next, tubes were centrifuged for 30 minutes at 4°C at a speed of 3000 rpm. The tubes were decanted except for the TC tube to remove any excess moisture. Afterwards, tubes were counted in the gamma counter for 1 minute. The intraassay CV was 7.1%, whereas the interassay CV was 11.9%. The detection limit of this assay is 0.67 pg estradiol/mL.

Plasma progesterone levels were measured using the Coat-A-Count Progesterone ^{125}I Radioimmunoassay kit per manufacturer's instructions (Siemens Medical Solutions Diagnostics, Los Angeles, CA). The sensitivity of this assay was determined to be 0.038 ng/mL, the interassay CV was 5% , and the intrassay CV was 11%.

All other serum analytes, minerals, and blood cell types were analyzed by the University of Kentucky Livestock Disease Diagnostic Center. For serum enzymes, the following specific activities were assayed: ALP, E.C. 3.1.3.1; ALT, E.C. 2.6.1.2; AST, E.C. 2.6.1.1; γ-glutamyltransferase, E.C. 2.3.22; creatine kinase, E.C. 2.7.3.2; LDH, E.C. 1.1.1.27.

Liver Tissue Biopsy

On d 16 and 28, hepatic tissue was collected by a modification of the aspiration technique in cattle (Brown et al., 2009). Briefly, the area from the 10^{th} to 12^{th} intercostal spaces and 10 to 30 cm from the dorsal median plane on the right side of each animal was clipped free of hair and cleansed with povidine-iodine and two subsequent 70% ethyl alcohol solution washes. The remaining 70% ethyl alcohol solution was dried with gauze. Lidocaine (Lidocaine 2% Injectible; The Butler Company, Dublin, OH; 1.6 mL per biopsy site) was subcutaneously injected between the 12^{th} and 13^{th} ribs approximately 10 cm from the dorsal medial plane. A topical anesthetic spray (CETACAINE 300 mg; Cetylite Industries, Pennsauken, NJ) was administered to the skin 20 cm from the dorsal median plane at the 12^{th} intercostal space for 2 seconds and an incision made with a scalpel. A trocar (7 mm diameter) was used to obtain the tissue from the liver. The collected tissue was weighed and separated for RNA (400 mg wet tissue) and protein extraction (200 mg wet tissue). Samples were placed in foil packs, snap frozen in liquid nitrogen and stored at -80°C. After completion of biopsy, the incision side was treated with a topical broad spectrum antibacterial spray (FURAZOLIDINE 4%; Veterinary Products Laboratories, Phoenix, AZ).

Immunoblot Analysis

Liver was (approximately 200 mg) was homogenized on ice for 30 s (setting 11, POLYTRON Model PT10/35, Kinematic, Inc., Switzerland) in 1.5 mL of 4°C sample extraction buffer solution (0.25 mM sucrose, 10 mM HEPES-KOH pH 7.5, 1 mM EDTA) that contained protease inhibitor cocktail (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, 434 ng/ml; aprotinin, 0.0123 ng/ml; bestatin, 11.6 ng/ml; L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine, 3.92 ng/ml; leupeptin, 4.21 ng/ml; pepstatin A, 2.19 ng/ml; Sigma, St. Louis, MO) to prevent proteolysis. Protein was quantified by a modified Lowry assay, using bovine serum albumin as a standard (Kilberg, 1989). After protein quantification, 60 μg/lane of protein homogenates were separated by 12% SDS-PAGE under reducing conditions, followed by electrotransfer of the proteins to a 0.45-μm nitrocellulose membrane (BioRad, Hercules, CA) using SDS-PAGE standard protocols (Howell et al., 2001, 2003).

The relative liver protein expression was evaluated using a general immunoblot regimen, described previously (Howell et al., 2001; 2003). Relative content of GS, ALT, AST, and GPR30, and β-catenin was evaluated in 60, 60, 60, and 90 μg/lane, respectively. For the detection of ALT and β-catenin, blots were hybridized with 40 μg of IgG anti-porcine ALT (Brown et al., 2009; United States Biological, Swampscott, MA) and 14.5 μg of IgG anti-chicken β-catenin (Abcam Inc., Cambridge, MA) respectively, per mL of blocking solution (1.5% nonfat dry milk (wt/vol) in 30 mM Tris-Cl [pH 7.5], 200 mM NaCl, 0.1% Tween-20) for 1.5 h at room temperature with gentle rocking. G protein coupled receptor 30 was probed (Chapter 3) using 0.4 μg of IgG anti-human

GPR30 polyclonal antibody (Abcam Inc.) per mL of blocking solution [1% nonfat dry milk (wt/vol), 30 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (vol/vol)] overnight at 4°C with gentle rocking. Glutamine synthetase was probed (Brown et al., 2009) using 1.25 µg of IgG anti-sheep polyclonal antibody (BD Biosciences, San Jose, CA) per mL of blocking solution (5% nonfat dry milk (wt/vol), 10 mM Tris-Cl [pH 7.5], 100 mM NaCl, 0.1% Tween 20 (vol/vol)) for 1 h at 37°C with gentle rocking.

All protein-primary antibody binding reactions were visualized with a chemiluminescence kit (Pierce, Rockford, IL) after hybridization of primary antibodies with horseradish peroxidase-conjugated goat anti-mouse IgG (BD Biosciences, San Jose, CA; GS, 1:5,000, and β-catenin, 1:10,000); horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam Inc.; GPR30, 1:5,000) or horseradish peroxidase-conjugated rabbit anti-sheep IgG (Santa Cruz Biotechnology, Santa Cruz, CA; ALT, 1:5,000).

The use of GS and ALT for cattle tissue previously was described by our laboratory (Brown et al., 2009), and use of anti-GRP30 was validated as discussed in Chapter 3. Use of the mouse monoclonal anti-chicken β-catenin against bovine β-catenin was justified given that Madin-Darby Bovine Kidney cell lysate is the positive control for this antibody (Abcam), whereas the use of relative β-catenin content to describe activation of the β-catenin/Wnt pathway by sex steroid receptor has recently been described for cattle skeletal muscle cells (Zhao et al., 2011). When probed with the mouse monoclonal anti-chicken β-catenin and horseradish peroxidase-conjugated goat anti-mouse IgG pair (Figure 4.3) a proportional increase in a single immunoreaction product (~ 94 kDa) was observed in response to increasing amounts of bovine liver tissue

homogenates. Conversely, no immunoreaction products were found when homogenates were probed with only the horseradish peroxidase-conjugated goat anti-mouse IgG.

Densitometric analysis of immunoreactive products was performed as described previously (Howell et al., 2003; Fan et al., 2004). Briefly, after exposure of autoradiographic film (Amersham), digital images of all observed immunoreactive species were recorded and quantified (Yamin et al., 1996; Dehnes et al., 1998; Ding et al., 1998) using the BioRad Versadoc imaging system and the Quantity One Program (Version 4.2.3, BioRad). Data were collected as arbitrary densitometric units and then were corrected for unequal loading and/or transfer of proteins by normalization to densitometric values of Fast-Green-stained (Fisher Scientific, Pittsburg, PA) proteins common to all immunoblot lanes/samples. For all results, densitometric values were normalized to young control animals by obtaining an average young control densitometric value on 16 d and dividing all results by this value. Digital images were prepared with PowerPoint (Microsoft PowerPoint 2003, Bellvue, MA).

Extraction of Total RNA and Relative Real-Time Reverse-Transcriptase-Polymerase Chain Reaction Assay

RNA Extraction and Purification. Liver (approximately 200 mg) was homogenized on ice for 30 s (setting 11, Polytron® Model PT10/35, Kinematic, Inc., Switzerland) in 2 mL of 4°C TRIzol® Reagent (Invitrogen, Carlsbad, CA). Total RNA was obtained by an acidic phenol-chloroform extraction as per instruction of the manufacturer (Invitrogen). Briefly, chloroform (3 mL or volume to volume) was added to each Trizol® homogenate sample in sterile 7-mL polypropylene tubes (Fisher Scientific)

and samples were shaken vigorously. After 3 min incubation in ice, samples were spun at 12,000 x g for 10 min at 4°C. The aqueous phase containing RNA was transferred to a fresh, sterile 7-mL polypropylene tube. After the addition of an equal volume of ice-cold isopropanol, samples were precipitated at -80°C for 10 min, and then at -20°C overnight. Samples were then thawed and spun at 12,000 x g for 10 min at 4°C and the supernatant was removed. Each RNA-containing pellet then was washed gently with 75% ethanol (0.5 mL) and spun at 7,500 x g for 5 min at 4°C. After removing the supernatant, each tube was allowed to dry for 10 min. Pellets were resuspended in 100 µL of DNAse/RNAse-free water, transferred to 1.5 mL microcentrifuge tubes, and stored at -80°C.

After the crude RNA was recovered, a purification procedure was performed using RNeasy Mini Kit (Qiagen, Valencia, CA) to minimize genomic DNA contamination (Applied Biosystems, 2004) and enrich all the mRNA longer than 200 nucleotides in molecular size. Purified RNA was then eluted with 60 µL of RNase-free distilled H2O and stored at –80°C.

Purified RNA was then eluted with 60 µL of RNase-free distilled H_2O and stored at –80°C. The integrity of the purified RNA was examined by gel electrophoresis using Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA) at the University of Kentucky Microarray Core Facility. Visualization of the gel images and electropherograms showed that all RNA samples had high quality with RNA integrity number greater than 8.0 and 28S/18S rRNA ratio greater than 1.8. The purity and concentration of purified RNA samples were analyzed by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), which revealed that all

the samples were of high purity with 260/280 absorbance ratios greater than 2.0 and 260/230 absorbance ratios greater than 1.75.

Reverse Transcription (RT). Approximately 3 μg of crude RNA was first treated with DNase I enzyme (amplification grade) in accordance with the manufacturer's instructions (Invitrogen) to ensure that no DNA was present. Briefly, one RNA sample was combined with 1 μL of 10x reaction buffer, 1 μL of DNase I (1 U/μL), and DEPC-treated H2O up to 10 μL, incubated at room temperature for 15 min, and then 1 μL of 25 mM EDTA was added to stop the reaction by incubating at 65°C for 10 min. Then the DNase-treated RNA samples were reverse transcribed to cDNA by using SuperScript III First-Strand Synthesis System in accordance with the manufacturer's instructions (Invitrogen). Briefly, a solution of hexamers (50 ng/μL) and oligo (dT)20 primer (50 μM) mix (1 μL each) was added to one DNase-treated sample (7 μL in volume), incubated at 70°C for 10 min, and then chilled on ice for 1 min. A solution containing 2 μL of RT buffer (10x), 2μL of dithiothreitol (0.1 M), 4 μL of MgCl2 (25 mM), 1 μL of dNTP (10 mM each), and 1 μL of RNAse Out was then added to the reaction. After incubation at 37°C for 2 min, the reaction was incubated with 1 μL reverse transcriptase at room temperature for 10 min, and then incubated at 50°C for 50 min. To stop the reaction, the reaction mixture was incubated at 70°C for 10 min and then chilled on ice. The resulting reaction products, cDNA, were stored at –20°C until used in real-time RT-PCR.

Real-Time RT-PCR. Before conducting real-time RT-PCR with ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), primer and probe sets for GS, ALT, and 18S cDNA were designed and manufactured using ABI Assays-by-Design Service (Applied Biosystems). Bovine-specific nucleotide sequences

were obtained from previously published Genbank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) sequences or from the bovine-specific database (Cattle Gene Index database) of The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/tgi/) by using the Basic Local Alignment Search Tool (BLAST) function and the appropriate human ortholog nucleotide sequence (Genbank). These specific parameters including the sequences of the forward and reverse primers and probes for relative real-time polymerase chain reaction are provided (Table 4.2). Each Assays-by-Design primer and probe set consists of 2 unlabeled PCR primers and one TaqMan Minor Groove Binding probe with FAM, a reporter dye labeled at 5' end. Components of a 25-µL real-time PCR reaction were an Assays-by-Design Primer and Probe set (1.25 µL), TaqMan Universal PCR Master Mix-No AmpErase UNG (12.5 µL), cDNA template (1.0 to 2.0 µL), and DNase/RNase free H_2O (9.25 to 10.25 µL). The PCR conditions used for the amplification and quantification were an initial denaturing stage (95°C for 10 min), followed by 40 cycles of 2 amplification stages for denaturing (95°C for 15 s) and annealing/extension (60°C for 1 min), with a melting curve program (60 to 95°C), a heating rate of 0.15°C/s, and continuous fluorescence measurements. To establish mRNA relative quantification methodology, the real-time PCR products were validated by DNA sequence verification, using the general protocol (Liao et al., 2008).

Relative mRNA Quantification Methods. For the relative quantification of GS and ALT mRNA expression levels, real-time quantitative RT-PCR methodology that used 2-step regimen was developed in accordance with ABI guidelines (Applied Biosystems, 2004; Liao et al., 2008). In the first step, all the RNA samples were reverse-transcribed to cDNA as described above for RT reaction. In the second, real-time RT-

PCR step, relative standard curve methods were established for both GS and ALT cDNA. For each mRNA quantified, ribosomal 18S RNA was selected as an endogenous control to normalize the variations in sample preparation, mRNA inputs, and RT efficiencies (Liao et al., 2008). Briefly, bovine liver total RNA was reverse-transcribed, the cDNA sample was serially diluted 2.5×, 5×, 25×, 125×, 625×, 3,125×, 15,625×, 78,125×, and 390,625×, and the linear range for target mRNA quantification was established to ascertain an appropriate amount of cDNA to be used for a standard curve method. For each cDNA sample the real-time RT-PCR reactions (as described above) were conducted in triplicate to average out the potential pipeting, mixing, or plate setting-up errors. The minimal threshold (CT) values detected using these dilutions were approximately 35 and 21 for the target and 18S cDNA, respectively. As a result, the optimal detection of GS, ALT and 18S cDNA were achieved by using 1:5, 1:5 and 1:15,625 dilutions of the RT product stocks, respectively.

The estradiol treatment effect on expression of 18S rRNA by the liver was evaluated by comparing the CT values obtained from the real-time PCR reactions (Applied Biosystems, 2004). The relative quantities of GS and ALT mRNA expression were normalized to the relative 18S quantities by calculating the target gene:18S relative quantity ratios, and these 18S-normalized quantity ratios were used for estradiol treatment effect on GS and ALT mRNA expression. The 18S-normalized ratio from the control animals (i.e., sham-implanted) was designated as a calibrator. Then the 18S-normalized ratios for the estradiol-implanted cows, as well as the control animals, were divided by the ratio of the calibrator, respectively. The CT values for 18S rRNA

quantities and the calibrated values for SH infusion treatment effect were all subjected to statistical analysis.

Statistical Analysis

Individual cows were the experimental units. Data are presented as least square means (± SEM). The effect of treatment on all measured parameters (non-implanted vs implanted) was evaluated by ANOVA, using the MIXED procedure of SAS with REPEATED MEASUREMENT (version 8.01, SAS Inst. Inc., Cary, NC). The main effects of estradiol treatment (TRT), time after implant (DAY), age of cow (AGE), and their interaction (TRT x DAY, TRT x AGE, AGE x DAY, TRT x AGE x DAY) were assessed. DAY was included in the REPEATED statement and cow was included random statement. Kenward-Roger adjustment was used to calculate the denominator df (Kenward and Roger, 1997).

RESULTS

Plasma Hormones

The potential effect of estradiol treatment on plasma estradiol and progesterone was determined (Table 4.3). Age did not affect (P = .336) plasma estradiol concentrations, whereas plasma estradiol concentrations of estradiol implanted cows were 47.3% higher (P = 0.025) than non-supplemented cows. Also, there was a day effect (P = 0.001) reflecting a 48% higher plasma estradiol concentration for both estradiol treatment groups on d 28 vs d 16. For progesterone, neither estradiol implantation, age, or day of experiment affected ($P \geq 0.285$) plasma levels.

Body Weight and Average Daily Gain

By design, initial BW within each age group did not differ (P = 0.628) between the young control (645 kg) and implant (663 kg) cows. Similarly, the initial BW did not differ (P = 0.765) between control (770 kg) and estradiol (760 kg) treatment groups of old cows. Over the experiment (Table 4.4), BW increased (P = 0.001) for both age groups, but BW was not affected by estradiol implantation (P = 0.689). In contrast, the average daily gain was higher (P = 0.061) in estradiol-supplemented cows, reflecting a 50% higher ADG by old, and 20% higher ADG by young, estradiol-supplemented vs non-supplemented old and young cows. Feed intake of control and implant animals range from 8 to 12 kg with orts ranging from 0 to 6 kg.

ALT and GS hepatic protein content

To determine if the ALT and GS expression was affected by estradiol supplementation, their relative protein content was measured in the liver homogenates (Table 4.5). For GS, the livers of old cows expressed 45% less (P = 0.015) GS protein than young cows. A treatment x day interaction (P = 0.024) was observed, apparently reflecting the relatively high day 16 expression of GS by young and old cows treated with estradiol vs lower day 28 GS expression by young and old cows treated with estradiol and day 16 and 28 by young and old cows not treated with estradiol. Moreover, the ratio of old:young cow GS expression within each of these estradiol x age treatment groups was 90, 41, 27 and 37%, respectively, suggesting that the relatively high old:young GS

expression ratio (90%) for estradiol treated old cows on day 16 had diminished to 41% by day 28.

In contrast to GS, hepatic ALT content was not affected by cow age ($P \geq 0.623$), estradiol treatment ($P \geq 0.324$), day of experiment ($P \geq 0.658$), or their interactions ($P \geq 0.224$).

ALT and GS hepatic mRNA content

To determine potential transcription regulatory effects of estradiol on ALT and GS, the relative content of ALT and GS mRNA in liver tissue was determined by real-time RT-PCR (Table 4.6). Old cows had more ($P = 0.012$) ALT mRNA than young adult cows. In both treatment groups of old cows, ALT mRNA content was 43% higher when compared to hepatic ALT expression of the young control and young implant groups. Although ALT mRNA expression was not affected by estradiol treatment ($P = 0.331$), a treatment x age interaction was found ($P = 0.035$), with ALT mRNA expression by old estradiol-treated cows being higher ($P \leq 0.029$) than both the control age groups and the young implant group. Also, there was no difference among the two control groups and the young implant group ($P \geq 0.387$).

For GS mRNA, although there was no age ($P = 0.851$) or day ($P = 0.921$) effect, there was an age x day interaction ($P = 0.008$), seemingly reflecting the lower expression of GS mRNA on d 28 than d 16 of young cows. Estradiol treatment stimulated ($P = 0.072$) GS mRNA expression by 38%, but there were no ($P \leq 0.286$) treatment x other main effect interactions.

Regulatory Protein Expression

To detect a potential relationship between GS expression and known regulatory proteins affected by estradiol receptor stimulation, and to establish if the hepatic expression was affected by estradiol implantation, the relative protein content of total β-catenin or GPR30 was determined by immunoblot analysis of liver homogenates (Table 4.7). Neither age, treatment, nor their interaction affected ($P \geq 0.492$) β-catenin expression. However, there was a day effect with β-catenin protein content increasing ($P = 0.054$) 17% from 16 d to 28 d. There were no ($P \geq 0.476$) main or interaction effects on GPR30 expression.

Serum and Plasma Analytes

Common blood metabolytes were determined (Table 4.8) to elaborate protein expression results. Although plasma ammonia levels were not affected by estradiol treatment, age, or day ($P \geq 0.660$), urea N levels were 12 to 25% higher ($P = 0.021$) in old vs young cows and consistently increased ($P = 0.001$) on day 28 vs day 16. Similarly, γ-glutamyltransferase activities tended ($P = 0.086$) to be about 15% higher in old vs young cows, whereas ALT and AST activities were not affected ($P \geq 0.222$) by age. Whereas γ-glutamyltransferase activities were lower ($P = 0.01$) on day 28 than 16, ALT and AST activities were 29 and 18% higher ($P \leq 0.012$) on 28 d than on 16 d. Estradiol treatment did not affect ($P \geq 0.392$) γ-glutamyltransferase, ALT, or AST activity.

Old cows had lower (~8%) total protein levels, which were not affected ($P = 0.582$ by estradiol treatment). Albumin, globulin, and total bilirubin contents were not affected ($P \geq 0.151$) by age, day, or estradiol treatment. Estradiol treatment did not affect

total protein, albumin, nor globulin levels ($P \geq 0.208$). A treatment × age × day interaction ($P = 0.064$) was observed for total bilirubin content, but its meaning is not apparent. ALP and creatinine levels were not affected ($P \geq 0.182$) by estradiol treatment or trial day, but ALP levels were 45 to 60% lower ($P = 0.001$) in old cows, whereas creatinine levels were 7 to 19% lower in old vs young cows. In contrast, creatine kinase was not affected by any main effect or interaction ($P \geq 0.264$).

In terms of energy status markers, neither glucose, LDH, triglycerides, nor cholesterol levels were affected ($P \geq 0.302$) by estradiol treatment or age of cows. Triglyceride levels also were not affected by day of experiment or any interactions among main effects. However, a day ($P = 0.003$) and day x estradiol treatment interaction ($P = 0.015$) was found for glucose, the later apparently reflecting the higher level of glucose at day 28 for estradiol-treated cows. Similarly, a day ($P = 0.067$) and day x estradiol treatment interaction ($P = 0.023$) was found for plasma LDH activity, the later apparently reflecting the higher level of activity at day 28 in young cows. For cholesterol, a day ($P = 0.024$) and estradiol treatment x day interaction ($P = 0.009$) was found, with the later apparently reflecting the lower level of cholesterol for non-estradiol supplemented young cows.

Serum Minerals

The effect of cow age, estradiol supplementation, and day of experiment on serum minerals was determined (Table 4.9). Old cows had less ($P \leq 0.044$) chloride (1%) and phosphorus (20%) than young cows. Estradiol treatment alone did not affect ($P \geq 0.323$) mineral levels but an estradiol treatment x age interaction was found ($P = 0.038$) for

chloride, apparently reflecting a higher chloride level for old control cows. In addition, a treatment × age × day interaction was observed for sodium levels (P = 0.033), whereas there was only a tendency for potassium (P = 0.105) and magnesium (P = 0.117) levels to be affected by this interaction. On day 28, cows had 1.4% less (P = 0.002) chloride than on day 28.

Blood Cell Types and Parameters

The effect of cow age, estradiol supplementation, and day of experiment on blood cell types was determined (Table 4.10). Old cows had 6.21% less (P = 0.058) RBC than did young cows. Estradiol supplementation did not affect (P ≥ 0.419) the abundance of any blood cell types, nor were any estradiol supplementation interactions found (P ≥ 0.123). The experimental day affected RBC (P = 0.001), hemogloblin (P = 0.001), packed cell volume % (P = 0.001), monocytes (P = 0.001), and resulting in 4.6, 4.0, 4.6%, and 64.8%, respectively, lower values on day 28 than day 16. An observed age x day interaction (P = 0.063) appears to reflect more lymphocytes on day 28 than day 16 in old cows.

DISCUSSION

Aging is associated with decreased metabolic functions and decreased gene expression of proteins responsible for intermediary metabolism in the liver (Serstè and Bourgeois, 2006). In fact, many enzymes become less active or functionally inactive due to aging, ostensibly resulting from the accumulation of reactive oxygen species-directed

oxidative damage associated with aging (Kregel and Zhang, 2007). Importantly, the activity of GS is highly susceptible to oxidative inactivation (Stadtman, 1990) and GS activity is decreased (21%) in the liver of old rats (Danh et al., 1985). Regarding the effect of aging on GS expression, GS mRNA content in the liver of old mice is decreased (Dhahbi et al., 1999; Spindler, 2001).

In cattle, little information is known about the relationship between hepatic function and aging. However, previous research in our lab has shown that the content of GS protein in the liver of old (≥ 10 years old), mature cows is decreased by 46 to 71% compared with that of young (3-5 years old) adult cows, whereas ALT content was 61 to 73% less in old cows (Matthews and Sipe, 2006). In the previous trial (Chapter 3), it was determined that 17β-estradiol (estradiol) supplementation (COMPUDOSE implants; Vetlife, Des Moines, IA) for 28 days increased the basal content of GS mRNA and protein, but not ALT, in the liver tissue of old beef cows with unknown genetic (breed) backgrounds, and having had ad libitum access to an alfalfa hay-based diet.

These findings were exciting because they demonstrated the potential applicability of estradiol supplementation in a typical, commercial beef cattle production regimen. However, not knowing the actual age, feed intake, and breed type, may have confounded or masked treatment effects. Therefore, to test the effect of estradiol supplementation using more defined cattle phenotypes, the first goal of the present trial was to determine if the same estradiol supplementation regimen equally affected GS expression in old and young adult Angus cows with known ages and pedigrees, receiving a known amount of a corn silage-based diet. In the previous trial, the exact ages of the old cows were not known. However, they were determined to be greater than 10 years of

age (Johnson, 1959). In this trial, the exact ages of cows were known. Also, another difference between this trial and the previous trial was the breed differences. In the earlier trial, the experimental animals represented a composite of many breeds including Angus, Brangus, and other crosses, whereas, in this trial the cows were predominantly Angus.

In the present trial, estradiol supplementation with COMPUDOSE implants equally increased plasma estradiol levels in both the young and old cows. For the old cows, this result is consistent with the 221% more plasma estradiol of old cows of an unknown exact age and breed type implanted with COMPUDOSE (Chapter 3). For young adult cows, our current findings are thought to be novel. Also consistent with our previous trial (Chapter 3), the increase in plasma estradiol was not accompanied by a change in progesterone concentrations, suggesting that a normal reproductive cycle was occurring in the presence of continuously elevated estradiol.

The ability of estradiol, either alone or in combination with progesterone, to stimulate the efficiency of BW gain in heifers and cows has been well documented and reviewed (Reinhardt, 2007). Consistent with previous observations, the ADG was higher for both young and old cows receiving the estradiol supplement. Interestingly, these differences were delineated even though only full body weights (not shrunk) were measured.

Estradiol Supplementation Transiently Increases Hepatic GS mRNA and Protein Content in both Young and Old Adult Cows

As noted above, the whole-animal effect of steroidal implants on growth and carcass parameters have been well elucidated. However, little is known regarding how

estradiol administration affects specific mechanisms in the liver, the tissue responsible for coordinating alteration of whole-body nutrient fluxes. However, findings from exploratory research conducted in our laboratory suggested that liver GS is up-regulated by SYNOVEX-S (20 mg 17β-estradiol benzoate + 200 mg progesterone) in finishing steers (Sipe et al., 2004). Also, GS protein and activity are increased in rat C6-glioma cells by supplemental estrogen (Haghighat, 2005), whereas estradiol up-regulates GS mRNA and protein content of the hypothalamus and hippocampus of humans (Blutstein et al., 2006).

The activities of GS, ALT, and other anionic amino acid-metabolizing enzymes are essential to facilitate metabolism of glutamine and aspartate (Heidger and Welbourne, 1999; Welbourne and Nissim, 2001). In the liver, glutamine arriving from the portal vein is absorbed by the periportal hepatocytes by the hepatic system N activity (Kilberg et al., 1980) and converted to L-glutamate and ammonia by mitochondrial glutaminase. Mitochondrial ALT also can produce glutamate by converting skeletal muscle-derived alanine to glutamate and pyruvate in the periportal hepatocytes. Glutaminase- and ALT-derived glutamate can be metabolized by periportal hepatoycte-localized glutamate dehydrogenase to ammonia and α-ketoglutarate, or glutamate can be exported into the sinusoid and then absorbed by pericentral hepatocyte-localized EAAC1 or GLT-1 glutamate transporter. Once absorbed by the periportal hepatocytes, sinusoidal glutamate and ammonia can be conjugated by GS to glutamine. In cultured rat glial cells (Hagheighat, 2005) and mouse brain tissue (Blutstein et al., 2006), estradiol is known to upregulate GS content and function. In contrast, estrogen did not affect GS activity in cultured primary rat hepatocytes (Sirma et al., 1996).

In cattle, little information is known about the relationship between hepatic function and aging. The first hypothesis tested in this experiment was that COMPUDOSE supplementation of estradiol to young and old adult cows would increase hepatic content of GS. The livers of old control cows expressed less GS protein than did livers of young, mature control cows (Table 4.5). Interestingly, exogenous estradiol supplementation increased hepatic content of GS mRNA and protein in both young and old adult cows by d 16. However, by d 28, this stimulation was diminished, suggesting that the estradiol stimulatory effect may become refractory with time. In contrast, previous research from our laboratory (Chapter 3) found that hepatic GS mRNA expression was increased 34 and 99% at d 14 and 28, respectively, by estradiol-supplemented vs non-supplemented old cows of uncertain age, feed intake, and breed history. Concomitantly, GS content was increased 350% by d 14 and 200% by d 28. Collectively, these studies demonstrate that estradiol supplementation by ear implants can stimulate GS expression in old and young beef cows. Because of the lower GS expression in old cows, these findings indicate that estradiol supplementation may be most effective in old cows, as suggested by the greater increase in ADG for old than young estradiol-supplemented cows (Table 4.4). However, because GS activity was not measured, confirmation that estradiol supplementation-mediated increase in GS protein actually results in increased GS function, awaits validation by future trials.

Previous research in our laboratory with adult Angus cows indicated that hepatic ALT protein also was reduced in the liver of old cows (Matthews and Sipe, 2006). In the present trial, however, a difference in hepatic ALT protein content was not observed between young and old cows. At the transcriptional level, old cows actually possessed

more hepatic ALT mRNA content than young mature cows. However, this greater ALT mRNA content did not result in the increase of ALT protein expression and suggests that ALT protein content may be post-transcriptionally regulated.

Elevated Plasma Estradiol Is Not Associated with Altered Hepatic GPR30 or β-catenin Content

The second goal of this experiment was to gain insight into potential mechanisms by which estradiol may upregulate GS mRNA and protein content, as has been reported in the hypothalamus and hippocampus of humans (Blutstein et al., 2006). As presented in the Introduction, estrogen-activated events can be mediated through estradiol binding of -α and ER-β receptors in the "classical" pathway (Figure 4.1), or by binding non-ER receptors, including GPR30- and β-catenin, as part of the "non-classical" pathway (Figure 4.2). In cattle, the mRNA for ERα and ERβ mRNA are expressed by the liver (Pfaffl et al., 2001). However, we attempted to validate the commercial anti-ERα antibodies in cow liver homogenates but failed. In contrast, we successfully validated the use of anti-β-catenin (Chapter 3) and anti-GPR30 (Figure 4.3). Moreover, evidence exists that β-catenin may be a modulator of estradiol stimulation of GS expression in pericentral hepatocytes (Burke and Tosh, 2006).

Accordingly, we tested the hypothesis that estradiol supplementation would increase hepatic expression of GRP30, β-catenin, or both, in estradiol-supplemented young and old adult cow. However, the relative content of neither GPR30 nor β-catenin was affected. These results indicate that stimulation of hepatic GS expression by exogenous estadiol was not associated with altered liver content of GPR30 and β-catenin.

Effect of Age and Estradiol Supplementation on Blood Biochemical and Clinical Parameters

The third hypothesis tested was that estradiol supplementation would alter blood biochemical and clinical indicators of whole-body physiological status. In terms of the blood metabolites, however, no differences were observed in response to estradiol treatment. However, age differences were observed for some of the metabolites. The old cows experienced higher serum blood urea nitrogen levels, but lower total protein levels in the blood, thus possibly indicating an increase in protein catabolism in these animals. Serum ALP levels were higher in the old animals. In contrast, it has been reported that serum ALP activity is lowly expressed in aged rats (Sakamoto et al., 2005; Hashimoto et al., 2008). As for γ-glutamyltransferase activity, the old cows exhibited higher levels than the young cows. Also, γ-glutamyltransferase activity has been shown to be higher in aged rats, but these rats were male and produced lower levels of estradiol compared to the young rat group (Hamden et al., 2007). In addition, chloride and phosphorus levels were decreased in our old cows. In aged rats, Hashimoto et al. (2008) reported higher levels of serum aspartate aminotransferase, alanine aminotransferase, total bilirubin, total cholesterol, globulin, and creatinine, but observed lower levels of triglyceride, and albumin/globulin. Furthermore, it has been reported that sodium and chloride levels are higher in aged rats, but the phosphorus levels were low (Hashimoto et al., 2008).

In terms of the blood cell types, estradiol implants did not affect any blood cell parameter. Similarly, RBC was the only blood cell type to be affected by age, with RBC abundance tending to be lower in old cows. In aged humans, red blood cell count and

hemoglobin are reported to be lower (Coppola et al., 2000). Also, packed cell volume is lower in aged humans (Gelmini et al., 1989). This result may indicate a reduced oxygen carrying capacity in aged cows.

Conclusions

The first primary hypothesis tested in this experiment was that exogenous estradiol supplementation by implant (estradiol) to young and old adult cows would increase hepatic content of GS is accepted, whereas the second primary hypothesis that estradiol supplementation would increase hepatic expression of β-catenin or, GRP30, and the third hypothesis that estradiol supplementation would alter blood biochemical and clinical indicators of whole-body physiological status are rejected.

Table 4.1: Proximate and mineral analysis of corn silage-based diet fed to young and old cows throughout the trial (dry matter basis)[1]

Item	
Proximate analysis	
DM, %	41.6
CP, %	9.6
Soluble Protein % CP	61
ADF, %	22.6
NDF, %	38.7
Lignin, %	3.2
NFC, %	43.6
Starch, %	35.3
Crude Fat, %	2.7
TDN, %	71
NEL, Mcal/lb	0.74
NEM, Mcal/lb	0.74
NEG, Mcal/lb	0.46
Mineral analysis	
Ash, %	5.42
Calcium, %	0.30
Phosphorus, %	0.32
Magnesium, %	0.13
Potassium, %	1.22
Sodium, %	0.458
Iron, ppm	473
Zinc, ppm	94
Copper, ppm	26
Manganese, ppm	79
Molybdenum, ppm	0.4

[1] Proximate and mineral values are an average of 3-4 samples collected throughout the trial and presented on a DM basis.

Table 4.2: Primer and probe sets used for the real-time quantitative PCR analyses of metabolic enzyme mRNA and 18S rRNA

Primer and Probe[a]	Location on template (bp)	Sequence[b]	Amplicon size (bp)
GS (TIGR TC274284)			
Forward	702-720	5'-CACGAATGCCGAGGTCATG-3'	
Probe (fwd.)	726-739	5'-FAM-ACAGTGGGAATTCC-3'	62
Reverse	740-763	5'-CGATTCCTTCACAGGGTCCTATCT-3'	
ALT (TIGR TC310617)			
Forward	975-996	5'-CCTCCTTCCACTCGATCTCCAA-3'	
Probe (rev.)	1002-1017	5'-FAM-CCGCACTCGCCCATGT-3'	79
Reverse	1031-1053	5'-TCCATATTCACCACCTCCACGTA-3'	
18S (GenBank DQ222453)			
Forward	548-572	5'-CCCTGTAATTGGAATGAGTCCACTT-3'	
Probe (rev.)	593-611	5'-FAM-CCAGACTTGCCCTCCAATG-3'	100
Reverse	625-647	5'-ACGCTATTGGAGCTGGAATTACC-3'	

[a]GS = glutamine synthetase; ALT = alanine transaminase; 18S = 18S ribosomal RNA. The contents in the parentheses associated with each protein are the accession numbers for the reported sequences retrieved from the public databases (TIGR or GenBank) and used as templates for designing primers and probes. The custom TaqMan probes were supplied in either the forward (fwd.) or the reverse (rev.) orientation as indicated in the parentheses for each probe.

[b]"FAM" labeled at the 5' end of the TaqMan probe is six-carboxy-fluorescein which is used as a reporter dye in the real-time PCR procedure.

Table 4.3: Plasma estradiol and progesterone concentrations of young and old adult cows receiving Control or Estradiol treatment[1]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item	Young	Old	Young	Old	Young	Old	Young	Old	SEM[2]
Estradiol, pg/mL	2.26	2.44	4.03	4.16	3.27	4.90	5.15	5.67	0.75
Progesterone, ng/mL	3.53	2.63	1.96	1.77	2.34	1.66	1.63	2.18	0.84

	Overall P-value						
	TRT	DAY	AGE	TRT × DAY	TRT × AGE	AGE × DAY	TRT × AGE× DAY
Estradiol	0.025	0.001	0.336	0.542	0.473	0.412	0.446
Progesterone	0.285	0.308	0.525	0.384	0.619	0.448	0.844

[1]Data are presented as least square means (n = 4-6) ± SEM for hormone concentrations of Control and Implant (25.7 mg estradiol) young vs. old treatment cows collected at 16 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.

Table 4.4: Bodyweight changes of young and old adult cows receiving Control or Estradiol treatment[1]

	Treatment												
	Control						Estradiol						
	0 d		16 d		28 d		0 d		16 d		28 d		
Item	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old	SEM
BW, kg	645	770	667	780	673	785	663	760	680	779	694	793	26

	Overall				
	Control		Implant		
	Young	Old	Young	Old	SEM
ADG, kg	1.00	0.55	1.20	1.11	0.43

	Overall P-Value						
	TRT	DAY	AGE	TRT × DAY	TRT × AGE	AGE× DAY	TRT× AGE× DAY
BW	0.689	0.001	0.001	0.160	0.714	0.549	0.267
ADG	0.061	N/A[2]	0.316	N/A[2]	0.137	N/A[2]	N/A[2]

[1]Values are least square means (n = 5-6) ± SEM for weight of Control and Implant (25.7 mg estradiol) treatment cows collected at 0, 16, and 28 d after implantation.

[2] For ADG, day effect and its interactions are not applicable for the statistical analysis.

Table 4.5: Normalized densitometric analysis of relative liver content of ALT and GS in young and old adult cows receiving Control or Estradiol treatment[1,2]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item	Young	Old	Young	Old	Young	Old	Young	Old	SEM[3]
ALT	1.00	1.02	1.07	1.05	1.00	1.00	0.98	0.79	0.12
GS	1.00	0.27	0.84	0.31	1.14	1.03	0.75	0.44	0.19

	Overall P-value						
	TRT	DAY	AGE	TRT × DAY	TRT × AGE	AGE × DAY	TRT × AGE × DAY
ALT	0.324	0.658	0.623	0.224	0.619	0.378	0.594
GS	0.151	0.005	0.015	0.024	0.197	1.000	0.254

[1]Values (normalized arbitrary units) are least square means (n = 5-6) ± SEM from liver biopsy samples of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implanting. Densitometric evaluation of all immunoreactive species was performed. Data were analyzed as a repeated measure as described in Materials and Methods.

[2]Values were normalized to the average young control value at 16 d for each protein by dividing the actual value for the animal by the average young control value. Average control values (in arbitrary units) ± SE used for normalization: ALT, 1785 ± 296;GS, 3073 ± 457.

[3]Most conservative error of the mean.

Table 4.6: Relative quantity of hepatic ALT and GS mRNA expression in old and young adult cows receiving Control or Estradiol treatment[1,2]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item	Young	Old	Young	Old	Young	Old	Young	Old	SEM[3]
ALT	1.00	0.68	0.83	1.25	0.68	1.26	0.80	1.52	0.21
GS	1.00	0.48	0.68	0.81	1.07	1.00	0.83	1.17	0.18

	Overall P-value						
	TRT	DAY	AGE	TRT × DAY	TRT × AGE	AGE × DAY	TRT × AGE × DAY
ALT	0.331	0.230	0.012	0.965	0.035	0.187	0.379
GS	0.072	0.921	0.851	0.782	0.286	0.008	0.522

[1]Data are the least square means (n = 5-6) ± SEM of the relative mRNA quantities from liver biopsy samples of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implanting.
[2]The normalized quantities of the control and estradiol treatment groups from both ages on 16 and 28 d calibrated to the young control group on 16 d.
[3]Most conservative error of the mean.

Table 4.7: Normalized densitometric analysis of relative liver content of β-catenin and GPR30 in young and old adult cows receiving Control or Estradiol treatment[1,2]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**SEM**
β-Catenin	1.00	0.86	1.03	1.07	0.91	0.78	1.11	0.95	0.19
GPR30	1.00	0.66	1.07	0.96	0.81	0.76	0.89	0.77	0.29

	Overall P-value						
	TRT	**DAY**	**AGE**	**TRT × DAY**	**TRT × AGE**	**AGE × DAY**	**TRT × AGE × DAY**
β-Catenin	0.755	0.054	0.572	0.670	0.784	0.599	0.492
GPR30	0.607	0.476	0.495	0.676	0.745	0.795	0.637

[1]Values (normalized arbitrary units) are least square means (n = 5-6) ± SEM from liver biopsy samples of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implanting. Densitometric evaluation of all immunoreactive species was performed.

[2]Values were normalized to the average young control value at 16 d for each protein by dividing the actual value for the animal by the average young control value. The following is a list of those average control values (in arbitrary units) ± SE used for normalization: Total β-catenin, 3540 ± 453; GPR30, 2822 ± 670.

Table 4.8: Serum and plasma analytes of young and old adult cows receiving Control or Estradiol treatment[1]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item[2]	Young	Old	Young	Old	Young	Old	Young	Old	SEM[3]
Ammonia, mM[4]	0.0708	0.0792	0.0800	0.0819	0.0975	0.0772	0.0782	0.0784	0.0128
Urea nitrogen, mg/100 mL	5	6	6	7	4	5	6	7	1
ALT, U/L	27	26	33	32	27	22	33	32	2
AST, U/L	83	72	113	71	68	74	83	84	11
γ-glutamyl-transferase, U/L	19	22	17	16	19	23	15	18	2
Total protein, g/100 mL	7.9	8.3	7.9	8.3	7.7	8.2	8.0	8.2	0.2
Albumin, g/100 mL	3.3	3.3	3.4	3.4	3.3	3.6	3.5	3.5	0.1
Globulin, g/100 mL	4.6	5.0	4.5	4.9	4.4	4.7	4.5	4.6	0.2
Albumin/Globulin ratio	0.7	0.7	0.8	0.7	0.8	0.8	0.8	0.8	0.0
Total bilirubin, mg/100 mL	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0
ALP, U/L	58	30	62	34	66	31	63	25	5
Creatinine, mg/100 mL	1.6	1.3	1.6	1.3	1.5	1.4	1.5	1.4	0.1
Creatine kinase, U/L	119	101	125	119	142	107	141	112	23
Glucose, g/100 mL	67	81	67	77	83	90	73	76	9

Table 4.8 (con't): Serum and plasma analytes of young and old adult cows receiving Control or Estradiol treatment[1]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item[2]	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**SEM[3]**
LDH, U/L	1017	1122	1173	1108	1084	1053	1170	1039	66
Triglycerides, g/100 mL	22	24	18	25	21	21	21	20	3
Cholesterol, mg/100 mL	65	83	50	89	87	73	76	60	9

Table 4.8 (con't): Serum and plasma analytes of young and old adult cows receiving Control or Estradiol treatment[1]

	Overall P-value						
	TRT	**DAY**	**AGE**	**TRT × DAY**	**TRT × AGE**	**AGE × DAY**	**TRT × AGE × DAY**
Ammonia, mM[4]	0.660	0.765	0.824	0.150	0.493	0.498	0.197
Urea nitrogen, mg/100 mL	0.545	0.001	0.021	0.094	0.922	0.857	0.657
ALT, U/L	0.592	0.001	0.325	0.290	0.638	0.312	0.435
AST, U/L	0.392	0.012	0.220	0.839	0.101	0.083	0.190
γ-glutamyltransferase, U/L	0.842	0.001	0.086	0.890	0.329	0.071	0.521
Total protein, g/100 mL	0.582	0.582	0.032	0.383	0.898	0.204	0.243
Albumin, g/100 mL	0.208	0.186	0.379	0.727	0.196	0.103	0.401
Globulin, g/100 mL	0.367	0.756	0.151	0.321	0.634	0.755	0.504
Albumin/Globulin ratio	0.125	0.336	0.285	0.162	0.427	0.292	0.706
Total bilirubin, mg/100 mL	0.929	0.233	0.482	0.557	0.236	0.895	0.064
ALP, U/L	0.942	0.838	0.001	0.067	0.268	0.681	0.692
Creatinine, mg/100 mL	0.530	0.182	0.018	0.793	0.197	0.513	0.336
Creatine kinase, U/L	0.621	0.545	0.264	0.647	0.597	0.685	0.863
Glucose, g/100 mL	0.395	0.003	0.302	0.015	0.678	0.306	0.919
LDH, U/L	0.745	0.067	0.584	0.529	0.379	0.023	0.538
Triglycerides, g/100 mL	0.590	0.427	0.350	0.900	0.241	0.645	0.442
Cholesterol, mg/100 mL	0.731	0.024	0.389	0.276	0.009	0.177	0.102

[1]Data are presented as least square means (n = 5-6) ± SEM for serum enzymes of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase.
[3]Most conservative error of the mean.
[4]Plasma.

Table 4.9: Serum minerals of young and old adult cows receiving Control or Estradiol treatment[1]

	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item	Young	Old	Young	Old	Young	Old	Young	Old	SEM[2]
Calcium, mg/100 mL	8.9	8.9	8.9	9.0	8.9	8.8	9.0	8.9	0.2
Chloride, mmol/L	108	110	106	108	108	108	107	107	1
Magnesium, mg/100 mL	2.1	1.9	2.1	2.0	2.0	2.1	2.1	2.1	0.1
Phosphorus, mg/100 mL	6.0	4.6	5.7	5.1	5.5	4.2	6.3	4.8	0.5
Potassium, mmol/L	3.9	3.9	4.0	3.9	3.8	3.5	4.0	4.0	0.1
Sodium, mmol/L	142	142	141	142	141	142	142	139	1

	Overall P-value						
	TRT	DAY	AGE	TRT × DAY	TRT × AGE	AGE × DAY	TRT × AGE × DAY
Calcium	0.944	0.536	0.763	0.595	0.624	0.881	0.520
Chloride	0.346	0.002	0.044	0.375	0.038	0.197	0.716
Magnesium	0.578	0.325	0.146	0.942	0.083	0.740	0.117
Phosphorus	0.744	0.122	0.005	0.176	0.609	0.526	0.308
Potassium	0.323	0.018	0.358	0.065	0.686	0.593	0.105
Sodium	0.432	0.039	0.967	0.969	0.465	0.197	0.033

[1]Data are presented as least square means(n = 5-6) ± SEM for serum minerals of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.

Table 4.10: Blood cell types and parameters of young and old adult cows receiving Control or Estradiol treatment[1]

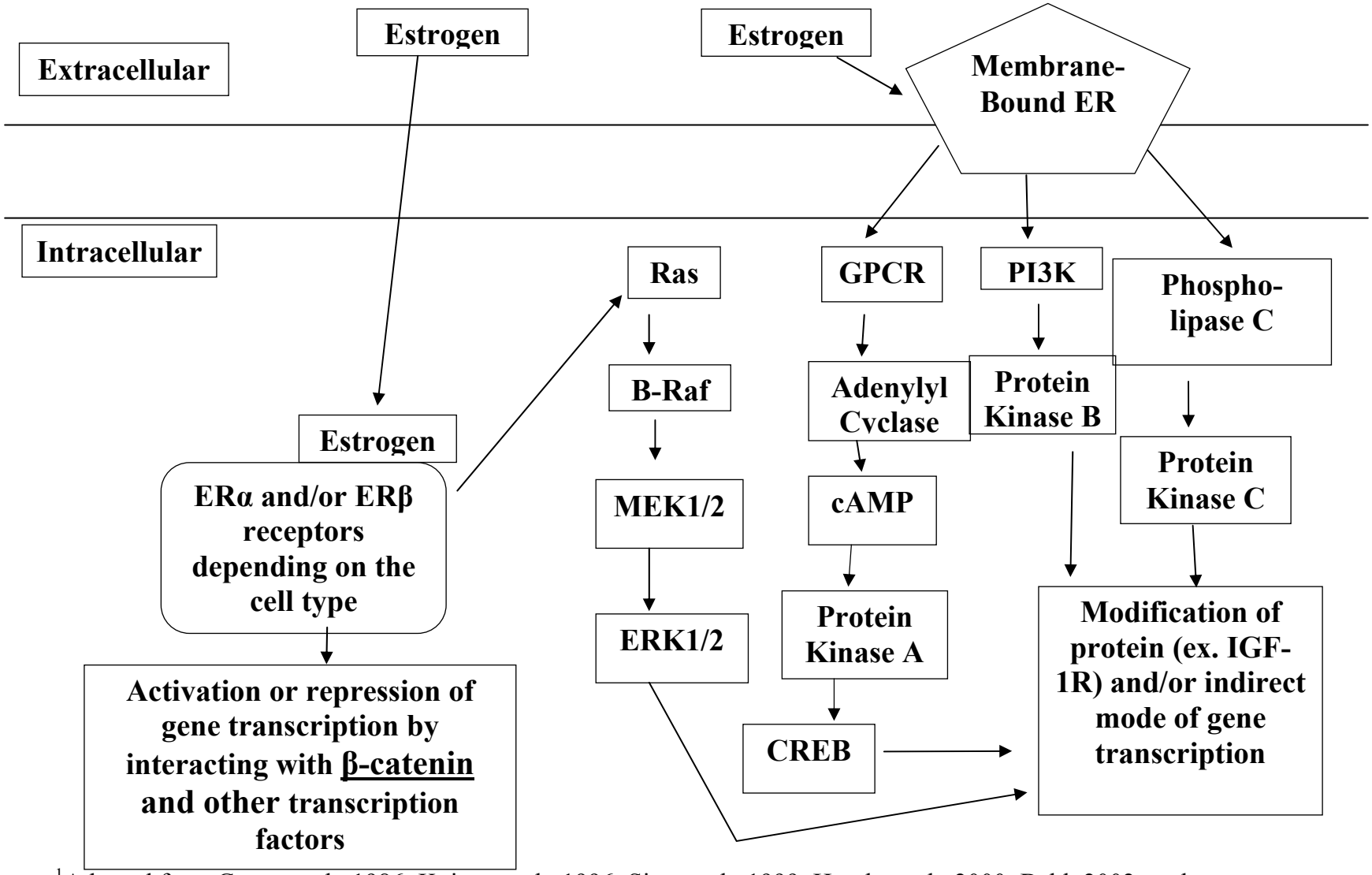
	Treatment								
	Control				Estradiol				
	16 d		28 d		16 d		28 d		
Item	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**	**SEM[2]**
RBC, $1\times10^6/\mu L$	7.54	6.86	7.24	6.66	7.30	6.91	6.77	6.62	0.25
Hemoglobin, g/dL	13.9	14.0	13.3	13.6	13.5	14.4	13.0	13.7	0.4
Packed cell volume, %	42.0	42.3	40.5	40.8	41.5	43.7	38.7	42.1	1.3
WBC, $1\times10^3/\mu L$	7.310	7.017	6.227	5.777	6.763	7.892	7.220	6.932	0.918
Neutrophils, $1\times10^3/\mu L$	1.885	2.493	1.694	2.496	2.317	2.075	2.660	2.516	0.416
Lymphocytes, $1\times10^3/\mu L$	4.050	3.449	3.847	2.737	3.462	4.874	3.741	4.008	0.682
Monocytes, $1\times10^3/\mu L$	0.854	0.771	0.213	0.208	0.648	0.570	0.372	0.250	0.150
Eosinophils, $1\times10^3/\mu L$	0.512	0.333	0.419	0.305	0.336	0.372	0.412	0.246	0.852

Table 4.10 (con't): Blood cell types and parameters of young and old adult cows receiving Control or Estradiol treatment[1]

	Overall P-value						
	TRT	**DAY**	**AGE**	**TRT × DAY**	**TRT × AGE**	**AGE × DAY**	**TRT × AGE × DAY**
RBC	0.441	0.001	0.058	0.289	0.438	0.267	0.641
Hemoglobin	0.910	0.001	0.120	0.659	0.333	0.814	0.523
Packed Cell Volume	0.940	0.001	0.192	0.431	0.288	0.516	0.554
WBC	0.429	0.097	0.975	0.274	0.611	0.342	0.445
Neutrophils	0.452	0.511	0.441	0.287	0.184	0.746	0.917
Lymphocytes	0.419	0.088	0.989	0.698	0.178	0.063	0.455
Monocytes	0.628	0.001	0.502	0.123	0.794	0.929	0.748
Eosinophils	0.470	0.346	0.139	0.699	0.558	0.451	0.151

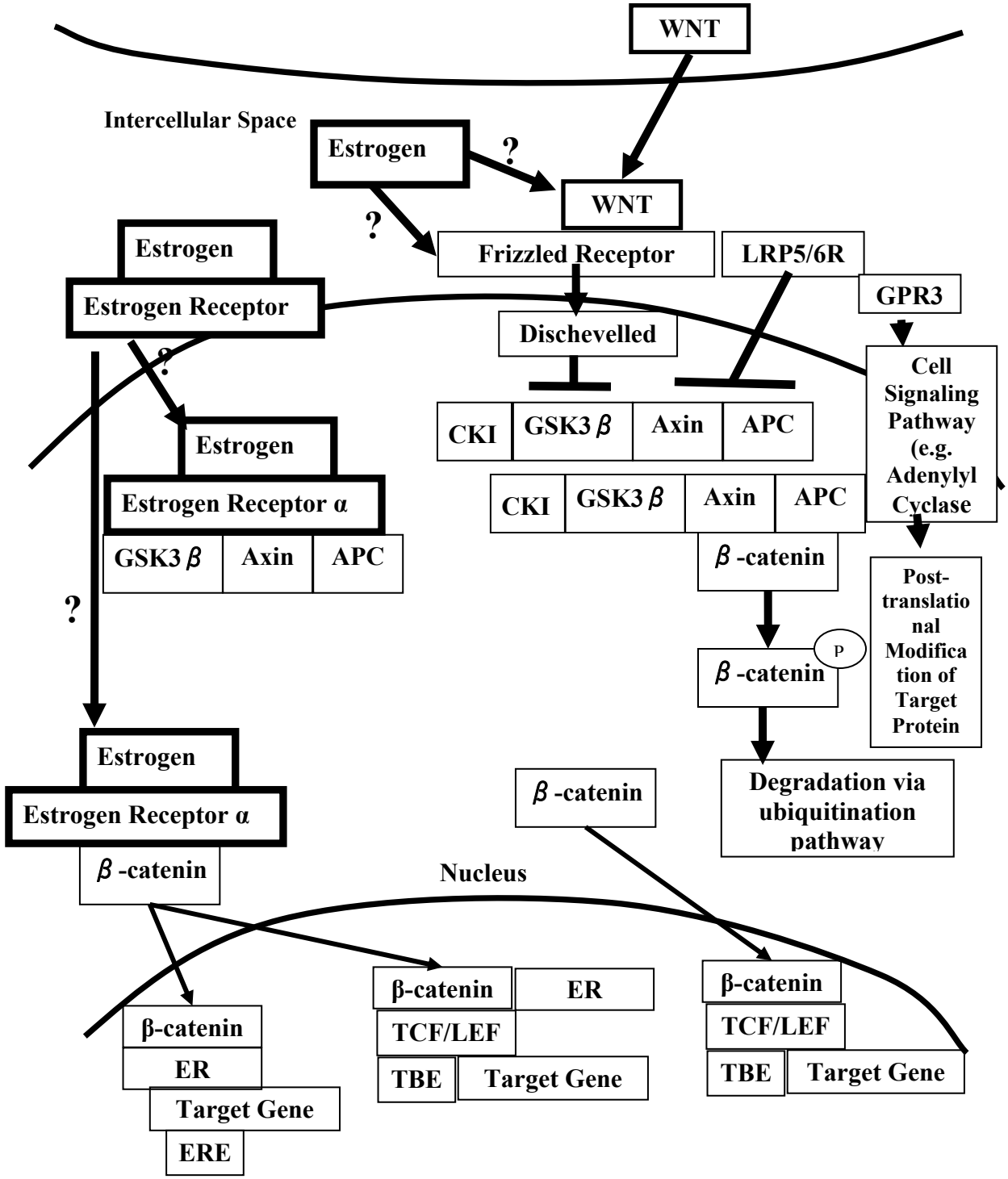
[1]Data are presented as least square means (n = 5-6) ± SEM for serum minerals of Control and Implant (25.7 mg estradiol) treatment cows collected at 16 and 28 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.

Figure 4.1: Potential Estrogen-Stimulated Pathways for Regulation of Glutamine Synthetase Expression[1]



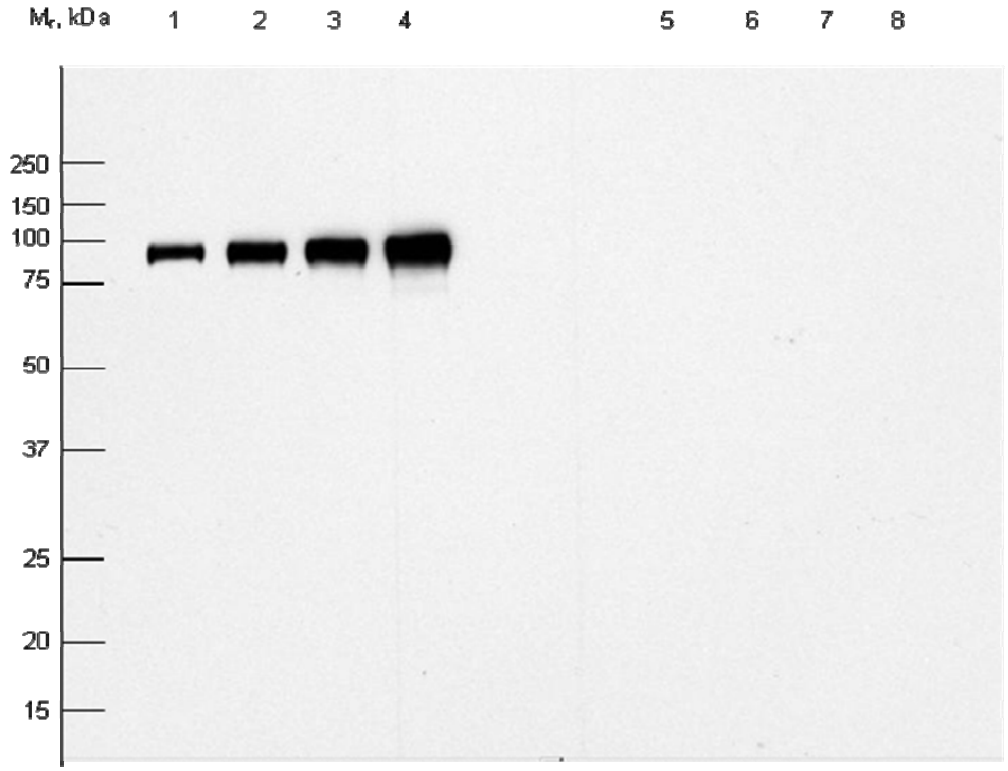
[1]Adapted from Green et al., 1986; Kuiper et al., 1996; Sing et al., 1999; Honda et al., 2000; Behl, 2002; and Kelly et al., 2005.

Figure 4.2: Potential Estrogen/β-Catenin-Stimulated Pathways for Regulation of Glutamine Synthetase Expression[1]



[1]Adpapted from Cardona-Gomez et al., 2004; Hou et al., 2004; Kouzmenko et al., 2004; and Eisenmann, 2005.

Figure 4.3: Immunoblot validation of mouse monoclonal anti-chicken β-catenin /goat anti-mouse Ig-horseradish peroxidase antibody for the detection of β-catenin in cow liver tissue



Companion immunoblots containing 15, 30, 60, or 90 µg of cow liver homogenate protein in lanes 1,5; 2,6; 3,7; and 4,8, respectively, were hybridized with either the primary/secondary pair (lanes 1-4) or only the secondary antibody (lanes 5-8). The M_r markers (kDa) are located to the left of the immunoblots.

Chapter 5

Glutamine synthetase and alanine transaminase were not affected by 17β-estradiol in developing heifers

INTRODUCTION

Past research in our lab has focused on studying the effect of exogenous estradiol on liver metabolism of aging cows. Specifically, we have observed how estradiol affects glutamine synthetase, an enzyme involved with nitrogen metabolism, in young and old cows. Also, we studied possible estradiol-mediated effects on blood metabolism in the old vs. young adult cow.

On the other side of the aging spectrum, we are also interested in studying the effect of estradiol and its combination with other sex steroids to promote cattle growth. Much research has been performed regarding the effects of anabolic implants on growth rate and carcass traits of growing steers and heifers (Heitzman, 1976). For instance, increased weight gain has been reported in steer and heifer calves treated with either estradiol 17 beta or zeranol, a non-steroidal estradiol agonist (Sawyer, 1987). However, on the cellular level, in the growing animal model, little is known about the effect of estradiol supplementation on blood metabolites and other parameters. For instance, even though blood estradiol levels of feedlot steers given Synovex-S (a combination of estradiol and progesterone) were reported to increase after 60 days after implantation (Rumsey and Beaudry, 1979), there is little research indicating the levels of blood estradiol in growing heifers given estradiol-only implants.

Currently, there is a need in the animal science industry to learn more about the underlying cellular and/or molecular mechanisms involved with growth and development

of food animals when given estradiol and other anabolic steroids (Chung and Johnson, 2008). Surprisingly, given the length of time that estradiol supplements have been used in cattle, little is known regarding the relationship between time of estradiol implantation and blood estradiol levels, and their correlation to biochemical and clinical blood parameters. To gain an understanding of these relationships, we conducted a trial to determine the effects of exogenous estradiol (Compudose® ear implants) on plasma estradiol levels and biochemical and clinical blood metabolites of growing beef heifers. (Trial 3).

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Nineteen young (8 to 10 months of age) non-pregnant beef heifers were weighed and using full BW were randomly allotted (0 d) to either a sham (Control; 0.5 mg oxytetracycline; BW = 293 kg) or Compudose® (Implant; 25.7 mg estradiol; Vetlife, Des Moines, IA; BW = 292 kg)) implant. Heifers were housed in dry lot conditions as four groups of 4-5 animals/pen, at the University of Kentucky Research and Education Center at Princeton, KY. Although housed in groups, heifers were individually fed through the use of a Calan gate system. Heifers had *ad libitum* access to water during the trial. Each animal was fed 105% of their previous week's average intake of a common late vegetative Bermudagrass hay/soybean hull/corn-based diet (Table 5.1) to achieve 1.1 kg BW gain/d for the duration of the trial (56 d). Full BW were taken on days 21 and 63 and shrunk BW determined on days 43 and 85.

Full BW were recorded on d 0, 28, and 56. Proximate analysis of the diet was determined by a commercial laboratory (Dairy One Forage Lab, Ithaca, NY; Table 1).

Compudose and Sham Implant Treatments

On day 1, Compudose® implants were inserted into the animal's left ear according to manufacturer's instructions. Briefly, the back of the ear was clipped, cleansed with 70% ethyl alcohol solution, and dried. The implant was placed subcutaneously in the dorsal-medial area of the ear using an implant device (Compudose® Implanter; Vetlife, Des Moines, IA). Sham implants were administered by injecting 5 mL of sterilized corn oil containing oxytetracycline in the same site of the ear as indicated above for the other treatments. Oxytetracycline was administered as part of the Control treatment to replicate the content of oxytetracycline supplied with each dose of Compudose.

Blood Collection and Analysis

Jugular venous blood samples were collected by venipuncture on d 14, 28, 43, and56. For preparation of plasma, 16 mL of blood was collected in EDTA-containing (0.9375 mg/mL) blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). For serum, 16 mL of blood was collected in serum blood collection tubes without an anticoagulant. For whole blood, 2 mL of blood was collected in EDTA containing (2.7 mg/mL) blood collection tubes, Becton Dickinson). Plasma and sera were recovered by refrigerated centrifugation at 3,000 × *g* for 10 min at 4°C and stored at −80°C.

Plasma estradiol levels (d 14, 28, 43, 56) were evaluated using the 3rd Generation Estradiol Radioimmunoassay kit (Diagnostic Systems Labs, Webster, TX) by Dr. Brian McBride (University of Guelph in Ontario, Canada). Briefly, plasma samples were

prepared for analysis by placing 800 µL of plasma in diethyl ether pre-rinsed glass 16×125 mm tubes. Diethyl ether (5 mL) was added to each tube and vortexed for 5 minutes. To freeze the aqueous layer, tubes were frozen in liquid nitrogen for approximately 60 seconds. The unfrozen ether top layer was immediately poured into respective glass 12×75 mm tubes. Tubes were allow to sit at room temperature in the fume hood for a minimum of 30 minutes to warm up and to allow the ether to evaporate about a half inch volume to avoid spillage and placed in the Savant vacuum for approximately 1 hour to allow tubes to be 95% dry. A second ether extraction was performed on the samples, using the same protocol as described previously, except that samples were completely dried by the Savant (1-2 hours) in the second extraction.

For RIA analysis, all samples were performed in duplicate. The ether-extracted samples were reconstituted using estradiol antiserum (primary antibody; 100 µL), vortexed for 1 minute, and then placed at 4°C for 19 hours. The standards used were 1.5, 3.25, 5, 7.5, 10, 15, 25, 50, 100, and 150 pg/mL. The standards were not prepared by ether extraction and were aliquoted (200 µL) to tubes appropriately labeled for each standard. Also, tubes were prepared to determine non-specific binding (NSB) and maximum binding (MB) of estradiol . In the MB and NSB tubes, 200 and 300 µL of the 0 pg/mL standard were aliquoted, respectively. Primary antibody was added to the MB and standards, vortexed for 60 seconds, and incubated for 19 hours at 4°C. Next, the tracer (^{125}iodine-labeled estradiol; 100 µL) was added to all tubes, vortexed for 60 seconds, and incubated for 19 hours at 4°C. Also, to determine total counts (TC), only the tracer was added to an empty tube. Next, the secondary antibody (1 mL) was added to all tubes except for the TC and was incubated for 1 hour at 4°C. After the secondary antibody

incubation, all tubes except for the TC were centrifuge at 2,400 rpm at 30 min at 4°C. All tubes were decanted, drained, and counted in a gamma counter for 1 minute. The intraassay and interassay CV of the RIA kit was 7.2 and 7.4%, respectively. The assay sensitivity is reported to be 0.54 pg/mL.

Plasma progesterone levels were measured using the Coat-A-Count Progesterone ^{125}I Radioimmunoassay kit per manufacturer's instructions (Siemens Medical Solutions Diagnostics, Los Angeles, CA). The sensitivity of this assay was determined to be 0.018 ng/mL. The interassay and intrassay CV was 5% and 5.86%, respectively. The methods of other serum analytes, minerals, and blood cell type evaluation by the University of Kentucky Livestock Disease Diagnostic Center has been described (Brown et al., 2009). For serum enzymes, the following specific activities were assayed: ALP, E.C. 3.1.3.1; ALT, E.C. 2.6.1.2; AST, E.C. 2.6.1.1; γ-glutamyltransferase, E.C. 2.3.22; creatine kinase, E.C. 2.7.3.2; LDH, E.C. 1.1.1.27.

STATISTICAL ANALYSES

Data are presented as least square means (± SEM). All experimental parameters between non-implanted and implanted animals were evaluated by ANOVA, using the MIXED procedure of SAS (version 8.01, SAS Inst. Inc., Cary, NC). Heifers were the individual experimental units. The effects of estradiol treatment(s), time after implant (DAY), and their interaction (TRT X DAY) were assessed. The statistical model used estradiol treatment as the fixed effect. Class variables were estradiol treatment (TRT) and animal, with animal included in the random statement. If a main treatment effect was observed, the protected Fisher's LSD procedure of SAS was used to separate treatment

means. Kenward-Roger adjustment was used to calculate the denominator df (Kenward and Roger, 1997). The GLM procedure of SAS with the MANOVA option was used to determine the strength of the relationship between plasma estradiol concentration and each blood parameter of all treatment groups and within each treatment group.

RESULTS

Animal Model. Before the start of the trial, all heifers were observed to exhibit behaviors of estrus, thus indicating these animals were cycling before the trial began. To validate and characterize the effect of a single dose of exogenous estradiol treatment (Implant) on circulating estradiol (pg/mL) and progesterone (ng/mL) over the 56 d experimental period, the concentrations of estradiol (d 14, 28, 43, 56) and progesterone (d 28, 56) in plasma were determined (Table 5.2). Plasma estradiol was 123, 60, 40, and 75% greater (P = 0.001) for Implant vs Control heifers at d 14, 28, 43, and 56, respectively. Despite the effect of Implant on plasma estradiol levels, plasma progesterone was not affected (P = 0.210). However, quantitatively, plasma progesterone levels were 38 and 47% less than Control at d 28 and 56, respectively.

The effect of Implant treatment (and its resulting elevated estradiol levels) on growth parameters was assessed (Table 5.3). By design, d 0 BW did not differ (P = 0.934) between heifers assigned to Control (293 kg) and Implant (292 kg) groups. Both groups of heifers gained BW at d 28 and 56 d (day effect, P = 0.001) but Implant did not affect (P = 0.704) BW gain. Overall average daily gain was 1.17 ±0.10 and 1.33 ± 0.09 kg for Control and Implant groups, respectively, and did not differ (P = 0.271). To determine if the efficiency of gain differed, the overall feed intake and feed:gain ratios

for each group was calculated and compared. Feed intake of the treated heifer group was not affected by exogenous estradiol (P = 0.453), whereas Implant heifers tended (P = 0.107) to have a greater efficiency of gain (3.57 vs 4.05). Feed intake of control and implant animals range from 19.5 to 27.7 kg/d.

Biochemical and Clinical Blood Profiles. To gain insight into the effects of implant-mediated elevated estradiol on metabolic capacities, profiles of serum enzymes and other blood constituents of Implant and Control heifers were compared after 28 and 56 d of treatment (5.4). Implant treatment did not affect (P > 0.06) any parameter. However, Implant heifers tended to have decreased (P = 0.077) creatinine levels on d 28 (11%) and 56 (7.8%), yet increased (P = 0.071) creatine kinase levels of 13 and 19% on d 28 and 56, respectively. Although the levels of certain metabolites (blood urea nitrogen, albumin, creatinine, and cholesterol) and enzymes (ALP, ALT, λ-glutamyltransferase,) differed (P ≤ 0.008) between d 28 and 56, the levels changed in the same manner for both Control and Implant heifers. In contrast, a treatment x d interaction was found for total protein (P ≤ 0.015) reflecting a 4.5% increase, but 3% decrease, from d 28 to 56 in Control vs Implant heifers. Likewise, triglyceride concentration decreased 19% in Control heifers but increased 13 % from d 28 to 56 in Implant heifers (P ≤ 0.005). Also, there was a tendency (P = 0.06) for globulin levels to increase 2.9% in Control heifers with time, whereas they decreased 8% from d 28 to 56 in Implant heifers.

The potential effect of exogenous estradiol on serum mineral levels also was examined (Table 5.5). Potassium levels were 9.9 and 2.4% lower (p = 0.029) on d 28 and 56, respectively, for Implant vs Control heifers. No other exogenous estradiol treatment effects were found. Although calcium and magnesium levels were decreased (P ≤ 0.042)

from 1.1 to 9.1% on d 28 to 56, both treatments responded in the same manner. No treatment x d interactions were found ($P \geq 0.164$).

The potential effect of exogenous estradiol on abundance of blood cell types was compared (Table 5.6). Although the amount of red blood cells, hemoglobin, and packed cell volume all consistently increased ($P \leq 0.041$) 3.3 to 5.9% from d 28 to d 56, estradiol treatment had no effect ($P \geq 0.194$). Similarly, estradiol treatment did not affect ($P \geq 0.137$) total white blood cell numbers, nor lymphocytes, monocytes, or neutrophils. However, estradiol-implant heifers had 118 and 235% more ($P = 0.057$) eosinophils at d 28 and 56, respectively, than did the control animals. No implant treatment x d interactions were found ($P \geq 0.126$).

Correlations between Plasma Estradiol and Biochemical and Clinical Blood Profiles. The strength of relationships between plasma estradiol levels and metabolic capacities, profiles of serum enzymes, and other blood constituents was determined through correlation analysis (Tables 5.7, 5.8, 5.9). Plasma estradiol-dependent relationships for combined (overall) and individual treatment (Control or Implant) were assessed. For blood, serum, and plasma analytes, no overall ($P \geq 0.175$), Control ($P \geq 0.096$), or Implant ($P \geq 0.412$) correlations were found, except for glucose (Table 5.7). For glucose, a moderate positive relationship ($r = 0.560$; $P = 0.047$) was observed between plasma estradiol concentrations and serum glucose levels in Control heifers, whereas no Implant or overall relationship was found ($P \geq 0.240$).

Potential relationships between plasma estradiol and blood minerals also were evaluated (Table 5.8). Neither an overall nor treatment-specific relationship was found ($P \geq 0.128$) for calcium, chloride, magnesium, phosphorus, or sodium. In contrast, a

moderate (r = 0.464) positive, overall relationship between estradiol levels and potassium levels was found (P = 0.020). Delineation of this effect into treatment-specific groups revealed that plasma estradiol and serum potassium levels of Implant heifers were strongly correlated (r = 0.697; P = 0.008), whereas no correlation (P = 0.274) was found for Control heifers.

For blood cell types and parameters (Table 5.9), no correlation (P ≥ 0.623) was found between plasma estradiol and red blood cells, hemoglobin, or packed cell volume. Similarly, no correlation was found (P ≥ 0.172) for total white blood cells or specific white blood cell type, except for monocytes. For monocytes, a moderate negative (r = -0.4135) relationship between monocyte abundance and estradiol level was observed overall (P = 0.045) and more strongly negative relationship (r = -0.605) for Implant heifers (P = 0.037). In contrast, there was no correlation (P = 0.673) between plasma estradiol levels and blood monocyte abundance in Control heifers.

Discussion

In general, mammals produce estradiol and, in females, the predominant source of estradiol is the ovaries. Specifically, the developing follicles of the ovaries produce estradiol (Hadley and Levine, 2007). Also, in the pregnant females, estradiol can be supplied by the placenta. The previous trials (Trial 1 and 2) involved using the old and young mature cow models. The main goal of Trial 3 was to establish a growing heifer cattle model to complete the young-to-old life spectrum for female cattle. Similar to Trial 1 (old cow model) and Trial 2 (old vs. young, mature cows), the plasma estradiol levels of the Compudose-implanted heifers increased. These results support our understanding

that plasma estradiol levels are affected by the presence of exogenous estradiol in the female cattle model despite the age of their body. In contrast to estradiol levels, progesterone levels were not affected by estradiol in the growing heifer model.

Previous research with growing cattle evaluated the plasma levels of estradiol, estrone, and estriol in response to Synovex-S (estradiol and progesterone) implanted feedlot steers (Rumsey and Beaudry, 1979). Plasma estradiol concentration was increased by implantation, whereas plasma estriol and estrone levels were not. Moreover, even though growth rate was determined to be higher in implanted steers, the rate of gain was not significantly correlated to estimated plasma estradiol levels. Interestingly, the study did not determine if the regulation of growth rate by Synovex-S is due to the synergistic effect of estradiol and progesterone or to the effects of estradiol or progesterone. However, in the present trial, in which exogenous estradiol increased defined plasma estradiol concentrations to levels consistent with in-estrus cattle, heifer growth rate was not affected.

In terms of nitrogen metabolism, blood urea nitrogen of growing heifers was not affected by estradiol, indicating other possible checkpoints in the nitrogen retention cycle to be affected by estradiol, similar to the estradiol-mediated effects observed during the previous trials. It was interesting to note that elevated plasma estradiol did not affect blood ALT or AST levels in this growing heifer model because it has been reported that zearalenone, a naturally-occurring estradiolic compound, can increase ALT and AST in the blood of rats (Stadnik and Borzecki, 2009).

Total bilirubin was decreased in response to the elevated estradiol levels in Implant heifers. This finding also differed from rat models in that ethynlestradiol, a

derivative of estradiol, was found to increase bilirubin and λ-glutamyltransferase levels in the blood of rats (Janssen et al., 2008). Also, creatinine levels of implant heifers were lower, thus indicating a possible increase in the removal of creatinine from the blood by the kidneys. The 16% increase in creatine kinase levels, is consistent with past research showing the increased activity of creatine kinase in the presence of increased estradiol-17 beta (Somjen et al., 2009).

In Trials 1 and 2, estradiol had an effect on potassium levels in the blood. However, potassium levels were increased in old cows given estradiol whereas increased estradiol in growing heifers resulted in decreased potassium levels and a strong positive relationship between estradiol and potassium. In ovariectomized female rats, 17beta-estradiol decreased plasma potassium levels in ovariectomized female rats (Zheng et al., 2006). The loss of potassium in the renin-angiotensin-aldosterone system in the kidney has been shown to be affected by estrogen and progesterone (Zheng et al., 2006). Also, the increased eosinophil levels of Implant heifers is consistent previous research with pigs, in which administration of 17beta estradiol increased eosinophil levels in ovariectomized pigs (Jayachandran et al., 2005).

Concluding Remarks

In cattle production, it is important to increase our understanding of the mechanism(s) involved in the economical production traits obtained by the implantation of sex steroids. With increasing environmental concerns (Kolok and Sellin, 2008), it is becoming more important to determine the exact mechanism(s) in which estradiol, progesterone, and other growth-promoting compounds work to improve production traits in cattle, thus leading to the creation of a non-steroidal implant.

The growing female heifer model helps to set the stage for future trials in which we can further delineate the mechanistic effects of estradiol and progesterone on hepatic and skeletal enzymes and transporters that support nitrogen metabolism and muscle growth at the molecular level.

Table 5.1: Formulation (as-fed basis) and selected nutrient composition (dry matter basis) of the basal diet fed to growing heifers

Item	Composition
Ingredients, %	
Bermudagrass hay, ground	25.0
Cracked corn	25.0
Soybean hulls	25.0
Soybean meal	8.00
Mineral mix	1.00
Nutrient composition[1,2]	
DM, %	89.2
CP, %	14.2
ADF, %	27.2
NDF, %	42.3
TDN, %	70.0
NE_m, Mcal/kg[1]	0.72
NE_g, Mcal/kg[1]	0.45
Ash, %	6.90

[1]The values were determined from 7 pooled samples collected throughout the trial.

[2]Calculated values.

Table 5.2: Plasma estradiol and progesterone concentrations of growing heifers receiving Control or Estradiol treatment[1]

	Treatment								
	Control				Estradiol				
Item	14 d	28 d	43 d	56 d	14 d	28 d	43 d	56 d	SEM[2]
Estradiol, pg/mL	1.85	2.37	2.80	1.91	4.14	3.79	3.90	3.34	0.493
Progesterone, ng/mL	N/A	2.56	N/A	4.07	N/A	1.86	N/A	2.22	0.918

	Overall P-value		
	TRT	DAY	TRT × DAY
Estradiol pg/mL	0.001	0.408	0.591
Progesterone, ng/mL	0.210	0.293	0.513

[1]Data are presented as least square means(n = 7-9) ± SEM for hormone concentrations of Control (sham) and Estradiol (25.7 mg estradiol) treatment heifers collected at 14, 28, 43, and 56 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.

Table 5.3: Growth performance of growing heifers receiving Control or Estradiol treatment[1]

	Treatment						
	Control			Estradiol			
Item	**0 d**	**28 d**	**56 d**	**0 d**	**28 d**	**56 d**	**SEM[2]**
BW, kg	293	328	359	292	332	366	10

	Overall Control	Estradiol	SEM
ADG[3]	1.17	1.33	0.10
Feed Efficiency[3]	4.05	3.57	0.20

	Overall P-value		
	TRT	**DAY**	**TRT × DAY**
BW, kg	0.704	0.001	0.532
ADG, kg	0.271	N/A	N/A
Feed Efficiency	0.107	N/A	N/A

[1]Values are least square means (n = 9-10) ± SEM for weight of Control and Estradiol (25.7 mg estradiol) treatment heifers collected at 0, 28, and 56 d after implantation.
[2]Most conservative error of the mean.
[3]Values are least square means (n = 9-10) ± SEM for ADG and feed efficiency of Control and Estradiol treatment heifers.

Table 5.4: Blood, serum, and plasma analytes of growing heifers receiving Control or Estradiol treatment[1]

	Treatment							
	Control		Estradiol			Overall P-value		
Item[2]	28 d	56 d	28 d	56 d	SEM[3]	TRT	DAY	TRT × DAY
ALP, U/L	127	202	115	219	27	0.943	0.001	0.433
ALT, U/L	32	42	31	43	1	0.791	0.001	0.452
AST, U/L	75	72	67	74	4	0.528	0.593	0.181
Blood urea nitrogen, mg/100 mL	9	11	9	11	1	0.691	0.006	0.920
Total protein, g/100 mL	6.6	6.9	7.0	6.8	0.1	0.278	0.416	0.015
Albumin, g/100 mL	3.2	3.3	3.1	3.3	0.0	0.564	0.004	0.240
Globulin, g/100 mL	3.5	3.6	3.8	3.5	0.1	0.199	0.265	0.060
Albumin/Globulin ratio	0.9	0.9	0.9	0.9	0.0	0.580	0.364	0.231
γ-glutamyltransferase, U/L	10	6	10	9	1	0.493	0.008	0.124
Total bilirubin, mg/100 mL	0.2	0.2	0.2	0.2	0.0	0.061	0.340	0.340
Creatinine, mg/100 mL	1.1	1.3	1.0	1.2	0.1	0.077	0.004	0.746
Creatine kinase, U/L	145	145	164	172	10	0.071	0.638	0.671
Glucose, g/100 mL	76	86	77	81	6	0.740	0.117	0.475
LDH, U/L	963	1015	991	995	37	0.912	0.439	0.507
Triglycerides, g/100 mL	27	22	23	26	2	0.904	0.333	0.005
Cholesterol, mg/100 mL	82	99	87	111	6	0.201	0.001	0.441

[1]Data are presented as least square means (n = 8-9) ± SEM for serum enzymes of Control and Estradiol (25.7 mg estradiol) treatment heifers collected at 28 and 56 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase.
[3]Most conservative error of the mean.

Table 5.5: Serum minerals of growing heifers receiving Control or Estradiol treatment[1]

	Treatment							
	Control		Estradiol			Overall P-value		
	28 d	56 d	28 d	56 d	SEM[2]	TRT	DAY	TRT × DAY
Calcium, mg/dL	9.6	9.3	9.6	9.5	0.1	0.251	0.042	0.164
Chloride, mmol/L	107	107	107	107	1	0.743	1.000	0.612
Magnesium, mEq/L	2.1	2.0	2.2	2.0	0.1	0.764	0.013	0.527
Phosphorus, mg/dL	7.1	7.1	6.5	7.1	0.3	0.248	0.208	0.290
Potassium, mmol/L	4.3	4.3	3.9	4.2	0.1	0.029	0.094	0.233
Sodium, mmol/L	139	138	138	138	1	0.383	0.397	0.669

[1]Data are presented as least square means(n = 8-9) ± SEM for serum minerals of Control and Estradiol (25.7 mg estradiol) treatment heifers collected at 28 d and 56 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.
[3]Taken from the University of Kentucky Livestock Disease Diagnostic Lab.

Table 5.6: Blood cell types and parameters of growing heifers receiving Control or Estradiol treatment[1]

	Treatment							
	Control		Estradiol			Overall P-value		
Item	28 d	56 d	28 d	56 d	SEM[2]	TRT	DAY	TRT × DAY
Red blood cells, 1 × 10^6/μL	8.20	8.60	8.00	8.26	0.31	0.525	0.038	0.649
Hemoglobin, g/dL	12.5	13.0	11.9	12.3	0.4	0.194	0.041	0.863
Packed cell volume, %	35.6	37.7	34.5	36.3	1.1	0.355	0.007	0.819
White blood cells, 1 × 10^3/μL	10.56	12.73	10.70	12.33	0.847	0.903	0.002	0.611
Neutrophils, 1 × 10^3/μL	2.670	3.287	4.001	3.633	0.447	0.137	0.688	0.126
Eosinophils, 1 × 10^3/μL	0.167	0.173	0.360	0.573	0.167	0.057	0.466	0.490
Lymphocytes, 1 × 10^3/μL	6.917	8.206	6.109	7.535	0.594	0.338	0.003	0.860
Monocytes, 1 × 10^3/μL	0.408	0.537	0.327	0.661	0.130	0.862	0.053	0.370

[1]Data are presented as least square means (n = 4-9) ± SEM for serum minerals of Control and Estradiol (25.7 mg estradiol) treatment heifers collected at 28 d and 56 d after implantation. Data were analyzed as a repeated measure as described in Materials and Methods.
[2]Most conservative error of the mean.
[3]Taken from the University of Kentucky Livestock Disease Diagnostic Lab unless noted otherwise.
[4]Taken from Duncan et al., 1994.

Table 5.7: Correlation of estradiol with blood, serum, and plasma analytes of growing heifers receiving Control or Estradiol treatment

	Correlation					
	Overall		Control		Estradiol	
Item[2]	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Urea nitrogen, mg/100 mL	-0.003	0.989	0.178	0.561	-0.104	0.723
ALP, U/L	0.113	0.582	0.103	0.739	0.120	0.683
ALT, U/L	0.274	0.175	0.482	0.096	0.175	0.549
AST, U/L	0.116	0.591	0.161	0.618	0.084	0.784
Total protein, g/100 mL	0.253	0.232	0.284	0.371	0.250	0.412
Albumin, g/100 mL	0.010	0.963	-0.284	0.347	0.164	0.575
Globulin, g/100 mL	0.201	0.335	0.269	0.373	0.161	0.599
Albumin/Globulin ratio	-0.132	0.520	-0.157	0.609	-0.119	0.685
γ-glutamyltransferase, U/L	-0.083	0.688	-0.410	0.164	0.117	0.689
Total bilirubin, mg/100 mL	-0.089	0.664	-0.158	0.606	-[2]	-
Creatinine, mg/100 mL	0.097	0.643	-0.131	0.684	0.235	0.419
Creatine kinase, U/L	0.032	0.884	0.327	0.299	-0.127	0.693
Glucose, g/100 mL	0.239	0.240	0.560	0.047	-0.224	0.440
LDH, U/L	-0.058	0.789	-0.296	0.351	0.030	0.924
Triglycerides, g/100 mL	0.053	0.795	-0.155	0.612	0.156	0.594
Cholesterol, mg/100 mL	-0.009	0.967	0.065	0.833	-0.071	0.810

[1]Data are presented as partial correlation coefficients between d 28 and d 58 plasma estradiol concentration (Table 5.2) and blood analytes of Control and Compudose (25.7 mg estradiol, Estradiol) treatment heifers (Table 5.4). Partial correlation coefficients were calculated as described in Materials and Methods.
[2]No value is present because the bilirubin values did not varied.

Table 5.8: Correlation of plasma estradiol with serum minerals of growing heifers receiving Control or Estradiol treatment

	Correlation					
	Overall		Control		Estradiol	
Item[2]	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Calcium, mg/dL	0.094	0.647	0.045	0.885	0.117	0.691
Chloride, mmol/L	0.063	0.761	0.404	0.171	-0.194	0.507
Magnesium, mEq/L	0.034	0.869	0.097	0.751	-0.003	0.992
Phosphorus, mg/dL	0.306	0.128	0.444	0.129	0.215	0.459
Potassium, mmol/L	0.464	0.020	0.328	0.274	0.697	0.008
Sodium, mmol/L	0.137	0.505	0.430	0.142	-0.138	0.639

[1]Data are presented as partial correlation coefficients between d 28 and d 58 plasma estradiol concentration (Table 5.2) and blood mineral content of Control and Compudose (25.7 mg estradiol, Estradiol) treatment heifers (Table 5.5). Partial correlation coefficients were calculated as described in Materials and Methods.

Table 5.9: Correlation of plasma estradiol with blood cell types and parameters of growing heifers receiving Control or Estradiol treatment

	Correlation					
	Overall		Control		Estradiol	
Item[2]	Coefficient	p-value	Coefficient	p-value	Coefficient	p-value
Red blood cells, $1 \times 10^6/\mu L$	0.101	0.623	0.095	0.758	0.109	0.712
Hemoglobin, g/dL	-0.027	0.896	-0.000	0.999	-0.050	0.866
Packed cell volume, %	-0.031	0.883	-0.045	0.889	-0.022	0.940
White blood cells $1 \times 10^3/\mu L$	-0.064	0.754	-0.403	0.172	0.347	0.224
Neutrophils, $1 \times 10^3/\mu L$	0.093	0.653	-0.276	0.362	0.311	0.280
Eosinophils, $1 \times 10^3/\mu L$	0.114	0.687	-0.193	0.618	0.143	0.760
Lymphocytes, $1 \times 10^3/\mu L$	-0.084	0.684	-0.335	0.264	0.183	0.532
Monocytes, $1 \times 10^3/\mu L$	-0.413	0.045	-0.130	0.673	-0.605	0.037

[1]Data are presented as partial correlation coefficients between d 28 and d 58 plasma estradiol concentration (Table 5.2) and blood cell types of Control and Compudose (25.7 mg estradiol, Estradiol) treatment heifers (Table 5.6). Partial correlation coefficients were calculated as described in Materials and Methods.

Chapter 6

General Summary

In this dissertation, three trials were conducted to study estrogen regulation of GS and ALT in young and old, mature cows. In Chapter 3, there are three salient observations from this study in which we were determining the effects of supplemental exogenous estradiol to old beef cows on (1) plasma estradiol concentration and (2) hepatic expression of GS, ALT and other proteins involved in hepatic glutamate/glutamine/alanine metabolism. First, the levels of plasma estradiol resulting from Compudose implantation in mature, cycling old beef cows, was characterized. Second, the defined increase in plasma estradiol of estrogen-implanted old cows is concomitant with increased expression of GS protein. Third, the identification that cattle liver expresses GRP30 protein is novel and enables subsequent research to identify how estradiol affects cellular metabolism.

In Chapter 4, our research tested the hypotheses that (1) glutamine synthetase expression will be up-regulated by estrogen in old and young mature cows and (2) that this regulation is affected by the β-catenin/Wnt pathway but not GPR30. Increased plasma estrogen levels were observed in both implanted young and old cows. In this trial, old cows expressed less GS expression by the liver compared to the young, mature cows. The relative hepatic protein content of glutamine synthetase was increased by estrogen in the old, mature cows, with more GS at day 14 than 28. In contrast, hepatic GS protein expression by young, mature cows was not affected by estrogen treatment. At the transcriptional level, GS mRNA was increased by estrogen. The protein expression of

both GPR30 and beta-catenin was not affected by estrogen implantation or age of the mature beef cows. In conclusion of Chapter 4 results, our hypothesis that GS will be up-regulated by estrogen for both age groups was partly confirmed because we observed a tendency of hepatic GS protein content in old cows to increase in the presence of estradiol and a treatment by day interaction, but not have hepatic GS protein content of young mature cows to be affected by exogenous estradiol. Also, the hypothesis that β-catenin/Wnt pathway is involved with estrogen-directed GS regulation was rejected.

In Chapter 5, the research goal was to evaluate the effects of estrogen on the clinical blood metabolites of growing heifers. The plasma estradiol levels increased in Compudose-implanted heifers. Blood urea nitrogen of growing heifers was not affected by estradiol. Estrogen did not affect blood ALT or AST levels of the growing heifers. We also observed that total bilirubin was decreased in heifers given Compudose implants. Furthermore, we noted that creatinine levels of growing heifers given Compudose were lower. A 16% increase in creatine kinase levels was observed. In growing heifers given estradiol, potassium levels decreased even though we noticed a strong positive relationship between estrogen and potassium. Also, eosinophil levels of Compudose-treated heifers were higher. The hypothesis was accepted in which certain blood metabolites of growing heifers are affected by exogenous estradiol, indicating that estradiol has many functional roles in the growing female model, other than the traditional reproductive capabilities.

Chapter 7

General Discussion

The results of these trials indicate that glutamine synthetase is negatively affected in the liver of old cows due to age, but not in young cows. Glutamine synthetase is an important nitrogen-metabolizing enzyme in the liver. In old cows, this low expression of glutamine synthetase can contribute to an impaired liver metabolism.

In Trial 1, estradiol levels in the blood was increased which supports our hypothesis that reduced GS expression can be “fixed” in old cows by estradiol implants. Also, in old cows, an elevated immune capacity is possible since we observed slight increased levels of total protein and globulin concentrations. As mentioned before, GS, ALT, and other anionic amino acid-metabolizing enzymes are essential to facilitate metabolism of glutamine and aspartate in peripheral tissues of old and young cows.

Estradiol supplementation seems to increase glutamine synthetase expression in the liver but not alanine transaminase in the liver of old cows but not young cows. Our lab has not analyzed other tissues including muscle to determine if glutamine synthetase protein expression is affected in a similar manner. It is an opportunity to evaluate the expression of GS and ALT in muscle since estradiol implantation is given to cattle to improve carcass quality, which is dependent on protein N biosynthesis. Glutamine synthetase mRNA and protein expression was increased in the old cows given supplemental estradiol, whereas other evaluated hepatic metabolic nitrogen-dependent enzymes were not affected. In terms of blood metabolites in old cows, estradiol supplementation increased levels of total protein, globulin, gamma-glutamyltransferase,

and potassium, indicating certain metabolites are sensitive to estrogen regulation. For total protein, estradiol affected the nitrogen content present in the blood. The increased content of globulin indicates the ability of estradiol to illicit a strong immune response in old cows, possiblyenhancing an old cow's ability to fight infections. Gamma-glutamyltransferase was increased in old cows in a estradiol treatment x day interaction, indicating that estrogen over time may have a negative impact on liver cell damage and cholestasis (the slowdown of bile moving from the liver). This idea can only be confirmed with future trials analyzing hepatocytes responsible for bile production and the gallbladder itself for its storage/release of bile produced in the liver. As indicative of reduced bile production, triglyceride concentration was high in old cows given estradiol supplementation, proving a reduced intestinal capacity to digest/absorb fats in the cows' diet. It would be interesting to see if the increased triglyceride concentration can contribute to muscle marbling in cows in future trials, in which possibly radiolabeled triglycerides can be tracked from entry via the diet to observe its establishment in the muscles of these old cows, thus increasing the profitability of old cows sent to market for their beef by producers. Another blood metabolite affected by estradiol was potassium. Cows given estradiol experienced an increased potassium level, thus indicating a possible connection between the absorption and excretion of potassium by the small intestine and kidney, respectively; and their regulation by estrogen because these tissues are responsible for maintaining potassium levels in the blood (Berne et al., 1998).

On the molecular level, our lab was interested in determining the potential regulation of glutamine synthetase in old cows by the GPR30 pathway. We were able to determine 1) identify GPR30 expression by cattle and 2) GPR30 protein content was not

affected by estradiol-directed regulation. It would be intriguing to determine if GPR30 activity and/or mRNA was affected to further delineate if GPR30 is part of the pathway that allows estradiol to positively affect glutamine synthetase, especially in our glutamine synthetase-containing cell lines (e.g. MDBK) were research can be performed to test GPR30 inbitiors.

In Trial 2, it was observed that increased plasma estradiol levels were present in both implanted young and old cows, as seen in the previous trial. In this trial, young vs. old, mature beef cows were used to determine if GS of young, cycling females function in a similar manner when exposed to estradiol. The novelty of this trial was that we observed a tendency for GS protein content to be increased in the liver of old cows indicating that estradiol seems to regulate GS expression by hepatocytes of old cows. Exogenous estradiol supplementation increased hepatic content of GS protein in both young and old cows on d16, but not on d 28, indicating that estradiol's stimulatory action may be refractory over time. Further trials will need to be conducted to determine if GS activity is also affected by estradiol regulation, since GS protein content is not completely indicative of GS activity in hepatocytes. It is interesting that hepatic GS of young cows were not affected by GS due to probably GS expression "running" at its optimal level due to the young cows being at their "peak" in terms of health. At the transcriptional level, GS mRNA was increased by estrogen, that resulted in a time-dependent increased GS protein expression, indicating that estrogen regulation may have occurred at the transcriptional level, but post-translational modifications occurred to negate the estradiol-directed GS mRNA synthesis. In concurrence with the tendency of GS to decrease in old cows, we did not observe any difference in GPR30 or beta catenin pathways, two

pathways used by estradiol to regulate GS, thus indicating that another trial will need to be completed comparing old and young, mature cows to validate the findings that GS of young cows are not affected by estradiol and both estrogen-directed cell signaling pathways are not involved with estradiol-directed GS regulation.

In Trial 3, we used a growing heifer cattle model to understand if young females are affected by estradiol in a similar fashion as old cows. Estradiol levels in blood was increased in heifers, similar to mature young and old cows, thus indicating that increased plasma estradiol levels can be connected to estrogen supplementation throughout the life spectrum of a female bovine in the beef industry. The hepatic GS and ALT expression were not measured at the time of this trial. However, samples of hepatic and skeletal tissues were collected for future analyses of GS and nitrogen-metabolizing enzymes of growing heifers. We did observed significant changes in blood metabolites. The levels of bilirubin were decreased in growing heifers. In humans and other species, aging red blood cells are destroyed by the reticuloendothelial cells, in which case, the porphryin moiety of hemoglobin is converted to bilirubin (Berne et al., 1998). Normally, bilirubin is released into the plasma, where it is bound to albumin for transport (Berne et al., 1998). In growing heifers, hepatocytes may be able to be more efficient in removing bilirubin from the blood to produce bilirubin glucuronides, a main component of bile in the presence of estradiol. Further research analyzing the hepatocytes responsible for synthesizing bile and storage of the bile by the gall bladder will need to be done to test this notion of efficient bilirubin removal. Also, creatinine levels were decreased in the growing heifer model. As a by-product of skeletal muscle creatine metabolism, the amount of creatinine is proportional to the muscle mass in humans, thus indicating that

estradiol implantation may have a potential effect with loss muscle mass in heifers. Creatine kinase levels were increased, thus indicating the "regenerative" properties of estradiol-directed creatine kinase to possibly re-establish the ATP pools during the contraction of skeletal muscles, in concurrent with humans and other species as indicated by Berne et al. (1998). Because blood potassium levels were decreased in the growing heifer model, estradiol has a negative impact on the retention of plasma potassium homeostasis in the blood and indicating a potential "dumping" of potassium by the small intestine and kidney into feces and urine, respectively. In this chapter and other chapters, we have observed an estradiol-directed immune response in our growing heifer model. Specifically, eosinophil levels were higher in estradiol-supplemented heifers, thus indicating estrogen's immunological "fighting" response has been examined in which these heifers have a potential efficient way to respond to the invasion of parasites and infections as they grow (Berne et al., 1998).

In conclusion, estrogen regulation increases glutamine synthetase expression in the liver but not for alanine transaminase in the liver of old cows. Our lab has not analyzed other tissues including muscle to determine if glutamine synthetase protein expression is affected in a similar manner. Extending the findings of this dissertation to that of determining the effect of estrogen supplementation on the expression of GS and ALT by muscle is the logical next step for this project, as estrogen implants typically are given to cattle to improve carcass mass, which is dependent on protein N biosynthesis.

Appendix: Examples of SAS Analyses and Outputs

A.1. Analysis of estradiol supplementation on the levels of plasma estradiol levels in the blood of old cows (Chapter 3)

A.1.1. Representative of SAS editor programming language using Proc Mixed (Plasma Estrogen Level of old cows) (Chapter 3)

```
filename new dde 'Excel|H:\[Estrogen Data.xls]SAS!R8C3:R35C6';
TITLE '601L Estrogen Data SAS- Repeated Measurements w/o Day 0
(Vanzant)';
data estrogen;
infile new missover;
INPUT COW $ TIME TREATMENT $ Estrogen;
run;
proc print;
run;
PROC MIXED  data=estrogen;
CLASSES TREATMENT COW TIME;
MODEL Estrogen =  TREATMENT TIME TREATMENT*TIME / DDFM=KR;
REPEATED TIME/type = ar(1) subject = COW(TREATMENT);
Lsmeans  TREATMENT TIME TREATMENT*TIME/diff;
quit;
```

A.1.2. SAS output

601L Estrogen Data SAS- Repeated Measurements w/o Day 0 (Vanzant) 1

14:58 Saturday, July 31, 2004

Obs	COW	TIME	TREATMENT	Estrogen
1	169	14	Control	1.500
2	169	28	Control	1.500
3	174	14	Control	2.572
4	174	28	Control	1.500
5	175	14	Control	1.482
6	175	28	Control	1.500
7	3/510	14	Control	1.511
8	3/510	28	Control	1.500
9	3/611	14	Control	1.500
10	3/611	28	Control	1.500
11	4/517	14	Control	1.500
12	4/517	28	Control	1.500
13	F068	14	Control	1.500
14	F068	28	Control	1.500
15	166	14	Implant	6.400
16	166	28	Implant	2.950
17	167	14	Implant	2.543
18	167	28	Implant	2.710
19	168	14	Implant	3.960
20	168	28	Implant	15.130
21	170	14	Implant	3.099
22	170	28	Implant	9.910

23	171	14	Implant	1.500
24	171	28	Implant	3.102
25	172	14	Implant	3.417
26	172	28	Implant	1.500
27	E8	14	Implant	8.081
28	E8	28	Implant	6.645

The Mixed Procedure

Model Information

Data Set	WORK.ESTROGEN
Dependent Variable	Estrogen
Covariance Structure	Autoregressive
Subject Effect	COW(TREATMENT)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Prasad-Rao-Jeske-Kackar-Harville
Degrees of Freedom Method	Kenward-Roger

Class Level Information

Class	Levels	Values

TREATMENT	2	Control Implant
COW	14	166 167 168 169 170 171 172 174 175 3/510 3/611 4/517 E8 F068
TIME	2	14 28

Dimensions

Covariance Parameters	2
Columns in X	9
Columns in Z	0
Subjects	14
Max Obs Per Subject	2
Observations Used	28
Observations Not Used	0
Total Observations	28

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	124.33302914	
1	1	124.27826928	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	COW(TREATMENT)	0.06748
Residual		7.5259

Fit Statistics

-2 Res Log Likelihood	124.3
AIC (smaller is better)	128.3
AICC (smaller is better)	128.8
BIC (smaller is better)	129.6

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	0.05	0.8150

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TREATMENT	1	12	10.68	0.0067
TIME	1	12	0.71	0.4146
TREATMENT*TIME	1	12	0.99	0.3386

Least Squares Means

Effect	TREATMENT	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control		1.5761	0.7555	12	2.09	0.0590
TREATMENT	Implant		5.0676	0.7555	12	6.71	<.0001
TIME		14	2.8975	0.7332	23.9	3.95	0.0006
TIME		28	3.7462	0.7332	23.9	5.11	<.0001
TREATMENT*TIME	Control	14	1.6521	1.0369	23.9	1.59	0.1242
TREATMENT*TIME	Control	28	1.5000	1.0369	23.9	1.45	0.1610
TREATMENT*TIME	Implant	14	4.1429	1.0369	23.9	4.00	0.0005
TREATMENT*TIME	Implant	28	5.9924	1.0369	23.9	5.78	<.0001

The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	TIME	_TREATMENT	_TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control		Implant		-3.4916	1.0685	12	-3.27	0.0067
TIME		14		28	-0.8487	1.0043	12	-0.85	0.4146
TREATMENT*TIME	Control	14	Control	28	0.1521	1.4203	12	0.11	0.9165
TREATMENT*TIME	Control	14	Implant	14	-2.4907	1.4664	23.9	-1.70	0.1024
TREATMENT*TIME	Control	14	Implant	28	-4.3403	1.4664	23.9	-2.96	0.0068
TREATMENT*TIME	Control	28	Implant	14	-2.6429	1.4664	23.9	-1.80	0.0841
TREATMENT*TIME	Control	28	Implant	28	-4.4924	1.4664	23.9	-3.06	0.0053
TREATMENT*TIME	Implant	14	Implant	28	-1.8496	1.4203	12	-1.30	0.2173

A.2. Analysis of estradiol supplementation on the expression of protein and for glutamate transporters and metabolizing enzymes of old cows (Chapter 3)

A.2.1. Representative of SAS editor programming language using Proc Mixed (GS protein content of old cows) (Chapter 3)

```
filename new dde 'Excel|H:\stuff 2\[601L GS Redo Densitometry
Calculations Normalized to d14 Ctrl.xls]SAS!R5C1:R32C4';
TITLE '601L GS Densitometry SAS without 173 without d0- Repeated
Measurements Normalized to d14 Ctrl (Vanzant)';
data protein;
infile new missover;
INPUT COW $ TIME TREATMENT $ SAS;
run;
proc print;
run;
PROC MIXED  data=protein;
CLASSES TREATMENT COW TIME;
MODEL SAS =  TREATMENT TIME TREATMENT*TIME / DDFM=KR;
REPEATED TIME/type = ar(1) subject = COW(TREATMENT);
Lsmeans  TREATMENT TIME TREATMENT*TIME/diff;
quit;
```

A.2.2. SAS output

601L GS Densitometry SAS without 173 without d0- Repeated Measurements Normalized to d14 Ctrl (Vanzan 13

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Obs	COW	TIME	TREATMENT	SAS
1	F068	14	Control	0.97
2	175	14	Control	0.58
3	172	14	Implant	2.97
4	169	14	Control	0.26
5	171	14	Implant	3.62
6	3/611	14	Control	3.31
7	170	14	Implant	6.55
8	3/510	14	Control	0.13
9	168	14	Implant	0.40
10	4/517	14	Control	1.56
11	167	14	Implant	7.01
12	174	14	Control	0.19
13	E8	14	Implant	4.24
14	166	14	Implant	6.74
15	F068	28	Control	0.06
16	175	28	Control	2.13
17	172	28	Implant	3.25
18	169	28	Control	0.31
19	171	28	Implant	1.21

20	3/611	28	Control	2.99
21	170	28	Implant	4.64
22	3/510	28	Control	0.08
23	168	28	Implant	0.22
24	4/517	28	Control	1.22
25	167	28	Implant	5.25
26	174	28	Control	0.00
27	E8	28	Implant	2.86
28	166	28	Implant	2.90

601L GS Densitometry SAS without 173 without d0- Repeated Measurements Normalized to d14 Ctrl (Vanzan 14

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The Mixed Procedure

Model Information

Data Set	WORK.PROTEIN
Dependent Variable	SAS
Covariance Structure	Autoregressive
Subject Effect	COW(TREATMENT)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Prasad-Rao-Jeske-Kackar-Harville

Degrees of Freedom Method Kenward-Roger

Class Level Information

Class	Levels	Values
TREATMENT	2	Control Implant
COW	14	166 167 168 169 170 171 172 174 175 3/510 3/611 4/517 E8 F068
TIME	2	14 28

Dimensions

Covariance Parameters	2
Columns in X	9
Columns in Z	0
Subjects	14
Max Obs Per Subject	2
Observations Used	28
Observations Not Used	0
Total Observations	28

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	101.75353916	
1	1	90.06468257	0.00000000

Convergence criteria met.

601L GS Densitometry SAS without 173 without d0- Repeated Measurements Normalized to d14 Ctrl (Vanzan 15

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The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	COW(TREATMENT)	0.7890
Residual		2.9374

Fit Statistics

-2 Res Log Likelihood	90.1
AIC (smaller is better)	94.1
AICC (smaller is better)	94.6
BIC (smaller is better)	95.3

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	11.69	0.0006

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TREATMENT	1	12	9.99	0.0082
TIME	1	12	6.71	0.0236
TREATMENT*TIME	1	12	6.23	0.0282

Least Squares Means

Effect	TREATMENT	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|

TREATMENT	Control	0.9850	0.6084	12	1.62	0.1314
TREATMENT	Implant	3.7043	0.6084	12	6.09	<.0001
TIME	14	2.7521	0.4581	14.8	6.01	<.0001
TIME	28	1.9371	0.4581	14.8	4.23	0.0008
TREATMENT*TIME	Control	14	1.0000	0.6478	14.8	1.54	0.1438
TREATMENT*TIME	Control	28	0.9700	0.6478	14.8	1.50	0.1553
TREATMENT*TIME	Implant	14	4.5043	0.6478	14.8	6.95	<.0001
TREATMENT*TIME	Implant	28	2.9043	0.6478	14.8	4.48	0.0005

601L GS Densitometry SAS without 173 without d0- Repeated Measurements Normalized to d14 Ctrl (Vanzan 16

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The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	TIME	_TREATMENT	_TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control		Implant		-2.7193	0.8604	12	-3.16	0.0082
TIME		14		28	0.8150	0.3146	12	2.59	0.0236
TREATMENT*TIME	Control	14	Control	28	0.03000	0.4449	12	0.07	

0.9474								
TREATMENT*TIME	Control	14	Implant	14	-3.5043	0.9161	14.8	-3.83
0.0017								
TREATMENT*TIME	Control	14	Implant	28	-1.9043	0.9161	14.8	-2.08
0.0555								
TREATMENT*TIME	Control	28	Implant	14	-3.5343	0.9161	14.8	-3.86
0.0016								
TREATMENT*TIME	Control	28	Implant	28	-1.9343	0.9161	14.8	-2.11
0.0522								
TREATMENT*TIME	Implant	14	Implant	28	1.6000	0.4449	12	3.60
0.0037								

A.3. Analysis of estradiol supplementation on the levels of plasma estradiol levels in the blood of old and young, mature cows (Chapter 4)

A.3.1. Representative of SAS editor programming language using Proc Mixed (Plasma Estrogen Level of old and young, mature cows) (Chapter 4)

```
filename new dde 'Excel|F:\602L only\Data\Blood Data\Estrogen\[602L
Estrogen Data-Matthews and Boling.xls]SAS!R4C2:R49C7';
TITLE '602L Estrogen Data SAS- Repeated Measurements w/o D0 (Dr.
Vanzant)';
data Estrogen;
infile new missover;
INPUT LabID COW $ AGE $ TIME TREATMENT $ ESTROGEN;
run;
proc print;
```

```
run;
PROC MIXED  data=Estrogen;
CLASSES TREATMENT COW AGE TIME;
MODEL ESTROGEN = TREATMENT TIME AGE TREATMENT*TIME TREATMENT*AGE
TIME*AGE TREATMENT*TIME*AGE/ DDFM=KR;
REPEATED TIME/type = ar(1) subject = COW(TREATMENT*AGE);
Lsmeans  TREATMENT TIME AGE TREATMENT*TIME TREATMENT*AGE TIME*AGE
TREATMENT*TIME*AGE/diff;
quit;
```

A.3.2. SAS output

602L Estrogen Data SAS- Repeated Measurements w/o D0 (Dr. Vanzant) 456

19:01 Friday, March 27, 2009

Obs	Lab ID	COW	AGE	TIME	TREATMENT	ESTROGEN
1	1	M119	Young	16	Control	1.779
2	4	M060	Young	16	Control	1.540
3	5	M068	Young	16	Control	3.490
4	6	N076	Young	16	Control	1.677
5	7	N088	Young	16	Control	1.196
6	10	N096	Young	16	Control	3.857
7	2	M002	Young	16	Implant	2.560
8	3	M034	Young	16	Implant	5.312
9	8	N087	Young	16	Implant	3.078
10	9	N082	Young	16	Implant	1.997
11	11	N106	Young	16	Implant	3.185

12	12	N069	Young	16	Implant	3.504
13	13	C221	Old	16	Control	1.211
14	15	D151	Old	16	Control	1.403
15	16	D229	Old	16	Control	1.886
16	19	C188	Old	16	Control	.
17	21	D255	Old	16	Control	1.663
18	23	G104	Old	16	Control	3.603
19	14	D029	Old	16	Implant	4.360
20	17	G025	Old	16	Implant	8.239
21	18	G027	Old	16	Implant	4.622
22	20	D222	Old	16	Implant	3.287
23	22	G094	Old	16	Implant	3.980
24	1	M119	Young	28	Control	3.303
25	4	M060	Young	28	Control	5.760
26	5	M068	Young	28	Control	6.599
27	6	N076	Young	28	Control	2.561
28	7	N088	Young	28	Control	2.818
29	10	N096	Young	28	Control	3.117
30	2	M002	Young	28	Implant	4.425
31	3	M034	Young	28	Implant	5.795
32	8	N087	Young	28	Implant	7.012
33	9	N082	Young	28	Implant	5.232
34	11	N106	Young	28	Implant	4.224
35	12	N069	Young	28	Implant	4.219
36	13	C221	Old	28	Control	2.871
37	15	D151	Old	28	Control	5.681
38	16	D229	Old	28	Control	2.698
39	19	C188	Old	28	Control	8.390

40	21	D255	Old	28	Control	1.933
41	23	G104	Old	28	Control	3.412
42	14	D029	Old	28	Implant	5.616
43	17	G025	Old	28	Implant	8.883
44	18	G027	Old	28	Implant	4.512
45	20	D222	Old	28	Implant	3.520
46	22	G094	Old	28	Implant	5.798

The Mixed Procedure

Model Information

Data Set	WORK.ESTROGEN
Dependent Variable	ESTROGEN
Covariance Structure	Autoregressive
Subject Effect	COW(TREATMENT*AGE)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Prasad-Rao-Jeske-Kackar-Harville
Degrees of Freedom Method	Kenward-Roger

Class Level Information

Class	Levels	Values
TREATMENT	2	Control Implant
COW	23	C188 C221 D029 D151 D222 D229 D255 G025 G027 G094 G104 M002 M034 M060 M068 M119 N069 N076 N082 N087 N088 N096 N106

AGE	2	Old Young
TIME	2	16 28

Dimensions

Covariance Parameters	2
Columns in X	27
Columns in Z	0
Subjects	23
Max Obs Per Subject	2

Number of Observations

Number of Observations Read	46
Number of Observations Used	45
Number of Observations Not Used	1

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	154.77542012	
1	2	150.33876997	0.00852430
2	1	149.93098699	0.00076921

The Mixed Procedure

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
3	1	149.89760526	0.00000704
4	1	149.89731500	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	COW(TREATMENT*AGE)	0.5711
Residual		2.8091

Fit Statistics

-2 Res Log Likelihood	149.9

AIC (smaller is better)	153.9
AICC (smaller is better)	154.3
BIC (smaller is better)	156.2

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	4.88	0.0272

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TREATMENT	1	16.3	6.07	0.0253
TIME	1	15.7	20.23	0.0004
AGE	1	16.3	0.98	0.3361
TREATMENT*TIME	1	15.7	0.39	0.5421
TREATMENT*AGE	1	16.3	0.54	0.4726
AGE*TIME	1	15.7	0.71	0.4118
TREATMENT*AGE*TIME	1	15.7	0.61	0.4457

The Mixed Procedure

Least Squares Means

Effect	TREATMENT	AGE	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control			3.2207	0.4304	16.6	7.48	<.0001
TREATMENT	Implant			4.7468	0.4458	16	10.65	<.0001
TIME			16	3.2157	0.3569	27.1	9.01	<.0001
TIME			28	4.7519	0.3506	26.1	13.55	<.0001
AGE		Old		4.2909	0.4509	16.6	9.52	<.0001
AGE		Young		3.6767	0.4251	16	8.65	<.0001
TREATMENT*TIME	Control		16	2.3462	0.5021	28.1	4.67	<.0001
TREATMENT*TIME	Control		28	4.0953	0.4838	26.1	8.46	<.0001
TREATMENT*TIME	Implant		16	4.0851	0.5074	26.1	8.05	<.0001
TREATMENT*TIME	Implant		28	5.4085	0.5074	26.1	10.66	<.0001
TREATMENT*AGE	Control	Old		3.3000	0.6160	17.3	5.36	<.0001
TREATMENT*AGE	Control	Young		3.1414	0.6012	16	5.23	<.0001
TREATMENT*AGE	Implant	Old		5.2817	0.6585	16	8.02	<.0001
TREATMENT*AGE	Implant	Young		4.2119	0.6012	16	7.01	<.0001
AGE*TIME		Old	16	3.6668	0.5249	27.9	6.99	<.0001
AGE*TIME		Old	28	4.9150	0.5074	26.1	9.69	<.0001
AGE*TIME		Young	16	2.7646	0.4838	26.1	5.71	<.0001

AGE*TIME	Young	28	4.5887	0.4838	26.1	9.48	<.0001
TREATMENT*AGE*TIME	Control	Old	16	2.4359	0.7351	29.9	3.31	0.0024
TREATMENT*AGE*TIME	Control	Old	28	4.1642	0.6842	26.1	6.09	<.0001
TREATMENT*AGE*TIME	Control	Young	16	2.2565	0.6842	26.1	3.30	0.0028
TREATMENT*AGE*TIME	Control	Young	28	4.0263	0.6842	26.1	5.88	<.0001
TREATMENT*AGE*TIME	Implant	Old	16	4.8976	0.7495	26.1	6.53	<.0001
TREATMENT*AGE*TIME	Implant	Old	28	5.6658	0.7495	26.1	7.56	<.0001
TREATMENT*AGE*TIME	Implant	Young	16	3.2727	0.6842	26.1	4.78	<.0001
TREATMENT*AGE*TIME	Implant	Young	28	5.1512	0.6842	26.1	7.53	<.0001

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	Estimate	Standard Error	DF
TREATMENT	Control			Implant			-1.5261	0.6197	16.3
TIME			16			28	-1.5362	0.3415	15.7

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	t Value	Pr > |t|
TREATMENT	Control			Implant			-2.46	0.0253
TIME			16			28	-4.50	0.0004

The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	Estimate	Standard Error	DF
AGE	Old			Young			0.6142	0.6197	16.3
TREATMENT*TIME	Control		16	Control		28	-1.7490	0.4813	16
TREATMENT*TIME	Control		16	Implant		16	-1.7389	0.7139	27.1
TREATMENT*TIME	Control		16	Implant		28	-3.0623	0.7139	27.1
TREATMENT*TIME	Control		28	Implant		16	0.01012	0.7011	26.1
TREATMENT*TIME	Control		28	Implant		28	-1.3132	0.7011	26.1
TREATMENT*TIME	Implant		16	Implant		28	-1.3234	0.4847	15.5
TREATMENT*AGE	Control	Old		Control	Young		0.1586	0.8607	16.6
TREATMENT*AGE	Control	Old		Implant	Old		-1.9817	0.9017	16.6
TREATMENT*AGE	Control	Old		Implant	Young		-0.9119	0.8607	16.6
TREATMENT*AGE	Control	Young		Implant	Old		-2.1403	0.8917	16
TREATMENT*AGE	Control	Young		Implant	Young		-1.0705	0.8502	16
TREATMENT*AGE	Implant	Old		Implant	Young		1.0698	0.8917	16
AGE*TIME		Old	16		Old	28	-1.2482	0.5030	16
AGE*TIME		Old	16		Young	16	0.9022	0.7139	27.1
AGE*TIME		Old	16		Young	28	-0.9220	0.7139	27.1
AGE*TIME		Old	28		Young	16	2.1504	0.7011	26.1
AGE*TIME		Old	28		Young	28	0.3262	0.7011	26.1
AGE*TIME		Young	16		Young	28	-1.8242	0.4622	15.5

TREATMENT*AGE*TIME	Control	Old	16	Control	Old	28	-1.7283	0.7067	16.5
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	16	0.1794	1.0043	28.1
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	28	-1.5904	1.0043	28.1
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	16	-2.4617	1.0499	27.9
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	28	-3.2299	1.0499	27.9
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	16	-0.8368	1.0043	28.1
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	28	-2.7153	1.0043	28.1
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	16	1.9077	0.9677	26.1
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	28	0.1378	0.9677	26.1
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	16	-0.7334	1.0149	26.1
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	28	-1.5016	1.0149	26.1
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	16	0.8915	0.9677	26.1
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	28	-0.9870	0.9677	26.1
TREATMENT*AGE*TIME	Control	Young	16	Control	Young	28	-1.7698	0.6536	15.5
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	16	-2.6411	1.0149	26.1
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	28	-3.4093	1.0149	26.1
TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	16	-1.0162	0.9677	26.1
TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	28	-2.8947	0.9677	26.1
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	16	-0.8713	1.0149	26.1
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	28	-1.6395	1.0149	26.1
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	16	0.7537	0.9677	26.1
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	28	-1.1248	0.9677	26.1
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Old	28	-0.7682	0.7160	15.5
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	16	1.6249	1.0149	26.1
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	28	-0.2536	1.0149	26.1
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	16	2.3931	1.0149	26.1

The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	t Value	Pr > |t|
AGE	Old			Young			0.99	0.3361
TREATMENT*TIME	Control		16	Control		28	-3.63	0.0022
TREATMENT*TIME	Control		16	Implant		16	-2.44	0.0217
TREATMENT*TIME	Control		16	Implant		28	-4.29	0.0002
TREATMENT*TIME	Control		28	Implant		16	0.01	0.9886
TREATMENT*TIME	Control		28	Implant		28	-1.87	0.0723
TREATMENT*TIME	Implant		16	Implant		28	-2.73	0.0152
TREATMENT*AGE	Control	Old		Control	Young		0.18	0.8560
TREATMENT*AGE	Control	Old		Implant	Old		-2.20	0.0425
TREATMENT*AGE	Control	Old		Implant	Young		-1.06	0.3045
TREATMENT*AGE	Control	Young		Implant	Old		-2.40	0.0289
TREATMENT*AGE	Control	Young		Implant	Young		-1.26	0.2260
TREATMENT*AGE	Implant	Old		Implant	Young		1.20	0.2477
AGE*TIME		Old	16		Old	28	-2.48	0.0246
AGE*TIME		Old	16		Young	16	1.26	0.2171
AGE*TIME		Old	16		Young	28	-1.29	0.2074
AGE*TIME		Old	28		Young	16	3.07	0.0050
AGE*TIME		Old	28		Young	28	0.47	0.6456
AGE*TIME		Young	16		Young	28	-3.95	0.0012

TREATMENT*AGE*TIME	Control	Old	16	Control	Old	28	-2.45	0.0260
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	16	0.18	0.8595
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	28	-1.58	0.1245
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	16	-2.34	0.0264
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	28	-3.08	0.0047
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	16	-0.83	0.4118
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	28	-2.70	0.0115
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	16	1.97	0.0594
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	28	0.14	0.8878
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	16	-0.72	0.4763
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	28	-1.48	0.1509
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	16	0.92	0.3653
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	28	-1.02	0.3171
TREATMENT*AGE*TIME	Control	Young	16	Control	Young	28	-2.71	0.0159
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	16	-2.60	0.0151
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	28	-3.36	0.0024
TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	16	-1.05	0.3033
TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	28	-2.99	0.0060
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	16	-0.86	0.3984
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	28	-1.62	0.1182
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	16	0.78	0.4431
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	28	-1.16	0.2556
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Old	28	-1.07	0.2998
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	16	1.60	0.1214
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	28	-0.25	0.8047
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	16	2.36	0.0261

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The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	Estimate	Standard Error	DF
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	28	0.5146	1.0149	26.1
TREATMENT*AGE*TIME	Implant	Young	16	Implant	Young	28	-1.8785	0.6536	15.5

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	t Value	Pr > |t|
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	28	0.51	0.6164
TREATMENT*AGE*TIME	Implant	Young	16	Implant	Young	28	-2.87	0.0113

A.4. Analysis of estradiol supplementation on the expression of protein and for glutamate transporters and metabolizing enzymes of old and young, mature cows (Chapter 4)

A.4.1. Representative of SAS editor programming language using Proc Mixed (GS protein content of old and young, mature cows) (Chapter 4)

```
filename new dde 'Excel|F:\602L
only\Data\Protein\Liver\Densitometry\GS\Data\Need to PRint\[602L Old
and Young Liver GS Protein Data Normalized to d16
YC.xls]SAS!R7C2:R52C6';
TITLE '602L GS Densitometry SAS- Repeated Measurements Normalized to
d16 YC (Dr. Vanzant)';
data protein;
infile new missover;
INPUT COW $ AGE $ TIME TREATMENT $ SAS;
run;
proc print;
run;
PROC MIXED  data=protein;
CLASSES TREATMENT COW AGE TIME;
MODEL SAS = TREATMENT TIME AGE TREATMENT*TIME TREATMENT*AGE TIME*AGE
TREATMENT*TIME*AGE/ DDFM=KR;
REPEATED TIME/type = ar(1) subject = COW(TREATMENT*AGE);
Lsmeans  TREATMENT TIME AGE TREATMENT*TIME TREATMENT*AGE TIME*AGE
TREATMENT*TIME*AGE/diff;
quit;
```

A.4.2. SAS output

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Obs	COW	AGE	TIME	TREATMENT	SAS
1	10	Young	16	Control	0.77

2	13	Old	16	Control	0.09
3	9	Young	16	Implant	0.75
4	17	Old	16	Implant	0.11
5	6	Young	16	Control	0.59
6	19	Old	16	Control	0.10
7	3	Young	16	Implant	0.88
8	18	Old	16	Implant	1.35
9	1	Young	16	Control	0.70
10	16	Old	16	Control	0.13
11	2	Young	16	Implant	0.62
12	14	Old	16	Implant	1.63
13	7	Young	16	Control	1.22
14	15	Old	16	Control	0.48
15	12	Young	16	Implant	1.67
16	5	Young	16	Control	1.22
17	23	Old	16	Control	0.81
18	11	Young	16	Implant	2.10
19	20	Old	16	Implant	1.18
20	4	Young	16	Control	1.51
21	21	Old	16	Control	0.00
22	8	Young	16	Implant	0.81
23	22	Old	16	Implant	0.88
24	10	Young	28	Control	0.73
25	13	Old	28	Control	0.33
26	9	Young	28	Implant	1.27
27	17	Old	28	Implant	0.01
28	6	Young	28	Control	.
29	19	Old	28	Control	0.06

30	3	Young	28	Implant	0.42
31	18	Old	28	Implant	0.78
32	1	Young	28	Control	0.83
33	16	Old	28	Control	0.10
34	2	Young	28	Implant	0.44
35	14	Old	28	Implant	1.12
36	7	Young	28	Control	0.95
37	15	Old	28	Control	1.02
38	12	Young	28	Implant	0.68
39	5	Young	28	Control	0.82
40	23	Old	28	Control	0.35
41	11	Young	28	Implant	1.12
42	20	Old	28	Implant	0.14
43	4	Young	28	Control	1.09
44	21	Old	28	Control	0.00
45	8	Young	28	Implant	0.58
46	22	Old	28	Implant	0.14

The Mixed Procedure

Model Information

Data Set	WORK.PROTEIN
Dependent Variable	SAS
Covariance Structure	Autoregressive

Subject Effect	COW(TREATMENT*AGE)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Prasad-Rao-Jeske-Kackar-Harville
Degrees of Freedom Method	Kenward-Roger

Class Level Information

Class	Levels	Values
TREATMENT	2	Control Implant
COW	23	1 10 11 12 13 14 15 16 17 18 19 2 20 21 22 23 3 4 5 6 7 8 9
AGE	2	Old Young
TIME	2	16 28

Dimensions

Covariance Parameters	2
Columns in X	27
Columns in Z	0
Subjects	23
Max Obs Per Subject	2

Number of Observations

Number of Observations Read	46
Number of Observations Used	45
Number of Observations Not Used	1

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	55.49588136	
1	2	48.76806958	0.00000000

The Mixed Procedure

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	COW(TREATMENT*AGE)	0.5598
Residual		0.1809

Fit Statistics

-2 Res Log Likelihood	48.8
AIC (smaller is better)	52.8
AICC (smaller is better)	53.1
BIC (smaller is better)	55.0

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	6.73	0.0095

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TREATMENT	1	18.9	2.24	0.1510
TIME	1	18.3	9.98	0.0053
AGE	1	18.9	7.18	0.0149
TREATMENT*TIME	1	18.3	6.05	0.0241
TREATMENT*AGE	1	18.9	1.79	0.1974
AGE*TIME	1	18.3	0.00	0.9998

TREATMENT*AGE*TIME	1	18.3	1.39	0.2535	

Least Squares Means

Effect	TREATMENT	AGE	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control			0.6045	0.1090	19.2	5.54	<.0001
TREATMENT	Implant			0.8395	0.1130	18.6	7.43	<.0001
TIME			16	0.8596	0.08897	28.4	9.66	<.0001
TIME			28	0.5844	0.09057	29.2	6.45	<.0001
AGE		Old		0.5116	0.1130	18.6	4.53	0.0002

The Mixed Procedure

Least Squares Means

Effect	TREATMENT	AGE	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
AGE		Young		0.9324	0.1090	19.2	8.55	<.0001
TREATMENT*TIME	Control		16	0.6350	0.1228	28.4	5.17	<.0001
TREATMENT*TIME	Control		28	0.5740	0.1274	30.1	4.51	<.0001
TREATMENT*TIME	Implant		16	1.0842	0.1288	28.4	8.42	<.0001

TREATMENT*TIME	Implant	28	0.5948	0.1288	28.4	4.62	<.0001
TREATMENT*AGE	Control	Old	0.2892	0.1523	18.6	1.90	0.0733
TREATMENT*AGE	Control	Young	0.9198	0.1561	19.8	5.89	<.0001
TREATMENT*AGE	Implant	Old	0.7340	0.1669	18.6	4.40	0.0003
TREATMENT*AGE	Implant	Young	0.9450	0.1523	18.6	6.20	<.0001
AGE*TIME	Old	16	0.6492	0.1288	28.4	5.04	<.0001
AGE*TIME	Old	28	0.3740	0.1288	28.4	2.90	0.0071
AGE*TIME	Young	16	1.0700	0.1228	28.4	8.71	<.0001
AGE*TIME	Young	28	0.7948	0.1274	30.1	6.24	<.0001
TREATMENT*AGE*TIME	Control	Old	16	0.2683	0.1737	28.4	1.55	0.1334
TREATMENT*AGE*TIME	Control	Old	28	0.3100	0.1737	28.4	1.79	0.0849
TREATMENT*AGE*TIME	Control	Young	16	1.0017	0.1737	28.4	5.77	<.0001
TREATMENT*AGE*TIME	Control	Young	28	0.8379	0.1864	31.6	4.49	<.0001
TREATMENT*AGE*TIME	Implant	Old	16	1.0300	0.1902	28.4	5.41	<.0001
TREATMENT*AGE*TIME	Implant	Old	28	0.4380	0.1902	28.4	2.30	0.0289
TREATMENT*AGE*TIME	Implant	Young	16	1.1383	0.1737	28.4	6.55	<.0001
TREATMENT*AGE*TIME	Implant	Young	28	0.7517	0.1737	28.4	4.33	0.0002

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	Estimate	Standard Error	DF
TREATMENT	Control			Implant			-0.2350	0.1570	18.9
TIME			16			28	0.2752	0.08709	18.3
AGE		Old			Young		-0.4208	0.1570	18.9
TREATMENT*TIME	Control		16	Control		28	0.06105	0.1227	18.6

TREATMENT*TIME	Control	16	Implant	16	-0.4492	0.1779	28.4	

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	t Value	Pr > |t|
TREATMENT	Control			Implant			-1.50	0.1510
TIME			16			28	3.16	0.0053
AGE		Old			Young		-2.68	0.0149
TREATMENT*TIME	Control		16	Control		28	0.50	0.6246
TREATMENT*TIME	Control		16	Implant		16	-2.52	0.0175

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The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	Estimate	Standard Error	DF
TREATMENT*TIME	Control		16	Implant		28	0.04017	0.1779	28.4
TREATMENT*TIME	Control		28	Implant		16	-0.5102	0.1811	29.2
TREATMENT*TIME	Control		28	Implant		28	-0.02088	0.1811	29.2
TREATMENT*TIME	Implant		16	Implant		28	0.4893	0.1237	18
TREATMENT*AGE	Control	Old		Control	Young		-0.6306	0.2181	19.2

TREATMENT*AGE	Control	Old	Implant	Old	-0.4448	0.2260	18.6	
TREATMENT*AGE	Control	Old	Implant	Young	-0.6558	0.2154	18.6	
TREATMENT*AGE	Control	Young	Implant	Old	0.1858	0.2285	19.1	
TREATMENT*AGE	Control	Young	Implant	Young	-0.02521	0.2181	19.2	
TREATMENT*AGE	Implant	Old	Implant	Young	-0.2110	0.2260	18.6	
AGE*TIME	Old	16	Old	28	0.2752	0.1237	18	
AGE*TIME	Old	16	Young	16	-0.4208	0.1779	28.4	
AGE*TIME	Old	16	Young	28	-0.1456	0.1811	29.2	
AGE*TIME	Old	28	Young	16	-0.6960	0.1779	28.4	
AGE*TIME	Old	28	Young	28	-0.4208	0.1811	29.2	
AGE*TIME	Young	16	Young	28	0.2752	0.1227	18.6	
TREATMENT*AGE*TIME	Control	Old	16	Control	Old	28	-0.04167	0.1667	18
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	16	-0.7333	0.2456	28.4
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	28	-0.5696	0.2548	30.1
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	16	-0.7617	0.2576	28.4
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	28	-0.1697	0.2576	28.4
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	16	-0.8700	0.2456	28.4
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	28	-0.4833	0.2456	28.4
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	16	-0.6917	0.2456	28.4
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	28	-0.5279	0.2548	30.1
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	16	-0.7200	0.2576	28.4
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	28	-0.1280	0.2576	28.4
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	16	-0.8283	0.2456	28.4
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	28	-0.4417	0.2456	28.4
TREATMENT*AGE*TIME	Control	Young	16	Control	Young	28	0.1638	0.1800	19.1
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	16	-0.02833	0.2576	28.4
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	28	0.5637	0.2576	28.4
TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	16	-0.1367	0.2456	28.4

TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	28	0.2500	0.2456	28.4
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	16	-0.1921	0.2664	30
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	28	0.3999	0.2664	30
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	16	-0.3004	0.2548	30.1
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	28	0.08624	0.2548	30.1
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Old	28	0.5920	0.1826	18
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	16	-0.1083	0.2576	28.4
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	28	0.2783	0.2576	28.4
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	16	-0.7003	0.2576	28.4
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	28	-0.3137	0.2576	28.4
TREATMENT*AGE*TIME	Implant	Young	16	Implant	Young	28	0.3867	0.1667	18

The Mixed Procedure

Differences of Least Squares Means

Effect	TREATMENT	AGE	TIME	_TREATMENT	_AGE	_TIME	t Value	Pr > |t|
TREATMENT*TIME	Control		16	Implant		28	0.23	0.8230
TREATMENT*TIME	Control		28	Implant		16	-2.82	0.0086
TREATMENT*TIME	Control		28	Implant		28	-0.12	0.9090
TREATMENT*TIME	Implant		16	Implant		28	3.96	0.0009
TREATMENT*AGE	Control	Old		Control	Young		-2.89	0.0093

TREATMENT*AGE	Control	Old	Implant	Old	-1.97	0.0641
TREATMENT*AGE	Control	Old	Implant	Young	-3.04	0.0068
TREATMENT*AGE	Control	Young	Implant	Old	0.81	0.4261
TREATMENT*AGE	Control	Young	Implant	Young	-0.12	0.9092
TREATMENT*AGE	Implant	Old	Implant	Young	-0.93	0.3624
AGE*TIME	Old	16	Old	28	2.23	0.0391
AGE*TIME	Old	16	Young	16	-2.36	0.0251
AGE*TIME	Old	16	Young	28	-0.80	0.4280
AGE*TIME	Old	28	Young	16	-3.91	0.0005
AGE*TIME	Old	28	Young	28	-2.32	0.0273
AGE*TIME	Young	16	Young	28	2.24	0.0373
TREATMENT*AGE*TIME	Control	Old	16	Control	Old	28	-0.25	0.8055
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	16	-2.99	0.0058
TREATMENT*AGE*TIME	Control	Old	16	Control	Young	28	-2.24	0.0329
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	16	-2.96	0.0062
TREATMENT*AGE*TIME	Control	Old	16	Implant	Old	28	-0.66	0.5154
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	16	-3.54	0.0014
TREATMENT*AGE*TIME	Control	Old	16	Implant	Young	28	-1.97	0.0589
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	16	-2.82	0.0087
TREATMENT*AGE*TIME	Control	Old	28	Control	Young	28	-2.07	0.0469
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	16	-2.80	0.0092
TREATMENT*AGE*TIME	Control	Old	28	Implant	Old	28	-0.50	0.6231
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	16	-3.37	0.0022
TREATMENT*AGE*TIME	Control	Old	28	Implant	Young	28	-1.80	0.0828
TREATMENT*AGE*TIME	Control	Young	16	Control	Young	28	0.91	0.3742
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	16	-0.11	0.9132
TREATMENT*AGE*TIME	Control	Young	16	Implant	Old	28	2.19	0.0370
TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	16	-0.56	0.5822

TREATMENT*AGE*TIME	Control	Young	16	Implant	Young	28	1.02	0.3173
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	16	-0.72	0.4764
TREATMENT*AGE*TIME	Control	Young	28	Implant	Old	28	1.50	0.1437
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	16	-1.18	0.2476
TREATMENT*AGE*TIME	Control	Young	28	Implant	Young	28	0.34	0.7373
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Old	28	3.24	0.0045
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	16	-0.42	0.6772
TREATMENT*AGE*TIME	Implant	Old	16	Implant	Young	28	1.08	0.2890
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	16	-2.72	0.0111
TREATMENT*AGE*TIME	Implant	Old	28	Implant	Young	28	-1.22	0.2334
TREATMENT*AGE*TIME	Implant	Young	16	Implant	Young	28	2.32	0.0323

A.5. Analysis of estradiol supplementation on the levels of plasma estradiol levels in the blood of heifers (Chapter 5)

A.5.1. Representative of SAS editor programming language using Proc Mixed (plasma estradiol levels of heifers) (Chapter 5)

```
filename new dde 'Excel|D:\604P\Data\Blood data\Estrogen\[604P Plasma
Estrogen Data.xls]SAS!R4C2:R75C7';
TITLE '604 Estrogen Data SAS- Repeated Measurements w/o Day 0
(Vanzant)';
data estrogen;
infile new missover;
INPUT LabID HEIFER $ AGE $ TIME TREATMENT $ Estrogen;
run;
proc print;
run;
PROC MIXED  data=estrogen;
CLASSES TREATMENT HEIFER TIME;
MODEL Estrogen =  TREATMENT TIME TREATMENT*TIME / DDFM=KR;
REPEATED TIME/type = ar(1) subject = HEIFER(TREATMENT);
Lsmeans  TREATMENT TIME TREATMENT*TIME/diff;
quit;
```

A.5.2. SAS output

Obs	ID	Lab HEIFER	AGE	TIME	TREATMENT	Estrogen
1	2	251	Young	14	Control	1.409
2	4	694	Young	14	Control	1.864
3	5	268	Young	14	Control	2.056
4	7	274	Young	14	Control	1.028
5	10	271	Young	14	Control	3.519
6	11	50	Young	14	Control	1.628
7	14	247	Young	14	Control	1.338
8	15	2634	Young	14	Control	.
9	20	256	Young	14	Control	1.930
10	1	2624	Young	14	Implant	5.290
11	3	249	Young	14	Implant	4.433
12	6	2608	Young	14	Implant	3.676
13	8	301R1	Young	14	Implant	3.393
14	9	264	Young	14	Implant	2.397
15	12	252	Young	14	Implant	2.886
16	13	301R2	Young	14	Implant	1.260
17	18	311R	Young	14	Implant	6.025
18	19	705R2	Young	14	Implant	7.871
19	2	251	Young	28	Control	1.179
20	4	694	Young	28	Control	1.294

21	5	268	Young	28	Control	1.776
22	7	274	Young	28	Control	4.621
23	10	271	Young	28	Control	.
24	11	50	Young	28	Control	3.022
25	14	247	Young	28	Control	1.956
26	15	2634	Young	28	Control	2.512
27	20	256	Young	28	Control	.
28	1	2624	Young	28	Implant	3.174
29	3	249	Young	28	Implant	7.097
30	6	2608	Young	28	Implant	4.037
31	8	301R1	Young	28	Implant	2.787
32	9	264	Young	28	Implant	.
33	12	252	Young	28	Implant	.
34	13	301R2	Young	28	Implant	3.075
35	18	311R	Young	28	Implant	3.848
36	19	705R2	Young	28	Implant	2.916
37	2	251	Young	43	Control	2.950
38	4	694	Young	43	Control	2.760
39	5	268	Young	43	Control	4.739
40	7	274	Young	43	Control	2.339
41	10	271	Young	43	Control	3.105
42	11	50	Young	43	Control	3.709
43	14	247	Young	43	Control	1.667
44	15	2634	Young	43	Control	1.866
45	20	256	Young	43	Control	2.039
46	1	2624	Young	43	Implant	3.925
47	3	249	Young	43	Implant	6.610
48	6	2608	Young	43	Implant	3.822

49	8	301R1	Young	43	Implant	2.969
50	9	264	Young	43	Implant	6.406

Obs	Lab ID	HEIFER	AGE	TIME	TREATMENT	Estrogen
51	12	252	Young	43	Implant	2.177
52	13	301R2	Young	43	Implant	3.558
53	18	311R	Young	43	Implant	3.047
54	19	705R2	Young	43	Implant	2.597
55	2	251	Young	56	Control	2.353
56	4	694	Young	56	Control	1.877
57	5	268	Young	56	Control	1.987
58	7	274	Young	56	Control	1.786
59	10	271	Young	56	Control	1.880
60	11	50	Young	56	Control	2.569
61	14	247	Young	56	Control	.
62	15	2634	Young	56	Control	1.283
63	20	256	Young	56	Control	.
64	1	2624	Young	56	Implant	4.723
65	3	249	Young	56	Implant	3.480
66	6	2608	Young	56	Implant	3.074
67	8	301R1	Young	56	Implant	3.853
68	9	264	Young	56	Implant	.

69	12	252	Young	56	Implant	2.304
70	13	301R2	Young	56	Implant	1.655
71	18	311R	Young	56	Implant	3.026
72	19	705R2	Young	56	Implant	4.176

The Mixed Procedure

Model Information

Data Set	WORK.ESTROGEN
Dependent Variable	Estrogen
Covariance Structure	Autoregressive
Subject Effect	HEIFER(TREATMENT)
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Prasad-Rao-Jeske-Kackar-Harville
Degrees of Freedom Method	Kenward-Roger

Class Level Information

Class	Levels	Values

TREATMENT	2	Control Implant
HEIFER	18	247 249 251 252 256 2608 2624 2634 264 268 271 274 301R1 301R2 311R 50 694 705R2
TIME	4	14 28 43 56

Dimensions

Covariance Parameters	2
Columns in X	15
Columns in Z	0
Subjects	18
Max Obs Per Subject	4

Number of Observations

Number of Observations Read	72
Number of Observations Used	64
Number of Observations Not Used	8

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	204.78750554	

1 2 203.61988307 0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	HEIFER(TREATMENT)	0.1795
Residual		1.6873

Fit Statistics

-2 Res Log Likelihood	203.6
AIC (smaller is better)	207.6
AICC (smaller is better)	207.8
BIC (smaller is better)	209.4

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	1.17	0.2799

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TREATMENT	1	20.9	18.56	0.0003
TIME	3	40.4	0.99	0.4083
TREATMENT*TIME	3	40.4	0.64	0.5914

Least Squares Means

Effect	TREATMENT	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control		2.2325	0.2593	21.6	8.61	<.0001
TREATMENT	Implant		3.7933	0.2530	20.1	14.99	<.0001
TIME		14	2.9932	0.3158	54.1	9.48	<.0001
TIME		28	3.0811	0.3483	55.7	8.85	<.0001
TIME		43	3.3492	0.3062	53.7	10.94	<.0001
TIME		56	2.6281	0.3368	54.7	7.80	<.0001
TREATMENT*TIME	Control	14	1.8497	0.4598	54.4	4.02	0.0002

TREATMENT*TIME	Control	28	2.3694	0.4926	55.7	4.81	<.0001
TREATMENT*TIME	Control	43	2.7971	0.4330	53.7	6.46	<.0001
TREATMENT*TIME	Control	56	1.9137	0.4922	55	3.89	0.0003
TREATMENT*TIME	Implant	14	4.1368	0.4330	53.7	9.55	<.0001
TREATMENT*TIME	Implant	28	3.7928	0.4926	55.7	7.70	<.0001
TREATMENT*TIME	Implant	43	3.9012	0.4330	53.7	9.01	<.0001

The Mixed Procedure

Least Squares Means

Effect	TREATMENT	TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT*TIME	Implant	56	3.3426	0.4598	54.4	7.27	<.0001

Differences of Least Squares Means

Effect	TREATMENT	TIME	_TREATMENT	_TIME	Estimate	Standard Error	DF	t Value	Pr > |t|
TREATMENT	Control		Implant		-1.5609	0.3623	20.9	-4.31	0.0003
TIME		14		28	-0.08788	0.4350	36.1	-0.20	0.8411

TIME		14		43	-0.3559	0.4391	55.3	-0.81	0.4210	
TIME		14		56	0.3651	0.4635	55.5	0.79	0.4343	
TIME		28		43	-0.2680	0.4273	34.9	-0.63	0.5345	
TIME		28		56	0.4530	0.4832	54.9	0.94	0.3527	
TIME		43		56	0.7210	0.4180	35.5	1.73	0.0932	
TREATMENT*TIME	Control	14	Control	28	-0.5197	0.6260	37.4	-0.83	0.4117	
TREATMENT*TIME	Control	14	Control	43	-0.9474	0.6305	55.3	-1.50	0.1386	
TREATMENT*TIME	Control	14	Control	56	-0.06402	0.6761	55.6	-0.09	0.9249	
TREATMENT*TIME	Control	14	Implant	14	-2.2871	0.6316	54.1	-3.62	0.0006	
TREATMENT*TIME	Control	14	Implant	28	-1.9431	0.6738	55.2	-2.88	0.0056	
TREATMENT*TIME	Control	14	Implant	43	-2.0515	0.6316	54.1	-3.25	0.0020	
TREATMENT*TIME	Control	14	Implant	56	-1.4929	0.6503	54.4	-2.30	0.0256	
TREATMENT*TIME	Control	28	Control	43	-0.4277	0.6043	34.9	-0.71	0.4838	
TREATMENT*TIME	Control	28	Control	56	0.4557	0.6946	54.9	0.66	0.5145	
TREATMENT*TIME	Control	28	Implant	14	-1.7674	0.6558	55	-2.69	0.0093	
TREATMENT*TIME	Control	28	Implant	28	-1.4234	0.6966	55.7	-2.04	0.0458	
TREATMENT*TIME	Control	28	Implant	43	-1.5318	0.6558	55	-2.34	0.0232	
TREATMENT*TIME	Control	28	Implant	56	-0.9732	0.6738	55.2	-1.44	0.1543	
TREATMENT*TIME	Control	43	Control	56	0.8834	0.6040	36.4	1.46	0.1521	
TREATMENT*TIME	Control	43	Implant	14	-1.3397	0.6123	53.7	-2.19	0.0331	
TREATMENT*TIME	Control	43	Implant	28	-0.9957	0.6558	55	-1.52	0.1347	
TREATMENT*TIME	Control	43	Implant	43	-1.1041	0.6123	53.7	-1.80	0.0770	
TREATMENT*TIME	Control	43	Implant	56	-0.5455	0.6316	54.1	-0.86	0.3916	
TREATMENT*TIME	Control	56	Implant	14	-2.2231	0.6555	54.5	-3.39	0.0013	
TREATMENT*TIME	Control	56	Implant	28	-1.8791	0.6963	55.4	-2.70	0.0092	
TREATMENT*TIME	Control	56	Implant	43	-1.9875	0.6555	54.5	-3.03	0.0037	
TREATMENT*TIME	Control	56	Implant	56	-1.4289	0.6736	54.7	-2.12	0.0384	
TREATMENT*TIME	Implant	14	Implant	28	0.3440	0.6043	34.9	0.57	0.5729	

TREATMENT*TIME	Implant	14	Implant	43	0.2356	0.6112	55.3	0.39	0.7014
TREATMENT*TIME	Implant	14	Implant	56	0.7942	0.6342	55.3	1.25	0.2158
TREATMENT*TIME	Implant	28	Implant	43	-0.1084	0.6043	34.9	-0.18	0.8587
TREATMENT*TIME	Implant	28	Implant	56	0.4502	0.6719	54.8	0.67	0.5056
TREATMENT*TIME	Implant	43	Implant	56	0.5586	0.5779	34.5	0.97	0.3404

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