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Effects of Dietary Conjugated Linoleic Acid Supplementation on Bovine Oocyte Lipid Metabolism, Lipid Composition and Embryo Cryotolerance

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EFFECTS OF DIETARY CONJUGATED LINOLEIC ACID SUPPLEMENTATION
ON BOVINE OOCYTE LIPID METABOLISM, LIPID COMPOSITION
AND EMBRYO CRYOTOLERANCE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program of
Animal and Dairy Sciences

by

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LIST OF ABBREVIATIONS

ACC β	acetyl-CoA carboxylase β
AI	artificial insemination
BCS	body condition score
BW	body weight
$^{\circ}\text{C}$	degree Celsius
CLA	conjugated linoleic acid
COC	cumulus-oocyte complex
CPT1	carnitin palmitoyl transferase 1
d	day
DDG	dry distillers grains
DESI-MS	desorption electrospray ionization mass spectrometry
DFR	dominant follicle removal
DHA	docosahexaenoic acid
DIM	days in milk
DM	dry matter
DMI	dry matter intake
ECM	energy corrected milk
FCM	fat corrected milk
Fr	French
FADS2	fatty acid desaturase 2
FRET	fluorescence resonance energy transfer
FSH	follicle-stimulating hormone
FTAI	fixed-time artificial insemination
g	gram
ga	gauge
GnRH	gonadotropin-releasing hormone
h	hour
ha	hectare
hd	head
HEPES	hydroxyethyl piperazineethanesulfonic acid
IETS	International Embryo Transfer Society
im	intramuscular
IVF	<i>in vitro</i> fertilization
LH	luteinizing hormone
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
min	minute
mRNA	messenger ribonucleic acid
NA	numerical aperture
NRC	National Research Council
OCM	oocyte collection medium
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PES	phenazine euthosulfate

PGF_{2α}..... prostaglandin F_{2α}
 PPARα..... peroxisome proliferator-activated receptor α
 PUFA..... polyunsaturated fatty acid
 s..... second
 SCC..... somatic cell count
 SE..... standard error
 SREBP1..... sterol response element-binding protein 1
 TALP..... Tyrode's albumen lactate pyruvate
 TMR..... total mixed ration
 TUGA..... transvaginal ultrasound-guided oocyte aspiration
 TUNEL..... terminal deoxynucleotidyl transferase dUTP nick-end labeling

ABSTRACT

Variation in cryotolerance exists between embryos from different animal breed, species and management conditions. Reduced tolerance to chilling and cryotolerance of oocytes and embryos has been associated with greater cytoplasmic lipids (Kim et al., 2001; Seidel, 2006). Previous studies in the cow have demonstrated nutrition-induced modification of follicular components. *Trans*-10, *cis*-12 conjugated linoleic acid (CLA) was identified as a potent inhibitor of milk fat synthesis in lactating cows (Baumgard et al., 2000) and inclusion of CLA in bovine embryo culture medium improved post-thaw embryo survival (Pereira et al., 2007). Dietary supplementation of cows with CLA could alter oocyte fatty acid metabolism, oocyte lipid composition and embryo cryotolerance, and responses may be different between *Bos indicus* and *Bos taurus* breeds of cattle. Therefore, a series of experiments were conducted to evaluate effects of dietary CLA supplementation of cows on (1) milk fat depression in lactating Holstein cows, (2) follicle and oocyte production and lipid content of oocytes from Brahman and Holstein cows, (3) mRNA expression of genes involved in lipid metabolism in oocytes from Brahman and Holstein cows and (4) cryosurvival of *in vitro*-produced embryos from CLA-supplemented oocyte donor cows.

Milk fat was depressed by 10.1% in lactating Holstein cows fed CLA. Follicle, oocyte and embryo production of cows were not influenced by CLA supplementation. Dietary supplementation of cows with CLA before oocyte collection did not influence cryotolerance of *in vitro*-produced embryos or

expression of genes in oocytes involved in lipid metabolism. Lipid content of oocytes was not influenced by CLA supplementation. The ovarian response to dietary CLA was similar among Brahman, Holstein and crossbred beef cows. The highly regulated mechanisms involved in fatty acid uptake by ovarian components may help explain the lack of ovarian response to dietary CLA in the current study.

CHAPTER I INTRODUCTION

The successful cryopreservation of embryos is vital for embryo transfer to make genetic improvement while being economically viable in a particular species. Embryo cryopreservation has become an important and rather routine practice in bovine embryo transfer. There are many reasons bovine embryos are cryopreserved for short or long-term storage in liquid nitrogen. Embryos are cryopreserved rather than immediately transferred into recipient females when greater-than-expected embryos are produced or recipient females are unavailable. Producers may want to cryopreserve embryos and transfer at a later date so offspring are born at a desired season due to weather, economic or marketing constraints. Cryopreserved embryos are often marketed by cattle producers, similar to selling cryopreserved semen of genetically superior bulls. Shipment of cryopreserved embryos across great distances is very efficient and economical and presents less risk of disease transmission compared with live animal transport. Embryo cryopreservation is also important in germplasm preservation programs.

The International Embryo Transfer Society (IETS) Data Retrieval Committee produces an annual report detailing embryo collection and transfer activities worldwide. In 2012, over 1.1 million embryos were collected or produced globally with embryos transferred to over one million cattle. In North America, 72% of the 355,866 *in vivo* embryos collected in 2012 were cryopreserved, while 57% of the 235,344 *in vivo*-produced embryos transferred

in 2012 were cryopreserved and thawed embryos. The trend of more embryos transferred after cryopreservation compared with fresh-transferred embryos has occurred annually for more than a decade in the cattle embryo transfer industry.

Cryopreservation of *in vivo*-produced embryos has become widely adapted and successful in cattle embryo transfer. Pregnancy rates after transfer of *in vivo*-produced, frozen-thawed embryos are reportedly 80 to 90% of the pregnancy rates achieved after transfer of fresh embryos (Hasler, 2001). However, variation in cryotolerance exists between *in vivo*-produced embryos from different species, breeds and management conditions.

Reduced tolerance to chilling and cryotolerance of oocytes and embryos has been associated with greater cytoplasmic lipids (Kim et al., 2001; Abe et al., 2002; Seidel, 2006). Nagashima et al. (1994) provided clear evidence for reducing cytoplasmic lipids by improving cryopreservation of porcine embryos after physical delipidation. Cooling and cryopreservation of lipid-rich porcine oocytes causes lipid droplets to coalesce and increase in size, which alters the structural organization within the cytoplasm of the oocyte (Edidin and Petit., 1977). The movement and structural alteration of lipid droplets during cooling may irreversibly damage bovine embryos by decreasing cytoplasmic organization of organelles (Mohr and Trounson, 1981).

The concentration and type of fat included in ruminant diets may influence the composition of oocytes and embryos, and therefore, their cryotolerance. Fatty acid composition of follicular fluid in dairy cows is sensitive to dietary supply of polyunsaturated fatty acids (PUFA) when feeding soybean oil (Batista et al.,

2010) and flaxseed oil (Zachut et al., 2010). *In vivo*-produced embryos from Nellore heifers fed PUFA had reduced cryotolerance (Guardieiro et al., 2014).

Another interesting area of study has identified specific conjugated linoleic acids (CLA) isomers that inhibit milk fat synthesis in dairy cows (Lor and Herbein, 1998; Chouinard et al., 1999a; Chouinard et al., 1999b). The *trans*-10, *cis*-12 CLA isomer was identified as a potent inhibitor of milk fat synthesis in lactating cows (Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002). Addition of *trans*-10, *cis*-12 CLA into culture media reduced lipid accumulation and improved cryotolerance of *in vitro*-produced bovine embryos (Pereira et al., 2007). Although the *trans*-10, *cis*-12 CLA isomer appears to influence both milk fat synthesis and embryo lipid accumulation *in vitro*, it is unknown whether oocyte and embryo lipid composition could be altered *in vivo* by feeding *trans*-10, *cis*-12 CLA. Research is warranted to elucidate possible relationships between dietary CLA supplementation of cows and oocyte and embryo composition, with potential implications for embryo cryopreservation.

Although cryopreservation of *in vivo*-produced bovine embryos is common, *in vitro*-produced embryos are more sensitive to chilling and cryopreservation than *in vivo*-produced embryos (Leibo and Loskutoff, 1993; Pollard and Leibo, 1993; Farin et al., 1999). Only 13% of the 40,546 *in vitro*-produced embryos transferred in North America in 2012 were cryopreserved embryos (IETS, 2012). In Brazil, the global leader of bovine *in vitro* embryo production, 303,168 fresh *in vitro*-produced embryos were transferred, while only 28,314 cryopreserved and thawed embryos were transferred (8.5%) in 2012.

Embryo transfer practitioners are reluctant to cryopreserve *in vitro*-produced bovine embryos at a rate similar to *in vivo* embryos because *in vitro* bovine embryos have not produced consistent satisfactory pregnancy rates after transfer. Research has closed the gap in cryosurvival between *in vivo* and *in vitro* bovine embryos, yet there remains opportunity for improvement before the widespread adoption of *in vitro* embryo cryopreservation in cattle.

Successful cryopreservation of embryos from *Bos indicus* cattle is variable (Visintin et al., 2002). Cytoplasmic lipid content is greater in *Bos indicus* oocytes compared with *Bos taurus* oocytes (Ballard, 2006), likely contributing to reduced cryotolerance in embryos from *Bos indicus* cattle. *Bos indicus* cattle also have greater milk fat compared with other breeds of cattle.

Additional research is necessary to elucidate mechanisms contributing to variation in cryotolerance associated with individual animal, breed, species, nutrition and season of the year (Seidel, 2006). Previous studies in the cow have demonstrated nutrition-induced modification of follicular components. Dietary supplementation of cows with CLA could alter oocyte fatty acid metabolism, oocyte lipid composition and embryo cryotolerance. The response to CLA supplementation may be different between *Bos indicus* and *Bos taurus* breeds of cattle. Better understanding of mechanisms contributing to variation in cryotolerance of embryos will likely continue to improve success of embryo cryopreservation. Research models that evaluate variation in lipid composition of oocytes and embryos have the potential to impact biological disciplines beyond cryopreservation.

CHAPTER II REVIEW OF LITERATURE

Cryotolerance of Embryos

Cryotolerance and cryosurvival are terms used in the literature that describes the ability of cells to survive a particular cryopreservation process. The most accurate test for the survival of embryos to cryopreservation is the birth of healthy offspring resulting from transfer of frozen and thawed embryos. The next best measure of embryo survival is a confirmed pregnancy in recipients after transfer of frozen- thawed embryos. However, applying statistical methods to observed differences in live offspring or pregnancy rates is very challenging. To show a statistical difference ($P \leq 0.05$) based on a power of 0.90 and pregnancy rates of 60% and 70% for control and treatment groups using a two-tailed test requires 496 embryo transfers in each treatment group (Chapman and Seidel, 2007; Hasler, 2010). A change in pregnancy rate of 10% in commercial embryo transfer would be financially significant, but it is very difficult and expensive to design practical studies to evaluate these differences (Hasler, 2010). Pregnancy rate differences of much less (e.g., 5% points) would be financially important, but would require an even greater number of embryo transfers than specifications listed above.

Researchers have developed techniques for the evaluation of cryosurvival of embryos that require fewer resources. Although many of these techniques are less robust than comparing pregnancy rates or live offspring, they allow for some inference into cryosurvival in experimental settings. One such method is a post-

thaw analysis followed by an *in vitro* survival assay. *In vitro* survival assays have been used to evaluate post-thaw survival in a number of published studies involving different *in vitro* culture conditions or cryopreservation techniques for bovine (Pugh et al., 1998; Enright et al., 2000; Abe et al., 2002; Lopatářová et al., 2002; Rizos, 2003; Pereira et al., 2007; Pereira et al., 2008; Al Darwich et al., 2010; Ruiz et al., 2013) and porcine embryos (Men et al., 2006). Generally, these researchers evaluated survival or re-expansion based on a defined set of criteria at the time of thawing and again at 12 h or 24 h intervals through 24 h to 72 h of *in vitro* culture. The number of embryos hatching was also reported in a number of the aforementioned studies. Most evaluated cryosurvival in moderate to low numbers of embryos ($n \leq 50$) per treatment group.

A number of research groups have transferred d-7 to d-8 *in vitro*-produced embryos and recovered them on d 14 to d 16 to evaluate embryonic survival, development and/or gene expression. After transferring d-7 embryos, d-14 to d-16 embryos were recovered and embryonic development was evaluated between *in vivo*- and *in vitro*-produced embryos (Bertolini et al., 2002; Angulo-Campos, 2010), *in vitro* and somatic cell nuclear transfer embryos (Alexopoulos and French, 2009) and *in vitro* culture conditions (Block et al., 2007; Clemente et al., 2009). Day-14 bovine embryos are spherical, ovoid or elongated (Alexopoulos and French, 2009; Clemente et al., 2009) and this stage immediately precedes important embryonic events, such as maternal recognition of pregnancy and the formation of extra-embryonic membranes and the embryo proper (Bertolini et al., 2002).

Embryo development was evaluated by comparative morphology and the presence of an embryonic disk (Bertolini et al., 2002; Alexopoulos and French, 2009). A more detailed assessment of d-16 embryos was reported using histological evaluation (Alexopoulos and French, 2009). Recovery rates at d 14 to d 16 after embryo transfer on d 7 ranged from 20% to 70% (Bertolini et al., 2002; Block et al., 2007; Alexopoulos and French, 2009). Clemente et al. (2009) evaluated development after *in vitro* culture with progesterone by transferring d-7 embryos (20 embryos per recipient) and recovering d-14 embryos from reproductive tracts after recipient heifers were slaughtered. Clemente et al. (2009) noted large variation in conceptus size on d 14, even within recipients, and recovery rate of d-14 embryos was 31% (1425 embryos transferred).

The period from d 8 to d 16 is also the time of greatest embryonic loss in cattle (Diskin and Sreenan, 1980; Roche et al., 1981). Embryonic loss during this period ranged from 40% to 60% in studies involving artificial insemination (Diskin and Sreenan, 1980; Roche et al., 1981; Dunne et al., 2000) and transfer of *in vitro*-produced embryos (Farin and Farin, 1995; Peterson and Lee, 2003; Farin et al., 2004). Embryonic loss after natural mating or AI from d 24 to term is only 5% to 8% in beef cows (Sreenan and Diskin, 1986). Transfer of d-7 embryos and recovery at d 14 may be a useful assay to evaluate early embryonic survival and development of cryopreserved embryos. The number of embryos recovered at d 14 and their morphology may allow some comparison of cryosurvival between experimental treatments.

Factors Contributing to Variation in Cryotolerance of Embryos

Modification of cryopreservation procedures has historically been the most common approach to improve success of cryopreservation of certain cell types (Leibo, 1981). Optimizing procedures (e.g., cryoprotectant type and concentration, cooling rate, warming rate) usually results in improvements, but they are often limited. Therefore, an alternative approach is to modify the cells themselves to improve their cryotolerance (Seidel, 2006). The remainder of this review will focus on biological, rather than procedural, factors which contribute to variation in cryotolerance of embryos.

Cryopreservation procedures exert considerable physical stress on cells; a major stress is osmotic due to movement of water and cryoprotectants across cell membranes, sometimes resulting in significant changes in volume (Leibo, 1981). Therefore, cells with relatively flexible membranes that are highly permeable to water and cryoprotectants may undergo less membrane damage than cells with more rigid membranes (Seidel, 2006).

Membrane Composition

Cell membranes properties are mediated by membrane composition. Cell membranes are composed mainly of phospholipids, cholesterol, other lipids and proteins. Except for proteins, the concentrations of these components in cell membranes of oocytes and embryos can potentially be altered by nutrition of the donor animal and/or composition of culture media (Seidel, 2006). Cell membranes that maintain a relatively fluid state allow for more effective diffusion of water and cryoprotectants. Some cell membranes change from fluid to a gel

state at decreased temperatures (Zeron et al., 2001), reducing membrane permeability and increasing cell damage upon warming. Membrane phase transition is also dependent on the composition of the cell membrane (Zeron et al., 2001). Therefore, research designed to better understand factors influencing cell membrane composition will likely lead to practices that improve membrane characteristics and decreased damage during cryopreservation.

Intracytoplasmic Lipid Composition

In addition to cell membrane properties, high concentrations of cytoplasmic lipid droplets have been associated with decreased cryotolerance. Triglyceride is the major lipid component of bovine and porcine oocytes (Homa et al., 1986; McEvoy et al., 2000; Kim et al., 2001) and is used as an energy source for embryo maturation both *in vitro* (Kim et al., 2001; Sturmey and Leese, 2003) and *in vivo*. Nagashima et al. (1994) provided clear evidence for reducing cytoplasmic lipids by improving cryopreservation of porcine embryos after physical delipidation. The sensitivity of porcine embryos to chilling may result in irreversible changes in membrane structure when porcine embryos are cooled below a critical temperature (Nagashima et al., 1994). Cooling causes lateral phase separation of membrane lipids in artificial and bacterial membranes (Petit and Edidin, 1974) and ram spermatozoa (Holt and North, 1986). Additionally, lipid droplets in porcine oocytes generally have close spatial relationship with smooth endoplasmic reticulum (Hyttel and Niemann, 1990) and are believed to be involved in modifying the physical properties and functions of mammalian cell

membranes (Stubbs and Smith, 1984). Therefore, modification of cytoplasmic lipids may influence membrane composition, which can influence cryotolerance.

Another theory is that the actual presence of lipid droplets may directly influence embryo survival during chilling (Nagashima et al., (1994). Cooling and cryopreservation of porcine oocytes causes lipid droplets to coalesce and increase in size, which alters the structural organization within the cytoplasm (Edidin and Petit., 1977). The movement and structural alteration of lipid droplets during cooling may irreversibly damage bovine embryos by decreasing cytoplasmic organization of organelles (Mohr and Trounson, 1981).

Triglyceride is metabolized by mitochondria through β -oxidation and the TCA cycle within the mitochondria matrix. Nonphysiological *in vitro* culture conditions (specifically those containing serum) are reported to decrease concentration of mitochondria and alter their ability to metabolize lipids by β -oxidation (Crosier et al., 2001b; Abe et al., 2002). Therefore, studies have focused on increasing lipid metabolism *in vitro* in an effort to mimic metabolic conditions *in vivo*. Additionally, embryo lipid metabolism may differ between donor animal or between breeds, leading to variation in both embryo lipid content and cryotolerance.

The quantity of unsaturated fatty acids rather than gross lipid content may be more critical to chilling sensitivity and cryopreservation of oocytes and embryos from domestic livestock species (McEvoy et al., 2000). Analysis of fatty acid composition of bovine (Zeron et al., 1999a; McEvoy et al., 2000; Kim et al., 2001) and porcine oocytes (Homa et al., 1986) showed palmitic (16:0), oleic

(18:1) and stearic (18:0) acids were the three most abundant fatty acids, listed in order. However, Sata et al. (1999) reported that myristic acid (14:0) was the most abundant fatty acid in bovine embryos, and indicated that fatty acid composition may differ by species or between age or breed of cow.

Stage of Development

The origin of oocyte lipids and the timing of lipid deposition are not fully understood. It appears that oocytes acquire and/or deposit lipids between the time of primordial follicle activation and the antral stage (Fair et al., 1997). It is not clear whether lipids pass into oocytes via junctions between the oocyte and cumulus cells or if they are taken directly from follicular fluid. A variety of pathways are likely involved in oocyte lipid metabolism and deposition during follicle development (Algriany et al., 2007).

Follicle formation in ruminants begins during fetal development and primordial follicle activation occurs over a long period of time, allowing groups of follicles to be activated and leave the resting pool while other follicles remain inactive (Hirshfield, 1991; Fortune et al., 2000; Fortune, 2003). Early growth of primordial follicles begins slow, the time needed to double the number of granulosa cells in early developmental stages takes ~7 d in the rat (Hirshfield, 1989) and 189 h in the rabbit (Mariana and De Pol, 1986). The rate of oocyte growth and granulosa cell proliferation increases with increasing follicle size (Hirshfield, 1991). It likely takes longer for a follicle to grow from 4 cells to 32 cells than it takes to grow from 32 cells to 2500 cells in the rat (Hirshfield, 1991).

The time necessary for a follicle and developing oocyte to advance from primordial stage to the antral follicle stage is likely highly variable because of the many factors that influence this process (Hirshfield, 1991). Although it is difficult to estimate, the course of follicular growth takes a long time relative to the reproductive cycle of the animal; several weeks in rodents, and perhaps several months in larger animals (Hirshfield, 1991; Fortune, 2003).

Lipid content of porcine embryos gradually declines during development *in vivo*, which may explain why expanded and hatched blastocyst stage embryos have a higher tolerance to cooling compared with morulae and early blastocysts (Nagashima et al., 1988). Bovine embryos follow a similar pattern, as lipid droplets diminish as the bovine embryo develops from early cleavage stages to the blastocyst stage (Mohr and Trounson, 1981; Pryor et al., 2008a). This demonstrates that lipids are utilized by the developing embryo, likely as a source of energy. The presence of relatively high concentrations of intracellular lipids in immature oocytes of most mammals likely contributes to limited success with oocyte cryopreservation.

In the mouse, *in vivo*-matured ovulated oocytes had significantly greater lipid concentrations and distinct droplet distribution patterns compared with immature oocytes (Yang, 2010). Higher lipid content in mature oocytes of the mouse could be due to uptake from follicular fluid or from the oviduct, *de novo* lipid synthesis or relocalisation of diffuse lipid stores into larger droplets (Yang, 2010). Dynamic reorganization of lipid droplets occurs along with oocyte maturation, likely in response to the LH surge (Yang, 2010).

Additionally, membranes of human zygotes and oocytes at different developmental stages have different membrane lipid phase transitions, suggesting a variation in their composition (Ghetler et al., 2005). Finally, oocytes from abbatoir-derived heifer ovaries (breed not specified) produced blastocysts with greater lipid content than those from mature cow-derived ovaries (Barceló-Fimbres and Seidel, 2008).

Species

Porcine oocytes and embryos are darker than those of ovine and bovine, indicating greater lipid content (Sturmey and Leese, 2003). Uneven intracellular ice crystal formation was noted when porcine embryos were cooled (Nagashima et al., 1988). Porcine oocytes contain greater lipid stores than bovine oocytes, which may help explain the limited success with cryopreservation of porcine oocytes and embryos (McEvoy et al., 2000). A great deal of research has been devoted to reducing intracellular lipids of porcine oocytes and embryos in an effort to improve success of cryopreservation. Triglyceride concentrations of porcine oocytes are greater than bovine and ovine (McEvoy et al., 2000), however, relative fatty acid compositions seem rather similar between these species.

Cattle Breed

Cryopreservation of embryos from Jersey donors resulted in reduced pregnancy rates compared with those from Holstein donors (Steel and Hasler, 2004), and this observation is correlated with greater fat content in milk from Jersey cows. Cytoplasmic lipid content is greater in *Bos indicus* oocytes

compared with *Bos taurus* oocytes (Ballard, 2006), likely contributing to reduced cryotolerance of *in vivo*-produced and *in vitro*-produced embryos of *Bos indicus* cattle. More Holstein Friesian embryos were classified as dark (measure as light, medium, or dark with stereomicroscope at 40x) compared with Belgian Blue embryos (Leroy, 2004). Holstein embryos contained greater intracellular lipids than Nelore embryos (Vistin, 2002); however, experimental numbers were relatively small (n = 20 per breed) and description of donor animal management and number of donors used was minimal. The previous study evaluated semi-thin sections of embryos stained with toluidine blue using light microscopy and ultrathin sections of embryos stained with uranyl acetate evaluated with transmission electron microscopy (Visintin, 2002). Embryos from *Bos indicus* and Jersey donors produce pregnancy rates similar to other breeds when collected and transferred fresh; however, successful embryo cryopreservation with these breeds is variable. In addition to greater embryo lipid content, *Bos indicus* and Jersey cattle generally have high milk fat content compared with other breeds of dairy and beef cattle.

Season

Seasonal effects on reproductive performance of cattle have been attributed to variation in temperature, humidity, photoperiod and nutrition (Thatcher, 1974). Many mechanisms contributing to sub-optimal reproductive efficiency during heat stress have been elucidated. Elevated ambient temperatures causing heat stress in cattle decreased the duration and intensity of estrus (Gwazdauskas et al., 1981), impaired follicular development and altered

dominance of first wave and pre-ovulatory dominant follicles (Wolfenson et al., 1995), increased the number of abnormal bovine embryos (Putney et al., 1989) and decrease the quality and developmental competence of bovine oocytes (Rocha et al., 1998). The physical properties of lipids in biological membranes and changes in lipid composition of membranes may help explain decreased fertility during heat stress (Quinn, 1985). Ambient temperature regulates transition from the lipid crystalline to the gel phase, known as lipid phase transition (Crowe et al., 1989), and injury to bovine oocytes occurs near the lipid phase transition (Arav et al., 1999).

Oocytes collected from Holstein cows during summer months had greater nonhomogeneous dark regions, possibly due to changes in lipid composition and content with season (Zeron et al., 2001). Increasing concentrations of unsaturated fatty acids in biological membranes is associated with increased membrane fluidity (Crowe et al., 1989) and the phospholipids of bovine oocyte membranes contained over twice the concentration of polyunsaturated fatty acids in winter compared with summer (Zeron et al., 2001). In addition, the T_m value (midpoint lipid phase transition) for bovine oocytes decreased from 19.5°C in summer to 13.5°C in winter (Zeron et al., 2001), which is likely explained by changes in relative and/or absolute concentrations of fatty acids in membranes and their corresponding chain lengths and number and position of double bonds. Together, these data indicate ambient temperature and/or season, directly or indirectly influences the composition of cell membranes of oocytes, which likely influences their cryotolerance.

Donor Nutrition

Important interactions between nutrition and reproduction have been given a great deal of attention in Scientific Literature. Recent research has focused on nutritional mechanisms influencing oocyte developmental competence and embryo quality. However, minimal data exists regarding nutrition potentially influencing embryo composition and cryopreservation. A possible source of variation in embryo cryopreservation may be dietary fat supplementation of donor animals.

Fats are included in ruminant diets primarily to provide energy, as fat is caloric dense (NRC, 1996). Positive effects on reproductive processes in fat supplemented beef cattle have been attributed to altering unsaturated fatty acid status rather than changes in energy status (Hess et al., 2008). Considerable research has focused on improving fertility of cattle through dietary fatty acid supplementation. Strategies for improving fertility in dairy cows, such as dietary fatty acid supplementation, was previously reviewed by Thatcher (2006).

Reproductive responses after feeding supplements high in polyunsaturated fatty acids (PUFA) has been investigated in both dairy and beef cows. The composition of bovine follicular fluid is sensitive to changes in dietary supply of PUFA (Batista et al., 2010). However, feeding PUFA failed to influence oocyte quality in lactating Holstein cows, suggesting that benefits of PUFA are a result of other biological mechanism (Bilby et al., 2006). Similarly, PUFA supplementation of beef heifers increased concentrations of PUFA of both plasma and uterine fluid, yet did not influence superovulation response, embryo

recovery rate, embryo quality or gene expression related to early embryo development (Childs et al., 2008). These results concurred with those of Bilby et al. (2006), who concluded that the potential positive effects of dietary PUFA on reproductive performance in dairy cattle were not a result of altering the early developing embryo.

Fouladi-Nashta et al. (2007) reported inclusion of calcium salts of palm oil (rich in PUFA) in the diet of lactating dairy cows did not influence oocyte quality or cleavage rate; however, cows fed a diet with high calcium salts of palm oil (800 g/d) had superior blastocyst production *in vitro* compared with cows fed a diet low in calcium salts of palm oil (200 g/d). Also, lactating Holstein cows supplemented with PUFA accelerated early embryonic development *in vivo* (Thangavelu et al., 2007). A contrasting report by Wakefield et al. (2008) in the mouse demonstrated that high dietary PUFA reduced normal embryo development; authors suggested that this could be associated with altered mitochondrial function. Feeding dairy cows a source of n-3 fatty acids (whole flax seed) compared with calcium salts of palm fatty acids decreased embryo quality yet did not influence pregnancy success after embryo transfer (Petit et al. 2008). Thatcher et al. (2006) indicated that inclusion of PUFA in ruminant diets during late gestation and early postpartum may accelerate uterine recovery and restoration of the reproductive system to support embryo development. Furthermore, PUFA supplementation (fish oil enriched calcium salts) altered fatty acid composition of the endometrium in a manner that would reduce secretion of $\text{PGF}_{2\alpha}$ (Bilby et al., 2006b).

Inconsistency in response in ruminants are likely a result of supply of specific unsaturated FA for absorption in the small intestine (Santos et al., 2008b), which is influenced by the efficacy of methods used to protect the supplement from biohydrogenation in the rumen. Additionally, the effects of fat supplementation on bovine oocyte quality depend on body condition of cows (Adamiak et al., 2005; Adamiak et al., 2006). Dietary fat induced alterations in fatty acid composition of bovine cumulus-oocyte complexes (COC) were influenced by carbohydrate source (high starch compared with high fiber) fed to cows (Adamiak et al., 2006). Further discrepancies between experiments could be due to the presence or lack of a negative control treatment (no fat diet), the use of transvaginal ultrasound-guided oocyte aspiration (TUGA) and *in vitro*-fertilization compared with collection of *in vivo* matured d-7 embryos, differences in energy required by donor animals (i.e., lactation in dairy cattle compared with beef cattle or ewes) or the timing of dietary treatments relative to oocyte/embryo recovery.

Although the effects of dietary fat supplementation on oocyte developmental competence and embryo quality appear negligible, the concentration and type of fat included in ruminant diets may influence the composition of oocytes and embryos, and therefore, their cryotolerance. Zeron et al. (2002a) reported that ewes supplemented with calcium salts of fish oil (high PUFA) increased the number of high quality oocytes compared with control-supplemented ewes (74% and 57%, respectively). Fish oil supplementation of ewes increased the proportion of long-chain unsaturated fatty acids in plasma

and cumulus cells and increased chilling resistance of ewe oocytes (Zeron et al., 2002a). However, changes in fatty acid composition of oocytes in the previously mentioned research were relatively small (not statistically different from controls), indicating that uptake of PUFA by bovine oocytes is likely selective or highly regulated in the cow (Bilby et al., 2006) and/or that tools used to evaluate lipid composition were not robust enough for microscopic samples, such as oocytes and embryos.

Cryotolerance of *in vivo*-produced embryos decreased when Nellore heifer diets were supplemented with calcium salts of palm oil, a rumen-protected fat high in PUFA (Guardieiro, 2010; Guardieiro et al., 2014). Pregnancy rate after transfer of cryopreserved bovine embryos was not influenced by fat source fed to Holstein cows (whole flax seed high in n-3 fatty acids compared with calcium salts of palm fatty acids), suggesting that these supplements likely did not significantly alter embryo lipid composition and therefore, cryotolerance of embryos was similar (Petit et al., 2008). However, the number of embryos transferred was relatively small (n = 60/treatment) and neither embryo cryotolerance nor embryo lipid composition were measured in the study by Petit et al. (2008).

Collectively, these studies indicate that both membrane and intracellular lipid composition of oocytes and embryos can be altered by diet of the donor animal. However, this area remains understudied, likely due to the complexity of these experiments. Furthermore, composition of developmentally competent embryos, produced either *in vivo* or *in vitro*, may differ from the composition of

embryos that are most cryotolerant. Therefore, nutritional and management recommendations for donor animals may differ when embryos collected are cryopreserved rather than transferred fresh.

Culture Environment

Culture of embryos *in vitro* results in fundamental differences compared with *in vivo*-produced embryos (Lonergan et al., 2003). *In vitro*-produced porcine embryos differed morphologically and contained greater cytoplasmic lipid droplets compared with those produced *in vivo* (Kikuchi, 2002). Ovine embryos cultured in medium containing blood serum resulted in lambs with heavier birth weights compared with embryos produced in medium containing serum albumin (Thompson et al., 1995). It has been reported several times that bovine embryos cultured in serum-containing media have altered ultrastructural morphology (Abe et al., 1999; Crosier et al., 2000, 2001a), more intracellular lipid droplets (Abd El Razek, 2000; Abe et al., 2002) and decreased cryotolerance (Abe et al., 2002; Rizos, 2003) compared with embryos cultured in serum-free media. Lipid composition of embryos is also influenced by the presence of serum in the medium (Sata et al., 1999; Abd El Razek, 2000).

Three mechanisms have been suggested to interfere with lipid metabolism of *in vitro*-produced bovine embryos: serum may increase lipid synthesis in the embryo (Abd El Razek, 2000), lipoproteins present in serum could be internalized by the cells (Ferguson and Leese, 1999; Sata et al., 1999) or lipid accumulation results because nonphysiological culture conditions containing serum decrease the concentration of mitochondria and alter their ability to metabolism lipids by

β -oxidation (Dorland et al., 1994; Crosier et al., 2001b; Abe et al., 2002). Crosier et al. (2001b) concluded that embryonic development of *in vitro*-produced bovine embryos may be negatively influenced as a result of both increasing lipids and decreasing mitochondria concentrations. Although removing serum from culture media decreases cytoplasmic lipid accumulation compared with controls, *in vivo*-matured bovine embryos still contain less cytoplasmic lipids than *in vitro*-produced bovine embryos cultured in serum-free medium (Rizos, 2003; De La Torre-Sanchez et al., 2006a).

Modification of Oocytes and Embryos to Improve Cryotolerance

To improve cryopreservation of certain cell types you must either modify cryopreservation procedures or modify the cells themselves to make them better suited to survive cryopreservation (Seidel, 2006). The first method is the historical basis for cryopreservation methods used today, however, further modification of current methods often result in limited improvement. Therefore, recent research has focused on methods that modify cells in an effort to improve their compatibility with cryopreservation procedures. These methods include physical modification by centrifugation with or without physical delipidation, altering the composition of culture medium and modifying donor nutrition.

Physical Modification

Nagashima et al. (1994) was the first to use centrifugation and micromanipulation to delipidate porcine embryos, providing clear evidence that the low tolerance of porcine embryos to chilling is associated with their high lipid content and that chilling sensitivity can be reduced by decreasing lipid content in

the embryo. Similarly, physical delipidation improved survival rate after freezing of porcine oocytes (Hara et al., 2005) and embryos (Ushijima et al., 2004) and *in vitro*-produced bovine embryos (Ushijima et al., 1999; Diez et al., 2001). Additionally, removal of cytoplasmic lipids from oocytes of domestic cat did not impair development after cryopreservation (Karja et al., 2006). Preliminary results indicate centrifugation and treatment of *in vitro*-produced bovine embryos with cytochalasin B (microfilament inhibitor) before cryopreservation, combined with laser-assisted hatching post-thaw improves cryotolerance (Pryor et al., 2008b). However, centrifugation of immature bovine oocytes produced *in vitro* with or without lipid removal (Murakami et al., 1998) and centrifugation of mature bovine oocytes produced *in vitro* (Otoi et al., 1997) did not influence cryosurvival. Nagashima et al. (1994) concluded the development of a noninvasive method to remove lipids may improve cryopreservation of porcine embryos and warrants further attention.

Cell membranes with greater concentrations of cholesterol are more fluid at lower temperatures, and therefore improve tolerance to chilling (Horvath and Seidel, 2006). The addition of cholesterol to sperm improved cryotolerance of bovine (Purdy and Graham, 2004), equine (Moore et al., 2005) and porcine (Moraes et al., 2010) sperm. Cholesterol-enhanced bovine oocytes had moderate improvement in development to the 8-cell stage compared with controls (55% vs. 41%; $P < 0.05$) after they were thawed and fertilized *in vitro* (Horvath and Seidel, 2006). These studies all used methyl- β -cyclodextrin, a hydrophilic molecule with a hydrophobic center, to transfer cholesterol to cell

membranes. Zeron et al. (2002b) modified bovine oocytes by incorporating liposomes containing egg-phosphatidylcholine into cell membranes. This treatment altered membrane phase transition and reduced chilling injuring, likely due to alterations in cell membrane composition.

Modification of Culture Systems

Modification of *in vitro* embryo culture systems has received a great deal of research attention in an effort to improve the efficiency and success of these systems. Although serum was historically an important component of bovine *in vitro* culture systems, it has been associated with abnormal pregnancies and offspring and increased embryo lipid accumulation (Young et al., 1998; Hasler, 2000; Farin et al., 2001; Thompson et al., 2007) and has largely been discontinued in bovine culture medium (Hasler, 2010). Removing serum from culture decreases cytoplasmic lipid accumulation, yet bovine embryos produced in serum-free medium contain greater cytoplasmic lipids compared with *in vivo*-produced embryos (Rizos, 2003; De La Torre-Sanchez et al., 2006a). Therefore, studies have been conducted to bridge this gap, which will likely improve cryopreservation of *in vitro*-produced embryos.

Dorbrinsky et al. (2000) treated porcine embryos with cytochalasin B before vitrification in an effort to limit cytoskeletal damage. Cytochalasin B treatment improved blastocyst developmental competence after cryopreservation and normal piglets were produced after embryo transfer (Dobrinsky et al., 2000). However, cytochalasin B treatment before vitrification did not improve post-thaw

viability of bovine oocytes (Mezzalana et al., 2002) or caprine parthenogenic blastocysts (Nims et al., 2004).

Experiments to improve the composition of bovine embryos by altering cell metabolism *in vitro* indicated that inclusion of phenazine ethosulfate (PES) into embryo culture medium decreased embryo lipid content (Barceló-Fimbres and Seidel, 2005; De La Torre-Sanchez et al., 2006a). Phenazine ethosulfate is an electron acceptor that oxidizes NADPH to NADP, which is necessary for conversion of glucose-6-phosphate into 6-phosphogluconate. Thus, PES increases flux of glucose through glycolysis and the pentose phosphate pathway (De La Torre-Sanchez et al., 2006a; De La Torre-Sanchez et al., 2006b), decreases lipid accumulation in bovine embryos produced *in vitro* (De La Torre-Sanchez et al., 2006a; De La Torre-Sanchez et al., 2006b; Barceló-Fimbres and Seidel, 2007a; Sudano et al., 2010), and improved cryotolerance of bovine embryos (Barceló-Fimbres and Seidel, 2007a).

Inclusion of PES into embryo culture medium did not prevent apoptosis after embryo cryopreservation (measured using the TUNEL Assay). The TUNEL assay is a method used to detect DNA fragmentation resulting from apoptotic signals by labeling the terminal end of nucleic acids (Gavrieli et al., 1992; Negoescu et al., 1996). Furthermore, PES supplementation reduced *in vitro* blastocyst development and reduced post-thaw hatching rates (Ruiz et al., 2013). Barceló-Fimbres et al. (2009) recently reported that embryos cultured in the presence of PES did not influence pregnancy rates between d 35 and d 98 of pregnancy, and had no detectable effect on postnatal fetal development.

Treatment of porcine embryos with the lipolytic agent forskolin during *in-vitro* maturation increased intracellular lipolytic activity and improved cryosurvival, likely due to reduction in intracellular lipid content (Men et al., 2006). Briefly, forskolin activates adenylate cyclase (Seamon et al., 1981), resulting in activation of lipase through the cAMP/protein kinase pathway (Strayfors and Belfrage, 1983; Honnor et al., 1985), which ultimately stimulates lipolysis through phosphorylation. Current literature is inconsistent regarding effects forskolin as a lipolytic agent in bovine embryos. The addition of 10 μ M and 40 μ M forskolin failed to decrease intracellular lipids of *in vitro*-produced bovine embryos in one trial (Pryor et al., 2008a), yet successfully decreased lipid content in another trial (Barceló-Fimbres and Seidel, 2008). Both of these authors measured intracellular lipids using a similar Nile Red staining procedure. Addition of 10 μ M forskolin increased survival and blastocyst hatching rates after slow-cool cryopreservation of *in vitro*-produced *Bos indicus* embryos (Pryor et al., 2010; Sanches et al., 2010). However, inclusion of 10 μ M forskolin in culture had no effect on bovine embryo development or cryosurvival (Ruiz et al., 2013).

Inclusion of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) in bovine embryo culture medium improved post-thaw embryo survival compared with controls, without influencing bovine blastocyst production (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013). This improvement in cryosurvival may be due to reduced accumulation of lipids in *trans*-10, *cis*-12 CLA cultured embryos, evaluated by Nomarski differential interference contrast microscopy. Additionally, results may be explained by increased membrane fluidity due to

incorporation of *trans*-10, *cis*-12 CLA into membranes of bovine embryos (Pereira et al., 2007). In contrast, post-thaw *in vitro* survival of bovine embryos was not improved after co-culture with 100 μ M *trans*-10, *cis*-12 CLA (Al Darwich et al., 2010). The primary difference between the previous two studies is the cryopreservation method used. Embryo survival may not have been improved in the study by Al Darwich et al. (2010) because embryos were cryopreserved using vitrification, while Pereira et al. (2007) cryopreserved embryos using a slow-cooled method. However, Pereira et al. (2008) reported bovine embryos cultured with *trans*-10, *cis*-12 CLA had improved survival after biopsy and vitrification. Multiple mechanisms are likely involved in *trans*-10, *cis*-12 CLA improving cryotolerance of embryos, requiring further research. With particular interest is the influence of *trans*-10, *cis*-12 CLA on oocyte lipid metabolism and embryo lipid composition.

In vitro embryos cultured with L-carnitine, a rate-limiting enzyme of β -oxidation, improved embryo development and increased metabolic activity via increased β -oxidation resulting in reduced embryo lipid concentration measured by fluorescence (Sutton-McDowall et al., 2011). Embryos cultured with L-carnitine did not improve embryo development but increased post-thaw reexpansion and hatching rates compared with control cultured embryos (Ruiz et al., 2013). Linoleic acid (18:2 n-6) supplementation during bovine *in vitro* oocyte maturation reduced maturation rate and subsequent embryo development to the blastocyst stage in a dose dependent manner (Marei et al., 2010). However, the supplement used in by Marei et al. (2010) did not include *trans*-10 *cis*-12 and

linoleic acid inclusion rates were much greater than most trials involving fatty acid supplementation of bovine culture medium.

Although significant research has focused on improving the cyrotolerance of *in vitro*-produced bovine embryos, a gap remains between the cryosurvival of *in vivo* and *in vitro* bovine embryos. Researchers will continue to modify *in vitro* culture systems toward conditions similar to those found *in vivo*. Commercial production of bovine embryos *in vitro* has the potential to significantly impact both genetic advancement and economic development, especially if further improvement in cryopreservation of these embryos is achieved.

Modification of Donor Nutrition

Supplementation of stallion diets with a nutraceutical containing docosahexaenoic acid (DHA) improved motion characteristics of cool-stored stallion semen and improve cryotolerance (Brinsko et al., 2005). Addition of dietary fish oil to boar diets increased the content of DHA in spermatozoa but did not improve cryotolerance (Maldjian et al., 2005). Supplementation of ewe diets with PUFA altered phospholipid fatty acid composition of follicular components and improved oocyte quality and resistance to chilling (Zeron et al., 2002a; Wonnacott et al., 2010). Oocytes from ewes fed PUFA had a lower temperature of lipid phase transition, leading to superior membrane integrity after chilling compared with control oocytes (Zeron et al., 2002a). Additionally, *in vitro*-produced embryos from Nellore heifers were more sensitive to cryopreservation when diets included rumen-protected PUFA (Guardieiro, 2010; Guardieiro et al., 2014).

Although long chain unsaturated fatty acids comprise a relatively small portion of the phospholipid portion of oocytes, slight increases in concentrations of these fatty acids could drastically improve membrane fluidity. These studies demonstrate potential means for modification of bovine oocyte or embryo composition and membrane fluidity *in vivo*, which could have implications for embryo cryotolerance.

Conjugated Linoleic Acid Supplementation

Another interesting area of study has identified specific conjugated linoleic acids (CLA) that inhibit milk fat synthesis in lactating dairy cows (Lor and Herbein, 1998; Chouinard et al., 1999a; Chouinard et al., 1999b). The *trans*-10, *cis*-12 CLA isomer was identified as a potent inhibitor of milk fat synthesis in lactating cows (Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002). The *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers result from natural biohydrogenation of dietary unsaturated fatty acids by rumen bacteria (Bauman et al., 1999; von Soosten et al., 2013). De Veth et al. (2004) analyzed relationships between abomasal infusion of *trans*-10, *cis*-12 CLA and milk fat across seven published studies. Significant milk fat depression (20 to 40%) occurs at a very minimal doses of abomasal infusion (2 to 6 g/d), while doses of *trans*-10 *cis*-12 greater than 10 g/d result in little or no further reduction in milk fat synthesis (de Veth et al., 2004). Transfer efficiency of abomasally infused *trans*-10, *cis*-12 into milk was relatively consistent with mean transfer efficiency of 22% across seven abomasal infusion studies in Holstein cows compared by de Veth et al. (2004).

Mechanisms Involved in CLA-Induced Milk Fat Depression in Dairy Cows

The *trans*-10, *cis*-12 CLA isomer is reported to exert specific effects on adipocytes that reduce the uptake of fatty acids without increasing lipolysis (Pariza et al., 2001). *Trans*-10, *cis*-12 CLA has inhibited both gene expression and Δ^9 -desaturase activity in the mammary gland (Lee et al., 1998; Breillon et al., 1999; Park et al., 2000; Baumgard et al., 2002). De Veth et al. (2004) indicated that alterations in the desaturase ratio are not essential to significantly reduce milk fat secretions, and the mechanism by which *trans*-10, *cis*-12 inhibits milk fat synthesis involves the coordinated regulation of lipogenic enzymes. Mammary tissue from cows abomasally infused with *trans*-10, *cis*-12 CLA had decreased mRNA abundance for lipogenic genes involved in lipid uptake and transport, *de novo* synthesis, desaturation and triglyceride synthesis (Baumgard et al., 2002). Peterson et al. (2004) indicated that the signal by which *trans*-10, *cis*-12 CLA regulates milk fat depression involves the sterol response element-binding protein regulatory pathway (SREBP-1) and a reduction in transcriptional activation of lipogenic genes.

Partially Rumen-Protected CLA Supplementation

A partially rumen-protected CLA supplement was developed (Lutrell[®], BASF) containing CLA isomers known to suppress milk fat. This product suppressed milk fat when fed to lactating sheep (Lock et al., 2006; Lock et al., 2008) and cows (Perfield et al., 2004; Castañeda-Gutiérrez et al., 2007a; Pappritz et al., 2011). Milk fat yield was significantly decreased by 14.1% in cows fed a rumen-protected CLA containing 7.1 g/d of each *cis*-9, *trans*-11 and

trans-10, *cis*-12 CLA (Castañeda-Gutiérrez et al., 2007a). Pappritz et al. (2011) reported milk fat depression was 14.8% in cows fed a lipid encapsulated CLA (Lutrell® Pure) containing 8 g per hd/d *trans*-10, *cis*-12 CLA.

The efficacy of a rumen-protected *trans*-10, *cis*-12 CLA supplement is measured by the degree of milk fat depression (Perfield et al., 2004). The transfer efficiency of CLA from a supplement into milk fat is influenced by efficacy of the protection from rumen metabolism as well as postruminal availability (Wu et al., 2010). Transfer efficiencies of CLA from rumen-protected supplements are generally much lower than the ~20% transfer efficiencies found in abomasal infusion studies (Chouinard et al., 1999a; Chouinard et al., 1999b; Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002).

Comparing the transfer of *trans*-10, *cis*-12 CLA from rumen-protected supplements with that from abomasal infusion (average 22%) provides an estimate of rumen biohydrogenation and a measure of the efficacy of rumen protection (de Veth et al., 2005). The efficiency of transfer to milk across 14 treatments supplementing Ca salts of CLA averaged 3.9% with a median value of 3.3% (de Veth et al., 2005). When compared with 100% protection with abomasal infusion, these values represent 83% to 85% of CLA metabolized in the rumen and 15% to 17% protected from rumen biohydrogenation (de Veth et al., 2005). The transfer efficiency of *trans*-10, *cis*-12 CLA into milk fat across eight published studies supplementing cows with Ca salts of CLA ranged from 1.9% to 7.4%, equating to 9% to 34% protection from rumen biohydrogenation (de Veth et al., 2005). Transfer efficiency of *trans*-10, *cis*-12 CLA into milk fat or

cows was similar for formaldehyde-protected (de Veth et al., 2005), amide-protected and lipid-encapsulated CLA (Perfield et al., 2004); when compared with transfer efficiencies of abomasal infusion studies, these methods provided 32% to 36% protection of *trans*-10, *cis*-12 from rumen biohydrogenation.

Pappritz et al. (2011) used duodenal fistulated cows to evaluate rumen metabolism and postruminal bioavailability of a lipid-encapsulated CLA supplement manufactured by BASF (Luttrell[®], BASF Ludwigshafen, Germany). Only 0.4 g of the daily consumed 8 g of *trans*-10, *cis*-12 CLA reached the duodenum, while transfer efficiency of the isomer into milk was 3%; this suggests a low (5%) ruminal protection rate (Pappritz et al., 2011). Of the 0.4 g of CLA reaching the duodenum, 48% was transferred to milk (Pappritz et al., 2011). Assuming similar biohydrogenation conditions to the Pappritz et al. (2011) study, von Soosten et al. (2013) reported that 0.24 g to 1.04 g of *trans*-10, *cis*-12 were available for absorption in the small intestine after cows were fed a lipid encapsulated supplement containing 6 g of *trans*-10, *cis*-12 CLA. The majority of the CLA in rumen-protected supplements are either metabolized in the rumen or unavailable for post-ruminal absorption. However, as evidenced by significant milk fat depression in cows fed rumen-protected CLA supplements, CLA isomers can alter lipid metabolism in biological tissues after very minimal postruminal absorption and transfer into these tissues.

Increases in milk yield of 3% to 10% have been reported when cows were fed supplements containing *trans*-10, *cis*-12 CLA (Bernal-Santos et al., 2003; Mackle et al., 2003; de Veth et al., 2006; Castañeda-Gutiérrez et al., 2007a).

Similarly, milk yield was 13% greater in lactating ewes fed CLA compared with control-supplemented ewes (Sinclair et al., 2010). In studies where milk energy output and cow energy balance are similar between supplemental treatment groups, energy spared by milk fat depression is likely repartitioned for milk production (Bernal-Santos et al., 2003; de Veth et al., 2006; Castañeda-Gutiérrez et al., 2007a; Sinclair et al., 2010). However, milk production was not influenced by CLA supplementation in a study by Castañeda-Gutiérrez et al. (2007a). Factors such as the inclusion rate of CLA isomers, method used to protect the supplement from rumen biohydrogenation, length of dietary treatment and season may influence the effect of CLA supplementation on milk production.

Deposition of CLA into Animal Tissues and Products

Any CLA isomers that are not metabolized in the rumen are available for absorption in the duodenum and transferred into products of the cow (von Soosten et al., 2013). Tissue-specific *in-vivo* synthesis of *cis*-9, *trans*-11 CLA by Δ^9 -desaturase has also been reported (Bauman et al., 1999). *Trans*-10, *cis*-12 CLA increased in milk fat of cows fed a rumen-protected CLA containing 7.1g/d of each *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, while no other changes in the fatty acid profile of milk were detected (Castañeda-Gutiérrez et al., 2007a). In many of the CLA abomasal infusion studies, *trans*-10, *cis*-12 CLA supplementation significantly decreases the relative percentage of short chain saturated fatty acids (<C16) in milk and increases long chain fatty acids (>C16), many of which are unsaturated. If dietary CLA could alter fatty acid composition of developing oocytes in a manner similar to CLA-induced alterations in milk,

changes in membrane composition could improve membrane fluidity of oocytes and embryos.

The *trans*-9, *cis*-12 isomer was detected in bovine fat deposits (retroperitoneal, omental, mesenteric and subcutaneous) but composition of fat was not influenced by CLA supplementation (von Soosten et al., 2013). The *trans*-10, *cis*-11 isomer was transferred into fat deposits after supplementation with the isomer, but only in minimal amounts (von Soosten et al., 2013). Transfer efficiency of *trans*-10, *cis*-11 into fat deposits was negligible in lactating dairy cows fed a rumen-protected CLA at a rate of 6 g of *trans*-10, *cis*-11 per hd/d (von Soosten et al., 2013). *Trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers were increased in perirenal fat of lactating ewes after CLA supplementation (Sinclair et al., 2010). Wynn et al. (2006) also observed increased *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA in perirenal, omental and mesenteric fat deposits of ewe lambs. Supplementation with CLA increased *trans*-10, *cis*-12 and *cis*-9, *trans* 11 in Longissimus dorsi of lactating ewes (Sinclair et al., 2010) but transfer of CLA into meat of dairy cows did not occur in a study by von Soosten et al. (2013).

Endometrial tissue of cows supplemented with CLA had increased proportions of C16:1 and tended to have increase C18:1 trans 6 to 9, but *cis*-9, *trans*-11 CLA was not increased in endometrial tissue of CLA-supplemented cows and *trans*-10, *cis*-12 was undetected in endometrial tissue of both treatment and control-supplemented cows (Castañeda-Gutiérrez et al., 2007a). Harris et al. (2001) reported incorporation of *cis*-9 *trans*-11 and *trans*-10, *cis*-12 CLA into uterine tissue of pregnant rats fed CLA at 1.1% of the diet. Significantly greater

doses of CLA were fed in the mouse study by Harris et al. (2001) compared with most CLA studies reported in ruminants. Additionally, a significant portion of the lipid-encapsulated supplement was likely biohydrogenated in the rumen in the study by Castañeda-Gutiérrez et al. (2007a), making less CLA available for absorption and deposition into tissues.

Fatty acid composition of follicular fluid appears to be sensitive to dietary lipid supplementation. Follicular fluid linolenic acid (C18:3) and C20:2 were greater and *cis*-9, *trans*-11 CLA and C22:2 tended ($P \leq 0.09$) to increase in cows fed a rumen-protected CLA containing 7.1g/d of each *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA compared with control-supplemented cows (Castañeda-Gutiérrez et al., 2007a). However, proportions of saturated, monounsaturated or polyunsaturated fatty acids in follicular fluid were not influenced by CLA supplementation (Castañeda-Gutiérrez et al., 2007a).

Zeron et al. (2002a) reported an increase in C18:2n-6 and C22:6n-3 in the phospholipid portion of follicular fluid of PUFA (fish oil) supplemented ewes. Fatty acid composition of follicular fluid is sensitive to changes in dietary supply of PUFA using soybean oil (Batista et al., 2010). Heifers fed fish oil had greater proportions of EPA and DHA in follicular fluid (Childs et al., 2008). Feeding dairy cows an encapsulated fat rich in C18:3n-3 (flaxseed oil) change the fatty acid composition of milk, plasma, follicular fluid, cumulus-oocyte complexes (COC) and granulosa cells and (Zachut et al., 2010). The composition of fatty acids in follicular fluid and plasma are similar (Zachut et al., 2010) and composition was

comparable to values reported by Castañeda-Gutiérrez et al. (2007a) and Zachut et al. (2008).

The fatty acid composition of granulosa cells and COC are quite different than that found in plasma and follicular fluid of cows (Zachut et al., 2010). *Trans-10, cis-12* CLA was increased in both oocytes and cumulus cells after maturation with 100 µM of the CLA (Lapa et al., 2011). Uptake of fatty acids by the follicle and oocyte is likely highly selective in the cow as a protective mechanism to ensure fatty acid composition of oocytes remains safe or optimum (Bilby et al., 2006; Santos et al., 2008a; Fouladi-Nashta et al., 2009; Sturmeier et al., 2009). The highly regulated process of fatty acid uptake leads to relatively small, if any change in fatty acid composition of oocytes to different dietary treatments. Lapa et al. (2011) reported minimal changes in fatty acid concentration of bovine oocytes after maturation with *trans-10, cis-12* CLA, concluding that oocytes accumulated very small amounts of *trans-10, cis-12* in a selective manner but maintained relatively consistent fatty acid concentration to ensure cellular integrity.

Fouladi-Nashta et al. (2009) reported fatty acid composition of plasma and milk reflected composition of fatty acids supplemented to cows, but fatty acids in granulosa cells were not altered by dietary fatty acid supplementation. It was concluded that the bovine ovary has mechanisms to buffer the oocyte against major fluctuations in plasma n-3 and n-6 fatty acids, resulting in very small alterations on oocyte developmental potential (Fouladi-Nashta et al., 2009). Greater concentrations of free fatty acids in bovine follicular fluid resulted in

significant fatty acid accumulation in cumulus cells, but only marginal changes in oocyte fatty acid composition (Aardema et al., 2012). Aardema et al. (2012) concluded that cumulus cells protect bovine oocytes against elevated follicular fluid fatty acid concentrations by accumulation of these fatty acids. These results indicate that cumulus cells may indeed be the buffer mechanism in the cow that protect the oocyte from changes in the follicular environment brought on by a variety of conditions such as stress, diet, season or stage of production.

Differences in fatty acid composition between follicular fluid, granulosa cells and COC suggest a selective uptake of specific fatty acids in different ovarian compartments of ruminant livestock species (Zeron et al., 2002a; Adamiak et al., 2006; Zachut et al., 2010). Although there appears to be a selective uptake mechanism of fatty acids in bovine ovarian tissues, reported data suggests that specific dietary supplementation can alter the fatty acid profile of ovarian components in cattle (Zachut et al., 2010).

Castañeda-Gutiérrez et al. (2007a) indicated that the incorporation of CLA isomers into milk fat and tissues of animals may be influenced by the plasma lipid fraction in which they are transported. Specific isomers appear to have preferential incorporation into specific lipid fractions, which differs among tissues and species. In a study by (Kramer et al., 1998) involving CLA-supplemented pigs, *trans*-10, *cis*-12 CLA was primarily incorporated into the triglyceride fraction of liver and heart, while *cis*-9, *trans*-11 CLA was incorporated into phospholipids.

Reproduction Responses to CLA Supplementation

Studies have examined CLA-induced milk fat depression as an approach to lessen negative energy balance during early lactation in dairy cows. There appears to be a positive effect of CLA supplementation on reproduction in lactating dairy cows, although the specific mechanism of action is not well established. Supplementation with ≤ 10 g/d of each *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA appears to benefit reproduction in lactating dairy cows even with no change in energy balance (Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005). Castañeda-Gutiérrez et al. (2007a) reported increased plasma concentrations of IGF-I in lactating cows fed a rumen-protected CLA containing 7.1g/d each of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. Additionally, CLA-supplemented cows tended ($P = 0.08$) to have greater concentration of progesterone during the early luteal phase, d 6 to d 8 of the estrous cycle (Castañeda-Gutiérrez et al., 2007a). Beneficial reproductive effects in dairy cows continued after supplementation concluded in studies by Bernal-Santos et al. (2003) and Castañeda-Gutiérrez et al. (2005). Castañeda-Gutiérrez et al. (2007a) concluded that CLA supplementation may improve bovine embryo viability by supporting development of the ovarian follicle.

De Veth et al. (2009) used semi-parametric and parametric models to conduct an integrated analysis of five controlled studies involving CLA supplementation and reproduction in early lactating dairy cows. In this study, models concluded that supplementation with *trans*-10, *cis*-12 CLA was positively associated with the probability of pregnancy success and a decrease in the

number of days to pregnancy detection as dietary dose increased (de Veth et al., 2009). Pregnancy success increased as CLA dose increased up to an optimal dose of 10.1 g of *trans*-10, *cis*-12 CLA. Models predicted the probability of pregnancy increased by 26% and the median time to pregnancy detection was decreased by 34 d when cows were fed the optimal daily dose of 10.1 g (pregnancy) and 10.5 g (d to pregnancy detection) of *trans*-10, *cis*-12 CLA compared with control-supplemented cows (de Veth et al., 2009).

Supplementation of cows with 100 g of lipid-encapsulated CLA per hd/d increased the number of follicles present and oocytes collected per session, and improved cleavage and d-8 embryo production *in vitro* (Höffmann et al., 2008). Inclusion of 100 µM of *trans*-10, *cis*-12 CLA into bovine maturation media increased the rate of high quality embryos on d 8 *in vitro*, but did not influence oocyte maturation or embryo cleavage or blastocyst production (Lapa et al., 2011). Maturation of bovine oocytes with 100 µM of *trans*-10, *cis*-12 CLA suppressed arachidonic acid (C20:4n-6) concentrations in oocytes (Lapa et al., 2011).

Supplementation with *trans*-10, *cis*-12 CLA appears to alter fatty acid composition of a variety of tissues and animal products. Additionally, CLA alters embryos *in vitro* in a manner that decreased lipid content and improved cryosurvival. However, there are currently no reports evaluating the cryotolerance of embryos from cows fed CLA supplements. Research may be warranted to elucidate possible relationships between CLA supplementation and

ovarian lipid metabolism as it relates to oocyte and embryo lipid composition and cryotolerance of embryos.

Evaluation and Quantification of Lipids in Oocytes and Embryos

Several methods have been developed to evaluate the lipid content of embryos and oocytes. The first detailed lipid analysis of immature mammalian oocytes was conducted with porcine oocytes using gas-liquid chromatography (Homa et al., 1986). Thin-layer gas chromatography has been used to quantify the fatty acid composition of oocytes and embryos in domestic livestock species; however, these techniques require pooling 100 to 1,000 oocytes or embryos (Coull et al., 1998; Sata et al., 1999; McEvoy et al., 2000; Kim et al., 2001; Zeron et al., 2001). These researchers quantified the fatty acid composition of the total lipid portion of samples and/or the fatty acid composition within phospholipids. Kim et al. (2001) used a kit-based assay to determine composition of lipids in immature and *in vitro*-matured bovine oocytes and embryos; however, a pool of 100 oocytes or embryos was used to evaluate lipid content. Concentration of triglyceride, the major lipid component of embryos, has been measured using 1 to 3 embryos with an enzymatic approach in combination with microfluorescence techniques (Ferguson and Leese, 1999; Sturme y and Leese, 2003).

Ultrastructural morphometry of *in vitro* and *in vivo* embryos was compared using light microscopy after staining ultra-thin or semi-thin histological sections of bovine (Crosier et al., 2000, 2001a; Abe et al., 2002) and porcine (Kikuchi, 2002) embryos. These studies facilitated evaluation of the morphological composition of lipid droplets, cell organelles, and their relative spatial relationships. Sturme y

et al. (2006) used a fluorescence resonance energy transfer (FRET) technique to study the co-localization of mitochondria and lipid droplets in porcine embryos. This method labels organelles with fluorescent probes (Mitotracker Green and Nile Red) whose spectral profiles overlap. Results indicated that mitochondria and lipid droplets lie within the FRET-distance (6 to 10 nm) of each other in porcine oocytes. The number and size of lipid granules was evaluated in embryos cultured in different medium by staining with Sudan Black-B (Abe et al., 2002; De La Torre-Sanchez et al., 2006a).

Nile Red is a lipid specific fluorescent dye that can be used to evaluate the lipid content in single mammalian oocytes; peak fluorescence observed corresponds to neutral lipids, which are mainly triglycerides (Genicot et al., 2005). In a hydrophobic lipid environment, lipids stained with Nile Red fluoresce yellow to orange with (580-596, 590 nm peak fluorescence) (Genicot et al., 2005). Fluorescence intensity after staining with Nile Red was used to evaluate differences in cytoplasmic lipids of oocytes and embryos differing in donor breed (*Bos indicus* vs. *Bos taurus*) (Ballard, 2006), species (Genicot et al., 2005), *in vitro* culture conditions (Barceló-Fimbres and Seidel, 2008), or visual color (dark vs. light) (Leroy et al., 2005). A strong linear relationship ($r^2 = 0.910$) was found between number of small lipid droplets ($<2 \mu\text{m}$) and fluorescence intensity after staining with Nile Red. Fluorescent intensity is the preferred method as it is a faster, more objective quantification of lipid content (Barceló-Fimbres and Seidel, 2007b). This technique allows rapid, highly repeatable evaluation of lipid content for relative comparison of individual oocytes from different donors, culture

conditions, or other treatments. However, this method does not permit qualitative analysis for the composition of lipids, such as identification of fatty acids or different lipid fractions, nor does it allow absolute quantitative analysis.

Glycerol production was quantified as a measure of intracellular lipolytic activity in porcine oocytes (Men et al., 2006). Under physiological conditions, lipase catalyzes the hydrolysis of triacylglycerols into glycerol and fatty acids. This method is widely used to measure lipolytic activity in a broad range of cells in lipid research. Measuring glycerol production is a relatively straightforward technique that can measure lipolytic activity within embryos without the need to quantify lipid concentrations.

Direct lipid analyses of individual, intact embryos and oocytes was recently reported using a matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) method (Ferreira et al., 2010). MALDI-MS spectra were acquired in the positive ion using a mass spectrometer equipped with a 200-Hz solid-state laser in the m/z range of 700-950. Human, bovine, sheep and fish oocytes, as well as bovine and insect embryos were analyzed. This method allows the detection of intact lipids of individual oocytes without solvent extraction or pooling of samples. Results indicate MALDI-MS is an accurate and highly reproducible method for lipid analysis in embryos and oocytes, with high sample throughput (Ferreira et al., 2010). This method provides the ability to compare gamete lipid profiles across a multitude of different treatments and conditions. Most importantly, few samples are needed and oocytes can be evaluated on an individual basis.

A slightly different mass spectrometry technique known as desorption electrospray ionization (DESI-MS) has been used to evaluate embryo lipids in oocytes and embryos from cows (Ferreira et al., 2011, 2012b; González-Serrano et al., 2013a; González-Serrano et al., 2013b) and mice (Ferreira et al., 2012a). This is an ambient ionization used for MS analysis of lipids directly from biological samples with no sample preparation. DESI-MS allows direct analysis of individual intact embryos and provides detailed free fatty acid and complex lipid profiling (Ferreira et al., 2011, 2012b). Ferreira et al. (2011) concluded that DESI-MS is likely to become a routine analytical tool for lipid analysis of mammalian embryos and will contribute to the development of culture systems that produce embryos with improved cryotolerance.

Another approach has been to evaluate membrane properties and their influence on fertility and cryopreservation. Membrane phase transition, the transition of membrane lipids from the crystalline to the gel phase (Crowe et al., 1989), was evaluated in the bovine oocytes and spermatozoa (Zeron et al., 2001; Zeron et al., 2002b) and human oocytes (Ghetler et al., 2005) using a Fourier transform infrared (FTIR) analyzer connected to a FTIR microscope. This method measures the vibration frequency of methylene (CH_2) groups, which are the primary component of the hydrocarbon chain of lipid molecules (Ghetler et al., 2005). Chilling injury to oocytes occurs near the lipid phase transition (Arav et al., 1999), with maximum injury at a midpoint (T_m) lipid phase transition of 16°C (Zeron et al., 1999b). Changes in membrane phase transition are related

to membrane composition, which ultimately influences membrane permeability and likely alters cryotolerance of the embryo.

Expression of Metabolic Genes in Oocytes and Embryos

Energy demands within most mammalian cells are regulated through β -oxidation of fatty acids in the mitochondria. A host of genes are involved in transcriptional control of β -oxidation. Oocytes and early embryos must closely regulate the expression of metabolic genes to prepare for changes in demand for energy from lipids during oocyte maturation and early embryonic development. However, relatively little is known about oocyte control of fatty acid metabolism during important final stages of maturation (Algriany et al., 2007). Exogenous fatty acids are likely the preferred source of long-chain fatty acids and energy source during oocyte maturation (Algriany et al., 2007). Alterations in the expression of different lipid metabolic genes are likely initiated by direct contact with fatty acids, altering chromatin structure and transcription through histone deacetylase activity (P  gorier et al., 2004). Diet induced milk fat depression in dairy cows resulted in the coordinated down regulation of mRNA expression for genes involved in mammary lipid synthesis (Piperova et al., 2000; Peterson et al., 2003). Al Darwich et al. (2010) reported that PUFA were involved in the control of adipogenesis and events that alter gene expression of multiple enzymes involved in lipid metabolism of bovine embryos.

ACC β and CPT1

The enzyme AMP-activated protein kinase (AMPK), which is activated by low ATP/AMP, increases fatty acid oxidation in skeletal muscle (Bergeron et al.,

1999; Ihlemann et al., 1999; Russell et al., 1999) and inhibits acetyl-CoA carboxylase (ACC β) (Carling et al., 1994; Winder et al., 2000). Mitochondrial ACC β regulates fatty acid oxidation through carnitin palmitoyl transferases (CPT1), a group of enzymes involved in mitochondrial energy homeostasis. The CPT1 enzyme catalyzes a rate-limiting step in the transfer of long-chain fatty acyl-CoA from the cytosol to mitochondria for oxidation (Abu-Elheiga et al., 2000; Kerner and Hoppel, 2000). There are multiply mechanisms by which CPT1 regulates β -oxidation in different mammalian cell. Mechanisms include changes in activity and rate of transcription of CPT and/or its inhibitor malanyl-CoA, the end product of ACC β (Abu-Elheiga et al., 2000). Expression of CPT1 was up-regulated in liver and adipose by dietary PUFA supplementation in rats (Ikeda et al., 1998). During oocyte maturation, CPT1 mRNA increases indicating an increase in β oxidation during this stage (Algriany et al., 2007).

PPAR α

Peroxisome proliferator-activated receptor alpha (PPAR α) is a member of the PPAR family of ligand-dependent transcription factors (Rosen et al., 1999). Expression of PPAR α is greater in tissues that have high rates of fatty acid oxidation, and is the primary subtype that facilitates lipid-induced activation of fatty acid oxidation associated genes (Braissant et al., 1996). Dietary unsaturated fatty acids have been reported to alter gene expression through PPAR, leading to altered metabolism, growth and cell differentiation in different tissues of rats (Ide, 2000; Jump, 2004). Polyunsaturated fatty acids were described as a natural ligand or activator of PPAR, although PUFA mediated

genes involved in lipid metabolism through both PPAR dependent and PPAR independent pathways (Ren et al., 1997).

FADS2

The FADS2 gene is a member of the fatty acid desaturase (FADS) gene family. Enzymes resulting from FADS2 regulate unsaturation of fatty acids through the addition of double bonds. Bovine embryos cultured with 100 μ M of *trans*-10, *cis*-12 CLA had reduced FADS2 transcript level compared with control cultured embryos (Al Darwich et al., 2010). This result likely modified the saturated/unsaturated fatty acid balance in bovine embryos and could influence membrane fluidity (Al Darwich et al., 2010). However, post-thaw survival of *in vitro*-produced bovine embryos was not improved after co-culture with 100 μ M of *trans*-10, *cis*-12 CLA (Al Darwich et al., 2010).

SREBP1

Sterol response element-binding protein-1 (SREBP1) is a transcription factor that, when activated, stimulates lipogenic gene transcription (Peterson et al., 2004). Fatty acid synthesis was suppressed through inhibition of SREBP in the liver of mice supplemented with PUFA (Kim et al., 1999). *Trans*-10, *cis*-12 CLA suppressed SREBP1 maturation in adipose and liver of rats in a dose dependent post-translational manner (Xu et al., 1999), inducing a decrease in fatty acid synthesis. Treatment of bovine mammary gland cells with 75 μ M/L of *trans*-10, *cis*-12 CLA did not alter SREBP-1 mRNA expression, but abundance of the activated nuclear fragment of the SREB-1 protein was reduced by CLA treatment (Peterson et al., 2004). Peterson et al. (2004) concluded that *trans*-10,

cis-12 CLA reduces lipid synthesis in the bovine mammary gland by inhibition of the proteolytic activation of SREBP-1 and reduction in transcription of lipogenic genes (ACC, FAS, SCD). Day-7 to d-8 bovine embryos contained SREBP1 mRNA and expression was down-regulated by PUFA inclusion into culture media (Al Darwich et al., 2010).

Alteration in gene expression at the transcriptional level cannot be definitively equated to changes in functional pathways as transcripts must be translated and post-translational modifications may be necessary in order for proteins to be functional. Functional biochemical assays such as proteomic analysis are necessary to accurately validate changes in mRNA expression; however, these techniques require large quantities of oocytes, which can be difficult to obtain in many large animal experimental models.

In vitro culture of bovine embryos altered mRNA expression of genes coding for fatty acid utilization and synthesis, which likely help explain lower developmental competence of *in vitro* compared with *in vivo* embryos (Algriany et al., 2007). Furthermore, altered gene expressing and resulting modification in lipid metabolism likely changes the composition of oocytes and embryos, resulting in differences in embryo cryotolerance. *Trans*-10, *cis*-12 CLA appears to influence mRNA expression of genes involved in lipid metabolism in the bovine mammary gland, as well as in bovine embryos after culture with CLA.

Supplementation of cows with CLA may alter lipid composition of oocytes via modification of oocyte gene expression. To our knowledge there are no current

reports evaluating mRNA expression of genes involved in lipid metabolism in oocytes and embryos collected from cows fed CLA.

Summary

Better understanding of biological mechanisms contributing to variation in cryotolerance of embryos will likely continue to improve success of embryo cryopreservation. Research designed to better understand factors influencing the composition of oocytes may lead to practices that produce embryos better suited to survive cryopreservation. This research may be especially important for those breeds and/or species that have limited success with cryopreservation. Additionally, there is a lack of understanding of the mechanisms contributing to variation in cryotolerance of gametes between and within individuals of a particular species. Development of models that evaluate variation in lipid metabolism and lipid composition of oocytes and embryos have the potential to impact biological disciplines beyond cryopreservation.

CHAPTER III
MILK FAT, MILK PRODUCTION, FIRST SERVICE PREGNANCY RATE
AND OOCYTE LIPID CONTENT IN LACTATING HOLSTEIN COWS FED
***TRANS-10, CIS-12 AND CIS-9, TRANS-11* CONJUGATED LINOLEIC ACIDS**

Introduction

Dietary supplements containing CLA reduced milk fat synthesis when fed to lactating cows (Loror and Herbein, 1998; Chouinard et al., 1999a; Chouinard et al., 1999b). The *trans-10, cis-12* CLA isomer was identified as a potent inhibitor of milk fat synthesis in lactating cows (Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002). A rumen-protected CLA supplement has been developed (Lutrell[®], BASF) containing CLA isomers known to suppress milk fat. Lipid encapsulated CLA suppressed milk fat when fed to lactating sheep and goats (Lock et al., 2006; Lock et al., 2008) and cows (Perfield et al., 2004; Castañeda-Gutiérrez et al., 2007b; Pappritz et al., 2011). In addition, supplementation with ≤ 10 g/d each of *trans-10, cis-12* and *cis-9, trans-11* CLA appears to benefit reproduction in lactating Holstein cows even without significant changes in energy balance (Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005).

Tolerance to chilling and cryotolerance of oocytes and embryos has been correlated to their lipid content (Kim et al., 2001; Abe et al., 2002; Seidel, 2006), that is, more and larger cytoplasmic lipid droplets reduce cryotolerance. Nagashima et al. (1994) improved post-thaw survival of porcine embryos after centrifugation and removal of lipids at the 1-cell stage. Inclusion of *trans-10, cis-12* CLA (the same isomer which induces milk fat depression in dairy cows) in

bovine embryo culture media decreased lipid accumulation and improved post-thaw embryo survival (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013).

Intracellular lipid content of embryos can be qualitatively quantified and compared between treatments using Nile Red and fluorescence microscopy. Nile Red is a lipid specific fluorescent dye that can be used to evaluate the lipid content in single mammalian oocytes. Peak fluorescence observed correspond to neutral lipids, which are mainly triglycerides (Genicot et al., 2005).

Fluorescence intensity after staining with Nile Red was used to evaluate differences in cytoplasmic lipids of oocytes and embryos differing in donor breed (*Bos indicus* vs. *Bos taurus*, Ballard, 2006; Holstein Fresian vs. Belgian Blue (Leroy, 2004) domestic livestock species (Genicot et al., 2005), bovine *in vitro* culture conditions (Barceló-Fimbres and Seidel, 2008), or visual color (dark vs. light) (Leroy et al., 2005).

Additional research is necessary to elucidate mechanisms contributing to variation in cryotolerance associated with individual animal, breed, species and nutrition (Seidel, 2006). Better understanding of mechanisms contributing to variation in cryotolerance of embryos will continue to improve success of embryo cryopreservation. If dietary CLA could alter ovarian lipid metabolism in a manner similar to CLA-induced effects on the bovine mammary gland, it may alter oocyte lipid composition, which could influence subsequent embryo cryotolerance.

Therefore, objectives of this study were (1) to verify previously reported milk fat depression and reproductive responses in Holstein cows fed rumen-protected CLA and (2) to evaluate CLA supplementation on follicle and oocyte production

and lipid content of oocytes using Nile Red stain. This trial is intended to generate proof-of-principal data necessary to undertake a series of experiments investigating the effects of CLA supplementation on bovine oocyte composition and embryo cryotolerance.

Materials and Methods

Animals and Experimental Diets

Experimental procedures in this study were approved by the Louisiana State University Animal Care and Use Committee, and were conducted at the Louisiana State University Agricultural Center Dairy Research Farm (Baton Rouge, LA) from September through December, 2010. Primiparous (n = 15) and multiparous (n = 24) Holstein females in late gestation were randomly allotted to experimental diets after stratification by previous (cows) or expected (heifers) milk production, lactation number and expected date of parturition. During gestation cows were housed in groups according to expected calving date in sod-based pens and group fed a corn silage-based total mixed ration (TMR). After calving, cows were housed in partially shaded free-stall pens with fans and sprinklers and were allowed access to bermudagrass-based (*Cynodon dactylon*) paddocks (0.6 ha).

Cows received a corn silage-based TMR (Table 3.1) enriched with either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids (CLA, Lutrell®, BASF, Lampertheim, Germany) or a rumen-protected calcium salts of palm oil (Ca salts, Megalac®, Church and Dwight Co., Inc., Ewing, NJ). Diets were formulated to meet or exceed

Table 3.1 Experimental diets for primiparous and multiparous Holstein cows¹

Ingredient	Kg/DM ²	% DM
Corn silage	7.1	31.2
Alfalfa hay	2.8	12.2
Ground corn	3.6	15.8
Corn distillers with sol.	2.4	10.4
Soybean hulls	2.3	10.0
Soybean meal	1.6	7.1
Bakery waste	1.4	6.2
Molasses	0.3	1.1
Megalac/Lutrell ³	0.1	0.4
Mineral premix	1.3	5.6

¹Each included at 100 g per hd/d in respective treatments.

²Dry matter.

³Diets fed as a total mixed ration in two daily offerings.

NRC (2001) recommendations for early-lactation Holstein cows that weighed 600 kg and produced 35 kg of 3.5% fat corrected milk (FCM). Supplements were included at 100 g per hd/d to respective treatment diets from parturition through 98 ± 7 days in milk (DIM). Calculated CLA inclusion was 10 g per hd/d each of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA. The fatty acid composition of the supplements is detailed in Table 3.2. The diets included corn silage, alfalfa hay, ground corn, corn distillers with solubles, soybean hulls, soybean meal,

Table 3.2 Percentage of fatty acids from total fatty acids in supplement¹

Fatty acid isomer	CLA	Ca salts
12:0	-	0.2
14:0	-	1.6
16:0	1.3	50.8
18:0	61	4.1
18:1 <i>cis</i>	10.1	35.7
18:2	1.3	7.0
<i>cis</i> -9, <i>trans</i> -11	12.6	-
<i>trans</i> -10, <i>cis</i> -12	12.6	-
18:3	-	0.2
Other	1.1	0.4

¹Composition according to manufacturer formulation.

²CLA (Lutrell) and Megalac are rumen-protected supplements with fat contents of 79.5% (Lutrell) and 84.5% (Megalac).

bakery waste, molasses and a mineral premix. Diets were mixed in a vertical-mixing feed wagon and offered twice daily to allow 5 to 10% refusal (as-fed basis). Refusal from each treatment diet (pen) were collected and weighed daily. Dry matter (DM) of silage was monitored weekly to maintain the correct forage-to-concentrate ratio.

Milk production was assessed at each of two daily milkings (3 AM and 3 PM) by electronic meters and a mean weakly milk yield was calculated for analysis. Milk composition (fat, protein and SCC) was analyzed weekly from consecutive AM and PM milk samples. Weekly body weight (BW) was recorded after milking and before afternoon feeding.

Synchronization for Oocyte Collection and Timed Artificial Insemination

Oocytes were collected using transvaginal ultrasound-guided oocyte aspiration (TUGA) at 63 ± 7 and 77 ± 7 DIM. An outline of the synchronization procedures is presented in Figure 3.1. A dominant follicle removal (DFR) procedure was performed on each female to synchronize the follicular wave at 59 ± 7 DIM. Three injections (im) of FSH (Folltropin[®]-V, Bioniche Animal Health, Athens, GA) were administered at 12-h intervals beginning 36 h after the DFR. A descending-dose FSH schedule was used with injections of 4.5, 4.0 and 3.5 ml (20 mg/ml) equating to 90, 80 and 70 mg of FSH for each of the three injections, respectively. Cumulus-oocyte complexes (COC) were collected 36 h after the final FSH injection (4 d after DFR) at 63 ± 7 DIM. These procedures were repeated on each animal beginning with DFR 10 d following the first TUGA session. The interval between TUGA sessions was 14 d and the second TUGA session was conducted at 77 ± 7 DIM. If cows had an ovarian cyst at the initial DFR or any DFR or TUGA session they were excluded from the remaining oocyte collections and from analysis of pregnancy rate after AI (artificial insemination).

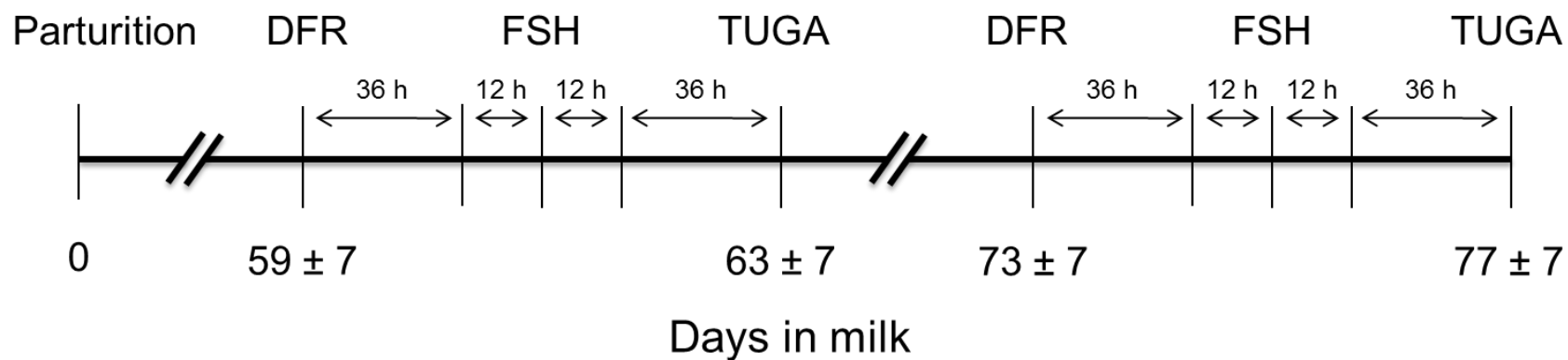


Figure 3.1 Synchronization schedule and procedures for transvaginal ultrasound-guided oocyte aspiration (TUGA). DFR is dominant follicle removal; FSH is Folltropin®-V, porcine follicle stimulating hormone, administered im.

Cows were synchronized for fixed-time AI (FTAI) using a modified OvSynch protocol in conjunction with the TUGA sessions (Figure 3.2). Cows were administered prostaglandin $F_{2\alpha}$ (Lutalyse[®], 5ml, 25 mg, im) at the time of both the first and second TUGA sessions. An injection of GnRH (Cysterellin, 2 ml, 100 μ g, im) was administered 10 d following the second $PGF_{2\alpha}$ and a third injection of $PGF_{2\alpha}$ 7 d following GnRH. A final injection of GnRH was administered ~56 h after the third $PGF_{2\alpha}$ injection. All cows were inseminated at 97 ± 7 DIM by the same experienced technician with Holstein semen of good fertility at 16 to 20 h following GnRH. Pregnancy status was assessed at 34 ± 5 d after AI by transrectal ultrasound or return estrus was recorded. Cows that had an ovarian cyst at any DFR or TUGA session were not included in pregnancy status analysis.

Oocyte Collection

Equipment and materials used for TUGA are presented in Appendix A. Cows were restrained in a manual squeeze chute and given local anesthesia via a caudal epidural injection of 5 ml of lidocaine (2%, Reproduction Resources, Walworth, WI). Follicles were visualized using a SonoSite[®] MicroMaxx[®] 8.5 MHz curved array transducer encased in a hard plastic probe equipped with a needle guide. Disposable needles (18 ga x 7.6 cm, Air-Tite Products Co., Inc., Virginia Beach, VA) were connected to polyethylene tubing (Becton Dickinson and Co., Sparks, MD) using metal hardware. The opposite end of the tubing was threaded into a urethral catheter connector (Cook Urological, Bloomington, IN)

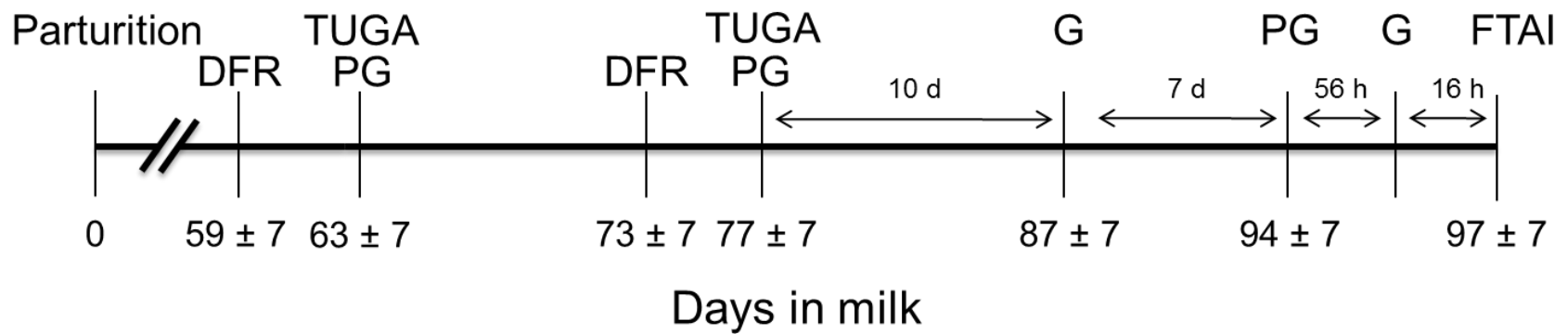


Figure 3.2 Synchronization schedule for fixed-time artificial insemination (FTAI). DFR is dominant follicle removal, PG is prostaglandin $F_{2\alpha}$ (Lutalyse[®], 5 ml, 25 mg, im), G is GnRH (Cysterellin[®], 2 ml, 100 μ g, im)

fitted to the plastic lid of filter (EmCon[®], Reproduction Resources), which is equipped with a 75 micron stainless steel mesh. A regulated vacuum pump (Watanabe Tecnologia Aplicada, Brazil) was connected to the filter creating a constant vacuum with aspiration rate of 15 to 20 ml/min when the foot pedal switch was engaged.

Follicular contents from follicles approximately ≥ 4 mm were collected into the filter containing 10 ml collection medium. The number of follicles targeted and CL present were recorded for each cow, in each TUGA session. Oocyte collection medium (OCM) was Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium containing 1% calf serum and heparin (10 IU/ml). Immediately following collection, filters were taken to the laboratory at the LSU AgCenter Dairy Research Farm to search for COC. Aspirate from each cow was transferred to a gridded dish containing OCM and searched for COC with a dissecting microscope. Recovered COC were graded according a four point scale (Table 3.3), similar to the procedures reported by Leibfried and First (1979).

Grade I, and II COC were washed in fresh OCM and cumulus cells were removed by high speed vortex 3 to 4 min in a 15 ml conical tube containing 1 ml of HEPES Talp medium (buffered culture medium, Appendix L) with 1 mg/ml of hyaluronidase. Completely denuded oocytes were washed in fresh OCM and divided for further processing. Half the oocytes were placed in 1.5 ml microtubules in a small drop (1 to 2 μ l) of 50% methanol in PBS and stored at -80°C for later mass spectrometry analysis. The remaining oocytes were placed

in a single well of a 4-well plate (Thermo Scientific, Rochester, NY) containing fixative medium for Nile Red analysis. All oocytes were held at 4°C until all cows were collected and transported on ice to the LSU Reproductive Biology Center for storage or further analysis.

Table 3.3 Classification protocol for evaluating bovine oocyte components¹

Component	Numerical score	Description
Investment (Cumulus Cell Status)	1	Complete, cumulus oophorus present; more than three layers thick; compact
	2	Partial, cumulus present; either not completely surrounding the oocyte or <3 layers thick; compact
	3	Expanded, cumulus present; cellular investment shows expansion; cumulus cells appear in scattered clumps in matrix
	4	Nude, cellular investment not present; oocyte only enclosed by zona pellucida
Ooplasm	1	Even granulation giving oocyte a dusty appearance; ooplasm fills the zona pellucida evenly
	2	Granules clumping or unevenly distributed in ooplasm, may be withdrawn to center of oocyte leaving clear periphery or coalescing into black bodies in ooplasm; ooplasm fills zona pellucida
	3	Ooplasm shrunk away from zona pellucida not evenly filling the zona; ooplasm degenerate, vacuolated, fragmenting or just left in remnants; empty zona pellucida

¹Modified from Leibfried and First (1979).

Nile Red Stain

Denuded oocytes were incubated in 4-well petri dishes containing gluteraldehyde/formaldehyde fixative medium (Appendix B) overnight (18 ± 2 h) at 4°C. Fixed oocytes were washed 4 times with PBS before Nile Red staining. Oocytes were rinsed 3 times in Nile Red working solution before staining in a single well of a 4-well plate containing Nile Red (1 µg/ml) working solution (Appendix C). Oocytes were incubated in Nile Red working solution at room temperature (~24°C) inside a sealed cardboard box (to ensure complete darkness) for 24 h. After incubation, oocytes were washed 3 times in PBS and suspended in 10 µl droplets of ProLong® Gold antifade reagent (Invitrogen Corporation, Carlsbad, CA) on a 25 x 55 mm glass slide. Slides were prepared with three droplets per slide containing 5 to 15 stained oocytes per droplet. Cover slips were carefully placed over the droplet of ProLong® Gold, lightly pressed down on all four corners and sealed with nail polish. Oocytes from each donor cow remained separated throughout fixing, staining and slide preparation.

Oocytes stained with Nile Red were evaluated with a Nikon Labophot microscope equipped with epifluorescent illumination and a FITC dichroic filter cube with long pass filter (excitation: 400-500 nm and emission: 515 nm). Images of each oocyte were captured with fluorescence microscopy at 20X objective (0.4 NA) using a QImaging Micropublisher 3.3 camera with QCapture Pro software and stored on a PC for analysis. After established, camera settings remained identical to capture each image. Prepared thin-layer slides of oyster ovarian tissue stained with hemotoxylin-eosin were used as control slides. These slides were used to account for possible variation in camera and

microscope settings or florescent bulb intensity between days. ImageJ software (imagej.nih.gov/ij/index.html) was used to evaluate mean florescence intensity of Nile Red stained oocytes in each image. Five samples from similar areas of each oocyte were measured to calculate mean florescence intensity. A total of 57 oocytes collected from cows fed CLA (n = 13 cows) and 54 oocytes from cows fed Ca salts (n = 12 cows) were used for Nile Red staining.

Statistical Analyses

The experimental unit in this study was pen as animals were fed on a pen (not individual) basis. Cow BW, mean weekly milk yield, milk composition data (fat, protein, ECM and FCM) were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC). The model included treatment (CLA and Ca salts) parity (primiparous and multiparous) and higher order interactions. Follicle and COC responses (number of follicles targeted, total COC and Quality COC recovered) were analyzed with the Mixed procedure of SAS. Main effects included dietary treatment, parity, collection and higher order interaction.

Nile Red intensity was analyzed with the GLM procedure of SAS. The model included supplemental treatment, collection and higher order interactions. If higher order interactions were not significant ($P > 0.07$) they were excluded from the model. When effects were significant ($P \leq 0.05$), least squares means (LSM) were compared using LSD (pdiff) of SAS. Recovery rate of COC and pregnancy rate between dietary treatments were analyzed using Chi Square with the frequency procedure of SAS.

Results

Body Weight and Dry Matter Intake

Multiparous cows weighed more (644, 632, 641, 646 and 659 ± 12 kg, $P \leq 0.001$) at each weigh period than primiparous cows (548, 551, 556, 564, 569 ± 14 kg), respectively. Body weight of cows was not influenced ($P \geq 0.77$) by dietary treatment and was 607, 600, 608, 614 and 623 ± 12 kg at 12, 32, 55, 74 and 94 ± 7 DIM, respectively. There was no difference ($P = 0.50$) in DMI between cows supplemented CLA (19.8 ± 0.3 kg) or Ca salts (19.5 ± 0.3 kg).

Milk Production and Composition

Mean weekly milk yield during the first 13 weeks of lactation was greater ($P = 0.0002$) for cows fed CLA (38.2 ± 0.4 kg) compared with cows fed Ca salts (35.4 ± 0.4 kg) (Figure 3.3). Milk yield and milk composition data are detailed in Table 3.4. Multiparous cows produced more milk ($P < 0.0001$) than primiparous cows (42.3 and 31.2 ± 0.5 , respectively).

Multiparous cows had greater milk fat yield ($P < 0.0001$) and percent milk fat ($P = 0.042$) than primiparous cows (1.56 ± 0.02 vs. 1.14 ± 0.03 kg; 3.69 ± 0.04 vs. $3.52 \pm 0.07\%$, respectively). Milk fat yield ($P = 0.0007$) and percent milk fat ($P < 0.0001$) were reduced (Figure 3.4) in cows fed CLA compared with cows fed Ca salts (1.28 ± 0.03 vs. 1.42 ± 0.03 kg; 3.28 ± 0.05 vs. $3.93 \pm 0.06\%$, respectively). There was a treatment by parity interaction with milk fat yield ($P < 0.025$); supplementation with CLA depressed milk fat yield in multiparous cows, but milk fat was not depressed in primiparous cows (Figure 3.5).

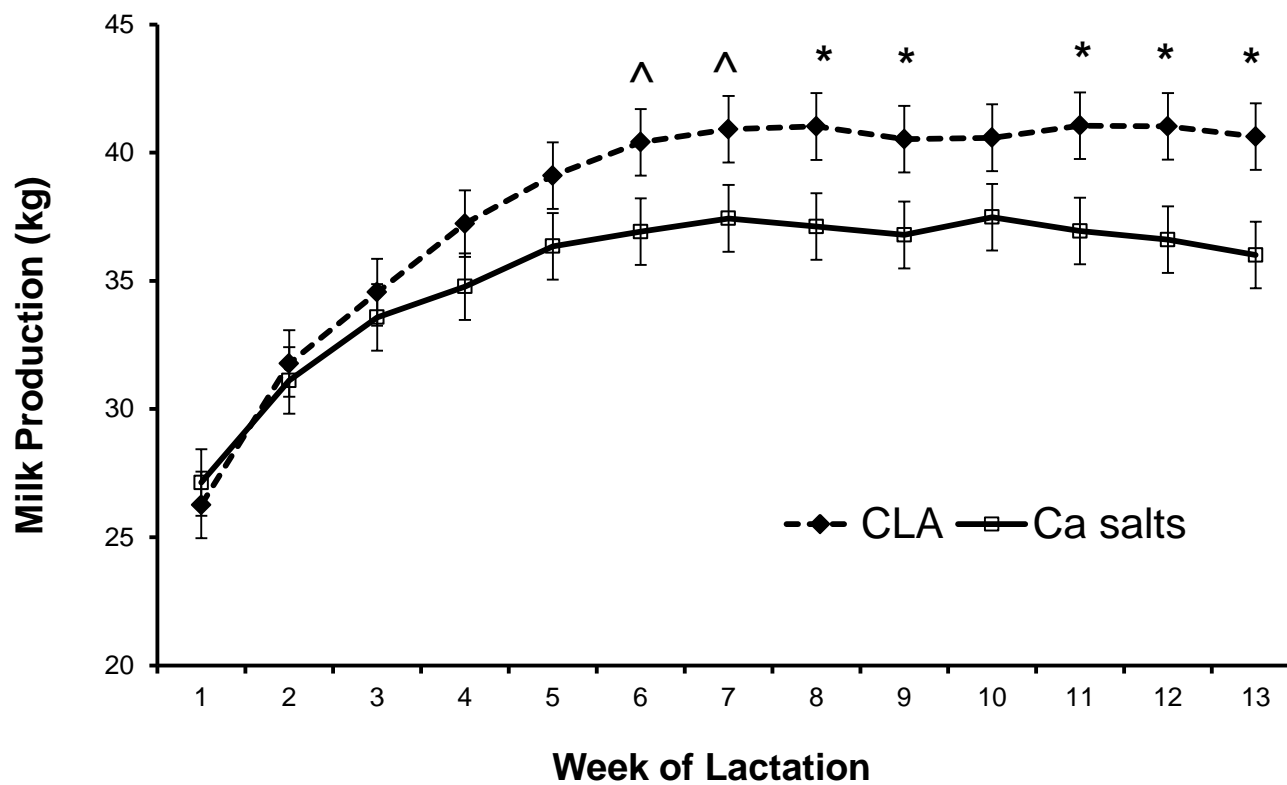


Figure 3.3 Mean weekly milk production of Holstein cows fed CLA or Ca salts. Means differ when signified with ^ ($P < 0.07$) and * ($P < 0.05$). Mean weakly milk production across all weeks was greater ($P = 0.0002$) for cows fed CLA compared with cows fed Ca salts (38.2 vs. 35.4 ± 0.4 kg, respectively).

Table 3.4 Milk production and milk composition of cows fed CLA or Ca salts

Item	Dietary Supplement ¹		SE	P value
	CLA	Ca salts		
Mean weekly milk yield, kg	38.2	35.4	0.4	< 0.0001
Milk fat yield, kg	1.28	1.42	0.03	0.0007
Milk fat, %	3.28	3.93	0.05	< 0.0001
Protein yield, kg	1.05	1.00	0.02	0.08
Milk protein, %	2.73	2.78	0.04	0.37
Energy corrected milk ² , kg	37.1	38.2	0.6	0.20
3.5% fat corrected milk ³ , kg	37.4	39.3	0.7	0.05

¹Partially rumen-protected supplements fed at 100g per hd/d. CLA cows consumed 8.6g per hd/d each of *trans-10*, *cis-12* and *cis-9, trans-11* CLA.

²Energy corrected milk (ECM) expresses the amount of energy in milk based on milk weight, fat and protein standardized to 3.5% fat and 3.2% protein. The formula used for ECM calculations (Dairy Records Management Systems DHI Glossary, 2013) was: (0.327 X milk kg) + (12.95 X fat kg) + (7.65 X protein kg).

³Fat corrected milk adjusts the pounds of milk produced to a standard fat (usually 3.5%) to calculate milk energy produced/excreted. The formula taken from the Dairy Records Management Systems DHI Glossary (2013) is: (milk kg X 0.432) + (fat kg X 16.216).

Protein yield was greater ($P < 0.0001$) in multiparous cows (1.16 ± 0.02 kg) compared with primiparous cow (0.90 ± 0.03 kg). Protein yield ($P = 0.08$) and percent protein ($P = 0.37$) in milk were not influenced by fatty acid supplementation (Table 3.4). Energy corrected milk (ECM) was greater ($P < 0.0001$) in multiparous compared with primiparous cows (43.1 ± 0.6 vs. 32.2 ± 0.8 kg, respectively), but was not influenced ($P = 0.20$) by dietary supplementation (Table 3.4).

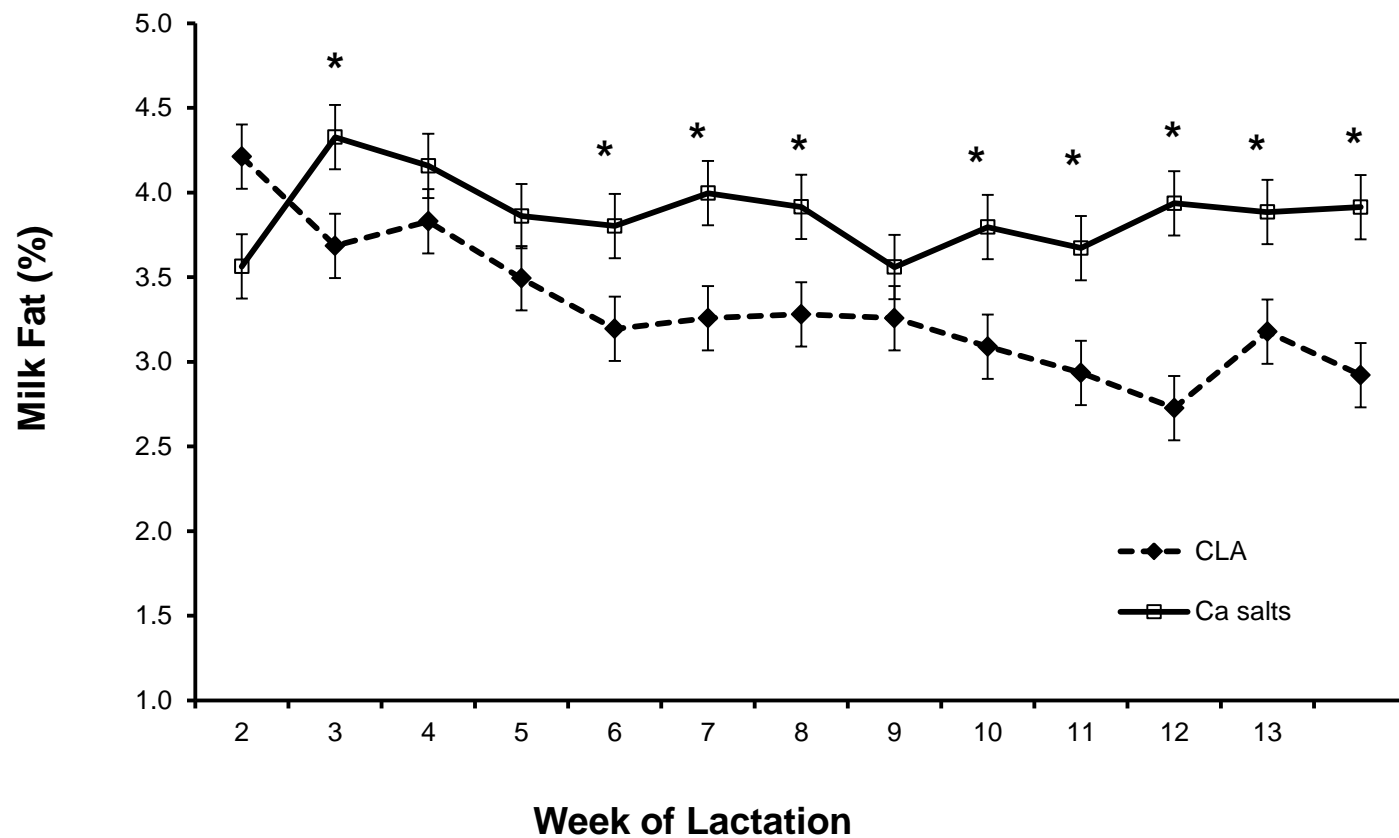


Figure 3.4 Percent milk fat of Holstein cows fed CLA or Ca salts. Means differ when signified with * ($P < 0.05$). Percent milk across all weeks was greater ($P < 0.0001$) for cows fed Ca salts compared with cows fed CLA (3.93 vs. 3.52 ± 0.05 %, respectively).

■ CLA multiparous ▨ CLA primiparous ■ Ca salts multiparous □ Ca salts primiparous

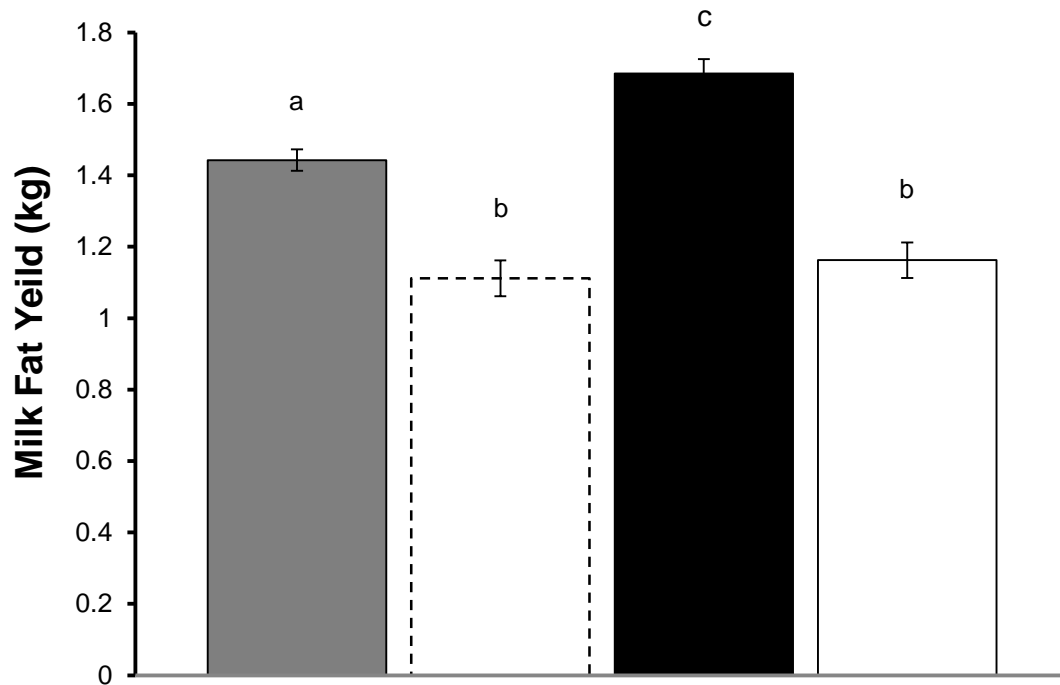


Figure 3.5 Milk fat yield of multiparous and primiparous Holstein cows fed CLA or Ca salts. A dietary treatment by parity interaction ($P < 0.025$) was identified. Different superscripts indicate mean differences ($P < 0.05$).

Multiparous cows had greater ($P < 0.0001$) 3.5% fat corrected milk (FCM) than primiparous cows (43.9 ± 0.6 vs. 32.8 ± 0.8 kg, respectively). Cows fed Ca salts had greater 3.5% FCM ($P = 0.05$; 39.3 ± 0.7 kg) than cows fed CLA (37.4 ± 0.7 kg).

Follicle, Oocyte and Pregnancy Responses

A total of 516 oocytes were collected from 32 Holstein cows after a total of 62 TUGA sessions ($n = 269$ CLA, $n = 247$ Ca salts). Follicle and oocyte responses of Holstein cows are presented in Table 3.5. No interactions were detected for collection ($P \geq 0.52$), treatment by collection ($P \geq 0.14$), treatment by

Table 3.5 Follicle and oocyte responses of Holstein cows fed CLA or Ca salts

Item	Dietary supplement ¹		SE	P value
	CLA	Ca salts		
Follicles targeted	17.0	15.7	1.6	0.54
COC recovered	8.2	7.6	1.0	0.69
Quality COC recovered ²	6.0	5.7	0.8	0.74
Recovery rate ³ (%)	47.9	48.3	3.0	0.88

No collection, treatment by collection or treatment by parity interactions were detected so mean response per cow per collection was reported.

¹Holstein cows were supplemented with either a supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids (CLA, Lutrell) or Ca salts of palm oil supplement (Ca salts, Megalac) at 150 g per hd/d, respectively.

²Quality Grade I and Grade II oocytes according to a four-point scale.

³Number COC recovered/number follicles targeted.

parity ($P \geq 0.36$) or collection by parity ($P \geq 0.74$) for the number of follicles targeted, COC and Quality COC recovered. Multiparous cows had greater follicles targeted per TUGA session ($P = 0.02$) than primiparous cows (19.2 ± 1.6 vs. 13.4 ± 2.0 follicles, respectively) (Table 3.6). Accordingly, multiparous cows produced more COC ($P = 0.009$) and quality (Grade I and II) COC ($P = 0.03$) per TUGA session compared with primiparous cows (9.9 ± 0.8 vs. 5.9 ± 1.3 COC; 7.2 ± 0.6 vs. 4.3 ± 1.0 Quality COC, respectively). Dietary treatment did not influence the number of follicles targeted ($P = 0.54$), COC recovered ($P = 0.69$) or Quality COC recovered ($P = 0.74$) per TUGA session (Table 3.5). Rate of COC recovery (48.1%) was similar ($P = 0.88$) between dietary treatments.

Table 3.6 Follicle and oocyte responses of multiparous and primiparous Holstein cows

Item	Parity		SE	<i>P</i> value
	Multiparous	Primiparous		
Follicles targeted	19.2	13.4	1.5	0.02
COC recovered	9.9	5.9	1.0	0.01
Quality COC recovered ²	7.2	4.4	0.8	0.03

No treatment or treatment by parity interactions were detected so mean response per cow per collection across dietary treatment groups (CLA and Ca Salts) was analyzed.

²Quality Grade I and Grade II oocytes according to a four-point scale.

First service pregnancy rate was similar ($P = 0.30$) for cows supplemented CLA ($n = 8/16$, 50.0%) and those supplemented with Ca salts ($n = 5/17$, 29.4%), respectfully (Figure 3.6).

Nile Red Stain

Intensity of control slides did not differ across day of image collection ($P = 0.68$). A total of 111 oocytes ($n = 57$ CLA, $n = 54$ Ca salts) from 25 cows ($n = 13$ CLA, $n = 12$ Ca salts) were stained and evaluated. There were no collection ($P = 0.34$) or treatment by collection ($P = 0.51$) interactions for Nile Red intensity. Mean Nile Red intensity was not influenced ($P = 0.88$) by dietary treatment (Figure 3.7). Representative images of Nile Red stained oocytes are available in Appendix D.

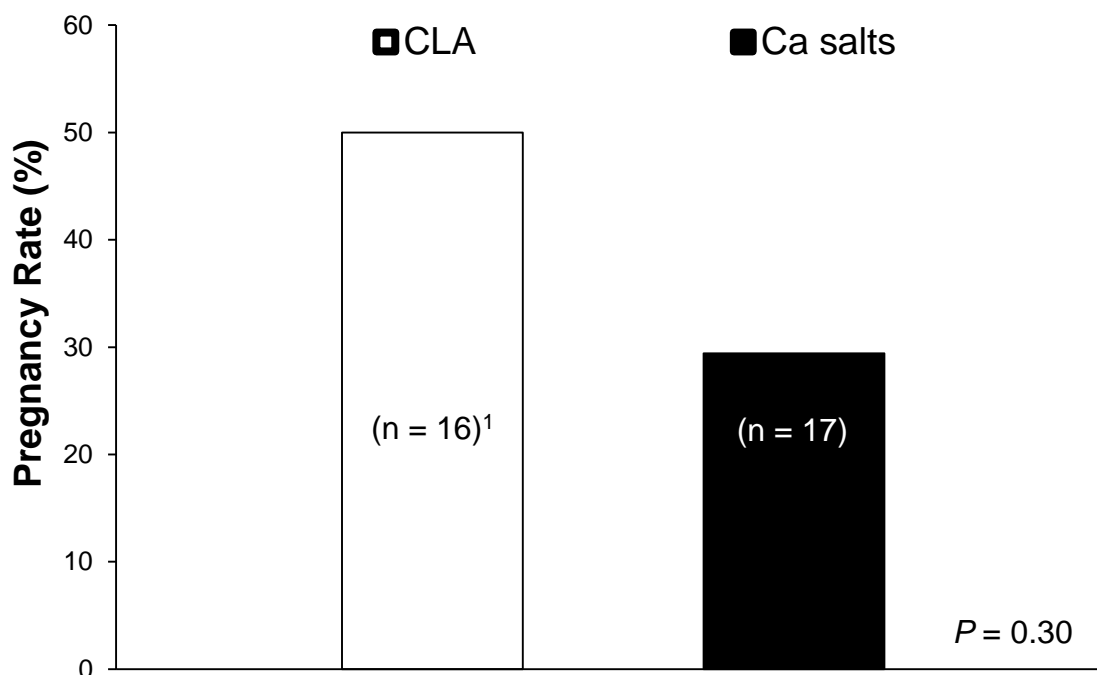


Figure 3.6 First service artificial insemination pregnancy rate of Holstein cows fed CLA or Ca salts. ¹Number of cows confirmed pregnant via ultrasound at 34 ± 5 d after AI over the number of cows inseminated.

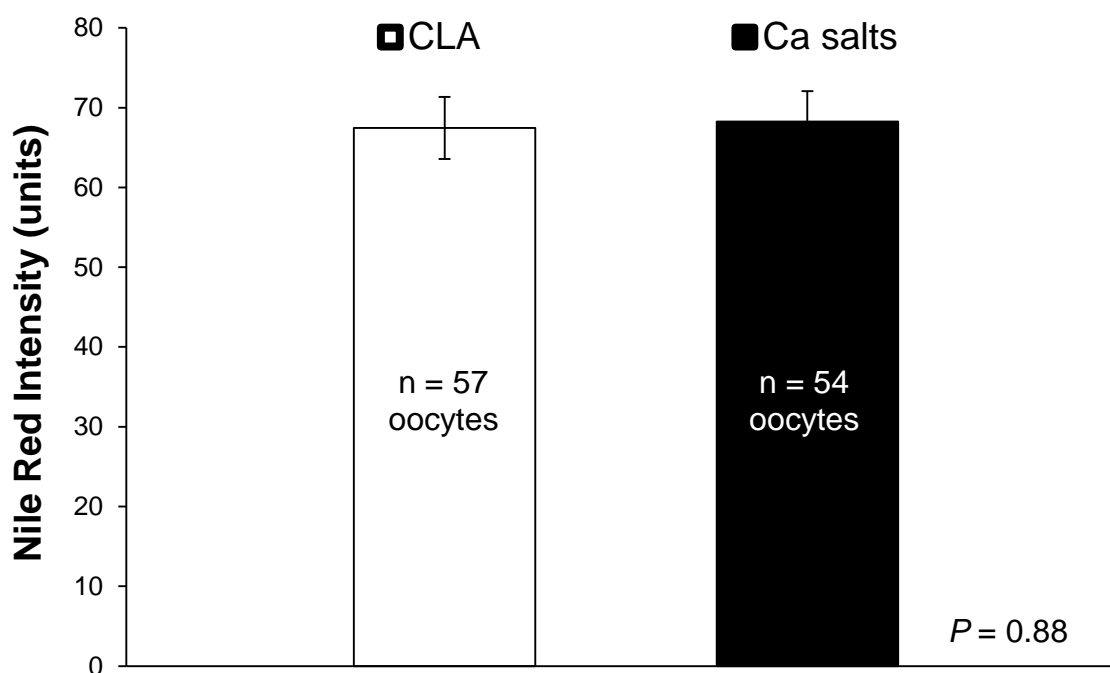


Figure 3.7 Mean Nile Red intensity of oocytes collected from Holstein cows fed CLA (n=57 oocytes, n=13 cows) or Ca salts (n=54 oocytes, n=12 cows).

Discussion

Mean animal feed refusal in this study was 13.5% (as fed) per d.

Therefore, actual intake of the CLA supplement was calculated at 86 g/hd per d (100 g/hd per d offered). This equates to 8.6 g each of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA. Mean weekly milk yield for cows fed CLA was 7.9% greater than cows fed Ca salts in this study. This is similar to reported increases in milk yield of 3 to 10% in cows fed supplements containing *trans*-10, *cis*-12 CLA (Bernal-Santos et al., 2003; Mackle et al., 2003; de Veth et al., 2006; Castañeda-Gutiérrez et al., 2007b). Similarly, milk yield was 13% greater in lactating ewes fed CLA compared with control fed ewes (Sinclair et al., 2010).

In studies where milk energy output and cow energy balance are similar between supplemental treatment groups, energy spared by milk fat depression is likely repartitioned for milk production (Bernal-Santos et al., 2003; de Veth et al., 2006; Castañeda-Gutiérrez et al., 2007b; Sinclair et al., 2010). However, milk production in Holstein cows was not influenced by CLA-supplementation in a study by Castañeda-Gutiérrez et al. (2007a). Milk production was also not influenced in dozens of studies involving very short-term abomasal infusion of CLA that significantly decreased milk fat. Factors such as the dose of CLA isomers, method used to protect the supplement from rumen biohydrogenation, length of dietary treatment, stage of lactation during treatment and season may influence milk production responses to CLA supplementation.

Milk fat yield was decreased by 9.9% in cows fed CLA compared with Ca salts in the current study. Milk fat percentage decreased from 3.93% in Ca salts

fed cows to 3.28% in CLA-fed cows. Sippel et al. (2009) reviewed CLA-induced milk fat depression using different doses of *trans*-10, *cis*-12 CLA in seven trials (Giesy et al., 2002; Perfield et al., 2002; Piperova et al., 2004; Selberg et al., 2004; de Veth et al., 2005; de Veth et al., 2006; Sippel et al., 2009). Sippel et al. (2009) plotted depression in daily milk fat yield over CLA dosage to develop a prediction equation for expected milk fat depression after dietary supplementation of CLA. Using that equation in the present study, dietary addition of 8.6 g *trans*-10, *cis*-12 CLA per hd/d resulted in an expected milk fat depression of 14.8%. This is slightly greater, but within reasonable range of the 9.9% milk fat depression in the current study.

Milk fat yield was significantly decreased by 14.1% in cows fed a rumen-protected CLA containing 7.1 g/d each of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Castañeda-Gutiérrez et al., 2007a). Pappritz et al. (2011) reported milk fat depression was 14.8% in cows fed a lipid encapsulated CLA (Lutrell®) at nearly the same inclusion rate (8.0 g of *trans*-10, *cis*-12 CLA) as the present study.

The efficacy of a rumen-protected *trans*-10, *cis*-12 CLA supplement is measured by the degree of milk fat depression (Perfield et al., 2004). The transfer efficiency of CLA from a supplement into milk fat is influenced by efficacy of the protection from rumen metabolism as well as postruminal availability (Wu and Papas, 1997). Transfer efficiencies of CLA from rumen-protected supplements are generally much lower than the ~20% transfer efficiencies found in abomasal infusion studies (Chouinard et al., 1999a; Chouinard et al., 1999b; Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002). The

majority of the CLA in rumen-protected supplements are either metabolized in the rumen or unavailable for postruminal absorption. However, as evidenced by significant milk fat depression in cows fed rumen-protected supplements, CLA isomers can alter biological tissues after very minimal absorption and transfer into these tissues.

Comparing the transfer of *trans*-10, *cis*-12 CLA from rumen-protected supplements with that from abomasal infusion (average 22%) provided an estimate of rumen biohydrogenation and, therefore, a measure of the efficacy of rumen protection (de Veth et al., 2005). The CLA in the current study was coated with hydrogenated vegetable fat consisting of palmitic and stearic acid linked to glycerin. Pappritz et al. (2011) used duodenal fistulated cows to evaluate rumen metabolism and postruminal bioavailability of a lipid-encapsulated CLA supplement manufactured by BASF (Lutrell[®], BASF Ludwigshafen, Germany). This supplement is the same encapsulation and CLA formulation used in the current study, and one of the inclusion rates in the Pappritz et al. (2011) study was 100 g of CLA per hd/d. Only 0.4 g of the daily consumed 8 g *trans*-10, *cis*-12 CLA reached the duodenum, while transfer efficiency of the isomer into milk was 3%; this suggests a low (5%) ruminal protection rate (Pappritz et al., 2011). Of the 0.4 g reaching the duodenum, 48% was transferred to milk (Pappritz et al., 2011).

Assuming similar biohydrogenation conditions to the Pappritz et al. (2011) study, von Soosten et al. (2013) reported that 0.24 g to 1.04 g of *trans*-10, *cis*-12 were available for absorption in the small intestine after cows were fed a lipid

encapsulated supplement containing 6 g of *trans*-10, *cis*-12 CLA. Although the current study used a similar approach to lipid encapsulate supplements, rumen protection may not have been as efficient in the current study compared with previously reported studies; resulting in less incorporation of *trans*-10, *cis*-12 into milk and less pronounced milk fat depression. Possible variation in individual animal DMI may also account for the less pronounced milk fat depression in the current study, as individual dietary intake of cows was not accessed.

A treatment by parity interaction was found with milk fat yield in the current study (Figure 5). Supplementation with CLA significantly decreased milk fat yield in multiparous cows, but milk fat yield of primiparous cows did not differ between CLA and Ca salts supplemented groups. Although very few studies involving milk fat depression have included primiparous cows, no report of this interaction could be found in the literature. Milk fat is often greater in multiparous compared with primiparous cows. Because individual intake was not assessed, DMI of primiparous cows may have been less than multiparous cows, resulting in less intake of CLA and little to no effect on milk fat depression. Additionally, low experimental numbers of primiparous cows may have influenced results. However, first lactation cows may indeed respond differently to CLA supplementation, with this finding needing further examination.

Cows fed Ca salts had significantly greater 3.5% FCM compared with CLA-supplemented cows, but ECM was not significantly altered by dietary supplementation. Fat corrected milk uses a standard formula to adjust milk yield to a standard fat test, usually adjusted to 3.5% FCM (DRMS DHI Glossary,

2013). Energy corrected milk calculates the amount of energy in milk based on milk weight, fat and protein standardized to 3.5% fat and 3.2% protein. These two measures are used to evaluate the total milk energy excreted in milk, and directly relates to profitability as dairy operations are compensated for both the quantity and quality (fat %) of their milk. In the current study, CLA-supplemented cows repartitioned a portion of the energy spared by milk fat depression into increased milk production. However, it appears that additional energy was retained and may have been repartitioned into other tissues or systems.

Multiparous cows had more follicles, COC recovered and Quality COC recovered per TUGA session compared with primiparous cows in this study. In contrast, Bilby et al. (2006) reported more visible follicles aspirated in primiparous compared with multiparous Holstein cows in a study feeding various dietary unsaturated fatty acids. However, only 4.6 ± 0.4 follicles were aspirated and 3.7 ± 0.3 oocytes recovered per cow in the Florida study using non-stimulated lactating Holstein cows collected every 3 d during summer months (Bilby et al. 2006). Differences in age, growing conditions, body condition and genetics of primiparous cows as well as season of experiment may have contributed to differences in parity effects for follicle and oocyte responses between studies.

Dietary treatment did not influence the number of follicles targeted, COC recovered or Quality COC recovered per TUGA session in the current study. Lactating Holsteins fed Lutrell (100 g/d) produced more follicles per collection compared with those produced from non-supplemented animals (Höffmann et al.,

2008). The previous report is an abstract that does not explain details regarding isoenergetic formulation of control and CLA fed cows. In the current study, energy spared by milk fat depression and not repartitioned into milk production of CLA fed cows does not appear to support the quantity of follicles nor the quantity and quality of COC produced by the ovary. Developmental potential of oocytes was not evaluated in the current study.

First service pregnancy rate was not influenced by dietary treatment in the current trial, as experimental numbers were too limited to establish a statistical difference. Supplementation with ≤ 10 g/d of each *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA appears to benefit reproduction in lactating Holstein cows even without significant changes in energy balance (Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005). Castañeda-Gutiérrez et al. (2007b) reported increased plasma concentrations of IGF-I in lactating cows fed a rumen-protected CLA containing 7.1g/d each of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. Additionally, CLA-supplemented cows tended ($P = 0.08$) to have greater concentrations of progesterone during the early luteal phase (d 6 to 8 of the estrous cycle; (Castañeda-Gutiérrez et al., 2007b). Beneficial reproductive effects were reported after supplementation of cows was terminated in studies by Bernal-Santos et al. (2003) and Castañeda-Gutiérrez et al. (2005). Castañeda-Gutiérrez et al. (2007b) concluded that CLA supplementation may improve embryo viability via improvement in the follicular environment. Supplementation of cows with 100 g of lipid-encapsulated CLA per hd/d improved cleavage rates and d-8 blastocyst rates *in vitro* (Höffmann et al., 2008).

Using semi-parametric and parametric models, de Veth et al. (2009) conducted an integrated analysis of five controlled studies involving CLA supplementation and reproduction in early lactating dairy cows. In this paper, prediction models concluded that supplementation with *trans*-10, *cis*-12 CLA was positively associated with the probability of pregnancy success and a decrease in the time to pregnancy as dietary dose increased (de Veth et al., 2009). Models predicted the probability of pregnancy increased by 26% and the median time to pregnancy was decreased by 34 d when cows are fed the optimal daily dose of 10.1 g (pregnancy) and 10.5 g (d to pregnancy) *trans*-10, *cis*-12 CLA compared with control-supplemented cows (de Veth et al., 2009).

Nile Red intensity was not influenced by dietary treatment in the current study. This indicates that concentration of intracytoplasmic neutral lipids was not significantly different in oocytes collected from cows fed CLA or Ca salts in this study. Nile Red staining allows evaluation of lipid content for relative comparison of individual oocytes from different donors or culture conditions. However, this method does not permit qualitative composition of lipids, such as identification of fatty acids or different lipid fractions, nor does it allow absolute quantitative analysis. Embryos co-cultured with *trans*-10, *cis*-12 CLA *in vitro* contained less lipids and post-thaw viability was improved compared with control cultured embryos (Pereira et al., 2007). However, there are currently no reports using Nile Red to evaluate lipid content of oocytes from cows fed CLA known to suppress milk fat. More dynamic quantitative lipid analysis techniques may have been beneficial to evaluate possible differences between dietary treatments.

Cows in different physiological stages may differ in the way they respond to CLA supplementation, particularly at the ovarian level.

Summary

Milk fat was depressed by 10.1% and milk production increased 7.3% in cows fed CLA compared with Ca salts. Supplementation with CLA did not influence follicle or oocyte production or the number of quality oocytes produced. Nile red intensity did not differ by dietary treatment, indicating no difference in lipid content of oocytes. However, further research is warranted to evaluate the influence of CLA supplementation on ovarian lipid metabolism and to elucidate mechanisms that may influence reproductive performance and embryo composition and cryosurvival. Better understanding of mechanisms contributing to variation in cryotolerance of embryos will continue to improve success of embryo cryopreservation.

CHAPTER IV
EXPRESSION OF GENES INVOLVED IN LIPID METABOLISM IN OOCYTES
FROM HOLSTEIN AND BRAHMAN COWS FED *TRANS*-10, *CIS*-12 AND
***CIS*-9, *TRANS*-11 CONJUGATED LINOLEIC ACIDS**

Introduction

Variation in cryotolerance exists between embryos from different breed, species and management conditions. Reduced tolerance to chilling and cryotolerance of oocytes and embryos has been associated with greater cytoplasmic lipids (Kim et al., 2001; Abe et al., 2002; Seidel, 2006). Nagashima et al. (1994) previously reported improved post-thaw survival of porcine embryos after centrifugation and removal of lipids at the 1-cell stage.

The concentration and type of fat included in ruminant diets may influence the composition of oocytes and embryos, and therefore, their cryotolerance. Feeding ewes diets high in polyunsaturated fatty acids (PUFA) altered fatty acid composition of follicular components (Wonnacott et al., 2010) and improved membrane integrity after chilling (Zeron et al., 2002a). Fatty acid composition of follicular fluid in dairy cows is sensitive to dietary supply of polyunsaturated fatty acids (PUFA) using soybean oil (Batista et al., 2010) and flaxseed oil (Zachut et al., 2010). *In vivo*-produced embryos from Nellore heifers were more sensitive to cryopreservation when diets included rumen-protected PUFA (Guardieiro, 2010; Guardieiro et al., 2014).

Another interesting area of study has identified specific conjugated linoleic acids (CLA) that inhibit milk fat synthesis in dairy cows (Lor and Herbein, 1998; Chouinard et al., 1999a; Chouinard et al., 1999b). The *trans*-10, *cis*-12 CLA

isomer was identified as a potent inhibitor of milk fat synthesis in lactating cows (Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002).

Additionally, inclusion of *trans*-10, *cis*-12 CLA in bovine embryo culture medium improved post-thaw embryo survival without influencing overall blastocyst production (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013).

The *trans*-10, *cis*-12 CLA isomer is reported to exert specific effects on adipocytes that reduce the uptake of fatty acids without increasing lipolysis (Pariza et al., 2001). *Trans*-10, *cis*-12 CLA altered expression of genes involved fatty acid oxidation and synthesis in the bovine mammary gland (Lee et al., 1998; Bretillon et al., 1999; Park et al., 2000; Baumgard et al., 2002) and in *in vitro* bovine embryos cultured with *trans*-10, *cis*-12 CLA (Al Darwich et al., 2010). Additionally, *In vitro* culture of embryos altered mRNA expression of genes coding for fatty acid utilization and synthesis; results likely help explain lower developmental competence of *in vitro* compared with *in vivo* embryos (Algriany et al., 2007). Altered gene expression and resulting modification in oocyte lipid metabolism likely alters the composition of oocytes, resulting in potential differences in the cryotolerance of embryos.

Carnitin palmitoyl transferase-1 (CPT-1) is an enzyme involved in mitochondrial energy homeostasis that catalyzes a rate-limiting step in the transfer of long-chain fatty acyl-CoA from the cytosol to mitochondria for oxidation (Abu-Elheiga et al., 2000; Kerner and Hoppel, 2000). During oocyte maturation, CPT1 mRNA increases indicating an increase in β -oxidation during this stage (Algriany et al., 2007). The FADS2 gene is a member of the fatty acid

desaturase (FADS) gene family. Enzymes resulting from FADS2 regulate unsaturation of fatty acids through the addition of double bonds. *In vitro* embryos cultured with 100 μ M *trans*-10, *cis*-12 CLA had reduced FADS2 transcript level compared with control cultured embryos (Al Darwich et al., 2010). Peroxisome proliferator-activated receptor alpha (PPAR α) is a member of the PPAR family of ligand-dependent transcription factors (Rosen et al., 1999) and expression of PPAR α is upregulated in tissues with high rates of fatty acid oxidation. Dietary unsaturated fatty acids have been reported to alter gene expression through PPAR, leading to altered metabolism, growth and cell differentiation in different tissues of rats (Ide, 2000; Jump, 2004).

Successful cryopreservation of embryos from *Bos indicus* cattle is variable (Visintin et al., 2002). Cytoplasmic lipid content is greater in *Bos indicus* oocytes compared with *Bos taurus* oocytes (Ballard, 2006), likely contributing to reduced cryotolerance in embryos from *Bos indicus* cattle. Cryopreservation of embryos from Jersey donors resulted in reduced pregnancy rates compared with those from Holstein donors (Steel and Hasler, 2004). In addition to greater embryo lipid content, *Bos indicus* and Jersey cattle have greater milk fat content compared with other breeds of cattle.

Intracellular lipid content of embryos can be qualitatively compared between treatments using Nile Red and fluorescence microscopy. Peak fluorescence observed correspond to neutral lipids, which are mainly triglycerides (Genicot et al., 2005). Fluorescence intensity after staining with Nile Red was used to evaluate differences in cytoplasmic lipids of oocytes and embryos

differing in donor breed (*Bos indicus* vs. *Bos taurus*, Ballard, 2006; Holstein Fresian vs. Belgian Blue (Leroy, 2004) domestic livestock species (Genicot et al., 2005), bovine *in vitro* culture conditions (Barceló-Fimbres and Seidel, 2008) or visual color (dark vs. light) (Leroy et al., 2005).

Additional research is necessary to elucidate mechanisms contributing to variation in cryotolerance associated with individual animal, breed, species and nutrition (Seidel, 2006). Previous studies in the cow have demonstrated nutrition-induced modification of follicular components, which may have implications for oocyte fatty acid metabolism, oocyte lipid composition and embryo cryotolerance. *Trans*-10, *cis*-12 CLA influences mRNA expression of genes involved in lipid metabolism in the bovine mammary gland, as well as in bovine embryos after culture with CLA. Supplementation of cows with CLA may alter lipid composition of oocytes via modification of oocyte gene expression. The response to CLA supplementation and/or oocyte lipid metabolism may be different between *Bos indicus* and *Bos taurus* breeds of cattle. To the author's knowledge there are no current reports evaluating mRNA expression of genes involved in lipid metabolism in oocytes and embryos collected from cows fed CLA.

Therefore, objectives of this study were to evaluate effects of CLA supplementation of Holstein and Brahman cows on (1) follicle, oocyte and embryo production (2) mRNA expression of CPT1, FADS2 and PPAR α in cumulus-oocyte complexes (COC) and (3) lipid content of Brahman oocytes using Nile Red stain.

Materials and Methods

Experiment 4.1: Holstein Cows

Experimental procedures in this study were approved by the Louisiana State University Animal Care and Use Committee and were conducted at the Louisiana State University Agricultural Center Dairy Research Farm (Baton Rouge, LA) from May through July 2012.

Animals and Experimental Diets

Nonlactating, nonpregnant Holstein cows ($n = 12$) aged 3 to 8 years were randomly allotted to experimental diets after stratification by age and body weight (BW). Cows were maintained on a 0.6 ha bermudagrass-based (*Cynodon dactylon*) paddock with minimal forage availability. Cows had access to shade structures and fresh water.

Cows were supplemented with a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids (Lutrell[®], BASF, Lampertheim, Germany) at 150 g per hd/d (High-CLA, $n = 4$ cows) 100 g per hd/d (Mod-CLA, $n = 4$ cows) or were not supplemented with fat (Control, $n = 4$ cows). Cows were gathered daily and individually fed in a locking headgate feeding system (Picture in Appendix E). Each cow was offered 1 kg per hd/d dry distillers grains (DDG) containing 150 g of CLA, 100 g of CLA or no CLA (DDG only). An open space between cows in the locking headgate system ensured cows only had access to the supplement intended for each animal. Calculated inclusion of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA was 15 g per hd/d (High-CLA) and 10 g per hd/d (Mod-CLA).

Supplement refusal was recorded, but generally all cows consumed the supplements immediately after it was offered. After supplements were consumed, cows were individually fed a total mixed ration (TMR) including corn silage and DDG (4.0 and 1.7 kg DM per hd/d for corn silage and DDG, respectively). The TMR and free-choice grass hay were fed in order to meet or exceed nutritional requirements. The headgate system was rinsed with a high-pressure water hose after each feeding to ensure no supplement carryover to the next day. Body weight of cows was recorded at 30 d intervals.

Synchronization for Oocyte Collection

An outline of the TUGA schedule is presented in Figure 4.1 and the ovarian stimulation schedule is presented in Figure 4.2. A dominant follicle removal (DFR) procedure was performed on each cow to synchronize the follicular wave 4 d before each TUGA session. Three injections (im) of FSH (Folltropin[®]-V, Bioniche Animal Health, Athens, GA) were administered at 12 h intervals beginning 36 h after the DFR. A descending-dose FSH schedule was used with injections of 3.5, 3.0 and 2.5 ml (20 mg/ml) equating to 70, 60 and 50 mg FSH for each of the three injections, respectively. Cumulus-oocyte complexes were collected 36 h after the final FSH injection (4 d after DFR).

Oocyte Collection

Oocytes were collected by TUGA in a similar manner as experiments in Chapter III and equipment and materials used for TUGA are presented in Appendix A. Oocytes were collected 11 d before the start of dietary supplementation and at 14, 28 and 42 d of dietary supplementation.

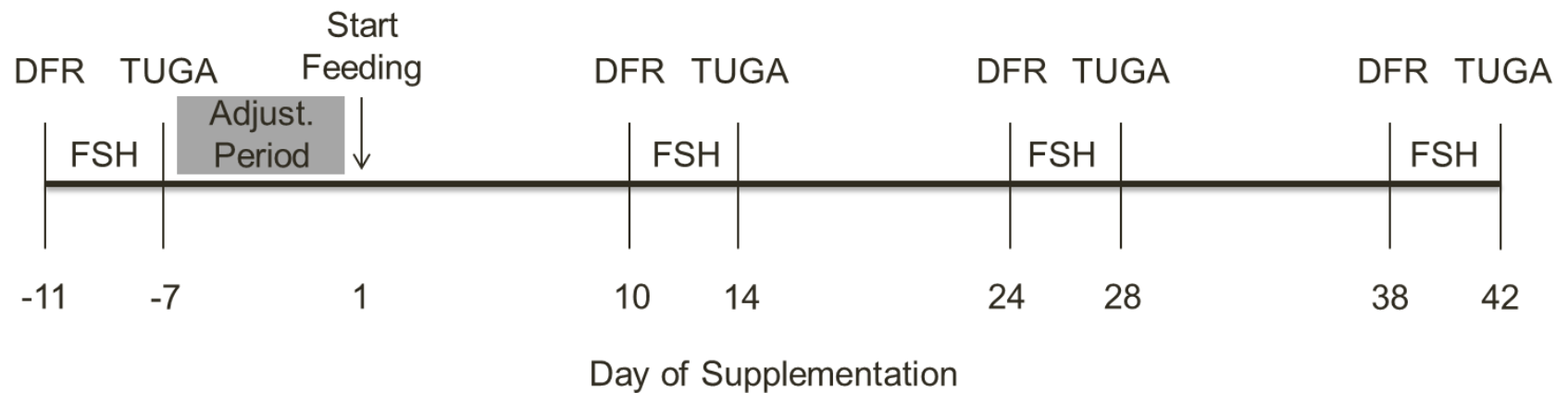


Figure 4.1 Schedule and procedures for trans-vaginal ultrasound-guided oocyte aspiration (TUGA) in Holstein cows (Experiment 4.1). DFR is dominant follicle removal; FSH is Folltropin®-V, follicle stimulating hormone.

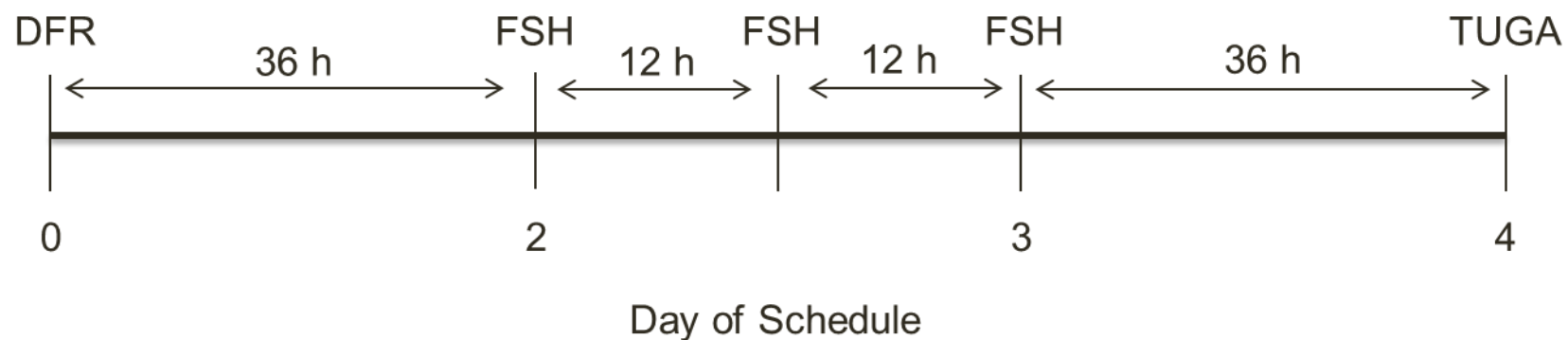


Figure 4.2 Ovarian stimulation schedule and procedures before transvaginal ultrasound-guided oocyte aspiration (TUGA) session in Holstein cows (Experiment 4.1). DFR is dominant follicle removal; FSH is Folltropin®-V, follicle stimulating hormone (20 mg/ml) administered in descending-dose schedule (injections of 3.5, 3.0 and 2.5 ml).

Contents from follicles approximately ≥ 4 mm were collected into the filter containing 10 ml collection medium. The number of follicles targeted was recorded for each cow, in each TUGA session. Oocyte collection medium (OCM) was Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium containing 1% calf serum and heparin (10 IU/ml). Immediately following collection, aspirate from each cow was transferred to a gridded dish containing OCM and searched for COC with a dissecting microscope. Recovered COC were graded according a four point scale similar to the procedures reported by Leibfried and First (1979). Grade I and Grade II COC were placed in 1.5 ml microtubules (2 COC/tube) in minimal volume (1 to 2 μ l) of PBS containing 0.1% polyvinyl alcohol (PVA) and stored at -80°C for later RNA isolation.

Synchronization and Ovarian Stimulation for Embryo Collection

Cows were subjected to ovarian stimulation and synchronization of estrus prior to *in vivo* embryo collection on d 69 of dietary supplementation (Figure 4.3). A DFR procedure was performed on each cow 14 d before embryo collection (d 55 of supplementation) and a controlled internal drug release device (CIDR) was inserted intra-vaginally. Eight injections (im) of FSH (Folltropin[®]-V, Bioniche Animal Health, Athens, GA) were administered at 12 h intervals beginning 36 h after the DFR. A descending-dose FSH schedule was used with injections of 3, 2.5, 2.5, 2, 2 1.5, 1.5 and 1 ml (20 mg/ml), equating to a total of 320 mg of FSH. Cows were administered (im) prostaglandin $\text{F}_{2\alpha}$ (Lutalyse[®], 5ml, 25 mg) at the time of the 7th and 8th FSH injection and CIDR were removed at the 8th FSH injection. Estrus of cows was detected and cows were inseminated with high

quality semen from the same Brangus sire at 12 ± 3 h (two 0.5 ml straws) and 24 ± 3 h (one 0.5 ml straw) after initiation of standing estrus.

Embryo Collection

Embryos were recovered via nonsurgical gravity flow embryo collection on d 69 of dietary supplementation by a single technician. Briefly, cows were restrained in a manual squeeze chute and given local anesthesia via a caudal epidural injection of 5 ml of lidocaine (2%, Reproduction Resources, Walworth, WI). The number of CL present was recorded for each cow. A Foley catheter (18 or 20 Fr, Allegro Medical, Bolingbrook, IL) was passed through the cervix into the uterine body and was connected to a 1 L bag of lactate ringers (containing 1% bovine calf serum as a surfactant) via Y-junction tubing (Reproduction Resources, Walworth, WI). The opposite end of the Y-tubing was connected to an E-Z Way filter (Reproduction Resources, Walworth, WI). Immediately following collection, filters were searched for ova with a dissecting microscope.

The number of recovered ova, degenerate/unfertilized ova, embryos and transferable quality embryos (TQE, Quality Grades 1 and 2) were recorded for each cow. Stage and Grade of embryos were assigned according to International Embryo Transfer Society (IETS) guidelines. All TQE were placed in 1.5 ml microtubes (2 embryos/tube) in minimal volume (1 to 2 μ l) of PBS containing 0.1% polyvinyl alcohol (PVA) and stored at -80°C for later RNA isolation or mass spectrometry analyses.

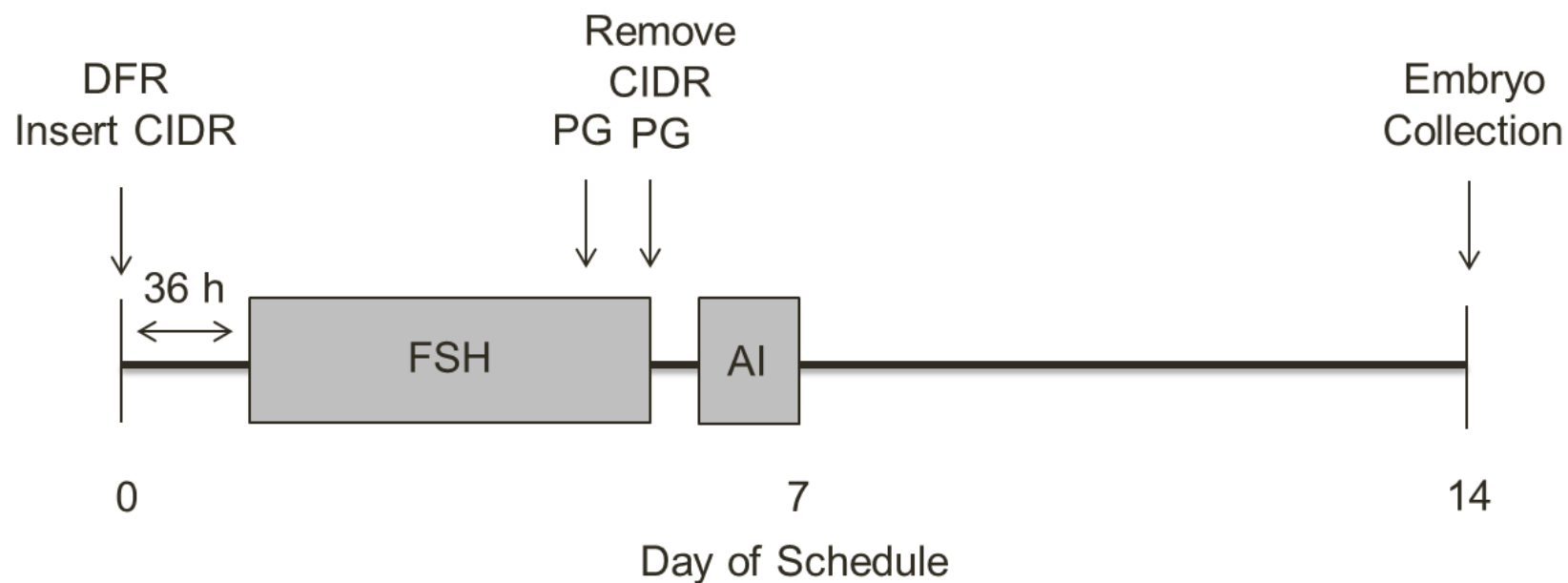


Figure 4.3 Synchronization schedule and procedures for ovarian stimulation before *in vivo* embryo collection in Holstein and Brahman cows (Experiment 4.1 and Experiment 4.2). DFR is dominant follicle removal; FSH is follicle stimulating hormone administered in desending-dose injections (Folltropin[®]-V in Experiment 4.1; Pluset[®] Original and Pluset[®] Flex H in Experiment 4.2). CIDR is controlled internal drug release device; PG is prostaglandin F_{2α} (Lutalyse[®], 5 ml, 25 mg, im). AI is artificial insemination at 12 h and 24 ± 3 h after standing estrus.

Experiment 4.2: Brahman Cows

Experimental procedures in this study were approved by the Louisiana State University Animal Care and Use Committee and were conducted at the Louisiana State University Agricultural Center Reproductive Biology Center (St. Gabriel, LA) from January through June 2013.

Animals and Experimental Diets

Nonlactating, nonpregnant Brahman cows ($n = 17$) aged 4 to 14 years were randomly allotted to experimental diets after stratification by age body weight (BW) and body condition (BCS). Cows were maintained on an 8 ha paddock consisting primarily of bermudagrass (*Cynodon dactylon*), blue grass (*Poa pratensis*) and white clover (*Trifolium repens*). Cows had access to a permanent shade structure, fresh water and free choice mineral supplement.

Cows were gathered daily, sorted into individual stalls in a barn and fed a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids (CLA, Lutrell[®], BASF, Lampertheim, Germany) or were not supplemented with fat (Control). Supplements were fed individually in rubber hook-over style feed pans (Little Giant[®] by Miller Manufacturing Co., Eagan, MN). The CLA (150 g per hd/d) was fed after incorporation with a pelleted soybean hull supplement (1 kg/hd) or the soybean hull supplement only was fed (Control). Cows were trained to eat out of feed pans for ~14 d before the start of the trial. Supplement refusal was recorded, but generally all cows consumed the supplements immediately after it was offered. Calculated CLA inclusion was 15 g per hd/d each of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA.

After supplements were consumed, cows were individually fed an additional 2 kg per hd/d soybean hull pellets from the start of the trial until d 36 of supplementation and 1 kg per hd/d soybean hulls from d 37 through d 74 of supplementation in order to meet or exceed nutritional requirements until adequate forage growth occurred. Cows were also offered free-choice grass hay from the start of the trial until d 36 of supplementation. Body weight and BCS of cows was recorded monthly.

Synchronization for Oocyte Collection

An outline of the TUGA schedule is presented in Figure 4.4. A dominant follicle removal (DFR) procedure was performed on each cow to synchronize the follicular wave 4 d before each TUGA session. Ovaries were not stimulated with FSH in Experiment 4.2.

Oocyte Collection

Procedures for oocyte collection were similar to those used in Experiment 4.1 and a list of equipment and materials used for TUGA is available in Appendix A. Oocytes were collected via TUGA 23 d before the start of supplementation and at 19, 40 and 106 d of dietary supplementation. The number of follicles targeted was recorded for each cow, in each TUGA session. Grade I and Grade II COC from the first three oocyte collections (d 19, d 40 and d 106 of supplementation) were placed in 1.5 ml microtubules (2 COC/tube) in minimal volume (1 to 2 μ l) of PBS containing 0.1% polyvinyl alcohol (PVA) and stored at -80°C for later RNA isolation. Grade I, and II COC from the final oocyte

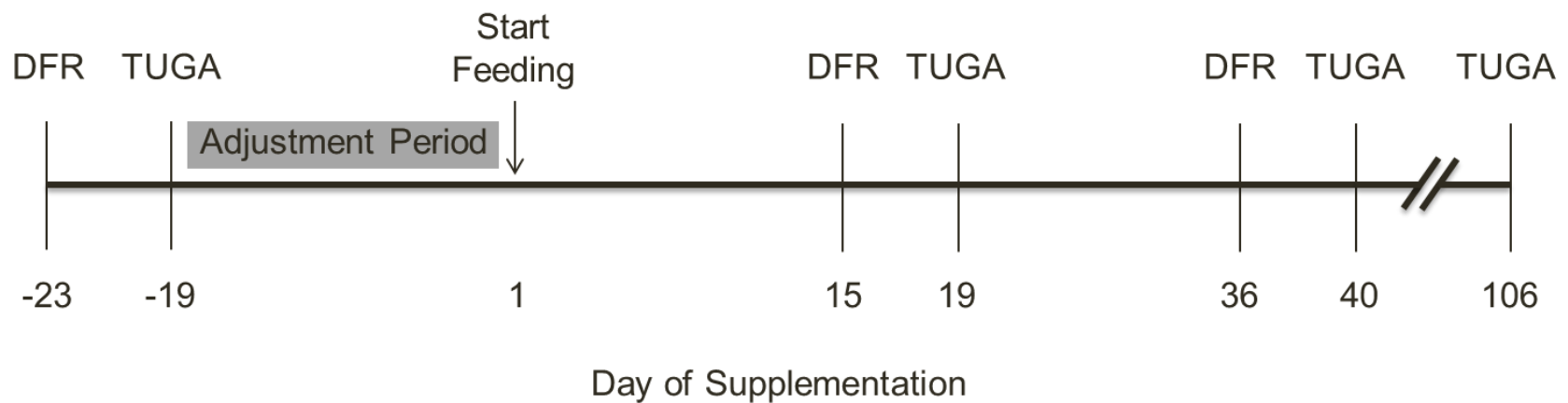


Figure 4.4 Schedule and procedures for trans-vaginal ultrasound-guided oocyte aspiration (TUGA) in Brahman cows (Experiment 4.2). DFR is dominant follicle removal. FSH was not used for ovarian stimulation before TUGA in Experiment 4.2.

collection (d 106 of supplementation) were washed in fresh OCM and cumulus cells were removed by high speed vortex 3 to 4 min in a 15 ml conical tube containing 1 ml of HEPES Talp medium with 1 mg/ml hyaluronidase. Completely denuded oocytes were placed in a single well of a 4-well plate (Thermo Scientific, Rochester, NY) containing fixative medium for Nile Red analysis.

Nile Red Stain

Denuded oocytes were incubated in 4-well petri dishes containing glutaraldehyde/formaldehyde fixative medium (Appendix B) overnight (18 ± 2 h) at 4°C. Fixed oocytes were washed 4 times with PBS before Nile Red staining. Oocytes were stained in a single well of a 4-well plate containing Nile Red (1 µg/ml) working solution (Appendix C) at room temperature (~24°C) in complete darkness for 24 h. After incubation, oocytes were washed 3 times in PBS and suspended in 10 µl droplets of ProLong® Gold antifade reagent (Invitrogen Corporation, Carlsbad, CA) on a 25 x 55 mm glass slide. Slides were prepared with three droplets per slide containing 5 to 15 stained oocytes per droplet. Cover slips were carefully placed over the droplet of ProLong® Gold, lightly pressed down on all four corners and sealed with nail polish. Oocytes from each donor cow remained separated throughout fixing, staining and slide preparation.

Oocytes stained with Nile Red were evaluated with a Nikon Labophot microscope equipped with epifluorescent illumination and a FITC dichroic filter cube with long pass filter (excitation: 400-500 nm and emission: 515 nm). Images of each oocyte were captured with fluorescence microscopy at 20x objective (0.4 NA) using a QImaging Micropublisher 3.3 camera with QCapture

Pro software and stored on a PC for analysis. After established, camera settings remained identical to capture each image. ImageJ software (imagej.nih.gov/ij/index.html) was used to evaluate mean florescence intensity of Nile Red stained oocytes in each image. Five samples from similar areas of each oocyte were measured to calculate mean florescence intensity. A total of 49 oocytes collected from CLA-fed cows (n = 9 cows) and 46 oocytes from Control-fed cows (n = 6 cows) were stained and evaluated with Nile Red.

Synchronization and Ovarian Stimulation for Embryo Collection

Cows were subjected to ovarian stimulation and synchronization of estrus prior to *in vivo* embryo collection on d 74 of dietary supplementation (Figure 4.3) and procedures used in Experiment 4.2 were similar to those in 4.2. A DFR procedure was performed on each cow 14 d before embryo collection (d 60 of supplementation) and a controlled internal drug release device (CIDR) was inserted intra-vaginally. Eight injections (im) of FSH (Pluset[®] Original and Pluset[®] Flex H, Minitube of America, Inc., Verona WI) were administered in a descending-dose at 12 h intervals beginning 36 h after the DFR. A total of 6.5 ml of Pluset[®] Flex H (FSH:LH = 3:1) and 6.25 ml of Pluset[®] Original (FSH:LH = 1:1) was injected per cow, delivering a total of 560 IU of FSH and 333 IU of LH based on labeled concentrations. Cows were administered (im) prostaglandin F_{2α} (Lutalyse[®], 5ml, 25 mg) at the time of the 7th and 8th FSH injection and CIDR were removed at the 8th FSH injection. Estrus of cows was detected and cows were inseminated with semen from a single fertile Brangus sire at 12 ± 3 h (two 0.5 ml straws) and 24 ± 3 h (one 0.5 ml straw) after initiation of estrus.

Embryo collection

Procedures for embryo collection were similar to those used in Experiment 4.1. The number of CL present and total recovered ova, degenerate/unfertilized ova, embryos and transferable quality embryos (TQE, Quality Grades 1 and 2) were recorded for each cow. Stage and Grade of embryos were assigned according to International Embryo Transfer Society (IETS) guidelines. All TQE were placed in 1.5 ml microtubules (2 embryos/tube) in minimal volume (1 to 2 μ l) of PBS containing 0.1% polyvinyl alcohol (PVA) and stored at -80°C for later RNA isolation and or mass spectrometry analyses.

mRNA Isolation and Reverse Transcription

Messenger RNA was isolated from COC (2 COC/sample) using RNeasy[®] Plus Micro Kit (Qiagen[®] Inc., Valencia, CA) (Appendix F). A total of 16 samples were isolated in Experiment 4.1 (Holstein cows) from COC collected on d 42 of supplementation and 28 samples were isolated in Experiment 4.2 (Brahman cows) from COC collected on d 40 of dietary treatment. The lysis buffer (Buffer RLT Plus) was added directly to the microtubules containing COC while at -80°C. Each sample mRNA was eluted in 17 μ l nuclease-free water and directly converted into cDNA using an iScript[™] cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA). The iScript[™] kit transforms mRNA into more stable cDNA using reverse transcriptase. The iScript reaction mix contained 4 μ l of iScript reaction mix, 1 μ l of reverse transcriptase and 15 μ l of mRNA elution (from Micro Kit). Reactions were placed into a thermocycler for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The resulting cDNA transcribed from each

sample was diluted with nuclease-free water and stored at -20°C until gene expression analysis.

Primer Validation and Calibrator Development

Primers were designed from bovine gene sequences using the BLAST function of GenBank (Table 4.1) and were purchased from Invitrogen™ (Carlsbad, CA). Bovine liver tissue was used as template cDNA for primer validation and calibrator development. Messenger RNA was isolated from bovine liver tissue using the Dynabeads® mRNA Direct Kit™ (Invitrogen, Carlsbad, CA) (Appendix G) and was immediately converted to cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad) (Appendix H).

Basic PCR reactions (Appendix I) were conducted using the JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma-Aldrich Inc., St. Louis, MO) to obtain an amplicon for each gene of interest, which were evaluated by gel electrophoresis using 2% agarose gel containing ethidium bromide. Gels were evaluated under UV light using a BIO-RAD Universal Hood II. Bands were compared with a 100 bp ladder to verify primer accuracy.

A calibrator was developed as a positive control for quantitative PCR (qPCR) using bovine liver mRNA converted to cDNA similar to above. The calibrator contained the cDNA from liver tissue and purified PCR product (QIAquick® PCR Purification Kit, Qiagen® Inc., Valencia, CA) encoding genes of interest (CPT1, FADS2 and PPARα) and internal reference genes (Poly A and GAPDH). Calculated concentrations of PCR products in the calibrator were

Table 4.1 PCR Primers

Primer ¹	GeneBank Accession Number	Sequence	Product size (bp)
CPT-1	NM_001034349.2	F 5' -CGACCCAAACAAGTACCCCA- 3' R 5' -CGCTGGGCATTTGTCTCTGA- 3'	156
FADS2	NM_001083444.1	F 5' -CCGACAAGTGGCTGGTCAT- 3' R 5' -GTGATCTGGGAATTCTTGCCG- 3'	232
PPAR α	NM_001034036.1	F 5' -TGGACGAATGCCAAGATCTGA- 3' R 5' -ATGACGAAAGGCGGGTTGTT- 3'	204
Poly A	X63436	F 5' -AAGCAACTCCATCAACTACTG- 3' R 5' -ACGGACTGGTCTTCATAGC- 3'	169
GAPDH	U85042	F 5' -CCTTCATTGACCTTCACTACATGGTCTA- 3' R 5' -TGGAAGATGGTGATGGCCTTTCCAT- 3'	127

¹*Bos taurus* poly(A) polymerase (Poly A) (reference gene), *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reference gene), *Bos taurus* carnitine palmitoyltransferase 1 (CPT-1), *Bos taurus* fatty acid desaturase 2 (FADS2), *Bos taurus* peroxisome proliferator-activated receptor α (PPAR α).

2 pg/μl for each gene of interest and 0.2 pg/μl for each reference gene. PCR was optimized for each gene using 10-fold dilutions of the calibrator to develop a standard curve using qPCR. Melt curves for each gene were evaluated to confirm only a single product was amplified. Results from PCR optimization and calibrator evaluates for each gene are presented in Appendix J. The first dilution of the calibrator for each genes was selected as the positive control for qPCR.

Quantitative PCR

Quantitative PCR was used to evaluate relative gene expression in COC collected from cows fed CLA or a Control supplement in both Experiment 4.1 and Experiment 4.2. Quantitative PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 20 μl reactions using 96-well plates and a Bio-Rad MyiQ thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) (Appendix K). Each reaction consisted of 10 μl of SsoFast, 4 μl of sample cDNA, 4 μl water, and 2 μl of forward and reverse primer pairs (10 pmol/μl) for each gene (Table 4.1). Measurements were conducted in triplicate (20 μl per reaction into designated wells of the 96-well plate) and each plate included a negative control (no template cDNA). The thermocycler procedure consisted of enzyme activation at 95°C for 1 min and 40 cycles of denaturing at 95°C for 5 s and annealing at 61°C for 30 s. A melt curve was conducted by increasing the temperature 0.5°C every 10 s from 61°C to 95°C (68 repeats). Amplification, standard curves and gene expression efficiency were normalized against internal reference genes PAP and GAPDH.

Statistical Analyses

Cow was considered the experimental unit in both experiments as dietary supplements were fed individually. Cow BW and BCS and all embryo responses (CL present, number of recovered ova, degenerate/unfertilized ova, embryos and TQE) were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) with dietary treatment in the model. Follicle and COC responses (number of follicles targeted, total COC and Quality COC recovered) were analyzed with the Mixed procedure of SAS. Main effects included dietary treatment, collection and the interaction. Nile Red fluorescence intensity was analyzed with the Mixed procedure of SAS. The model included dietary treatment with cow as a repeated measurement. Nonsignificant ($P > 0.07$) higher order interactions were excluded from the model. When effects were significant ($P \leq 0.05$), least squares means (LSM) were compared using LSD (pdiff) of SAS. Recovery rate of COC and total ova were analyzed with Chi Square using the Frequency procedure of SAS.

Relative gene expression was evaluated in COC collected from cows fed CLA (150 g per hd/d) or a Control supplements using the REST 2009 Software (Pfaffl et al., 2002). This software uses a mathematic model that accounts for different PCR efficiencies of the genes of interest (GOI) and reference genes and allows normalization of GOI against multiple references genes. The REST software calculated expression (relative to internal reference genes PAP and GAPDH) for each GOI in each sample and a geometric mean for each treatment group was calculated. Estimates of concentrations used the equation $c = A^*e^{CT}$ (where A^*e is the PCR efficiency) to allow exponential variation. The REST

software provided 95% confidence intervals for expression ratios and calculated a *P*-value to identify up-regulation or down-regulation for GIO between dietary treatment groups within experiments (Experiment 4.1 and 4.2).

Results

Experiment 4.1: Holstein Cows

Body weight of cows was not influenced ($P \geq 0.94$) by treatment at any time period throughout the trial. Cows weighted 695 ± 25 kg at the start of supplementation and 657 ± 18 kg at the end of the trial.

Follicle, Oocyte and Embryo Responses

A total of 394 oocytes were collected from 12 Holstein cows after a total of 48 TUGA sessions ($n = 105$ CLA-High, $n = 199$ CLA-Mod and $n = 114$ Control). Follicle and oocyte responses of Holstein cows are presented in Table 4.2. There were no collection ($P \geq 0.19$) or treatment by collection interactions ($P \geq 0.10$) for follicle and COC responses, therefore collection was removed from the model. Cows fed Mod-CLA had more follicles targeted ($P = 0.003$), COC ($P < 0.001$) and Quality COC ($P = 0.005$) recovered than cows fed High-CLA or Control supplements, respectively. However, Mod-CLA cows had more follicles present ($P = 0.02$) at the TUGA session before initiation of dietary treatments, indicating the differences in follicle and oocyte responses are likely not associated with dietary treatment. Cows fed High-CLA, Mod-CLA and Control supplements produced 12.4 , 18.2 and 10.5 ± 1.5 follicles and 6.6 , 13.3 and 7.6 ± 1.2 COC per cow per TUGA session, respectively. Oocyte recovery rate from

Table 4.2 Follicle and oocyte responses of Holstein cows fed CLA or no lipid supplement¹

Item	Dietary supplement ²			SE	P value
	High-CLA	Mod-CLA	Control		
Cows (n)	4	4	4		
Follicles targeted	12.4 ^a	18.2 ^b	10.5 ^a	1.5	0.003
COC recovered	6.6 ^a	13.3 ^b	7.6 ^a	1.2	<0.001
Quality COC recovered ³	6.1 ^a	10.3 ^b	6.1 ^a	1.0	0.005
Recovery rate ⁴ (%)	52.5 ^a	72.9 ^b	72.2 ^b		<0.001

¹No collection or treatment by collection interactions were detected so mean response per cow per collection was reported. Oocytes were collected 11 d before the start of feeding and at 14, 28 and 42 d of feeding (four collections).

²Nonlactating Holstein cows were supplemented with either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids at 150 g per hd/d (High-CLA) 100 g per hd/d (Mod-CLA) or were not supplemented with fat (Control).

³Quality Grade I and Grade II oocytes according to a four-point scale.

⁴Number COC recovered/number follicles targeted.

^{a,b} Means within row without a common superscript were different ($P \leq 0.05$).

cows fed High-CLA (52.5%) was reduced ($P < 0.001$) compared with recovery rates from Mod-CLA (72.9%) and Control (72.2%) fed cows.

In vivo embryo responses of Holstein cows are reported in Table 4.3. A total of 52 embryos (Grade I or Grade II) were collected from 11 cows (4.6 embryos/cow). One High-CLA fed cow was not collected because of poor ovarian stimulation and the presence of unovulated follicles at collection. Dietary treatment did not influence any of the embryo responses measured ($P \geq 0.30$).

Table 4.3 Embryo responses of Holstein cows fed CLA or no lipid supplement¹

Item	Dietary supplement ²			SE	P value
	High-CLA	Mod-CLA	Control		
Cows ³ (n)	3	4	4		
CL ⁴	9.5	8.8	6.3	1.9	0.48
Total ova	7.7	6.5	4.8	2.3	0.68
Degenerate/unfertilized	1.5	2.5	4.0	2.0	0.69
Embryos ⁵	6.7	5.3	2.8	1.7	0.30
Recovery rate ⁶ (%)	67.7	70.3	73.1		0.90

¹In vivo embryos were collected on d 69 of supplementation

²Nonlactating Holstein cows were supplemented with either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids at 150 g per hd/d (High-CLA) 100 g per hd/d (Mod-CLA) or were not supplemented with fat (Control).

³One cow in the High-CLA group was not collected because of poor ovarian stimulation and/or multiple unovulated follicles.

⁴Number corpora lutea present at the time of embryo recovery

⁵Quality Grade 1 and Grade 2 embryos collected (according to the International Embryo Transfer Society (IETS) guidelines.

⁶Number ova recovered/number of CL present.

Gene Expression

A total of 14 samples (n = 7 each from CLA-High and Control groups) consisting of 2 COC/sample collected on d 42 of feeding were used for gene expression analyses in Experiment 4.1. Dietary supplementation of Holstein cows did not influence the expression of CPT1 ($P = 0.95$), FADS2 ($P = 0.41$) or PPAR α ($P = 0.55$) in COC. Box plots representing the expression of each gene of interest evaluated in Experiment 4.1 are available in Figures 4.5, 4.6 and 4.7.

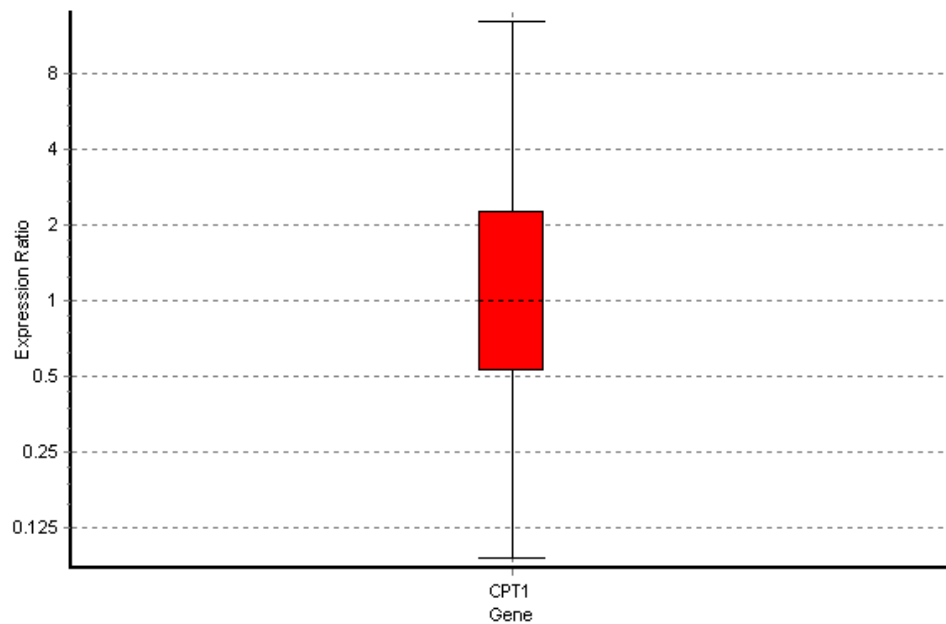


Figure 4.5 Expression ratio of CPT1 in cumulus-oocyte complexes collected from Holstein cows (Experiment 4.1) fed CLA or no lipid supplement. Boxes represent the interquartile range (middle 50%). Dashed line within box is the median. Whiskers are minimum and maximum.

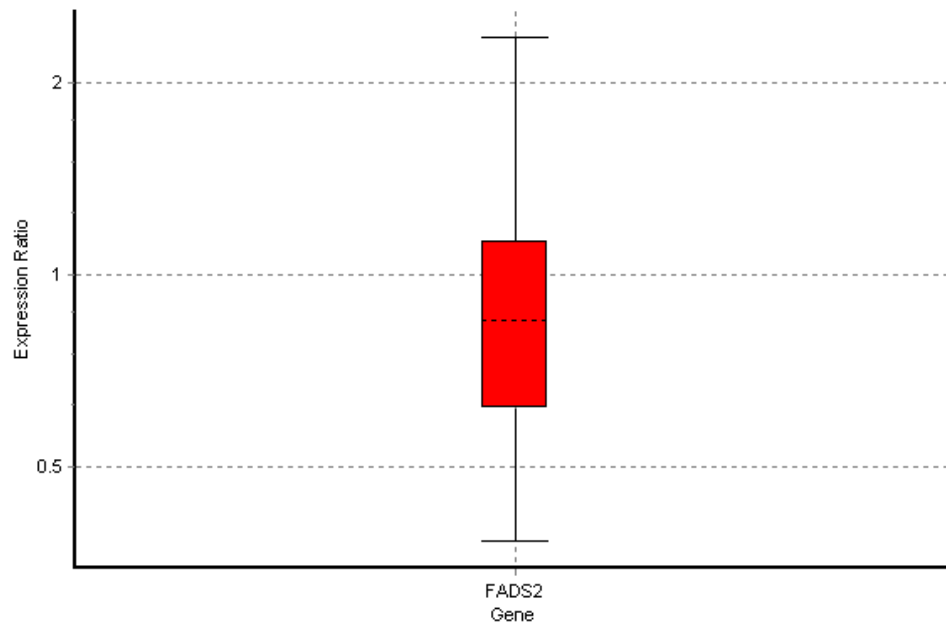


Figure 4.6 Expression ratio of FADS2 in cumulus-oocyte complexes collected from Holstein cows (Experiment 4.1) fed CLA or no lipid supplement. Boxes represent the interquartile range (middle 50%). Dashed line within box is the median. Whiskers are minimum and maximum.

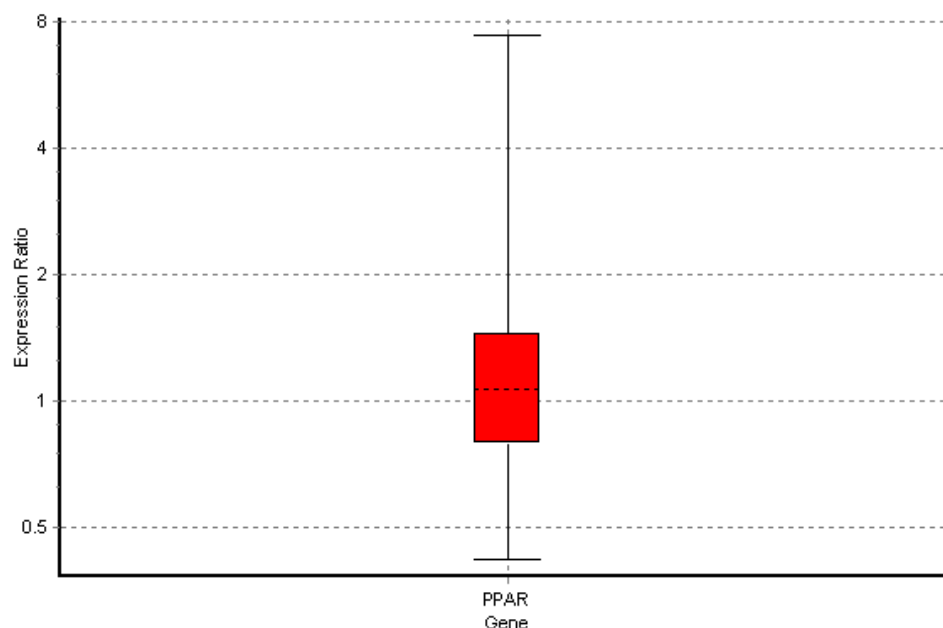


Figure 4.7 Expression ratio of PPAR α in cumulus-oocyte complexes collected from Holstein cows (Experiment 4.1) fed CLA or no lipid supplement. Boxes represent the interquartile range (middle 50%). Dashed line within box is the median. Whiskers are minimum and maximum.

Experiment 4.2: Brahman Cows

Dietary treatment did not influence body weight ($P \geq 0.79$) or BCS ($P \geq 0.78$) of cows at any time period throughout the trial. Cows weighed 605 ± 14 kg with at the start of supplementation and 670 ± 16 kg at the end of the trial. Cow BCS was 5.2 ± 0.2 at the start of the trial and 5.6 ± 0.2 at the end of the trial.

Follicle, Oocyte and Embryo Responses

A total of 774 oocytes were collected from 17 Brahman cows after a total of 51 TUGA sessions ($n = 406$ for CLA and $n = 368$ for Control). Follicle and oocyte responses of Brahman cows are presented in Table 4.4.

Table 4.4 Follicle and oocyte responses of Brahman cows fed CLA or no lipid supplement¹

Item	Dietary supplement ²		SE	P value
	CLA	Control		
Cows (n)	9	8		
Follicles targeted	17.3	17.0	1.7	0.90
COC recovered	15.6	15.3	2.4	0.93
Quality COC recovered ³	10.7	10.6	1.5	0.99
Recovery rate ⁴ (%)	91.0	90.0		0.63

¹No collection or treatment by collection interactions were. Oocytes were collected 23 d before the start of dietary supplementation and at 19 and 40 d of dietary supplementation (three collections).

²Nonlactating Brahman cows were supplemented with either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids at 150 g per hd/d (CLA) or were not supplemented with fat (Control).

³Quality Grade I and Grade II oocytes according to a four-point scale.

⁴Number COC recovered/number follicles targeted.

There were no collection ($P \geq 0.30$) or treatment by collection interactions ($P \geq 0.68$) for follicle and COC responses, therefore collection was removed from the model. Dietary CLA supplementation did not influence the number of follicles targeted ($P = 0.90$) COC recovered ($P = 0.93$) or number of Quality COC recovered ($P = 0.99$). Responses for CLA compared with Control-supplemented cows were 17.3 vs. 17.0 ± 1.7 follicles targeted, 15.6 vs. 15.3 ± 2.4 COC recovered and 10.7 vs. 10.6 ± 1.5 Quality COC recovered. Rate of COC recovery was similar ($P = 0.63$) between dietary treatments and total COC recovery rate across treatments was 90.5%.

In vivo embryo responses of Brahman cows are reported in Table 4.5. A total of 63 embryos (Grade I and Grade II) were collected from 12 cows (5.3 embryos/cow). One CLA fed cow and two Control fed cows were not collected because of poor response to ovarian stimulation and/or the presence of multiple

Table 4.5 Embryo responses of Brahman cows fed CLA or no lipid supplement¹

Item	Dietary supplement ²		SE	P value
	CLA	Control		
Cows ³ (n)	8	4		
CL ⁴	10.5	14.5	2.1	0.20
Total ova ⁵	4.1	7.5	1.9	0.27
Degenerate/unfertilized ⁶	4.0	4.7	0.9	0.62
Embryos ⁷	3.0	5.3	2.1	0.48
Recovery rate ⁸ (%)	39.3	51.7		0.14

¹*In vivo* embryos were collected on d 74 of dietary supplementation, responses are presented per cow.

²Nonlactating Brahman cows were supplemented with either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids at 150 g per hd/d (CLA) or were not supplemented with fat (Control).

³One CLA cow and two Control cows were not collected because of poor ovarian stimulation and/or multiple unovulated follicles and one Control cow had endometritis at the time of collection and was not collected.

⁴Number corpora lutea present at the time of embryo recovery.

⁵Total ova included fertilized embryos, degenerate and unfertilized ova

⁶Unfertilized and degenerate embryos were classified based on International Embryo Transfer Society (IETS) guidelines

⁷Quality Grade 1 and Grade 2 embryos collected (IETS) guidelines.

⁸Number ova recovered/number of CL present.

unovulated follicles at the time of collection. Additionally, one Control-supplemented cow had endometritis at the time of collection and was not collected. Dietary treatment did not influence any of the embryo responses measured ($P \geq 0.20$).

Nile Red

A total of 49 oocytes collected from CLA-fed cows ($n = 9$ cows) and 46 oocytes from Control-fed cows ($n = 6$ cows) were stained with Nile Red in Experiment 4.2. Mean Nile Red intensity was not influenced ($P = 0.79$) by dietary treatment (Figure 4.8). Representative images of Nile Red stained oocytes are available in Appendix D.

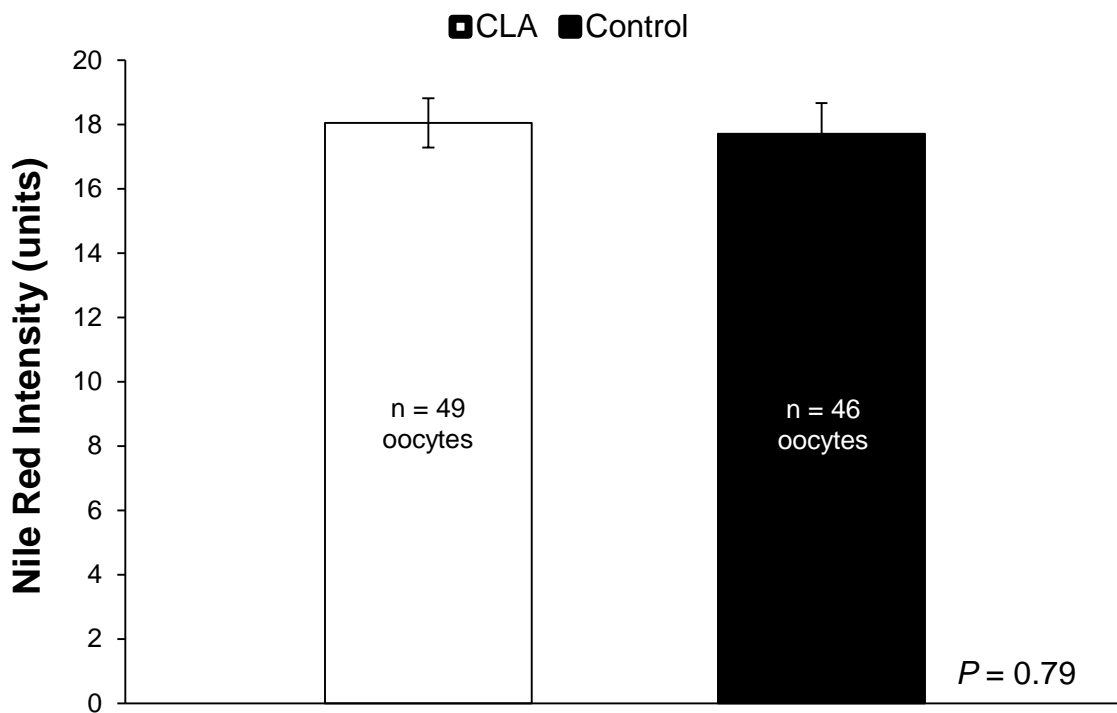


Figure 4.8 Mean Nile Red intensity of oocytes collected from Brahman cows supplemented with CLA ($n = 9$ cows) or no fat supplement (Control) ($n = 12$ cows). Oocytes were collected on d 109 of dietary supplementation.

Gene Expression

A total of 28 samples ($n = 14$ each from CLA and Control groups) consisting of 2 COC/sample collected on d 40 of dietary supplementation were used for gene expression analyses in Experiment 4.2. Feeding Brahman cows CLA compared with Control supplements did not influence the expression of CPT1 ($P = 0.32$), FADS2 ($P = 0.27$) or PPAR α ($P = 0.63$) in COC. Box plots representing the expression of each gene of interest evaluated in Experiment 4.2 are presented in Figures 4.9, 4.10 and 4.11.

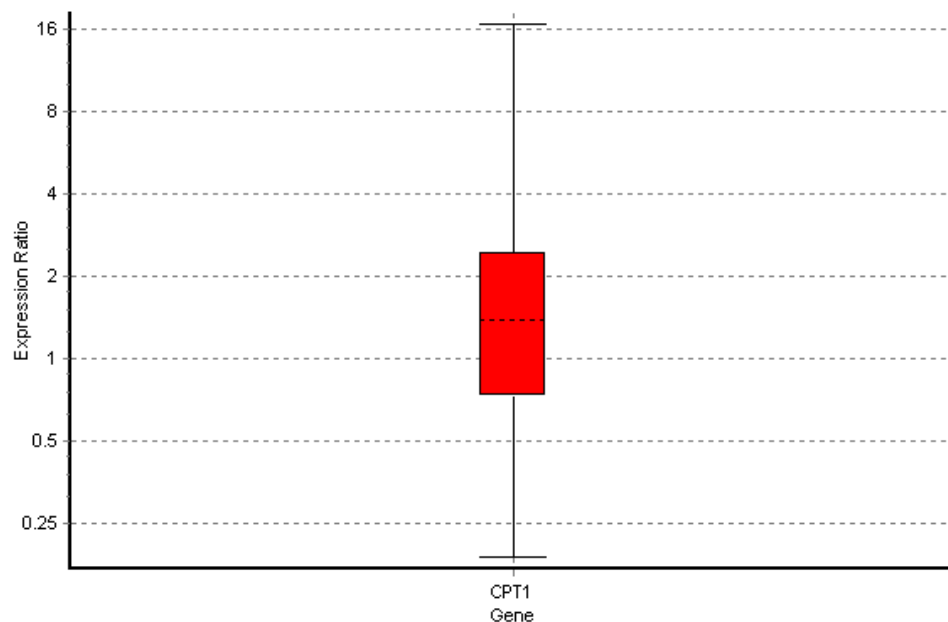


Figure 4.9 Expression ratio of CPT1 in cumulus-oocyte complexes collected from Brahman cows (Experiment 4.2) fed CLA or no lipid supplement. Boxes represent the interquartile range (middle 50%). Dashed line within box is the median. Whiskers are minimum and maximum.

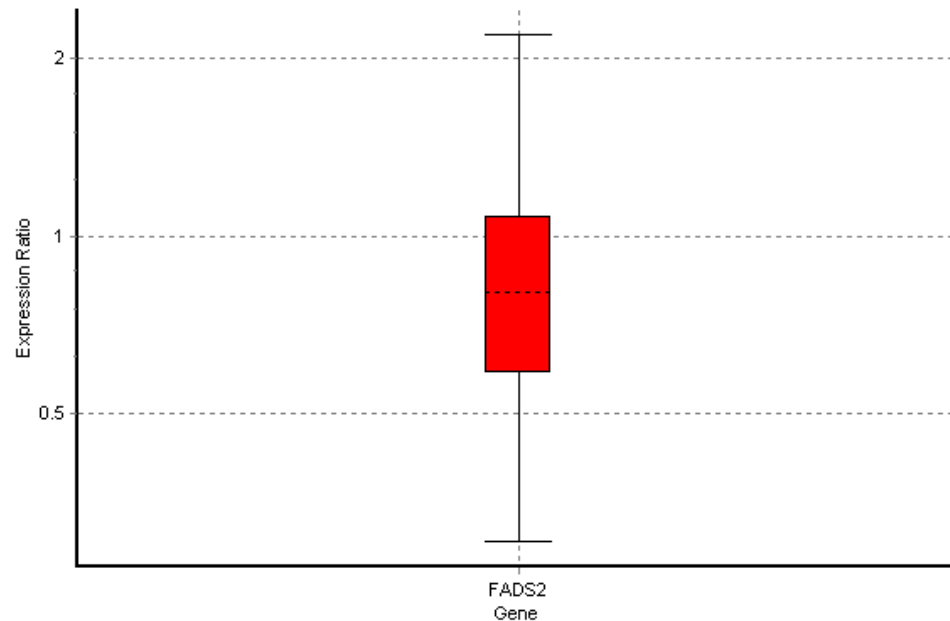


Figure 4.10 Expression ration of FADS2 in cumulus-oocyte complexes collected from Brahman cows (Experiment 4.2) fed CLA or no lipid supplement. Boxes represent the interquartile range (middle 50%). Dashed line within box is the median. Whiskers are minimum and maximum.

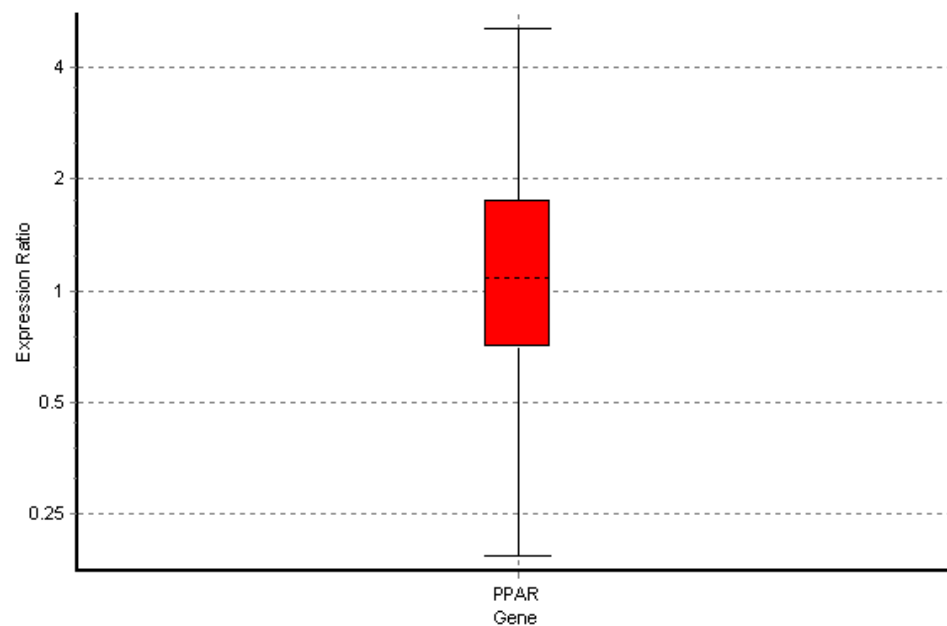


Figure 4.11 Expression ration of PPAR α in cumulus-oocyte complexes collected from Brahman cows (Experiment 4.2) fed CLA or no lipid supplement. Boxes represent the interquartile range (middle 50%). Dashed line within box is the median. Whiskers are minimum and maximum.

Discussion

Body weight of Holstein cows in Experiment 4.1 was not influenced by 70 d of CLA supplementation and body weight and BCS of Brahman cows in Experiment 4.2 was not influenced by 107 d of CLA supplementation. Results indicate that the small amount of energy supplied from the CLA supplement likely did not significantly alter net energy intake of cows. The CLA (80% fat) fed at 150 g per hd/d results in supplementation of 120 g of fat per hd/d. This contributed <4% of the metabolizable energy required for maintenance for nonlactating, nongestating cows of similar breed, BW, BCS and age to the cows used in the current study (NRC 1996, Level 1 Model).

Holstein cows fed Mod-CLA had greater follicles targeted ($P = 0.003$), COC and Quality COC recovered than cows fed High-CLA or Control supplements, respectively. However, Mod-CLA cows had more follicles present at the first TUGA session (before initiation of dietary treatments), indicating the differences in follicle and oocyte responses are likely not associated with dietary treatment. Mean follicle and oocyte responses are likely easily influenced by one or two cows with increased follicle counts as there were only four cows per treatment group. Cows were not stratified by follicle count before the experiment. Dietary CLA did not influence follicle and oocyte responses compared with controls in Brahman cows in Experiment 4.2.

Differences in oocyte recovery rate found in Experiment 4.1 could have been due to difficulty in collection of one cow in the High-CLA group. Inexperience or inconsistent technique by the technician(s) performing TUGA or

searching and processing oocytes may have also contributed to differences in oocyte recovery rate in Experiment 4.1. Oocyte recovery rate was not different between dietary treatment groups in Brahman cows in Experiment 4.2.

Höffmann et al. (2008) reported increased follicles present per collection when lactating Holsteins were fed CLA (Lutrell at 100 g/d) compared with unsupplemented cows. Ewes fed fish oil (high in PUFA) had increased follicle and oocyte production compared with unsupplemented ewes (Zeron et al., 2002a). Follicle responses after CLA supplementation may depend on physiological stage of production (lactation vs. nonlactating) in cows. Highly productive lactating Holsteins, like those in the study by Höffmann et al. (2008), likely repartitioned energy from milk fat depression into increased follicle production. Nonlactating cows in the current study may not experience the beneficial effects of CLA supplementation because they are not deficient in energy like high producing Holstein cows during early lactation. However, supplementation with ≤ 10 g/d of each *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA appears to benefit reproduction in lactating Holstein cows even without significant changes in energy balance (Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005).

Nile Red intensity was not influenced by dietary treatment of Brahman cows in Experiment 4.2. This indicates that concentration of intracytoplasmic neutral lipids did not differ in oocytes collected from cows fed CLA or Control supplements in the current study. Nile Red intensity of oocytes from lactating Holstein cows was not influenced by CLA supplementation in the study

presented in Chapter III of this dissertation. Nile Red staining allows evaluation of lipid content for relative comparison of individual oocytes from different donors or culture conditions. However, this method does not permit qualitative composition of lipids, such as identification of fatty acids or different lipid fractions, nor does it allow absolute quantitative analysis.

Embryos co-cultured with *trans*-10, *cis*-12 CLA *in vitro* contained less lipids and post-thaw viability was improved compared with control cultured embryos (Pereira et al., 2007). There are currently no reports using Nile Red to evaluate lipid content of oocytes from cows fed CLA. More dynamic quantitative lipid analysis techniques may have been beneficial to evaluate possible differences between dietary treatments.

Dietary supplementation of Brahman and Holstien cows with CLA compared with control supplementation did not influence COC expression of genes involved in lipid metabolism (CPT1, FADS2 and PPAR α). *In vitro* culture of embryos altered mRNA expression of genes coding for fatty acid utilization and synthesis (Algriany et al., 2007). Al Darwich et al. (2010) reported that PUFA were involved in the control of adipogenesis and events that alter gene expression of multiple enzymes involved in lipid metabolism of bovine embryos. Additionally, dietary CLA-induced milk fat depression in dairy cows resulted in the coordinated down regulation of mRNA expression for genes involved in mammary lipid synthesis (Piperova et al., 2000; Peterson et al., 2003).

Expression of CPT1 was up-regulated in liver and adipose by dietary PUFA supplementation in rats (Ikeda et al., 1998). During bovine oocyte

maturation, CPT1 mRNA increases indicating an increase in β oxidation during this stage (Algriany et al., 2007). The CPT1 enzyme catalyzes a rate-limiting step in the transfer of long-chain fatty acyl-CoA from the cytosol to mitochondria for oxidation (Abu-Elheiga et al., 2000; Kerner and Hoppel, 2000).

The FADS2 gene is a member of the fatty acid desaturase gene family. Enzymes resulting from FADS2 regulate unsaturation of fatty acids through the addition of double bonds. *In vitro* embryos cultured with 100 μ M of *trans*-10, *cis*-12 CLA had reduced FADS2 transcript level compared with control cultured embryos (Al Darwich et al., 2010). This result likely modified the saturated/unsaturated fatty acid balance in embryos and could influence membrane fluidity (Al Darwich et al., 2010). However, post-thaw *in vitro* embryo survival was not improved after co-culture with 100 μ M of *trans*-10, *cis*-12 CLA (Al Darwich et al., 2010).

Peroxisome proliferator-activated receptor alpha (PPAR α) is a member of the PPAR family of ligand-dependent transcription factors (Rosen et al., 1999). Expression of PPAR α is greater in tissues that have high rates of fatty acid oxidation, and is the primary subtype that facilitates lipid-induced activation of fatty acid oxidation associated genes (Braissant et al., 1996). Dietary unsaturated fatty acids have been reported to alter gene expression through PPAR, leading to altered metabolism, growth and cell differentiation in different tissues of rats (Ide, 2000; Jump, 2004). Polyunsaturated fatty acids were described as a natural ligand or activator of PPAR, although PUFA mediate

genes involved in lipid metabolism through both PPAR dependent and non-PPAR pathways (Ren et al., 1997).

Differences in fatty acid composition between follicular fluid, granulosa cells and COC after dietary fatty acid supplementation suggest a selective uptake of specific fatty acids in different ovarian compartments (Zeron et al., 2002; Adamiak et al., 2006; Fouladi-Nashta et al., 2009; Zachut et al., 2010; Aardema et al., 2012). Aardema et al. (2012) concluded that cumulus cells protect oocytes against elevated follicular fluid fatty acid concentrations by accumulation of these fatty acids. Uptake of fatty acids by the follicle and oocyte is likely highly selective as a protective mechanism to ensure fatty acid composition of oocytes remains safe or optimum (Bilby et al., 2006; Santos et al., 2008a; Fouladi-Nashta et al., 2009; Sturmey et al., 2009). The highly regulated mechanisms involved in fatty acid uptake by bovine ovarian components may help explain why gene expression of COC and lipid content of oocytes (measured by Nile Red intensity) were not influenced by CLA supplementation in the current study.

Oocytes were collected on d 42 (Experiment 4.1) and d 40 (Experiment 4.1) of dietary supplementation. This may not have been sufficient time for supplemental CLA to modify the follicular environment in a way that altered metabolic gene expression of COC. The origin of lipids and the timing of lipid deposition in bovine oocytes are not fully understood. It appears that oocytes acquire and/or deposit lipids between the time of primordial follicle activation and the antral follicle stage (Fair et al., 1997). Although it is difficult estimate, the course of follicular growth takes a long time relative to the reproductive cycle of

the animal; several weeks in rodents, and perhaps several months in larger animals (Hirshfield, 1991; Fortune, 2003).

Summary

Dietary supplementation of Brahman and Holstien cows with *trans*-10, *cis*-12 CLA compared with control supplementation did not influence COC expression of genes involved in lipid metabolism (CPT1, FADS2 and PPAR α). It appears that ovarian lipid metabolism was either not altered by CLA supplementation or that slight modifications were not enough to influence expression of the metabolic genes analyzed. Additionally, lipid content of oocytes measured by Nile Red was not influenced by CLA supplementation. The highly regulated mechanisms involved in fatty acid uptake by ovarian components may help explain why lipid content of oocytes and gene expression of COC was not influenced by CLA supplementation in the current study.

CHAPTER V
CRYOSURVIVAL OF *IN VITRO*-PRODUCED BOVINE EMBRYOS AFTER
COLLECTION OF OOCYTES FROM COWS FED *TRANS*-10, *CIS*-12
AND *CIS*-9, *TRANS*-11 CONJUGATED LINOLEIC ACIDS

Introduction

Cryotolerance and cryosurvival are terms used in the Scientific Literature that describe the ability of cells to survive a cryopreservation procedure. Variation in cryotolerance exists between embryos from different breed, species and management conditions. Reduced tolerance to chilling and cryotolerance of oocytes and embryos has been associated with greater cytoplasmic lipids (Kim et al., 2001; Abe et al., 2002; Seidel, 2006). Nagashima et al. (1994) previously reported improved post-thaw survival of porcine embryos after centrifugation and removal of lipids at the 1-cell stage. Cooling causes lateral phase separation of membrane lipids in artificial and bacterial membranes (Petit and Edidin, 1974) and ram spermatozoa (Holt and North, 1986). When porcine embryos were held at 15°C, structural changes within lipid droplets caused droplets to coalesce and form larger droplets (Edidin and Petit., 1977). The resulting loss or decrease in cytoplasmic organization of organelles may irreversibly damage bovine embryos (Mohr and Trounson, 1981).

Although cryopreservation of *in vivo*-produced bovine embryos is common place, *in vitro*-produced embryos are more sensitive to chilling and cryopreservation than *in vivo*-produced embryos (Leibo and Loskutoff, 1993; Pollard and Leibo, 1993; Farin et al., 1999). Non-physiological culture conditions alter *in vitro* embryo lipid metabolism resulting in increased embryo lipids, which

likely contributes to reduced cryotolerance of *in vitro* compared with *in vivo* bovine embryos (Dorland et al., 1994; Crosier et al., 2001b; Abe et al., 2002). Research has closed the gap in cryosurvival between *in vivo* and *in vitro* bovine embryos, yet there remains opportunity for improvement before the widespread adoption of *in vitro* embryo cryopreservation (Seidel, 2006).

The concentration and type of fat included in ruminant diets may influence the composition of oocytes and embryos, and therefore, their cryotolerance. Inclusion of calcium salts of palm oil in ewe diets altered phospholipid fatty acid composition of follicular components (Wonnacott et al., 2010). Oocytes from ewes fed fish oil had a lower temperature of lipid phase transition, leading to superior membrane integrity after chilling (Zeron et al., 2002a). Fatty acid composition of follicular fluid in dairy cows is sensitive to dietary supply of polyunsaturated fatty acids (PUFA) using soybean oil (Batista et al., 2010). Feeding dairy cows an encapsulated flaxseed oil altered the fatty acid composition of milk, plasma, follicular fluid, cumulus-oocyte complexes (COC) and granulosa cells (Zachut et al., 2010). *In vivo*-produced embryos from Nellore heifers were more sensitive to cryopreservation when diets included rumen-protected PUFA (Guardieiro, 2010; Guardieiro et al., 2014). The previous mentioned studies have demonstrated the potential means for modification of follicular fluid and oocyte composition *in vivo*, which may have implications for embryo cryosurvival.

Another interesting area of study has identified specific conjugated linoleic acids (CLA) that inhibit milk fat synthesis in dairy cows (Lor and Herbein, 1998;

Chouinard et al., 1999a; Chouinard et al., 1999b). The *trans*-10, *cis*-12 CLA isomer was identified as a potent inhibitor of milk fat synthesis in lactating cows (Baumgard et al., 2000; Baumgard et al., 2001; Peterson et al., 2002). A rumen-protected CLA supplement has been developed (Lutrell[®], BASF) containing CLA isomers known to suppress milk fat. Lipid encapsulated CLA suppressed milk fat when fed to lactating sheep and goats (Lock et al., 2006; Lock et al., 2008) and cows (Perfield et al., 2004; Castañeda-Gutiérrez et al., 2007b; Pappritz et al., 2011).

Inclusion of *trans*-10, *cis*-12 CLA in bovine embryo culture medium improved post-thaw embryo survival compared with control embryos without influencing overall blastocyst production (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013). This improvement in cryosurvival may be due to reduced accumulation of lipids in *trans*-10, *cis*-12 CLA cultured embryos, evaluated by Nomarski microscopy analysis. In addition, these results may be explained by increased membrane fluidity due to incorporation of *trans*-10, *cis*-12 CLA into bovine embryo membranes (Pereira et al., 2007). The author is unaware of any published data involving the effects of dietary CLA supplementation of cows on cryosurvival of embryos.

In vitro survival assays have been used to evaluate post-thaw survival of embryos in a number of published studies involving *in vitro* culture conditions and/or cryopreservation techniques for bovine (Pugh et al., 1998; Enright et al., 2000; Abe et al., 2002; Lopatářová et al., 2002; Rizos, 2003; Pereira et al., 2007; Pereira et al., 2008; Al Darwich et al., 2010; Ruiz et al., 2013) and porcine

embryos (Men et al., 2006). Generally, these researchers evaluated embryo survival or re-expansion based on a defined set of criteria at the time of thawing and again at 12 h or 24 h intervals through 24 h to 72 h of *in vitro* culture. The number and percentage of embryos hatching were also reported in a number of these studies.

A number of research groups have transferred d-7 or d-8 *in vitro*-produced bovine embryos and recovered them on d 14 to d 16 to evaluate embryonic survival, development and/or gene expression. These assays have been used to compare survival and/or gene expression between *in vivo*- and *in vitro*-produced bovine embryos (Bertolini et al., 2002; Angulo-Campos, 2010), *in vitro* and somatic cell nuclear transfer embryos (Alexopoulos and French, 2009) and to compare different *in vitro* culture conditions (Block et al., 2007). Day-14 bovine embryos are spherical, ovoid or elongated (Alexopoulos and French, 2009) and this stage immediately precedes important embryonic events such as maternal recognition of pregnancy and the formation of extra-embryonic membranes and the embryo proper (Bertolini et al., 2002). Transfer of d-8 embryos and their recovery at d 14 may be a useful assay to evaluate early embryonic survival and development of cryopreserved embryos. The number of embryos recovered at d 14 and their morphology may allow comparison of cryosurvival between experimental treatments.

Research is necessary to elucidate mechanisms contributing to variation in cryotolerance associated with individual animal, breed, species and nutrition (Seidel, 2006). Better understanding of mechanisms contributing to variation in

cryotolerance of embryos will continue to improve success of embryo cryopreservation. Previous studies in the cow have demonstrated nutrition-induced modification of follicular fluid and oocyte fatty acid composition, which may have implications for embryo cryotolerance. Furthermore, addition of *trans*-10, *cis*-12 CLA to bovine embryo culture media has improved cryotolerance of embryos (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013).

Further research is warranted to determine if supplementing cows with *trans*-10, *cis*-12 CLA could influence the fatty acid composition of oocytes *in vivo*, and to determine potential influence on subsequent cryosurvival of *in vitro*-produced embryos. Therefore, objectives of this study were to evaluate effects of CLA-supplementation of crossbred cows on (1) follicle and oocyte production and *in vitro* embryo production after TUGA and (2) cryosurvival of *in vitro*-produced embryos using post-thaw *in vitro* and *in vivo* survival assays.

Materials and Methods

Animals and Experimental Diets

Experimental procedures in this study were approved by the Louisiana State University Animal Care and Use Committee, and were conducted at the Louisiana State University Agricultural Center Reproductive Biology Center (St. Gabriel, LA) from March through August, 2013. Crossbred (Angus x Red Angus and Angus x Brangus) cows ($\leq 1/4$ Brahman) (n = 28) aged 4 to 7 years were randomly allotted to a 2 X 2 factorial experimental design after stratification by body weight and body condition score (BCS) (1 to 9). Cows were maintained on 5 ha paddocks of blue grass (*Poa pratensis*), bermudagrass (*Cynodon dactylon*)

and white clover (*Trifolium repens*). Groups of cows (n = 4 groups of 7 cows each) were randomly assigned to paddocks and were rotated every 14 d to reduce confounding paddock effects. Cows had access to clean water, mineral supplement and portable shade structures and/or shade trees in each paddock.

Cows were fed either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids (CLA, Lutrell[®], BASF, Lampertheim, Germany) or were not supplemented with fat (Control). All cows were group fed 1 kg per hd/d of a pelleted soybean hull supplement fed in feed bunks. The CLA was top-dressed (150 g per hd/d) or the soybean hull supplement only was fed (Control). Supplement refusal was noted but generally all cows consumed the supplements immediately after it was offered. Calculated CLA inclusion was 15 g per hd/d each of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA. Body weight (BW) and body condition score BCS of cows was recorded monthly.

Synchronization for Oocyte Collection

Oocytes were collected using transvaginal ultrasound-guided oocyte aspiration (TUGA) on d 129 (Collection 1) and d 143 (Collection 2) of feed supplementation. An outline of the synchronization and collection procedures is presented in Figure 5.1. A dominant follicle removal (DFR) procedure was performed on each female to synchronize the follicular wave at d 125 of supplementation (4 d before TUGA). Three injections (im) of FSH (Pluset[®] Original and Pluset[®] Flex H, Minitube of America, Inc., Verona WI) were

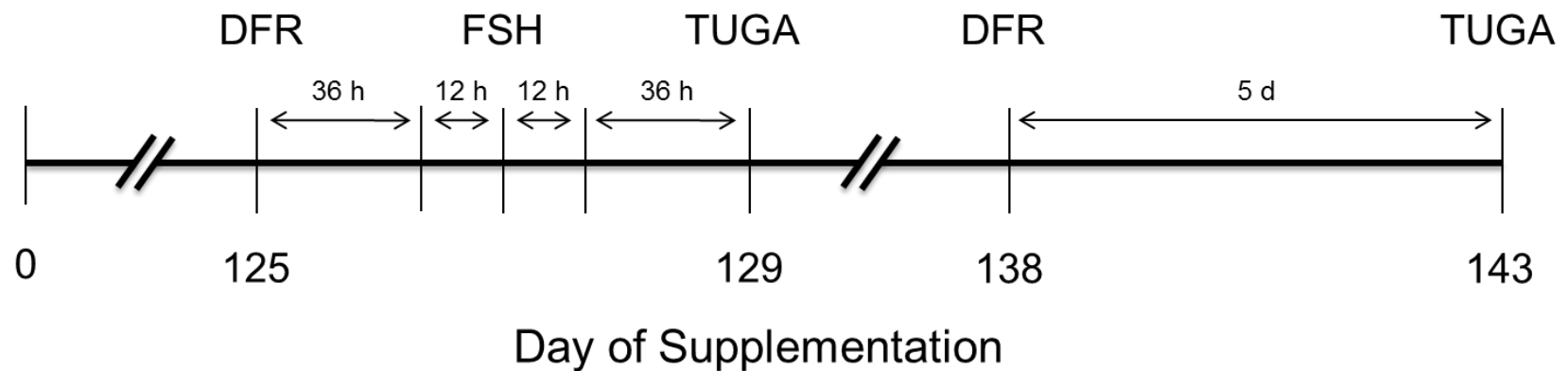


Figure 5.1 Synchronization schedule and procedures for trans-vaginal ultrasound-guided oocyte aspiration (TUGA) in crossbred beef cows. DFR is dominant follicle removal; FSH is Pluset[®] Original and Pluset[®] Flex H, follicle stimulating hormone, administered im. FSH was used in advance of the first TUGA (Collection 1, d 129) but not before the second TUGA (Collection 2, d 143).

administered in a descending dose at 12-h intervals beginning 36 h after the DFR. Based on labeled concentrations, injections delivered 87.5, 70 and 35 IU of FSH and 52.5, 70 and 35 IU of LH for the three injections, respectively. Oocytes were collected 36 h after the final FSH injection (4 d after DFR) on d 129 of feeding. A DFR was performed on each female 9 d following TUGA to synchronize the follicular wave for a second oocyte collection. Cows were not stimulated with FSH for Collection 2 and TUGA was performed 5 d after the DFR at d 143 of feeding. This resulted in a 14-d interval between oocyte collections.

Oocyte Collection Procedure

Oocytes were collected by TUGA on d 129 (Collection 1) and d 143 of feed supplementation using similar procedures as experiments in Chapter III and Chapter IV and a list of equipment and materials used for TUGA is presented in Appendix A. Follicular content from all visible follicles were collected into the filter containing 10 ml collection medium. The number of follicles targeted and CL present were recorded for each cow, in each TUGA session. Oocyte collection medium (OCM) was Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium containing 1% calf serum and heparin (10 IU/ml).

Immediately following collection, aspirate from each cow was transferred to a gridded dish containing OCM and searched for COC with a dissecting microscope. Recovered COC were graded according a four point scale (I through IV) similar to procedures reported by Leibfried and First (1979).

***In Vitro* Production and Cryopreservation of Embryos**

All COC of Grade III or better were washed in fresh OCM, moved through 3 drops of oocyte maturation medium and placed in microtubules containing maturation medium. The COC were matured by treatment group in microtubules (~50 COC/tube) in a battery powered shipment incubator maintained at 38.5°C. While maturing, COC were shipped overnight to Trans Ova Genetics (Centerville, TX). Maturation media was prepared by Trans Ova and shipped overnight on icepacks the day before oocyte collections. Oocytes were processed for *in vitro* fertilization (IVF) 20 ± 2 h after being placed in maturation medium. Semen from a single bull of known IVF success (laboratory standard) was used to fertilize oocytes.

Trans Ova conducted IVF and *in vitro* culture procedures according to standard operating procedures and all oocytes across treatment groups were handled the same with similar culture media and environments. After 7 d of culture, embryos produced were assigned a developmental stage and grade according to International Embryo Transfer Society (IETS) guidelines. All embryos of Grade 1 and Grade 2 were cryopreserved in individual 0.25 ml plastic straws containing 1.5 M ethylene glycol using a slow-cooled, controlled-rate embryo freezing system. A total of 198 (n = 94 CLA and 104 Control) transferable quality embryos (TQE)(Quality Grades I and II) were cryopreserved and shipped in liquid nitrogen to the LSU Reproductive Biology Center for analysis.

Post-thaw *In Vitro* Survival of Embryos

Embryos were thawed in groups according to treatment (n = 4), d of freezing (n = 2) and embryo stage and grade. Straws containing embryos were held in air at room temperature for 5 s and placed into a 37°C water bath for 30 s. After thawing, embryos were expelled into a dish and rinsed 3 times in HEPES Talp medium (Appendix L). Cryopreserved *In vivo*-produced bovine (Angus) embryos (n = 61) were used as a control for the *in vitro* survival assay. *In vivo* embryos were collected on the same ranch in Florida and were cryopreserved by a single IETS freeze code using a slow-cooled method in 1.5 M ethylene glycol.

The initial cryosurvival evaluation (8 ± 2 min post-thaw) was recorded and embryos were washed in modified synthetic oviductal fluid (mSOF) and placed in a single well of 4-well plates containing mSOF with 5% FBS (Appendix M) for culture. Embryos were cultured in groups (n = 7 to 12 embryos) in an incubator at 39°C under 5% CO₂ in air for 24 h. Cryosurvival was defined as an embryo with an intact zona pellucida and an expanded or re-expanding blastocoel cavity. Embryos were recorded as either survived or did not survive. Survival of embryos was evaluated at the time of thawing and at 12 h and 24 h \pm 0.5 h post-thaw by a single technician.

Post-thaw *In Vivo* Survival of Embryos

After 24 h of culture embryos were rinsed in Hepes Talp medium and loaded according to treatment group and d of freezing into 0.25 ml straws containing Syngro® Holding Medium (Bioniche Animal Health, Belleville, ON; distributed by Reproduction Resources, Walworth, WI). Embryos were

transferred into synchronous (8 d after the onset of standing estrus) recipient cows (n = 10) into the uterine horn ipsilateral to a palpable corpus luteum (CL). Embryos were transferred by treatment group into a single recipient cow (n = 10 recipients for the 4 dietary treatment pens on each of 2 freeze days and 2 *in vivo* control groups). A total of 231 embryos (n = 85, 92 and 54 for CLA, Control and *in vivo*, respectively) were transferred into 10 cows (n = 17 to 38 embryos/cow).

Embryos were collected from cows 6 d after transfer (d 14) via nonsurgical gravity flow embryo collection by a single experienced embryo transfer technician. Large Foley catheters (24 to 20 Fr, Allegro Medical, Bolingbrook, IL) were used to collect potentially large and/or elongated embryos. Recovered embryos that were the appropriate size/stage with a distinguishable inner cell mass (ICM) were considered viable. Embryos not recovered or those that did not advance in development were considered dead or dying. All recovered embryos were placed into Syngro[®] Holding Media until photos were captured.

Statistical Analyses

Cow BW and BCS were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) and the model included dietary treatment (CLA and Control) and pen was the experimental unit. Follicle, COC (number of follicles targeted and COC recovered) and embryo (number transferable quality embryos produced) responses were analyzed with the Mixed procedure of SAS. Main effects included dietary treatment, collection (1 and 2) and the interaction and the experimental unit was pen. Any nonsignificant effects ($P > 0.07$) were excluded from the model. When effects were significant ($P \leq 0.05$), least squares means

(LSM) were compared using LSD (pdiff) of SAS. Recovery rate of COC, embryo production rate (transferable quality embryos produced *in vitro*), post-thaw *in vitro* survival and hatching and *in vivo* survival of embryos were analyzed using Chi Square analyses with the frequency procedure of SAS. When Chi Square resulted in $P \leq 0.07$ for post-thaw and *in vitro* embryo survival analyses, a Tukey multiple comparisons of proportions test was used to determine differences among the three groups of embryos thawed (*in vitro* embryos from CLA and Control-supplemented oocyte donors and *in vivo* control embryos).

Results

Body Weight and Body Condition of Cows

Body weight of cows was not influenced ($P \geq 0.87$) by treatment at any time period throughout the trial. Cows weighted 623 ± 10 kg at the start of supplementation and 660 ± 9 at the end of the trial. Supplementation did not affect BCS of cows ($P \geq 0.67$) and was 5.6 ± 0.1 and 6.0 ± 0.1 at the start and end of supplementation, respectively.

Follicle, Oocyte and Embryo Production

Follicle and oocyte responses are presented in Table 5.1. A total of 775 oocytes were collected from 28 cows over two collections ($n = 357$ CLA, $n = 418$ Control). There were no collection ($P \geq 0.82$) or treatment by collection interactions ($P \geq 0.38$) for follicle and COC responses, therefore, means of responses for the two collections combined were analyzed. Supplemental treatment did not influence the number of follicles targeted ($P \geq 0.40$) or COC recovered ($P = 0.39$) per TUGA session.

Table 5.1 Follicle, oocyte and *in vitro* embryo production of crossbred cows fed CLA or no lipid supplement¹

	Dietary supplement ²			
Item	CLA	Control	SE	<i>P</i> value
Mean response per pen per collection ³				
Follicles targeted (per cow) ⁴	106 (15.2)	122 (17.4)	19	0.59
COC recovered (per cow) ⁵	89 (12.8)	105 (14.9)	18	0.58
COC Recovery rate ⁶ (%)	85.7	84.0		0.52
Embryos produced (per cow) ⁷	23.5 (3.4)	26 (3.7)	2.6	0.54
Embryo production rate ⁸ (%)	26.3	24.9		0.68

¹Oocyte donors were crossbred Angus x Red Angus and Angus x Brangus ($\leq 1/4$ Brahman) cows (aged 4 to 7 years).

²Oocyte donor cows were supplemented (150 g per head/d) with either a lipid-encapsulated supplement containing *trans*-10, *cis*-12 and *cis*-9, *trans*-11 conjugated linoleic acids (CLA) or were not supplemented with fat (Control).

³There were 7 cows/pen, 2 pens/trt (2 X 2 factorial arrangement) and oocytes were collected from each cow on d 129 (Collection 1) and d 143 (Collection 2) of supplementation.

⁴There were no collection ($P = 0.86$) or treatment by collection interactions ($P = 0.38$) for follicles targeted/pen, therefore collection (1 and 2) was eliminated from the statistical model.

⁵There were no collection ($P = 0.82$) or treatment by collection interactions ($P = 0.38$) for COC recovered/pen, therefore collection (1 and 2) was eliminated from the statistical model.

⁶Number cumulus-oocyte complexes (COC) recovered/number follicles targeted.

⁷There were no collection ($P = 0.13$) or treatment by collection interactions ($P = 0.08$) for TQE produced/pen, therefore collection (1 and 2) was eliminated from the statistical model.

⁸Number of embryos (Grade 1 and Grade 2) produced after 7 d of culture/number of oocytes fertilized.

Mean follicles targeted and COC recovered per pen across treatments for Collection 1 and Collection 2 were 116.8 and 111.5 \pm 19.3 follicles and 100.0 and 93.8 \pm 17.9 COC, respectively. Rate of COC recovery was similar ($P = 0.52$) between dietary treatments and total COC recovery rate across treatments was 84.9%. Means of responses for the two collections combined were analyzed (Table 5.1). The number of TQE produced per pen were not different ($P = 0.54$) between cows fed CLA ($n = 47$) compared with Control ($n = 52$) supplemented cows (Table 5.1). The rate of TQE produced (number of TQE frozen/number of oocytes fertilized) were not different between cows fed CLA or no lipid supplement ($P = 0.68$) and TQE production rate was 25.6% across dietary treatments (Table 5.1).

Quality grades of *in vitro*-produced embryos are presented by collection (1 and 2) and dietary treatment in Table 5.2. More Grade 1 embryos were produced ($P = 0.04$) after Collection 1 (FSH stimulated) compared with Collection 2 (not stimulated) (21.5 vs. 13.8 \pm 1.7 embryos/pen for collection 1 and 2, respectively). There was a dietary treatment by collection interaction ($P = 0.04$) for production of quality Grade 1 embryos (Table 5.2). Supplementation of CLA did not influence the number of Grade 1 ($P = 0.77$) or Grade 2 ($P = 0.09$) embryos frozen ($n = 72$ vs. 69 Grade 1 embryos and $n = 22$ vs. 35 Grade 2 embryos produced after collection of oocytes from cows fed CLA and Control, respectively).

Table 5.2 Quality grade of *in vitro* embryos produced after collection of oocytes from cows fed CLA or no lipid supplement¹

Item	Collection 1 ²			Collection 2			SE		P value		
	CLA	Control	Mean ⁴	CLA	Control	Mean	Dietary Trt ⁵	Collect. Mean ⁶	Trt	Collect.	Trt* Collection
Embryos/pen ³											
Grade 1	19	24	21.5 ^a	17	10.5	13.8 ^b	2.4	1.7	0.77	0.04	0.16
Grade 2	2 ^{bc}	11.5 ^a	6.8	9 ^{ad}	6 ^{bcd}	7.5	1.4	1.0	0.09	0.63	0.04
Total	21	35.5	28.3	26	16.5	21.3	3.7	2.6	0.54	0.13	0.08

¹Quality grades of embryos were assigned by experienced technicians according to International Embryo Transfer Society (IETS) guidelines. Oocytes for *in vitro* embryo production were collected from cows (Angus x Red Angus and Angus x Brangus) ($\leq 1/4$ Brahman) fed conjugated linoleic acids (CLA) or no fat (Control).

²Oocytes were collected on d 129 (Collection 1) and d 143 (Collection 2) of supplementation. Three injections of FSH (Pluset, im) were administered in a descending dose before Collection 1, while no FSH was used before Collection 2.

³There were 7 cows/pen, 2 pens/trt (2 X 2 factorial arrangement).

⁴Mean embryos per pen per collection (including both dietary treatments) were included to depict collection effects as there were no statistical dietary effects.

⁵Standard error for CLA and Control groups for each collection.

⁶Standard error for collection means (included both dietary treatments).

^{a,b,c,d} Means within row without a common superscript were different ($P \leq 0.05$).

***In Vitro* Culture and Cryosurvival of Embryos**

Embryos evaluated for post-thaw survival is listed by quality grade and developmental stage in Table 5.3. Post-thaw *in vitro* survival and hatching rates of *in vitro* embryos produced after collection of oocytes from donor cows fed CLA or no lipid supplement and *in vivo* control embryos are presented in Table 5.4. Supplemental treatment of oocyte donors did not influence post-thaw survival of embryos at the time of thaw ($P = 0.31$) or at 12 h ($P = 0.83$) or 24 h ($P = 0.55$) post-thaw. However, cryopreserved *in vivo*-produced control embryos had greater post-thaw survival compared with both *in vitro* embryo groups (CLA and Control) at the time of thaw ($P = 0.03$). The three groups did not differ in percent embryo survival at 12 h ($P = 0.36$) or 24 h ($P = 0.17$) post thaw. The percentage of embryos hatching after 24 h of *in vitro* culture did not differ ($P = 0.95$) between CLA (23.3%) and Control (22.8%) supplemented oocyte donor groups. However, *in vitro* embryos had a greater ($P = 0.005$) hatching rate compared with *in vivo* control embryos (3.6%).

Post-thaw survival and hatching rates are displayed by embryo quality grade (Grade 1 and Grade 2) in Table 5.5. Quality Grade 1 *in vivo* control embryos had greater post-thaw survival at the time of thaw ($P = 0.02$) and tended to be greater ($P = 0.07$) at 24 h post-thaw compared with *in vitro* embryos produced from oocyte donors fed CLA, while survival of *in vitro* embryos from Control-supplemented donors was intermediate at the time of thaw and at 24 h post-thaw.

Table 5.3 Stage and grade of embryos evaluated for post-thaw *in vitro* and *in vivo* survival¹

Item	Embryo source ²		
	CLA	Control	<i>In vivo</i> control ³
Totals embryos			
Grade 1	72	69	41
Grade 2	22	35	20
Embryo Stage (IETS stage code)			
Compact morula (4)	1	3	37
Early blastocyst (5)	19	12	24
Blastocyst (6)	22	23	0
Expanded blastocyst (7)	52	69	0

¹Stage and grade of embryos were assigned by experienced technicians according to International Embryo Transfer Society (IETS) guidelines.

²Oocytes for *in vitro* embryo production were collected from cows (Angus x Red Angus and Angus x Brangus) ($\leq 1/4$ Brahman) fed conjugated linoleic acids (CLA) or no fat (Control).

³Cryopreserved *in vivo* embryos were used as a control for *in vitro* and *in vivo* survival assays.

Table 5.4 Post-thaw *in vitro* survival and hatching of *in vitro* embryos produced after collection of oocytes from cows fed CLA or no lipid supplement¹

Item	Embryo source ¹		<i>In vivo</i> control ²	<i>P</i> value
	CLA	Control		
Post-thaw survival ³ (%)				
Collection 1 ⁴	87.5	93.7	96.4	0.34
Collection 2	81.3 ^a	80.0 ^a	100 ^b	0.048
Total	84.1 ^a	89.3 ^{ab}	98.2 ^b	0.029
Survival after 12 h culture (%)				
Collection 1	89.5	87.3	96.4	0.41
Collection 2	83.3	80.0	88.9	0.65
Total	86.1	85.0	92.7	0.36
Survival after 24 h culture (%)				
Collection 1	89.5	87.3	96.4	0.41
Collection 2	75.0	79.3	88.9	0.35
Total	81.4	84.8	92.7	0.17
Hatching rate (%)				
Collection 1	26.3	17.5	7.1	0.13
Collection 2	20.8 ^{ab}	34.5 ^a	0.0 ^b	0.004
Total	23.3 ^a	22.8 ^a	3.6 ^b	0.005

¹Oocytes for *in vitro* embryo production were collected from cows (Angus x Red Angus and Angus x Brangus) ($\leq 1/4$ Brahman) fed conjugated linoleic acids (CLA) or no fat (Control).

²Cryopreserved *in vivo* embryos were used as a control for *in vitro* survival assay.

³Embryos with an intact zona pellucida and expanded or re-expanding blastocoel cavity at evaluation were considered to have survived cryopreservation.

⁴There were 7 cows/pen, 2 pens/trt and 2 oocyte collections

^{a,b,c} Means within row without a common superscript are different ($P \leq 0.05$).

Table 5.5 Post-thaw *in vitro* survival and hatching rate of Grade 1 and Grade 2 *in vitro* embryos produced after collection of oocytes from cows fed CLA or no lipid supplement¹

Item	Embryo source ²			<i>P</i> value
	CLA	Control	<i>In vivo</i> control ³	
Grade 1 embryo survival ⁴ (%)				
Post-thaw	80.6 ^a	90.0 ^{ab}	100.0 ^b	0.02
12 h culture	83.9	83.3	97.1	0.13
24 h culture	80.7 ^a	86.4 ^{ab}	97.1 ^b	0.07
Hatching rate	17.7 ^a	18.6 ^a	2.9 ^b	0.07
Grade 2 embryo survival (%)				
Post-thaw	92.3	87.9	95.2	0.63
12 h culture	91.6	87.9	90.5	0.89
24 h culture	83.3	81.8	90.5	0.67
Hatching rate	37.5 ^a	30.3 ^a	4.8 ^b	0.03

¹Quality grade of embryos were assigned by experienced technicians according to International Embryo Transfer Society (IETS) guidelines.

²Oocytes for *in vitro* embryo production were collected from cows (Angus x Red Angus and Angus x Brangus) ($\leq 1/4$ Brahman) fed conjugated linoleic acids (CLA) or no fat (Control).

³Cryopreserved *in vivo* embryos were used as a control for *in vitro* survival assay.

⁴Embryos with an intact zona pellucida and expanded or re-expanding blastocoel cavity at the evaluation were considered to have survived cryopreservation.

There was no difference in survival of Grade 1 embryos between the three groups (CLA, Control and *in vivo* control) at 12 h post-thaw. Hatching rate of *in vitro*-produced Grade 1 embryos were not different ($P = 0.90$) between supplemental treatments (17.7% CLA vs. 18.6% for Control). However, hatching rates of *in vitro*-produced embryos (across supplemental treatment groups) tended to be greater ($P = 0.07$) than the hatching rate of *in vivo* control embryos (2.9% hatching rate).

There were no differences in survival of quality Grade 2 embryos between the three groups of embryos evaluated. Similar to Grade 1 embryos, hatching rates of Grade 2 *in vitro* embryos were similar between supplemental treatments ($P = 0.57$), but were significantly greater ($P = 0.03$) than *in vivo* control embryos (4.8%).

There were no dietary treatment effects for post-thaw survival or hatching of *in vitro*-produced embryos at any point, therefore data for the two *in vitro* embryo groups (CLA and Control-supplemented oocyte donors) were combined to evaluate the effect of embryo quality grade on post-thaw survival and hatching of *in vitro* embryos (Table 5.6). There were no significant differences ($P \geq 0.30$) in survival between Grade 1 and Grade 2 embryos at the time of thaw or at 12 h or 24 h post-thaw. A greater percentage ($P = 0.025$) of Grade 2 embryos (33.3%) hatched after 24 h of post-thaw *in vitro* culture compared with Grade 1 embryos (18.2%).

Table 5.6 Post-thaw *in vitro* survival of Grade 1 and Grade 2 *in vitro*-produced embryos¹

Item	<i>In vitro</i> embryos ²		<i>P</i> value
	Grade 1	Grade 2	
Survival, %			
Post-thaw	85.3	89.8	0.73
12 h culture	83.6	89.5	0.30
24 h culture	83.5	82.5	0.87
Hatching rate	18.2	33.3	0.025

¹Quality grades of embryos were assigned by experienced technicians according to International Embryo Transfer Society (IETS) guidelines.

²Oocytes for *in vitro* embryo production were collected from cows (Angus x Red Angus and Angus x Brangus) ($\leq 1/4$ Brahman) fed conjugated linoleic acids (CLA) or no fat (Control); there were no dietary treatment effects for survival or hatching at any point, therefore data was combined to evaluate the effect of embryo quality grade on survival and hatching.

***In Vivo* Survival of Embryos**

Only 17 embryos were recovered at d 14 after 6 d *in utero* (total recovery was 7.4% of embryos transferred at d 8). Recovery of expanded or elongated d 14 embryos produced from donor cows fed CLA (12.9%) was greater ($P = 0.03$) compared with *in vivo* control embryos (1.8%), while recovery of embryos produced from Control fed donor cows (5.4%) was intermediate and did not differ from CLA-supplemented group (Table 5.7). Pictures of representative d-14 embryos are presented in Appendix N.

Table 5.7 *In vivo* survival of *in vitro* embryos produced after collection of oocytes from cows fed CLA or no lipid supplement¹

Item	Embryo source ²			<i>P</i> value
	CLA	Control	<i>In vivo</i> embryos ³	
Embryos transferred, n	85	92	55	
Embryos recovered, n	11	5	1	
Recovery/Survival rate, %	12.9 ^a	5.4 ^{ab}	1.8 ^b	0.03

¹Embryos (d 8) were transferred into synchronous cows by treatment group and collected 6 d later on d 14; recovered embryos that were the appropriate size/stage with a distinguishable inner cell mass were considered to have survived.

² Oocytes for *in vitro* embryo production were collected from cows (Angus x Red Angus and Angus x Brangus) ($\leq 1/4$ Brahman) fed conjugated linoleic acids (CLA) or no fat (Control).

³Cryopreserved *in vivo* embryos were used as a control for *in vivo* survival assay.

Discussion

Body weight and BCS of cows was not influenced by 143 d of CLA supplementation, indicating that the small amount of energy supplied from the CLA supplement likely did not significantly alter net energy intake of cows. The CLA (80% fat) was supplemented at 150 g per hd/d, equating to 120 g of fat per hd/d. This contributes <4% of the metabolizable energy required for maintenance for nonlactating, nongestating crossbred beef cows of similar breed, BW, BCS and age to the cows used in the current trial (NRC 1996, Level 1 Model).

Dietary treatment did not influence the number of follicles targeted or COC recovered per TUGA session in the current study. Höffmann et al. (2008)

reported increased follicles present per collection when lactating Holsteins were fed CLA (Lutrell at 100 g/d) compared unsupplemented cows. Ewes fed fish oil (high in PUFA) had increased follicle and oocyte production compared with unsupplemented ewes (Zeron et al., 2002a). Follicle responses after CLA supplementation depend on physiological stage of production (lactation vs. nonlactating) of cows. Highly productive lactating Holsteins, like those in the study by Höffmann et al. (2008), likely repartitioned energy from milk fat depression into increased follicle production. Nonlactating beef cows may not see the same beneficial effects of CLA supplementation because they are not deficient in energy like high producing Holstein cows during early lactation. However, supplementation with ≤ 10 g/d of each *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA appears to benefit reproduction in lactating Holstein cows even without significant changes in energy balance (Bernal-Santos et al., 2003; Castañeda-Gutiérrez et al., 2005). Ewes fed fish oil (high in PUFA) had increased follicle and oocyte production (Zeron et al., 2002a).

The number of follicles present and COC recovered per collection was similar between Collection 1 (FSH stimulated) and Collection 2 (no FSH). Folltropin[®]-V (Bioniche Animal Health) is the most widely FSH product in the industry for bovine ovarian stimulation, which according to literature provided by Bioniche, has a very low LH:FSH ratio. The use of Pluset and Pluset Flex H for ovarian stimulation in the current study likely supplied more LH and less FSH than is typically used in ovarian stimulation protocols before TUGA, which may have contributed to similar oocyte and follicle production between collections.

A variety of ovarian stimulation protocols and FSH dosages increased the number of follicles present and COC recovered per TUGA session in problem (donors unsuccessful with conventional embryo collection) beef and dairy cows (Looney et al., 1994), Holstein Friesian heifers (Bungartz et al., 1995), Friesian x beef cows (Goodhand et al., 2000) and Angus-cross cows (Chaubal et al., 2006). In contrast, no increases in follicle and COC production per TUGA session were reported in Angus cows (Gibbons et al., 1994), Holstein cows (Stubbings and Walton, 1995) or Simmental heifers (Goodhand et al., 1999). It is difficult to compare results of the FSH ovarian stimulation studies due to variation in FSH composition between and within product brands and the use of a wide variety of ovarian stimulation protocols; the influence of biological factors such as breed, age, physiological stage of production and nutrition further complicate comparisons between studies (Chaubal et al., 2006).

Dietary treatment did not influence the number of Quality Grade 1, Grade 2 or total TQE produced in this experiment. The number of Quality Grade 1 embryos produced was almost identical between CLA (n = 72) and Control (n = 69) supplement groups. There was no difference ($P = 0.09$) in the number of Grade 2 embryos between cows supplemented CLA (n = 35 embryos) compared with Control-supplemented cows (n = 22 embryos). There was a dietary treatment by collection interaction for production of Quality Grade 2 embryos (Table 2). This outcome is likely a result of the small number of Grade 2 embryos produced in this trial, especially in the CLA group at Collection 1.

Supplementation of lactating dairy cows with 100 g of lipid-encapsulated CLA per hd/d improved cleavage rates and d-8 blastocyst rates *in vitro* (Höffmann et al., 2008). Lactating dairy cows fed CA salts of palm oil at a high inclusion rate (800 g/d) produced more blastocysts *in vitro* after TUGA compared with a low (200 g/d) Ca salts inclusion (Fouladi-Nashta et al., 2007). However, Bilby et al. (2006) reported no improvement in embryo production *in vitro* after TUGA from PUFA supplemented lactating Holstein cows. Although embryo production was not directly measured, Castañeda-Gutiérrez et al. (2007b) concluded that CLA supplementation of lactating dairy cows improved ovarian follicular steroidogenesis, supporting ovarian follicle development. Inclusion of *trans*-10, *cis*-12 CLA into bovine oocyte maturation medium increased the rate of high quality embryos on d 8 *in vitro*, but did not influence oocyte maturation or embryo cleavage or blastocyst production (Lapa et al., 2011). However, inclusion of *trans*-10, *cis*-12 CLA into embryo culture medium did not influence blastocyst production (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013).

More Grade 1 embryos were produced after FSH-treatment (Collection 1) compared with no FSH stimulation (Collection 2) across dietary treatment groups. Stimulating cows with FSH before TUGA increased the number of high quality oocytes recovered per TUGA session (Goodhand et al., 1999; Goodhand et al., 2000) and increased the number of *in vitro* embryos produced per TUGA session (Looney et al., 1994; Goodhand et al., 1999; Chaubal et al., 2006). Although total embryo production was not influenced by donor FSH treatment, Gibbons et al. (1994) reported significantly more Grade 1 *in vitro* embryos

produced after FSH treatment of Angus cows. The use of DFR and a follicle/oocyte coasting period was similar between Collection 1 and Collection 2 in the current study. Stimulation with increased dosages of LH (Pluset Flex H) in the second and third injections may have facilitated follicular growth and COC maturation before Collection 1 as ovarian follicles transitioned from FSH to LH dependence after the follicle recruitment phase.

In the present study, cryopreserved *in vivo*-produced control embryos had greater post-thaw survival at the time of thaw compared with *in vitro*-produced embryos from CLA and Control-supplemented oocyte donors. However, embryo survival did not differ between *in vitro*-produced embryos from CLA and Control-supplemented oocyte donors and *in vivo*-produced control embryos at 12 h or 24 h post-thaw, indicating comparable cryosurvival of *in vitro* and *in vivo* embryos after post-thaw culture in this study. *In vitro*-produced embryos are more sensitive to chilling and cryopreservation than *in vivo*-produced embryos (Leibo and Loskutoff, 1993; Pollard and Leibo, 1993; Farin et al., 1999). Research has closed the gap in cryosurvival between *in vivo* and *in vitro* bovine embryos, yet there remains opportunity for improvement before the widespread adoption of *in vitro* embryo cryopreservation (Seidel, 2006).

It is interesting to note that the percentage of embryos hatching after 24 h of culture in the current study was greater for *in vitro*-produced embryos compared with *in vivo* control embryos (22.8% vs. 3.6% for *in vitro* and *in vivo* embryos, respectively). This was likely attributed to the difference in embryo maturity between *in vitro* embryos and *in vivo* control embryos (Table 3). In the

current study, 83.8% of *in vitro* embryos were blastocyst or expanded blastocyst stage with fewer compact morula and early blastocysts. The *in vivo* control embryos were all compact morula and early blastocysts with no blastocyst or expanded blastocyst stage embryos.

Dietary supplementation of CLA before TUGA in the current study did not influence post-thaw survival of *in vitro*-produced embryos at the time of thaw or at 12 h or 24 h post-thaw compared with Control-supplemented cows. The percentage of embryos hatching after 24 h of culture was also not influenced by dietary treatment of oocyte donor cows. When quality Grade 1 and Grade 2 embryos were analyzed separately by dietary treatment, embryo survival and hatching followed similar trends. Ewe diets supplemented with calcium salts of fish oil (high PUFA) altered phospholipid fatty acid composition of follicular components and improved chilling resistance of oocytes (Zeron et al., 2002a). Conversely, cryotolerance of *in vivo*-produced embryos decreased when Nellore heifer diets were supplemented with calcium salts of palm oil, a rumen-protected fat high in PUFA (Guardieiro, 2010; Guardieiro et al., 2014).

Inclusion of *trans*-10, *cis*-12 conjugated linoleic acid in bovine embryo culture media improved post-thaw embryo survival compared with controls, without influencing blastocyst production (Pereira et al., 2007; Pereira et al., 2008; Ruiz et al., 2013). In contrast, post-thaw *in vitro* embryo survival was not improved after co-culture with 100 μ M of *trans*-10, *cis*-12 CLA (Al Darwich et al., 2010). Improvements in cryosurvival after inclusion of *trans*-10, *cis*-12 CLA into embryo culture medium does not appear to correlate to cryosurvival of *in vitro*

embryos after dietary supplementation of *trans*-10, *cis*-12 CLA oocyte donors.

However, the current study is the first known trial involving direct measurement of embryo cryosurvival after *trans*-10, *cis*-12 CLA supplementation in cows.

Fatty acid composition of follicular fluid and oocytes was not evaluated in the current study. However, it appears that follicular fluid and oocyte composition were either not altered by CLA supplementation or that slight modifications were not enough to influence cryotolerance of *in vitro* embryos. Castañeda-Gutiérrez et al. (2007a) reported very few alterations in follicular fluid fatty acid composition after CLA supplementation in cows. However, fatty acid composition of bovine follicular fluid was sensitive to changes in dietary supplementation of PUFA using soybean oil (Batista et al., 2010) and fish oil (Childs et al., 2008). Feeding dairy cows a rumen-protected flaxseed oil altered the fatty acid composition of plasma, follicular fluid, COC and granulosa cells (Zachut et al., 2010).

The fatty acid composition of granulosa cells and COC are quite different than that found in plasma and follicular fluid (Zachut et al., 2010). Differences in fatty acid composition between follicular fluid, granulosa cells and COC after dietary fatty acid supplementation suggest a selective uptake of specific fatty acids in different ovarian compartments (Zeron et al., 2002; Adamiak et al., 2006; Fouladi-Nashta et al., 2009; Zachut et al., 2010; Aardema et al., 2012). Lapa et al. (2011) reported minimal changes in fatty acid concentration of oocytes after *in vitro* maturation with *trans*-10, *cis*-12 CLA, concluding that oocytes accumulated very small amounts of *trans*-10, *cis*-12 in a selective manner but maintained relatively consistent fatty acid concentration to ensure cell integrity. Aardema et

al. (2012) concluded that cumulus cells protect oocytes against elevated follicular fluid fatty acid concentrations by accumulation of these fatty acids. Uptake of fatty acids by the follicle/oocyte is likely highly selective as a protective mechanism to ensure fatty acid composition of oocytes remains safe or optimum (Bilby et al., 2006; Santos et al., 2008a; Fouladi-Nashta et al., 2009; Sturmey et al., 2009). The highly regulated mechanisms involved in fatty acid uptake by ovarian components may help explain why cryotolerance of *in vitro* embryos was not influenced by CLA supplementation in the current study.

A lack of improvement in cryotolerance of embryos from CLA-supplemented cows in the current study may be a result of inconsistent or incomplete protection of the CLA supplement from rumen biohydrogenation. Inconsistency in reproductive responses to fatty acid supplementation is likely a result of supply of specific unsaturated FA for absorption in the small intestine (Santos et al., 2008b), which is influenced by the efficacy of methods used to protect the supplement from biohydrogenation in the rumen. Using duodenal fistulated cows, Pappritz et al. (2011) evaluated postruminal bioavailability of a lipid-encapsulated CLA supplement similar to the one used in the current study (Lutrell[®], BASF Ludwigshafen, Germany) and results indicated a relatively low rate of ruminal protection (5%).

There were no significant differences in survival between quality Grade 1 and Grade 2 *in vitro*-produced embryos across dietary treatments at any time point in the current trial. However, a significantly greater percentage of Grade 2 *in vitro*-produced embryos hatched after 24 h post-thaw culture compared with

Grade 1 embryos (33.3% vs. 18.2% for Grade 2 and Grade 1 embryos, respectively). This result is difficult to explain and may not be indicative of differences in embryo quality and/or predict differences in pregnancy success between embryo quality grades. Hatching rates provide inference on the survival of embryos and progression towards a viable pregnancy, but improved hatching rates do not necessarily result in more pregnancies after transfer. Recent reports evaluating both experimental (Spell et al., 2001) and commercial embryo transfer data sets indicate no difference in pregnancy rates after embryo transfer between quality Grade 1 and Grade 2 bovine embryos.

Embryo recovery rate at d 14 after 6 d in utero was extremely low and inconsistent in the current trial. Only 17 embryos were recovered from 10 recipient cows after transfer of 232 viable embryos on d 8 (recovery rate = 7.4%). Recovery rates at d 14 to d 16 after embryo transfer on d 7 ranged from 20 to 70% (Bertolini et al., 2002; Block et al., 2007; Alexopoulos and French, 2009). In the current study, recovery rate of d-14 embryos produced from oocytes collected from CLA-supplemented cows was greater than *in vivo* control embryos, while Control-supplemented group was intermediate. However, based on very poor recovery of embryos from all groups, it's difficult to make any strong conclusions regarding embryo survival based on these data. The number of embryos recovered on d 14 was 11, 5 and 1 for CLA, Control and *in vivo* control groups, respectively.

Recipient cows in the current study were in excellent body condition (BCS = 6) and nutrition. All recipients had an observed estrus and had a palpable and

ultrasound verified CL at the time of embryo transfer on d 8. Embryo recovery was performed by a single experienced embryo transfer professional. Large Foley catheters (24 to 20 Fr) were used for collections to accommodate the recovery of expanded/or elongated d-14 embryos. The technician used the largest catheter that would pass through the cervix for each cow.

Collection of large expanded d-14 embryos may require alternative recovery methods such as uterine horn flushing with the syringe method or modification of typical Foley Catheters by enlarging the ports to allow embryos to enter the catheter. The embryo transfer technician noted that the uterus of the d-14 recipient cows in the current study were flaccid and very difficult to manipulate compared with d 7 cows, likely contributing to poor recovery rates.

Summary

Dietary supplementation of cows with *trans*-10, *cis*-12 CLA before oocyte collection did not influence cryotolerance of *in vitro*-produced embryos. Although not directly measured, it appears that follicular fluid and oocyte composition were either not altered by CLA supplementation or that slight modifications were not enough to influence cryotolerance of *in vitro* embryos. The highly regulated mechanisms involved in fatty acid uptake by ovarian components may help explain why cryotolerance of *in vitro* embryos was not influenced by CLA supplementation in the current study. The current study is the first known trial involving direct measurement of embryo cryosurvival after dietary supplementation of *trans*-10, *cis*-12 CLA in cows.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Cryopreservation of embryos is an important tool used in the bovine embryo transfer industry to preserve superior genetics. Although cryopreservation of *in vivo*-produced bovine embryos has become widely adapted, variation in cryotolerance exists between embryos from different species, breeds and management conditions. In addition, there remains opportunity to improve the success of embryo cryopreservation of *in vitro*-produced bovine embryos and embryos from *Bos indicus* breeds of cattle. To the author's knowledge this is the first report evaluating effects of CLA supplementation in cattle on oocytes fatty acid metabolism, oocyte composition and embryo cryotolerance.

In Chapter III of the current study, milk fat was depressed by 10.1% and milk production increased 7.3% in lactation Holstein cows fed CLA. This study supported other published results regarding dietary CLA-induced milk fat depression in dairy cows. Results from Chapter III lead to a series of experiments to determine effects of CLA supplementation on oocyte lipid metabolism, lipid composition and embryo cryotolerance.

Supplementation with CLA did not influence follicle or oocyte production or the number of quality oocytes or embryos produced in any of the trials in this dissertation. Dietary supplementation of crossbred beef cows with *trans*-10, *cis*-12 CLA before oocyte collection did not influence cryotolerance of *in vitro*-produced embryos (Chapter V). Dietary supplementation of Brahman and

Holstien cows with *trans*-10, *cis*-12 CLA did not alter expression of genes in cumulus-oocyte complexes (COC) involved in lipid metabolism (Chapter IV). Lipid content of oocytes measured by Nile Red fluorescence in this study was not influenced by CLA supplementation in Holstein (Chapter III) or Brahman cows (Chapter IV).

Previous studies in the cow demonstrated nutrition-induced modification of follicular components (Childs et al., 2008; Batista et al., 2010; Zachut et al., 2010). However, results from the current research indicate that ovarian lipid metabolism and oocyte composition was either not altered by CLA supplementation or that slight modifications were not enough to alter expression of the metabolic genes analyzed and resulted in no improvement in cryotolerance of *in vitro*-produced embryos.

Differences in fatty acid composition between follicular fluid, granulosa cells and COC after dietary fatty acid supplementation suggest a selective uptake of specific fatty acids in different ovarian compartments (Zeron et al., 2002; Adamiak et al., 2006; Fouladi-Nashta et al., 2009; Zachut et al., 2010; Aardema et al., 2012). Uptake of fatty acids by the follicle/oocyte is likely highly selective as a protective mechanism to ensure fatty acid composition of oocytes remains safe or optimum (Bilby et al., 2006; Santos et al., 2008a; Fouladi-Nashta et al., 2009; Sturmey et al., 2009). The highly regulated mechanisms involved in fatty acid uptake by ovarian components may help explain why lipid content of oocytes, gene expression of COC and cryotolerance of *in vitro* embryos was not influenced by dietary CLA supplementation in the current study. In addition, the

lack of response in the current study may be a result of inconsistent or incomplete protection of the CLA supplement from rumen biohydrogenation.

The duration of CLA supplementation relative to oocyte collections in the current study may not have been sufficient for supplemental CLA to modify the follicular environment in a way that altered metabolic gene expression of COC. The origin of lipids and the timing of lipid deposition in bovine oocytes are not fully understood. It appears that oocytes acquire and/or deposit lipids between the time of primordial follicle activation and the antral follicle stage (Fair et al., 1997). Although it is difficult to estimate, the course of follicular growth takes a long time relative to the reproductive cycle of the animal; several weeks in rodents, and perhaps several months in larger animals (Hirshfield, 1991; Fortune, 2003). Dietary CLA could potentially influence ovarian lipid metabolism and composition of bovine oocytes if the duration of supplementation before oocyte or embryo collection were extended beyond the schedule used in the current trial. Oocytes were collected after ~40 d of supplementation for gene express analysis (Chapter IV) and ~140 d for *in vitro* embryo production and cryotolerance evaluations (Chapter V).

Recently, considerable research has focused on the follicular micro-environment and oocyte quality and fertility in lactating dairy cows, as reviewed by Leroy et al. (2012). Investigation in this area will likely continue to improve our understanding of lipid metabolism and lipid uptake by the oocyte during follicular development. Research designed to better understand factors influencing the composition of oocytes may lead to practices that produce embryos better suited

to survive cryopreservation. Although it appears difficult to alter bovine oocyte lipid composition *in vivo*, further research is necessary to better understand effects of both dietary CLA supplementation and *in vitro* culture CLA supplementation on bovine oocyte and embryo lipid metabolism, lipid composition and embryo cryotolerance.

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APPENDIX PROTOCOLS AND MATERIALS

Appendix A. Equipment and supplies for transvaginal ultrasound-guided oocyte aspiration (TUGA)

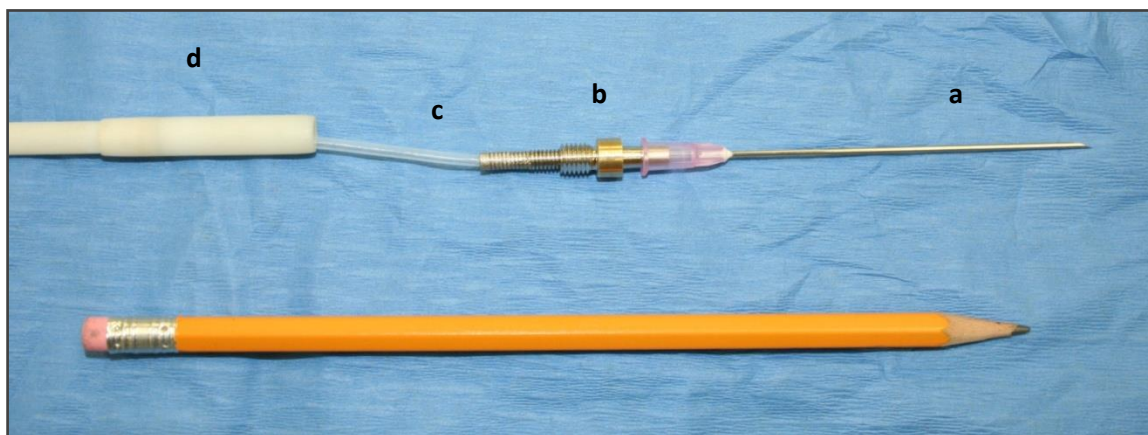
- SonoSite® MicroMaxx® ultrasound system
- 8.5 MHz curved array transducer (model C11e, SonoSite®)
- Hard plastic probe handle equipped with accessories to utilize short needle systems (Boland Vet Sales; Keller, Texas, www.bolandvetsales.com)
- Plastic probe covers are used to protect the transducer and probe during the procedure (distributed by Reproductive Resources, Walworth, WI; product # 273028-3436)
- Sterilized non-spermicidal lubricating jelly (Priority Care®, Vet One)
- Non-radiopaque polyethylene tubing (inner diameter x outer diameter = 1.67 x 2.42 mm, 7.3 French; Intramedic®, Clay Adams® Brand, Becton Dickinson and Co., Sparks MD)
- Ureteral catheter connectors (Part # 050010, Cook Urological®)
- EmCon® filters (Reproductive Resources, Walworth, WI)
- Epidural needles (18 ga X 3.8 cm, Reproductive Resources or Vet One)
- Lidocaine (2%, Reproductive Resources or Vet One)
- Collection needles (18 ga x 7.6 cm, Lot # 100107, Air-Tite Products Co., Inc., Virginia Beach, VA)
- Regulated vacuum pumps manufactured by Cook Veterinary Products (Bloomington, IN; VMAR-5000) and Watanabe Tecnologia Aplicada (Brazil; Pressure Max, BV-003)



Plastic probe (Boland Vet Sales; Keller, Texas) designed for 8.5 MHz curved array SonoSite® transducer, pictured with short disposable needle system (Bailey, 2011).



Short disposable needle system with needle guide (a), transducer (b), needle (c), Metal connector (d) and plastic manipulation tool (e) (Bailey, 2011).

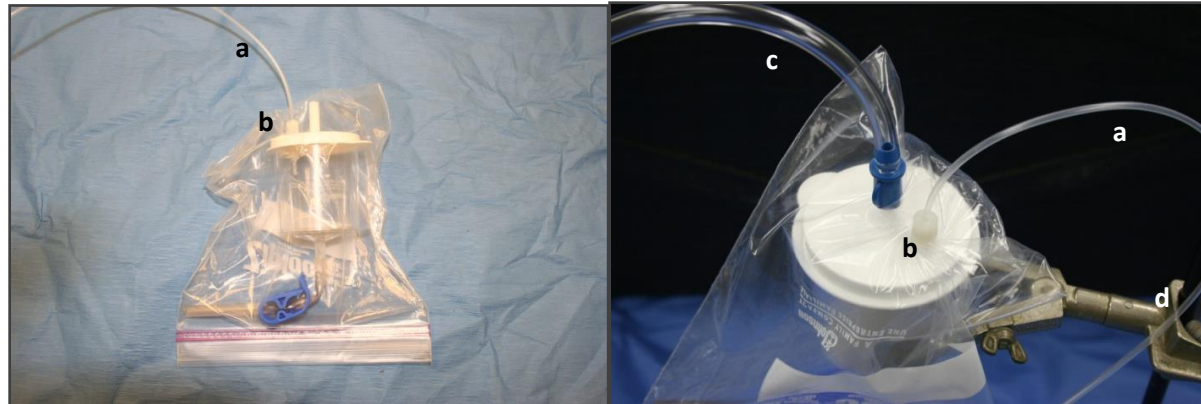


Short disposable needle (a) to metal connector (b), connected to plastic collection tubing (c) connector is threaded into manipulation tool (d) (Bailey, 2011).

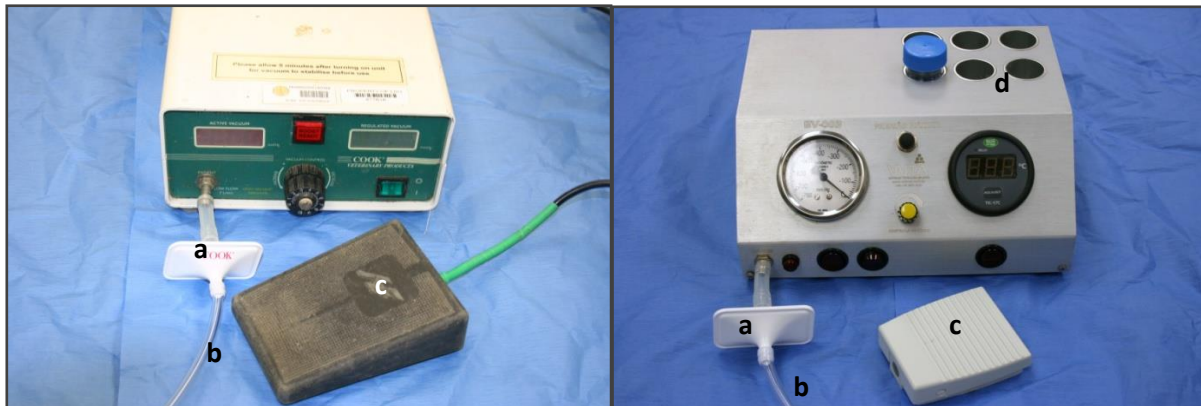


Figure 8. Ureteral catheter connector (Cook Urological) fixed to the lid of EmCon®

filter using epoxy glue, with collection tubing passing through connector (Bailey, 2011).



EmCon[®] filter placed inside a Ziploc[®] quart plastic bag to ensure consistent suction during TUGA; plastic oocyte collection tubing (a), ureteral catheter connector (b), tubing connected to vacuum pump (c), attached to a ring stand during aspirations (d) (Bailey, 2011).



Regulated vacuum pumps manufactured by Cook Veterinary Products (left; Bloomington, IN; VMAR-5000) and Watanabe Tecnologia Aplicada (right; Brazil; Pressure Max, BV-003); pumps equipped with air filter (a), vacuum tubing that attaches to top of oocyte filter (b), control pedal (c), and media tube warmer (Watanabe pump only) (d) (Bailey, 2011).



Oocyte collection at the LSU AgCenter Reproductive Biology Center, St. Gabriel, Louisiana.



Oocyte collection using transvaginal ultrasound guided oocyte aspiration (TUGA) at the LSU AgCenter Reproductive Biology Center, St. Gabriel, Louisiana.

Appendix B. Nile Red Fixative Medium

Fixative Medium

1. 216 µl Formaldehyde (16%)
2. 500 µl Glutaraldehyde (50%)
3. 3,284 µl Dulbecco's PBS

Medium needs to be made on the day of fixing samples and stored at 4°C

Appendix C. Nile Red Stain

Nile Red Stock:

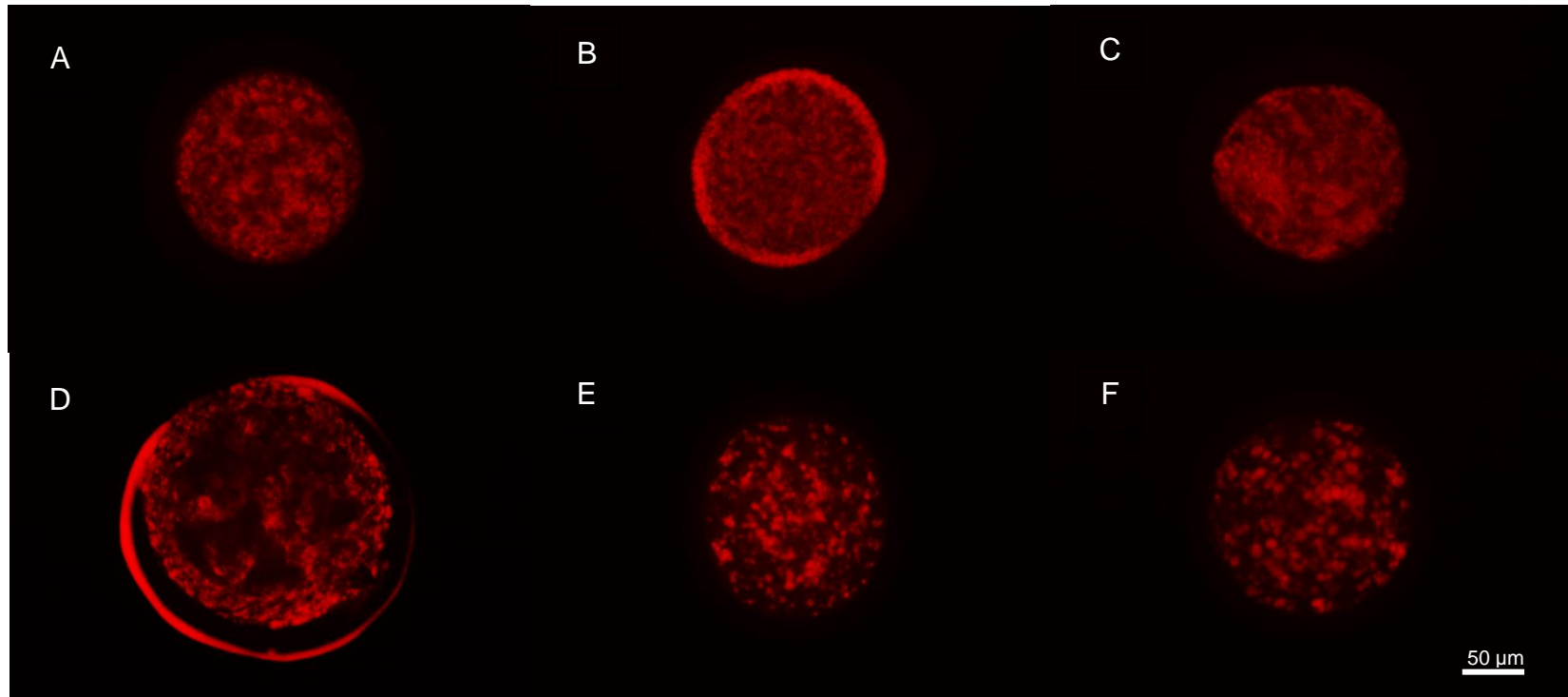
1. 10 mg Nile Red
2. 10 ml DMSO

Nile Red Working Solution:

1. 5 µl Nile Red Stock
2. 4,995 µl NaCl (0.9%)
3. 5 mg Polyvinyl-Pyrrolidone (PVP)

Preparing Nile Red Working Solution:

1. Prepare in 15 ml conical tube wrapped in tin foil as solution is light sensitive
2. Centrifuge at 2,500g for 10 min then filter solution using a 0.25 µm filter attached to a 25 ml embryo friendly (Norm-Ject) syringe
3. Oocytes incubated in a single well of a 4-well plate at room temperature for 24 h in complete darkness.
4. Place sterile H₂O (500-1,000µL) in middle of 4-well dish (between wells) to ensure Nile Red stain does not evaporate



Appendix D. Images of bovine oocytes stained with Nile Red dye (20x objective). These oocytes were stained for a course project (Bailey, 2010) and images were captured in the same manner as those presented in this dissertation. Images were adjusted for brightness and contrast to enhance differences in morphology and distribution of lipid droplets within the cytoplasm (these images not meant to compare intensity). Oocytes A and B have a relatively homogeneous distribution, C and D have areas of more localized lipids and oocytes E and F both have fewer, larger lipid droplets distributed throughout the cytoplasm.



Appendix E. The locking headgate feeding system used to feed Holstein cows in Experiment 4.1 (cows being trained with corn silage) at the LSU AgCenter Dairy Research Farm, Baton Rouge.

Appendix F. mRNA Isolation of Cumulus-oocyte complexes (COC)

RNeasy® Plus Micro Kit

Catalog number 74034 (QIAGEN® Inc., Valencia, CA)

1. Add 350 µl Buffer RTL Plus directly to microtubules containing COC while still at -80°C (add buffer while tube is still in freezer).
2. Vortex for 2 min at maximum speed.
3. Transfer lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30 s at 9,000 x g, discard the column and save the flow-through.
4. Transfer the sample to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 15 s at 9,000 x g. Discard the flow-through.
5. Add 700 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid, centrifuge for 15 s at 9,000 x g and discard flow-through.
6. Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid, centrifuge for 15 s at 9,000 x g and discard flow-through.
7. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid, centrifuge for 2 min at 9,000 x g to wash the spin column membrane. Discard the collection tube with the flow through.
8. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column and centrifuge at max speed for 5 min to dry the membrane. Discard the collection tube with the flow-through.
9. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube and add 17 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently and centrifuge for 1 minute at max speed to elute the RNA.
10. RNA from COC was directly converted into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Appendix G. mRNA Isolation of Bovine Liver Tissue

Dynabeads® mRNA Direct Kit™

Catalog number 61011/61012 (Invitrogen, Carlsbad, CA)

Procedures for “standard” version of manufacturer’s instructions

1. Add Liver sample (~20 mg sample) from -80°C into a 10 ml round bottom tube.
2. Add 1250 µl lysis/binding buffer directly to the tube containing the frozen liver sample while the sample is still in the -80°C freezer.
 - a. RNA is very sensitive, must add buffer in freezer and work quickly to prevent mRNA degradation.
3. Use sonication grinding tool to mince/homogenize liver tissue within the buffer inside the 10 ml tube.
 - a. Sonicate tissue for 1 to 2 min or until tissue is completely homogenized into the buffer.
 - b. There will be considerable bubbles and foam production so ensure the tube is large enough to contain everything while mixing.
4. Force lysate through a 20 or 21 gauge needle 3 to 5 times using a 2 ml syringe to shear DNA (as described by manufacturer’s instructions).
5. Prepare 250 µl Dynabeads Oligo (dT)₂₅ per manufacturer instruction.
6. Add sample lysate to pre-washed Dynabeads (washed in lysis/binding buffer) in 1.5 µl microtubule.
7. Resuspend beads completely into sample lysate and incubate with continuous mixing (roller mixer) for 10 min at room temperature to hybridize polyA to beads.
8. Place tubule on magnet for 2 min and remove supernatant. If solution is noticeably viscous, increase the time to ~10 min if necessary.
9. Wash beads/mRNA complex twice in 1250 µl Washing Buffer A at room temperature and place tubule on magnet to separate the beads from the solution between each washing step.

10. Wash beads/mRNA complex twice in 1 ml Washing Buffer B at room temperature. Place tubule in the magnet to separate the beads from the solution.
11. Elute mRNA from the beads by adding 27 μ l nuclease-free water, incubate at 70°C for 2 min and immediately place tubule on the magnet. Pipette supernatant containing mRNA to a new RNASE-free tubule.
12. RNA from Liver was directly converted into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Appendix H. cDNA Synthesis Protocol

iSCRIPT cDNA Synthesis Kit

Catalog number 170-8890 (Bio-Rad Laboratories, Inc., Hercules, CA, USA)

1. 20 μ l reactions were carried out in PCR tubes (Bio-Rad).
2. Add 4 μ l of iScript reaction mix and 1 μ l of reverse transcriptase to a 15 μ l RNA sample (bovine liver samples isolated by Dynabeads, bovine COC isolated by RNeasy® Plus Micro Kit.
 - a. If using less than 15 μ l RNA sample add nuclease-free water to a final volume of 20 μ l for each reaction.
3. Place PCR tubes into thermocycler and run cDNA protocol.
 - a. 5 min. at 25°C, 30 min. at 42°C and 5 min. at 85°C (hold at 4°C).

Appendix I. Quantitative PCR Protocol

SsoFast™ EvaGreen® Supermix

Catalog number 172-5200 through 172-5205 (Sigma-Aldrich Inc., St. Louis, MO)

1. Quantitative PCR was used to evaluate relative gene expression in COC collected from cows fed CLA or a Control supplement in both Experiment 4.1 and Experiment 4.2 using 20 µl reactions in 96-well plates.
2. Plate templates were set-up on the computer controlling the thermocycler.
3. One gene per plate per experiment was run (14 unknowns in Experiment 4.1 and 28 unknowns in Experiment 4.2).
4. Each reaction consisted of 10 µl of SsoFast, 4 µl of sample cDNA (isolated from COC using RNeasy® Plus Micro Kit), 4 µl water, and 1 µl of forward primer (10 pmol/µl) and 1 µl of reverse primer (10 pmol/µl).
 - a. Master mixes were prepared to avoid pipetting error and reduced contamination.
5. Added 20 µl of each reaction into designated well of a 96-well plate (samples run in triplicate), cover plate securely with sealing tape.
6. Placed 96-well plate into thermocycler with the following program:

Item	Temperature	Time
Enzyme activation	95°C	1 min
40 cycles:		
Denaturation	95°C	5 s
Annealing/extension	61°C	30 s
Melt curve (68 steps)	61°C-95°C	10 s/step

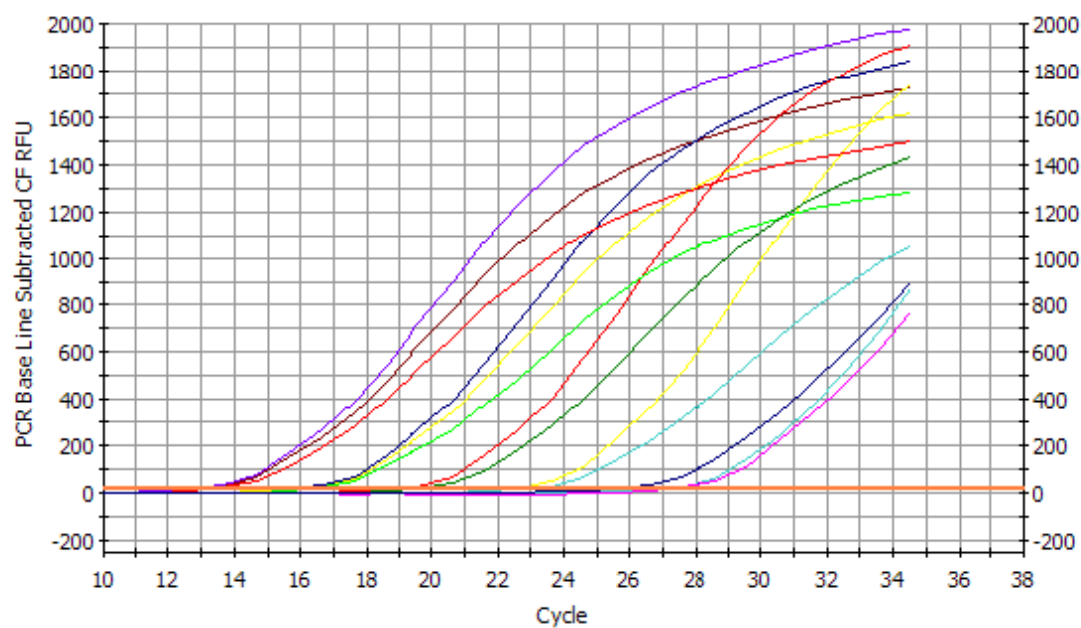
Annealing temperature is specific to primers used.

Appendix J. Primer Validation and Calibrator Evaluations

PCR Primers

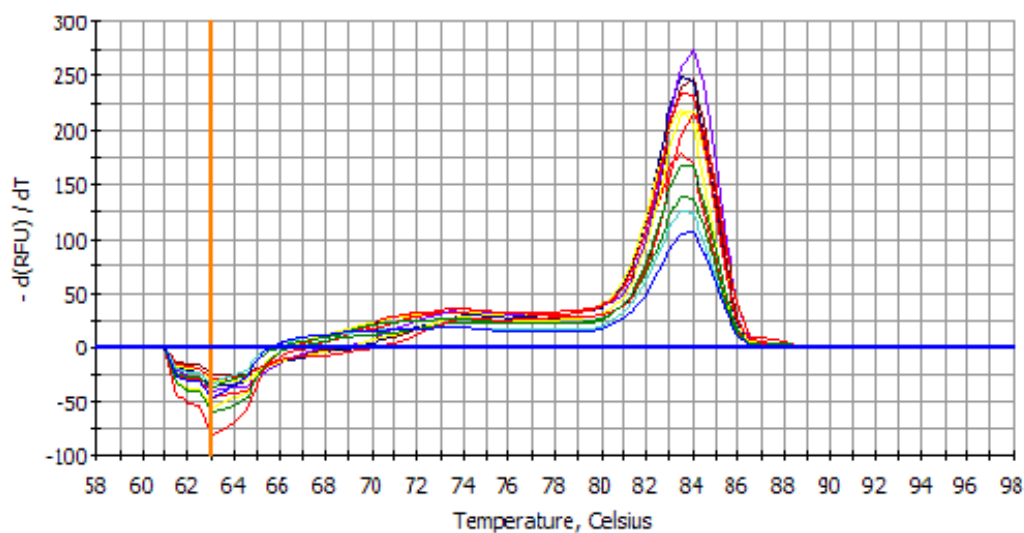
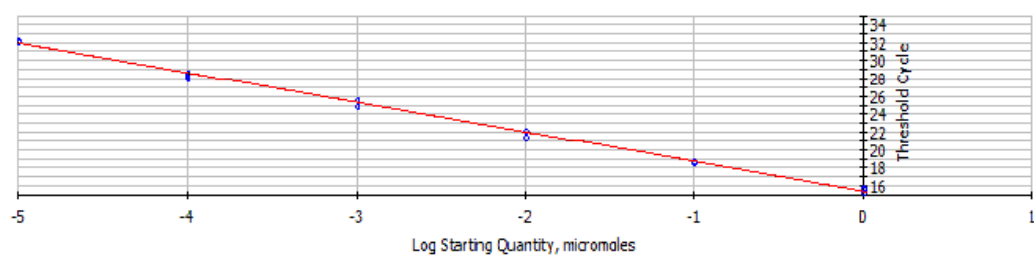
Primer ¹	GeneBank Accession Number	Sequence	Product size (bp)
CPT-1	NM_001034349.2	F 5' -CGACCCAAACAAGTACCCCA- 3' R 5' -CGCTGGGCATTTGTCTCTGA- 3'	156
FADS2	NM_001083444.1	F 5' -CCGACAAGTGGCTGGTCAT- 3' R 5' -GTGATCTGGGAATTCTTGCCG- 3'	232
PPAR α	NM_001034036.1	F 5' -TGGACGAATGCCAAGATCTGA- 3' R 5' -ATGACGAAAGGCGGGTTGTT- 3'	204
Poly A	X63436	F 5' -AAGCAACTCCATCAACTACTG- 3' R 5' -ACGGACTGGTCTTCATAGC- 3'	169
GAPDH	U85042	F 5' -CCTTCATTGACCTTCACTACATGGTCTA- 3' R 5' -TGGAAGATGGTGATGGCCTTTCCAT- 3'	127

¹*Bos taurus* poly(A) polymerase (Poly A) (reference gene), *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (reference gene), *Bos taurus* carnitine palmitoyltransferase 1 (CPT-1), *Bos taurus* fatty acid desaturase 2 (FADS2), *Bos taurus* peroxisome proliferator-activated receptor α (PPAR α).

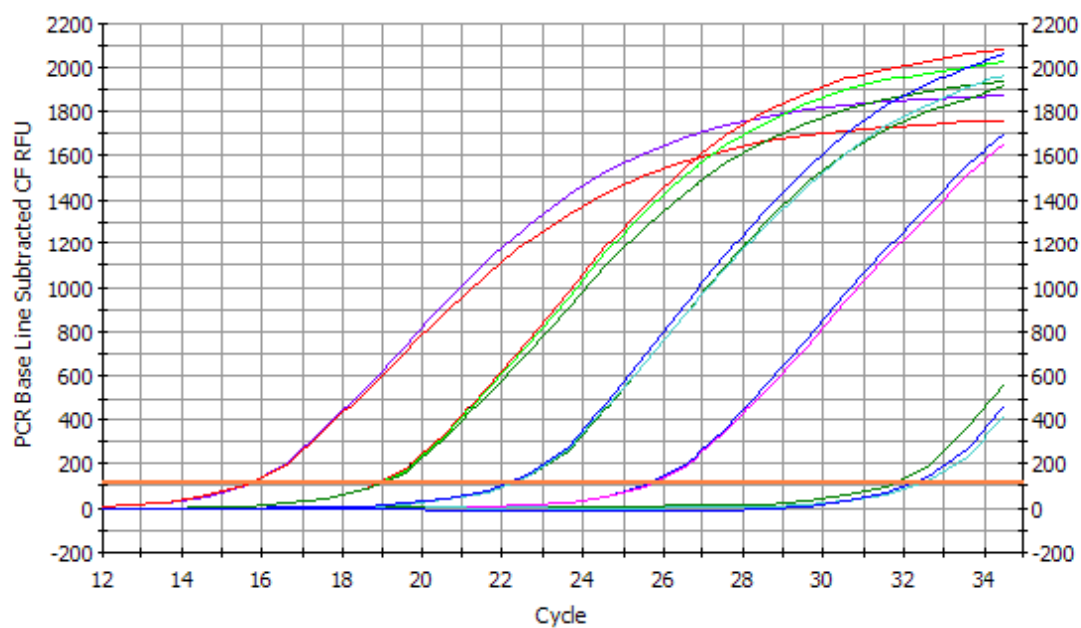


Correlation Coefficient: 0.999 Slope: -3.281 Intercept: 15.447 $Y = -3.281X + 15.447$
 PCR Efficiency: 101.7 %

Unknowns
 Standards

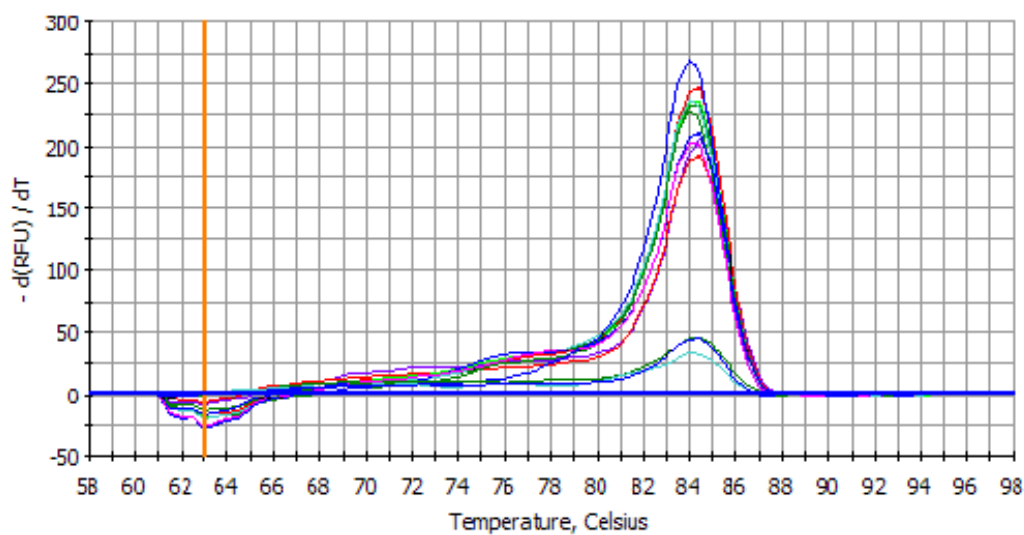
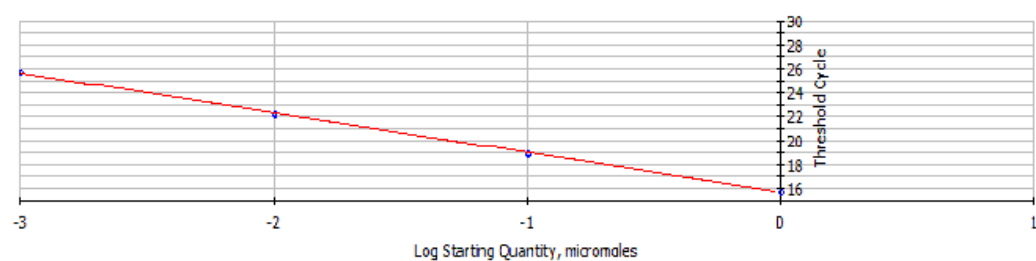


Amp/Cycle (top), Standard Curve (middle) and melting curve (bottom) for Poly A.

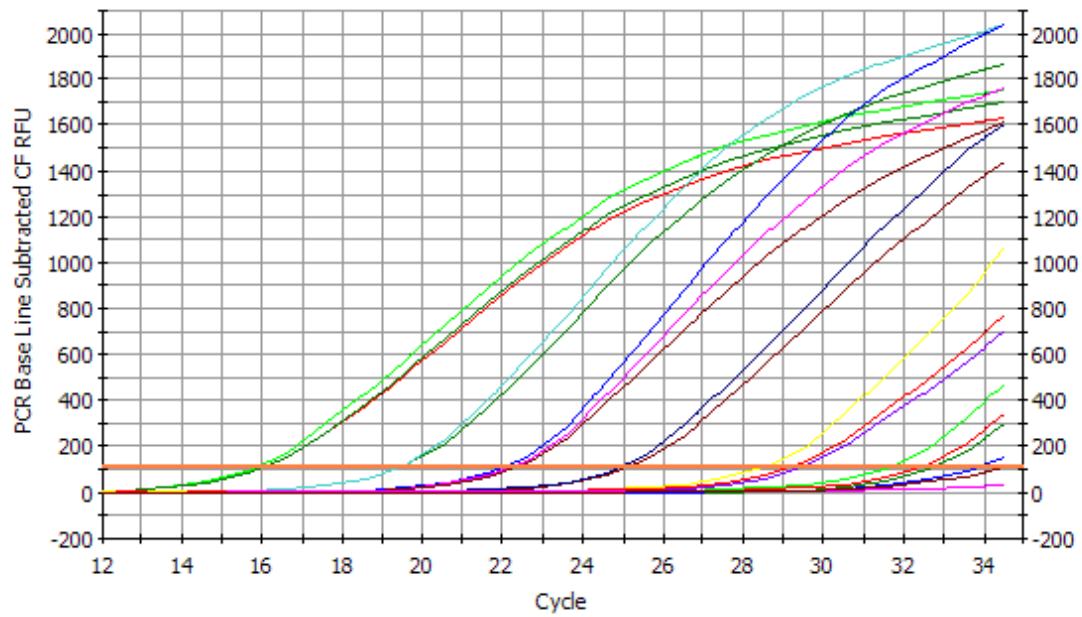


Correlation Coefficient: 1.000 Slope: -3.320 Intercept: 15.671 $Y = -3.320X + 15.671$
 PCR Efficiency: 100.1 %

Unknowns
 Standards

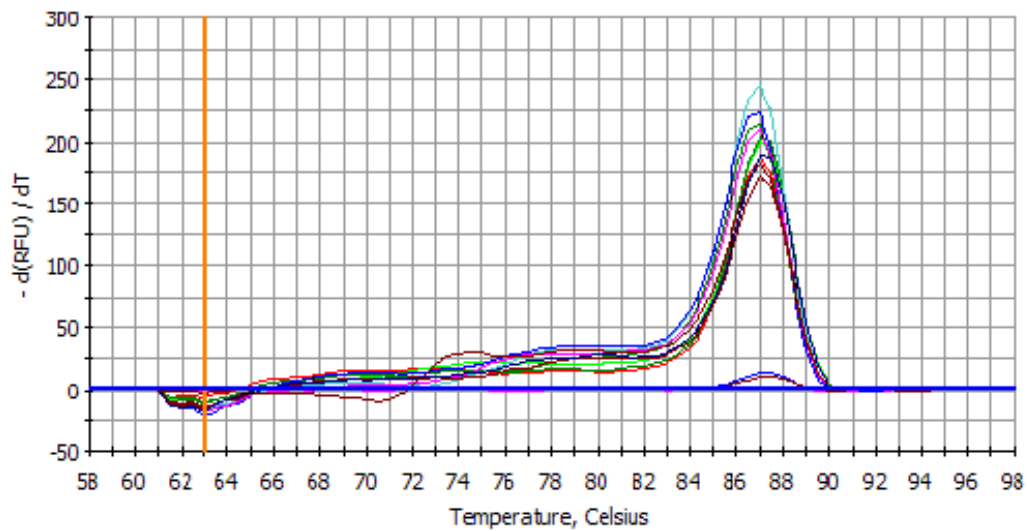
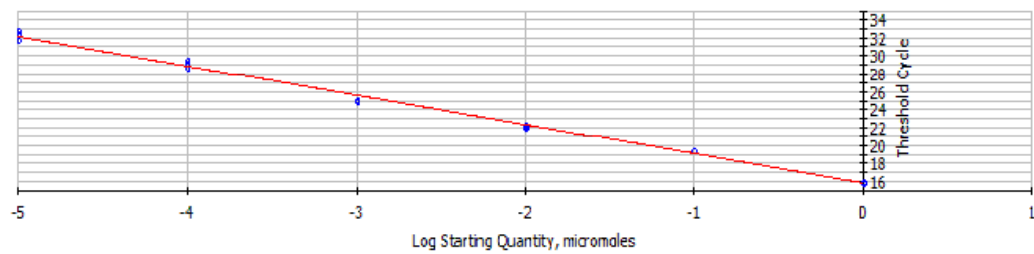


Amp/Cycle (top), Standard Curve (middle) and melting curve (bottom) for GAPDH.

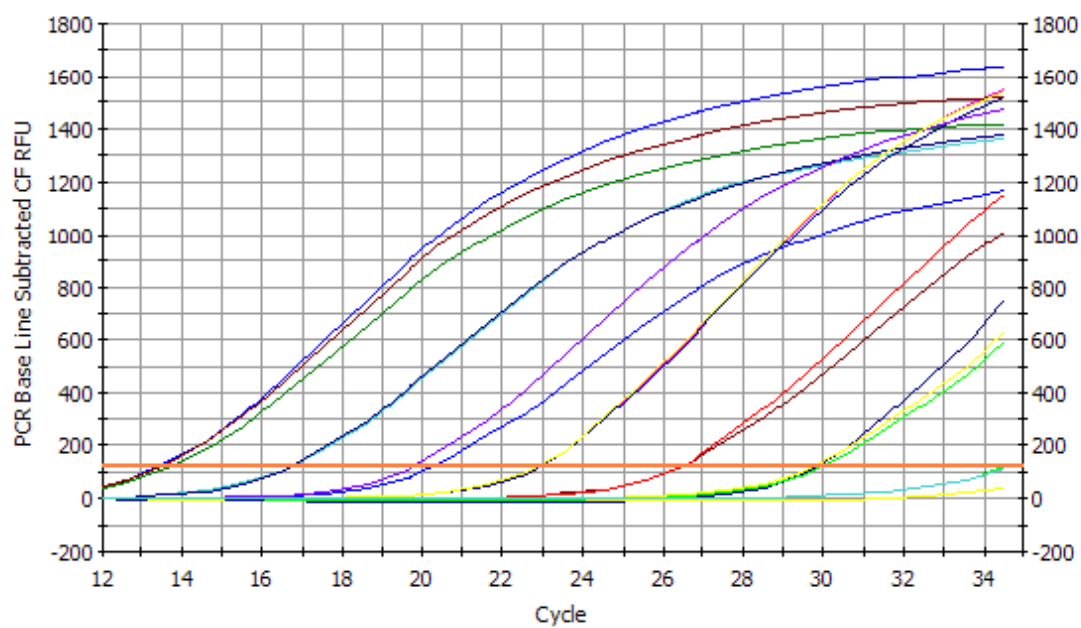


Correlation Coefficient: 0.998 Slope: -3.249 Intercept: 15.918 $Y = -3.249X + 15.918$
 PCR Efficiency: 103.2 %

Unknowns
 Standards

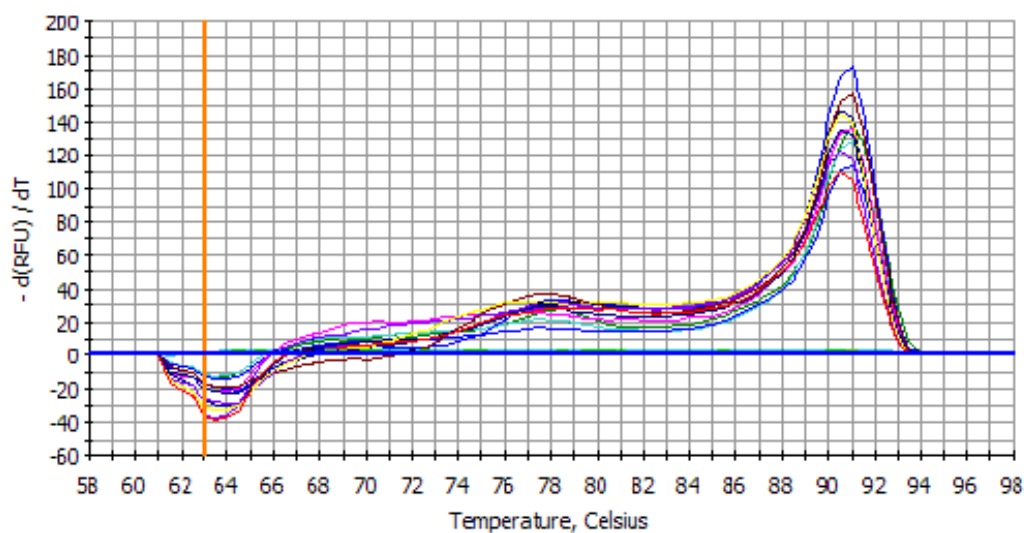
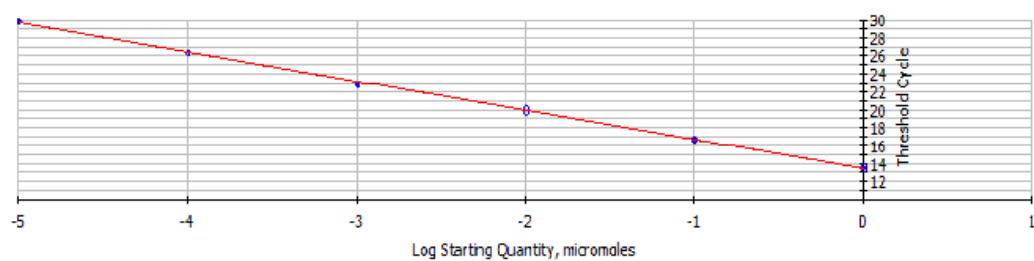


Amp/Cycle (top), Standard Curve (middle) and melting curve (bottom) for CPT1.

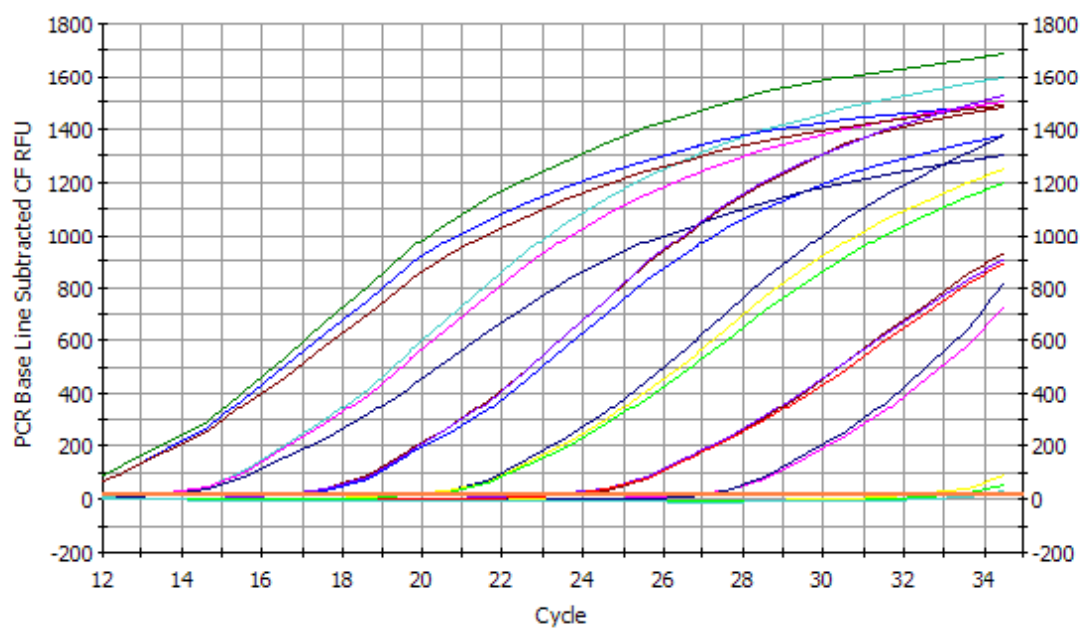


Correlation Coefficient: 0.999 Slope: -3.242 Intercept: 13.461 $Y = -3.242X + 13.461$
 PCR Efficiency: 103.4 %

Unknowns
 Standards

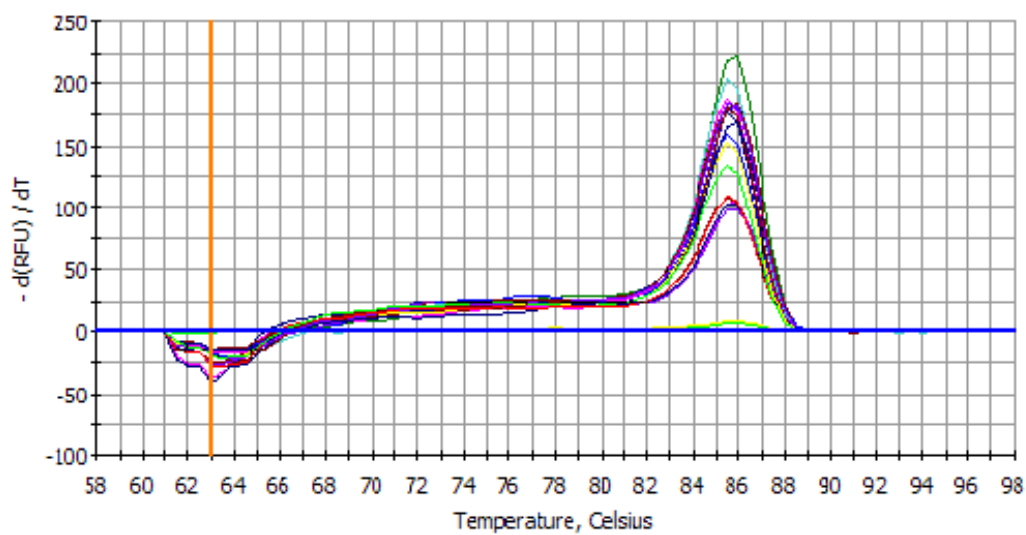
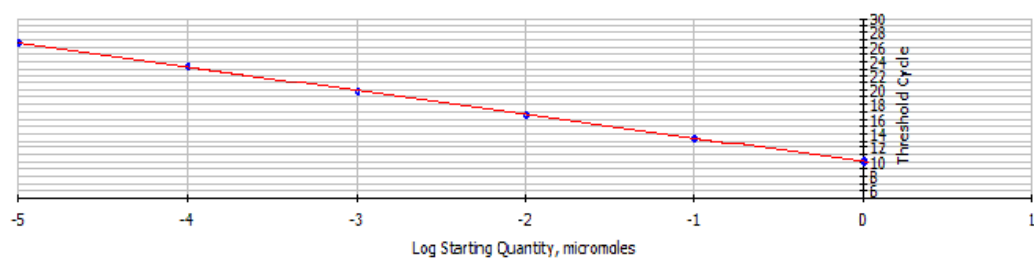


Amp/Cycle (top), Standard Curve (middle) and melting curve (bottom) for FADS2.



Correlation Coefficient: 1.000 Slope: -3.323 Intercept: 10.054 $Y = -3.323X + 10.054$
 PCR Efficiency: 100.0 %

Unknowns
 Standards



Amp/Cycle (top), Standard Curve (middle) and melting curve (bottom) for PPAR.

Appendix K. PCR Protocol

JumpStart™ REDTaq® ReadyMix™ Reaction Mix

Catalog number P0982 (Sigma-Aldrich Inc., St. Louis, MO)

1. Basic PCR reactions were conducted to validate each primer and for calibrator development using 50 µl reactions carried out in PCR tubes.
2. Each reaction consisted of 25 µl of JumpStart™ REDTaq® ReadyMix™, 2 µl of forward primer (10 mM), 2 µl of reverse primer (10 mM), 16 µl of water and 5 µl of sample cDNA (mRNA isolated from bovine liver).
 - a. Prepare master mixes when possible to avoid repeated pipetting and/or contamination.
3. Placed tubes into thermocycler with the following program:

Item	Temperature	Time
Hotstart	94°C	2 min
35 cycles:		
Denaturation	94°C	30 s
Annealing	61°C	30 s
Extension	72°C	1 minute
Final extension	72°C	5 min
Hold	4°C	indefinitely

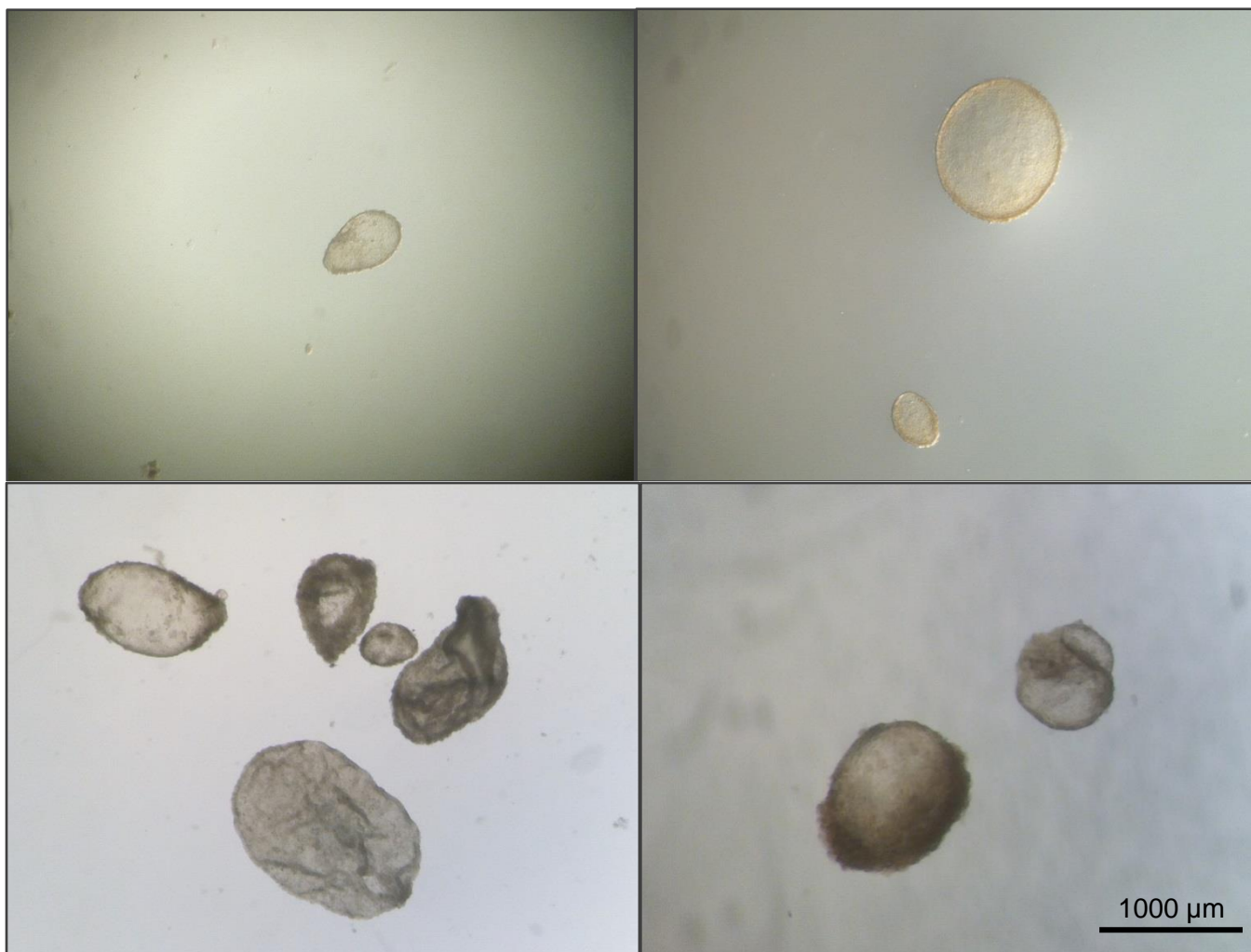
- a. Annealing temperature is specific to primers used.
4. Remove tubes from thermocycler and add 15 µl of each PCR product to a 2 % agarose gel containing ethidium bromide.
 5. Gels were evaluated under UV light using a BIO-RAD Universal Hood II. Bands for were compared with a 100 bp ladder to verify primer accuracy.

Appendix L. HEPES Talp Medium

Item	Product ID	Company	Amount
HEPES-TL			40 ml
BSA, Fraction V	A-4503	Sigma	120 mg
Na pyruvate (20 mM stock)			400 µl
Pen/Strep	15140-122	Gibco	200 µl

Appendix M. mSOF Medium

Item	Product ID	Company	Amount
SOF	IVL05-100ML	Caisson Labs	35.2 ml
FBS (5%)			2 ml
BME (essential a.acids)			800 µl
MEM (nonessential a. acids)			400 µl
Pen/Strep	15140-122	Gibco	400 µl
Glucose stock (50 µM)	G-7021	Sigma	1200 µl



Appendix N. Images of d-14 bovine embryos from *in vivo* survival assay in Chapter V (4X objective).



Brahman cow from Experiment 4.2 (Chapter IV) at the LSU AgCenter Reproductive Biology Center, St Gabriel, Louisiana.



Brahman cows from Experiment 4.2 (Chapter IV) heading toward the barn for their daily supplementation at the LSU AgCenter Reproductive Biology Center, St Gabriel, Louisiana.

VITA

Cody Bailey is the son of Gary and Val Bailey and younger brother to Cory Bailey. Cody was born and raised in Creston, Iowa and graduated from Creston High School in 2003. After high school, Cody attended Iowa State University where he received his B.S. in Agricultural Studies with minors in Animal Science and Agronomy in 2007. While at Iowa State, Cody was a member of the Alpha Gamma Rho fraternity, Eta Chapter.

Upon graduation from Iowa State, Cody interned at Trans Ova Genetics in Sioux Center, Iowa before beginning graduate studies at Oklahoma State University in the fall of 2007 under the guidance of Dr. Robert Wettemann. His M.S. research involved maintenance energy requirements of beef cows and estrus detection using rumen temperature in beef cows. He received his M.S. in Animal Science from Oklahoma State University in July 2009.

Cody moved to Baton Rouge, Louisiana in the fall of 2009 and entered the graduate program in reproductive physiology at Louisiana State University under Dr. Robert Godke. He is now a PhD candidate in reproductive physiology in the Department of Animal Sciences at Louisiana State University.