THE EFFECTS OF CONJUGATED LINOLEIC ACID ON SPERMATOGENESIS IN *DB/DB* MICE

by

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ABSTRACT

Reproductive function in human males is negatively correlated with obesity and diabetes mellitus (DM). Conjugated linoleic acid (CLA) has been shown to help increase insulin sensitivity and reduce adiposity in some animal models. Whether CLA influences obesity and DM associated infertility has not been studied. To investigate how obesity and DM influence male reproductive function and to determine whether CLA isomers are able to alter male fertility potential by improving spermatogenesis. Seven-week-old male obese and type 2 diabetic mice (*db/db*, n=40) were randomized to either an 8.5% (w/w) fat diet of *cis*-9, *trans*-11 CLA (0.4%, w/w), *trans*-10, *cis*-12 CLA or a control diet for 6 weeks. Lean (n=10) C57BLKS/J mice were fed a control diet and a paired-weight group received a restricted amount of control diet to match the body weight of the *trans*-10, *cis*-12 CLA group. Sperm counts, sperm morphology, testis lipids, including seminolipid, were analyzed. Genes affecting seminolipid synthesis and spermatogenesis were measured using q-RT-PCR. Obese and diabetic mice fed a control diet displayed significantly lower sperm counts ($p<0.0001$) and higher abnormal morphology ($p<0.0001$) than their lean counterparts. They also had significantly decreased *cst* ($p=0.003$) and *cgt* ($p=0.02$) gene expression, despite increased seminolipid concentration. Genes involved in spermatogenesis, including *Ccna*1, *Cdc*25*c* and *Hlf*3, were also significantly lower in the obese and diabetic mice in comparison to the lean control group. Both CLA isomers significantly increase normal sperm morphology ($p<0.0001$) to the level of the lean mice. In this study, neither CLA isomer was found into the testis triacylglycerols (TAG) or phospholipids (PL) and showed minor influences in the fatty acid composition of TAG and PL. The *trans*-10, *cis*-12 CLA isomer significantly

decreased seminolipid (per mg of lipid p=0.0351) to levels similar to the lean mice, and significantly increased *cgt* gene expression. Both CLA isomers also significantly increased *Ccna*1 gene expression (p=0.0002). These results indicate that seminolipid and genes associated with spermatogenesis play a role in DM and obesity induced reproductive dysfunction. Both dietary CLA isomers appear to be useful in increasing functionally viable sperm and therefore improve reproductive potential.

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8-OHdB	8-hydroxydeoxyguanosine
AA	arachidonic acid
ALA	α-linolenic acid
ANSA	4-amino-3-hydroxy-1-naphthalenesulfonic acid
ATP	adenosine triphosphate
BMI	body mass index
cgt	ceramide galactosyltransferase
CLA	conjugated linoleic acid
CPT	carnitine palmitoyl transferase
cst	cerebroside-3 sulfotransferase
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DM	diabetes mellitus
DPA	docosapentaenoic acid
DTA	docosatetraenoic acid
ED	erectile dysfunction
FSH	follicle stimulating hormone
GalEAG	galactosylalkylacylglycerol
GLA	γ-linolenic acid
GnRH	gonadotropin releasing hormone
HDL	high-density lipoprotein
HSL	hormone sensitive lipase

LA	linoelic acid
LDL	low-density lipoprotein
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MUFA	monounsaturated fatty acid
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PL	phospholipid
PPARα	peroxisome proliferator-activated receptor-α
PPARγ	peroxisome proliferator-activated receptor-γ
PUFA	polyunsaturated fatty acids
q-RT-PCR	quantitative reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
SAD	sagittal abdominal diameter
SAT	saturated fatty acid
Se	selenium
SHBG	sex hormone binding globulin
t-BOOH	tert-butylhydroperoxide
TAG	triacylglycerol
UCP	uncoupling protein
VLCFA	very long chain fatty acids
VLDL	very low-density lipoprotein

WHO	World Health Organisation
Zn	zinc

CHAPTER I

INTRODUCTION

Infertility is defined by Taber's Cyclopedic Medical Dictionary as the inability for a couple to conceive after one year of unprotected sexual intercourse (Venes and Taber, 2005). Infertility is a multi-factorial issue that is currently plaguing many North Americans. It affects approximately 15% of couples, with 40% of the cases being attributed to the male (Smith *et al.*, 2007). The etiology of male infertility is often multi-factorial: erectile dysfunction (ED), hypogonadotropic hypogonadism, retrograde ejaculation, abnormal semen production and hormone imbalances can be problematic on their own or in conjunction with each other. Furthermore, obesity, diabetes mellitus (DM), environmental toxins, and lifestyle factors can all influence male fertility.

As it is the site of steroidogenesis and spermatogenesis, the testis is central to the study of infertility. A unique lipid environment exists within the testis that may regulate its function. Recent understanding of lipid composition and function has been extended with the finding of seminolipid involvement in spermatogenesis. Since dietary lipid can influence membrane and tissue level, lipid composition and function, it is of interest to know if spermatogenesis can also be affected by dietary lipids, thereby improving infertility.

A review of the background related to male infertility will focus on testis function, lipid composition, and the effects of obesity and diabetes on fertility.

1. Issues in Male Fertility

Male infertility can arise from several different etiologies. ED, a mechanical etiology, is the inability for a man to achieve and maintain an erection (Jones and Lopez,

2006). While the incidence of ED increases with age, there are other confounding factors. As ED is correlated to microvascular issues commonly associated with related to glycemic control, 35-75% of diabetic men will experience some form of ED (McVary, 2008). ED is also found to directly correlate to body mass index (BMI) (Selvin *et al*., 2007). Retrograde ejaculation can be a cause of infertility for males, as the semen reverses through the urethra and enters the urinary bladder instead of exiting the penis (Jones and Lopez, 2006). Hypogonadotropic hypogonadism, another etiology in male infertility, is the diagnosis given to males that have decreased secretion of gonadotropic hormones, which will affect the hypothalamic-pituitary-testicular axis (Jones and Lopez, 2006). This leads to a cascade where testosterone and other androgen levels are insufficient for normal reproduction. This etiology is also commonly associated with obesity (Cohen, 1999).

Spermatogenesis, regulated by hormones, is central to fertility. Any shift in hormone levels, diet, or exercise can cause a change in semen quality and sperm morphology. Oligozoospermia, asthenozoospermia and azoospermia are three terms commonly used to describe semen analysis. Oligozoospermia is defined by the World Health Organization (WHO) as a semen concentration of less than $15x10^6$ sperm per millilitre of ejaculate, and azoospermia is defined as the absence of spermatozoa in the ejaculate (Cooper *et al.*, 2009). Asthenozoospermia is a semen sample where less than 32% of sperm exhibit forward progression, and less than 25% of sperm show rapid progression (Cooper *et al.*, 2009; World Health Organization, 1999). Sperm morphology is also important to semen analysis, and the WHO describes a man with abnormal sperm morphology as having less than four percent morphologically normal sperm (Cooper *et*

al., 2009). Many different abnormalities can occur in morphology, including abnormalities in the head neck or mid-piece, or tail (Kvist and Björndahl, 2002). These are reproductive challenges that need to be overcome for fertilization to occur. While the solution is not always obvious, some ways to improve the outcomes will be discussed.

2. Male Reproductive and Accessory Organs: Structure and Function

2.1. Testis

2.1.1. Structure

The testis is an oval shaped organ. Approximately 60-70% of its volume is occupied with seminiferous tubules, in which Sertoli and Leydig cells are housed (Bhasin and Jameson, 2008). These are the building blocks of the testis. Normal testicular volume is considered to be approximately 15-25 mL or 3 x 4 cm in humans (Smith *et al.*, 2007). Spermatogenesis is correlated to the size of the testis; a smaller testis size may be a sign of impaired spermatogenesis (Schorge and Williams, 2008), indicating that testis size is an important factor for normal sperm production.

2.1.2. Function

The testis has two major functions: steroidogenesis and spermatogenesis. Steroidogenesis is the synthesis of the commonly known 'male' hormones, androgens. The testis acts as an endocrine organ by secreting testosterone into the blood to be circulated to tissues, to stimulate growth and libido, among other things (Jones and Lopez, 2006). The testis acts as a paracrine organ by generating androgens to help regulate the second function of the testis, spermatogenesis. It is important to first understand how steroidogenesis and spermatogenesis are regulated before being able to answer problems surrounding fertility.

Gonadotropic releasing hormone (GnRH) is released from the hypothalamus and acts on the anterior pituitary to stimulate the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are two peptide hormones that are required for both steroidogenesis and spermatogenesis (Smith *et al.*, 2007).

2.1.2.1.Steroidogenesis

Androgens are synthesized from cholesterol in the Leydig cells of the testis. **Figure I-1** shows the metabolism of androgens within the testis. Once LH is released from the anterior pituitary, it proceeds to act on the Leydig cells to stimulate the production of testosterone (Spark, 1988). Steroidogenesis can occur through two different pathways, the first through the pregnenolone route, and the second through the progesterone route with the progesterone pathway being dominant (McPhee *et al.*, 2007). Insufficient steroidogenesis greatly affects fertility, as the testosterone functions in sexual responsiveness, libido, and spermatogenesis (McPhee *et al.*, 2007). Approximately 98% of circulating testosterone is bound to sex-hormone binding-globulin (SHBG) and albumin (Kronenberg and Williams, 2008). The Sertoli cells also secrete testicular fluid into the tubular cavity, in addition to androgen binding protein and inhibin (Jones and Lopez, 2006).

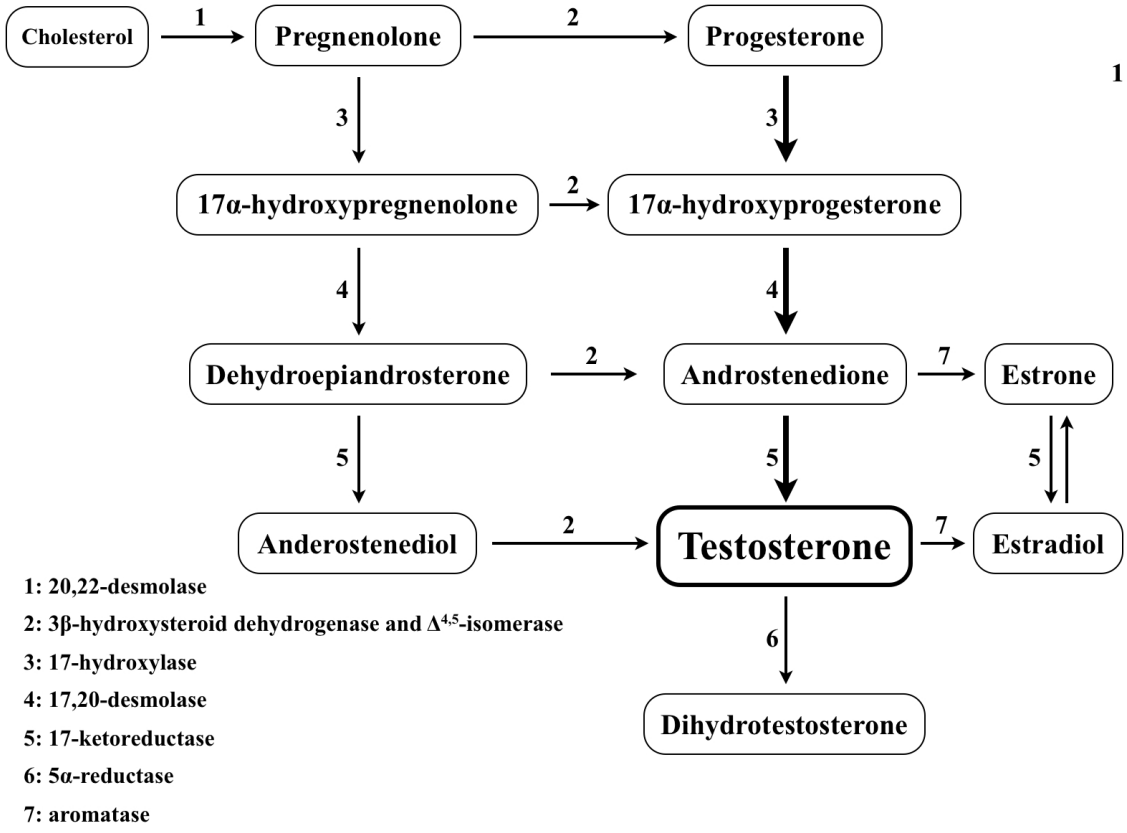

Figure I-1: Steroidogenesis – Production of Testosterone (Jones and Lopez, 2006).

2.1.2.2.Spermatogenesis

Spermatogenesis, the production of male gametes, begins at puberty. Spermatogenesis requires 65-75 days in humans (Jones and Lopez, 2006) and 34-38 days in mice (Oakberg, 1956) to proceed from the germ cell to spermatozoa. Prophase I requires the most amount of time in spermatogenesis at approximately ⅓ of the time in humans (Jones and Lopez, 2006), and ¼ of the time in mice (Oakberg, 1956). In order to ensure continual sperm production, a spermatogenic wave occurs whereby a new group of spermatogonia begin dividing every 16 days in humans (Jones and Lopez, 2006), and every 8-9 days in mice (Oakberg, 1956).

The spermatogonia are located between the Sertoli cells in the basal compartment (Widmaier, Raff and Strang, 2006), which ranges from the basement membrane to the tight junctions between the Sertoli cells that form the blood-testis barrier (Jones and Lopez, 2006). The initial step of spermatogenesis occurs when mitosis takes place in the prespermatogonium to yield a stem cell, and a type A spermatogonium (**Figure I-2**) (Kierszenbaum, 2007). The stem cells are used to initiate further cycles of spermatogenesis (Widmaier *et al.*, 2006).

The type A spermatogonium continues to divide, resulting in mitotic amplification, and after several amplification steps, the cells in the clone become type B spermatogonia (Kierszenbaum, 2007). The type B spermatogonia divide one more time and pass through the blood-testis barrier. The cells are no longer in direct contact with circulating blood, which protects the sperm from the body's immune response, as sperm beyond this point are considered foreign (Jones and Lopez, 2006). In the adluminal compartment, the cells are called primary spermatocytes (4N), and meiosis I is initiated (Kierszenbaum, 2007). After meiosis I is complete, the primary spermatocytes are now known as secondary spermatocytes (2N) (Kierszenbaum, 2007). There is one more division with meiosis II where the secondary spermatogcytes become spermatids (N), which are haploid (Jones and Lopez, 2006).

FSH acts on the Sertoli cells, and is required to complete sperm maturation (Burger and De Kretser, 1989). The spermatids then undergo spermiogenesis to become fully formed spermatozoa, but are not yet capable of fertilizing an ovum (Jones and Lopez, 2006). The final step is the phagocytosis of the residual bodies remaining after spermiogenesis, and is performed by the Sertoli cells (Kierszenbaum, 2007). There are

many factors that can influence spermatogenesis; therefore, any missed or altered step can hinder sperm morphology and quantity, leading to oligozoospermia, or asthenozoospermia and subsequently causing problems with fertilization.

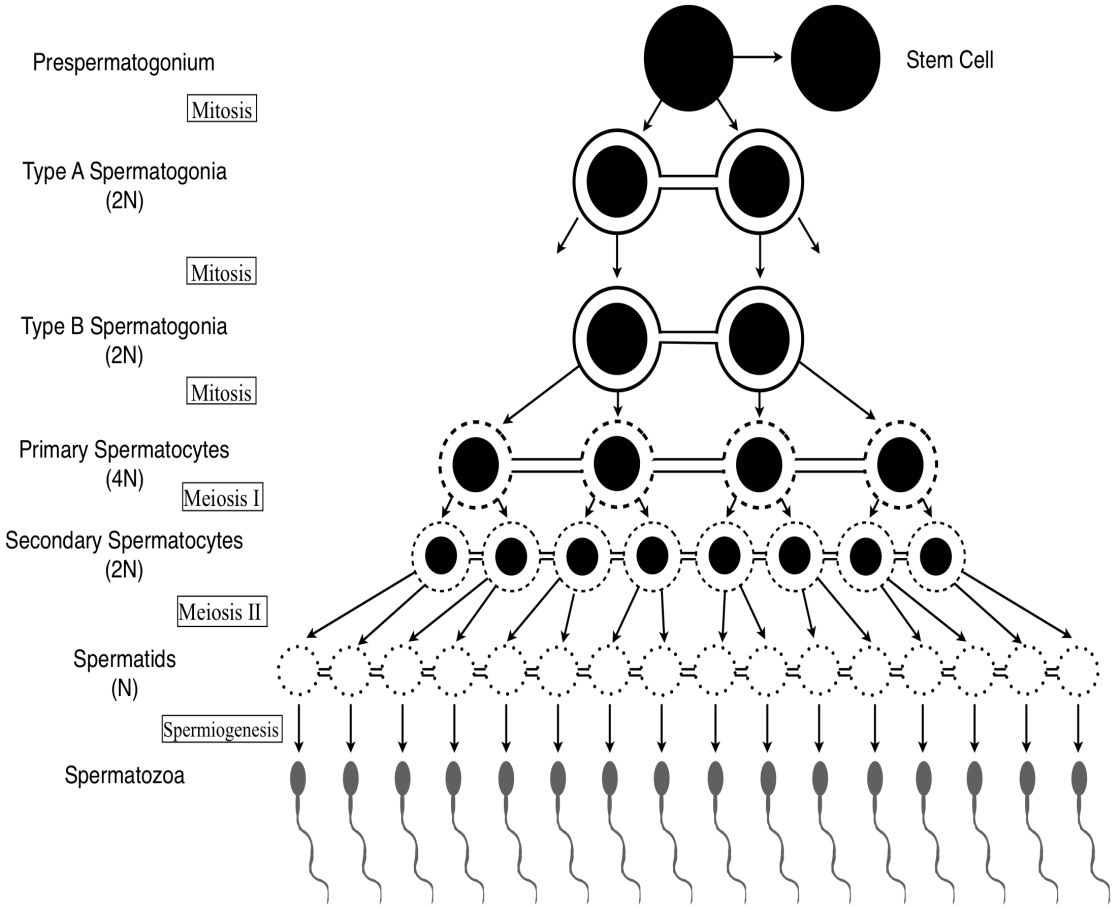

Figure I-2: Spermatogenesis in the seminiferous tubules.

2.2. Epididymis

After the sperm leave the seminiferous tubules of the testis, they enter and remain in the epididymis for at least 20 days (Jones and Lopez, 2006). While maturing in the epididymis passing through the caput, corpus and cauda, the sperm learn forward progression, and it is the secretions from the epididymis that stimulates this maturation process (Jones and Lopez, 2006). Furthermore, the fluids that initially surround the sperm are absorbed from the lumen, in order to concentrate the sperm (Widmaier *et al.*, 2006). Although spermatogenesis itself cannot falter here, if the epididymis is unable to properly concentrate the semen, oligozoospermia may result, despite normal spermatogenesis. Moreover, if the secretions are not adequate for sperm maturation, or if the epididymis is not long enough to give the sperm sufficient time to mature, this leads to sperm that are unable, or impaired in their ability, to swim toward the ovum.

2.3. Seminal Vesicles

The sperm then leaves the epididymis at ejaculation and enters the vas deferens where it eventually comes to the seminal vesicles. The seminal vesicle is responsible for approximately 2/3 of the seminal fluid. This fluid contains fructose that nourishes the sperm in the female system (Yen *et al.,* 2004), and vitamin C (Jones and Lopez, 2006), which is responsible for free radical scavenging. Smaller seminal vesicles or altered fluid components may mean that organ function is not optimal. Vitamin C is found in a higher concentration in the seminal plasma than in blood plasma (Lewis *et al.,* 1997). Therefore, vitamin C may function as an antioxidant to attenuate sperm cell death. In fact, low vitamin C in seminal plasma was associated with increased production of reactive oxygen species (ROS) in asthenozoospermic males (Lewis *et al.*, 1997).

Therefore, increased levels of ROS may cause oligozoospermia or azoospermia when vitamin C is inadequate in the seminal vesicle fluid. Furthermore, insufficient sugar may decrease the sperm virility after entering the female reproductive tract, due to decreased energy stores.

2.4. Prostate

The role of the prostate is to secrete an alkaline fluid that comprises 13-33% of the seminal fluid (Jones and Lopez, 2006). The excretion of the fluid is important for sperm survival in the acidic environment of the female genital tract (Jones and Lopez, 2006). In addition, the prostate is important in other aspects of sperm health. It aids in sperm nutrition as it contains a phosphatase that helps produce choline (Kumar and Majumder, 1995). As choline appears to be important in capacitation and the acrosomal reaction, it may be an important aspect of fertilizing an ovum (Zanetti *et al*., 2010). The prostate is also enriched in zinc (Zn), and it secretes Zn into the seminal plasma (Kumar and Majumder, 1995).

3. Lipid Composition in Testis and Sperm

3.1. Testis

The testis has a very unique lipid composition. It contains high polyunsaturated fatty acids (PUFAs), notably docosahexaenoic acid (DHA, C22:6(n-3)) in humans, and docosapentaenoic acid (DPA, C22:5(n-6)) in rodents. Approximately 8-24% of the lipid in the human testis is DHA (Bieri and Prival, 1965), and approximately 13-16% of total lipids (Bieri and Prival, 1965) and 22-25% of phosphatidylethanolamine (Merrells *et al*., 2009; Suh *et al*., 2011) in the rodent testis is DPA. From the time of birth to just after reproductive age, approximately 6 weeks, the amount of DPA in the rodent testis

increases significantly, indicating the necessity of DPA for spermatogenesis (Tam *et al*., 2008; Davis *et al*., 1966). DPA content also increases when rats are fed a diet rich in linoleic acid (LA, C18:2(n-6)) (Bieri *et al*., 1969), indicating that the lipid content of the testis can be altered based on dietary intake of lipids. The testes also include very long chain fatty acids (VLCFA) with carbon chain length longer than 22, in sphingomyelin and ceramide (Furland *et al*., 2007). Furthermore, they have sphingolipids, cholesterol, and seminolipids. Seminolipid is found only in the testis and spermatozoa, and therefore is thought to have a biological importance to spermatogenesis. Based on the lipid content and specific fatty acids found in the testis, it is reasonable to think that lipid can be a main determinant in normal testis development.

3.2. Seminolipids

Seminolipid (3-sulfogalactosyl-1-alkyl-2-acyl-*sn*-glycerol) is a major sulfoglycolipid in the mammalian testis and is associated with male infertility by controlling spermatogenesis. Seminolipid comprises approximately 3%, or 1.04 μmole/g of tissue, of the total lipids in spermatozoa (Ishizuka *et al.,* 1973). This is approximately three times higher than the amount of seminolipid found in the testis (Ishizuka *et al.*,1973). Although the quantity is lower, seminolipid still comprises 90% of the glycolipids found in the testis (Goto-Inoue *et al*., 2009). Seminolipid is synthesized from alkylacylglycerol in the testis. Ceramide galactosyltransferase (*cgt*) converts alkylacylglycerol to galactosylalkylacylglycerol (GalEAG). Cerebroside-3 sulfotransferase (*cst*) then converts GalEAG to seminolipid (**Figure I-3**). Seminolipid is required for sperm differentiation (Honke *et al.*, 2002), and if inadequate seminolipid is synthesized, spermatogenesis will not be able to proceed normally.

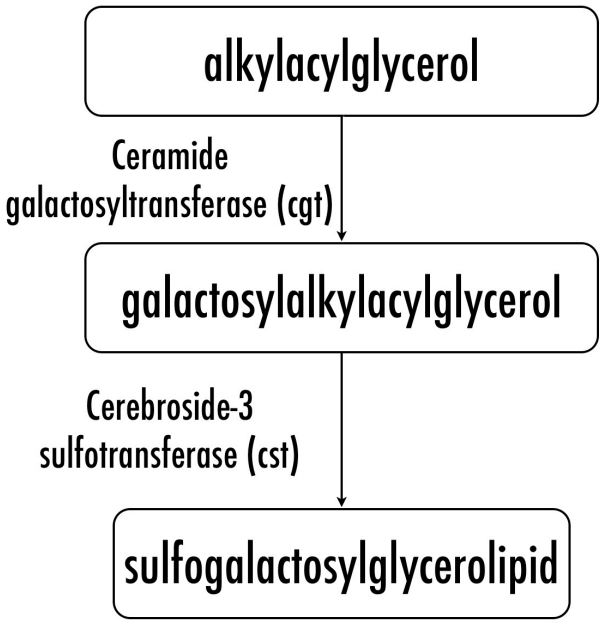

Figure I-3: Seminolipid Synthesis

3.3. Seminolipids in Spermatogenesis

Honke *et al.* (2002) used *cst* knockout mice (*cst*-/-), which have limited seminolipid production, to see how spermatogenesis was affected by the *cst* deletion. Semiquantitative RT-PCR showed that the absence of *cst* affected several the genes involved in the meiosis I of spermatogenesis (Honke *et al.*, 2002). *Hlf*3 and *Sprm*-1 were not affected by the *cst* deletion; however, *Ccna*1and *Cdc*25*c* were both significantly reduced (Honke *et al.*, 2002). *Ccna*1 is found just before the first meiotic division, which could indicate that meiosis I was not completed in a majority of the primary spermatocytes (Honke *et al.*, 2002). *Ccna*1 encodes the cyclin A1 protein that functions to phophorylate Cdc25 phosphatases, and helps to uregulate the cell cycle (Liu *et al.,* 2000). Reduced *Cdc*25*c*, expressed in the late pachytene to diplotene stage, would

perhaps indicate a problem in DNA tetrad formation (Honke *et al.*, 2002; Manipalviratn *et al*., 2007), but it still requires the phosphorylation of its protein by cyclin A1 proteins to become activated (Clarke *et al*., 1993). *Hlf*3 is expressed in the same time frame as *Cdc25c*, but was still expressed normally. This could indicate that there is another factor that helped upregulate *Hlf*3. Perhaps seminolipid is not required for spermatogenesis in the mid to late pachytene stage. *Prm*1 was not present in *cst*-/- mice (Honke *et al.*, 2002) indicating that there were problems in sperm chromatin condensation and chromatin stabilization, which is important for cell division to occur normally (Petronczki *et al*., 2003).

Histologically, *cst*-/- mice showed primary spermatocytes that had undergone early maturation arrest and not passed through meiosis I of spermatogenesis. Therefore the primary spermatocytes were multinucleated cells containing intact homologous chromosomes (Honke *et al.*, 2002; Kierszenbaum, 2007). Absence of secondary spermatocytes, reduced gene expression required for the progression of spermatogenesis. Finally, in *cst-/-* mice the absence of seminolipid from both the total and acidic fractions of the chromatogram (Honke *et al.*, 2002), presents strong evidence to point to the importance of seminolipid in spermatogenesis.

3.4. Sperm

Sperm also has a unique lipid composition. DHA comprises approximately 25-58% of the fatty acids (Aksoy *et al*., 2006; Nissen and Kreysel, 1983), and LA comprises approximately 5% of the fatty acids (Aksoy *et al.*, 2006). A few studies have shown that DHA and LA content of sperm is significantly reduced in the sperm of asthenozoospermic (Tavilani *et al*., 2007) and oligozoospermic (Zalata *et al*., 1998) men

in comparison to normozoospermic men. Therefore, the obvious conjecture is that insufficient intake or metabolism of essential fatty acids may hinder male fertility due to inadequate amounts of the fatty acids in the spermatozoa. Moreover, as asthenozoospermia is an issue of sperm movement, and is associated with reduced sperm DHA levels (Tavilani *et al.*, 2007), perhaps DHA is required to ensure sperm tail membrane fluidity. In monkeys, and presumably other species, DHA was found to be significantly higher in the sperm tail versus the sperm head based on 19.6% of total fatty acids in the sperm tail versus 1.1% of the total fatty acids in the sperm head (Connor *et al*., 1998). This large difference would suggest that DHA is involved in sperm movement. In rats, spermatids have a significantly higher proportion of DPA than do the spermatocytes (Beckman et al., 1978), which suggests an increased requirement of DPA during the sperm maturation process.

4. Factors Affecting Testis Function

4.1. Obesity

Obesity is becoming an increasingly large problem in North American society. The WHO defines a person as being overweight or obese when their BMI ≥25 kg/m^2 or ≥30 kg/m^2, respectively (World Health Organization, 2000). In 2004, 23.1% of adult Canadians were obese and 36.1% were overweight, and the prevalence is rising (Tjepkema, 2005). In the male adult population, 42.0% were overweight and 22.9% were obese (Tjepkema, 2005). Being obese is one risk factor for infertility in men that causes poor semen quality and decreased sperm number (Jensen *et al.,* 2004). This can be a result of changes in lipid metabolism (Suh *et al.*, 2011), or hormone fluctuations (Cohen,

1999; Chavarro et al., 2009), but obesity can also lead to ED (Selvin *et al.*, 2007), which is another cause of infertility in males.

4.1.1. Characteristics of Obese Testes and Accessory Sex Organs

Male reproductive organ size in *fa/fa* Zucker rats is significantly different from the size in the wild type rats (Suh *et al.*, 2011). This could indicate impaired organ function, and therefore decreased fertility.

Obese *fa/fa* Zucker rats showed a higher occurrence of unbalanced testes leaving one testis underdeveloped in comparison to the lean counterpart (Suh *et al.*, 2011). As seminiferous tubules comprise the majority of the testis volume (Bhasin and Jameson, 2008), the extent of spermatogenesis likely correlates with testis volume. Human studies investigating the volume of the testis in obesity have found that an increased BMI is not associated with a decrease in testis size (Jensen *et al.*, 2004; Denzer *et al*., 2007).

Fatty acid analysis, using gas chromatography, showed that while all other fatty acid levels were comparable, DPA content was significantly lower in the underdeveloped testis than in the normal testis (Suh *et al.*, 2011). Since DPA is required for normal spermatogenesis in rodents (Davis *et al.*, 1966), decreased DPA levels could be one cause of underdevelopment. Furthermore, the underdeveloped testes and normal size testes of *fa/fa* Zucker rats showed reduced *cst* and *cgt* gene expression, which results in diminished seminolipid levels (Kim *et al,*, unpublished data). As seminolipid levels are correlated to spermatogenesis, this decrease in testis size may be due to decreased seminiferous tubule volume resulting from impaired spermatogenesis (Honke *et al.*, 2002). Combining the low DPA levels with the low seminolipid levels, it would be expected that spermatogenesis will be impaired, and perhaps cause oligozoospermia.

The shortened epididymis found in the *fa/fa* Zucker rat in comparison to the lean rat (Suh *et al.*, 2011) may not allow sufficient time for the concentrating effects of the epididymis, or perhaps the sperm will not acquire the full extent of forward progression. This could imply that the sperm leaving the epididymis of the obese rat would not have the extent of forward progression or have as highly concentrated semen as the lean control. These two issues can perhaps hinder egg fertilization by decreasing sperm concentration and motility, possibly to the extent of asthenozoospermia and oligozoospermia, respectively.

The seminal vesicle is smaller in the obese rat in comparison to the lean rat (Kim *et al.*, unpublished data). Decreased volume of seminal vesicle secretions may include a decrease in vitamin C or fructose amount, leading to increased ROS production and reduced energy stores for the sperm. Perhaps inadequate secretion from the smaller seminal vesicle will impair sperm survival in the female reproductive tract.

The *fa/fa* Zucker rat had an enlarged prostate in comparison to the lean rat (Kim *et al.*, unpublished data). This was also found to be true in humans, that as BMI or waist circumference increases, so does the size of the prostate (Park *et al*., 2009). An enlarged prostate may lead to or be indicative of prostate cancer or of hyperplasia (Jones and Lopez, 2006), therefore, obesity in itself may be a risk factor in prostate cancer. Men who have prostate cancer or other prostate issues are more likely to have ED (Selvin *et al.*, 2007), which hinders sperm transport.

4.1.2. Hormone Imbalance

Studies have shown that the hormone profile changes when a person is obese. In obese men who were experiencing infertility, circulating levels of total testosterone,

SHBG, FSH and inhibin were all significantly lower than in lean counterparts (Chavarro *et al.*, 2009). In contrast, estradiol, free androgen and LH increase in obesity (Chavarro *et al.*, 2009). Testosterone and estradiol imbalances are possibly due to changes in steroidogenesis. Aromatase converts androstenedione and testosterone to estrone and estradiol, respectively. In obesity, aromatase activity increases in adipocytes, hepatocytes, and the Leydig cells, leading to an increase in testosterone-to-estradiol conversion (Cohen, 1999). This conversion is directly related to the extent of obesity (Schneider *et al*., 1979).

If adiposity decreases, less testosterone would convert to estradiol, causing an increase in serum testosterone levels and a decrease in serum estradiol. In fact, after weight loss, circulating total testosterone, free testosterone, and SHBG levels increase (Jensen *et al.*, 2004), which may be due to a decrease in aromatase activity.

Hypogonadotropic hypogonadism may be the cause of the hormone imbalances associated with obesity. The increase in fat mass is the basis for decreased GnRH and LH response to low testosterone levels (Cohen, 1999). Although adiposity can result in low circulating testosterone levels, the reverse is true in that low levels of testosterone can cause adiposity (Cohen, 1999). A subsequent increase in aromatase and estradiol levels that further depress LH levels (Cohen, 1999), and propagate the cycle. If these testosterone levels are inadequate for normal spermatogenesis, oligozoospermia may result from decreased sperm production, and would appear approximately 3 months later.

4.1.3. Semen Profile

The majority of studies to date have indicated a relationship showing a negative correlation between BMI and semen quality in humans (Jensen *et al.*, 2004). Sperm

concentration and total sperm count decreased as the BMI increased. The inverse correlation between BMI to spermatogenesis may be from decreased seminolipid or testosterone levels, but this has not been tested. However, they did note that sperm morphology and motility were normal (Jensen *et al.*, 2004). Although the epididymis was shown to be shorter in obese *fa/fa* Zucker rats (Suh *et al.*, 2011), motility was still normal, most likely indicating that the sperm had forward progression in this human study (Jensen *et al.*, 2004).

4.2. Diabetes Mellitus

Type 2 Diabetes Mellitus (DM-2) is related to lifestyle issues, such as obesity, poor diet, and lack of exercise (Runge *et al*., 2009). In this case, the β-cells are overworked, and become enlarged to ensure enough insulin is being produced to normalize blood glucose levels. If this occurs for a prolonged period of time, the body's cells become insulin-resistant due to the constant abnormally high levels of circulating insulin (Runge *et al.*, 2009). Fertility issues for male patients with DM can include: ED, impaired semen parameters, and hormone imbalances.

4.2.1. Characteristics of Diabetic Testes and Accessory Sex Organs

Hassan *et al.* (1993) found that both the epididymis and testis were lower in weight in Sprague Dawley rats treated with streptozotocin than in the control rats. This may be related to neuropathy, as 60.0% of patients with DM-1 and 57.3% of patients with DM-2 had a reduced testis size associated with neuropathy (Ali *et al*., 1993). These studies, and many others, show that there is a discrepancy in the gross anatomy of male reproductive organs between normal and diabetic males. In the same way that a smaller testis and epididymis in the obese male can produce reproductive problems, so can these

organ sizes in the diabetic male, although the etiology may be different. Insulin is required for spermatogenesis (Shirneshan *et al*., 2008). Therefore, insulin receptor insensitivity to the high levels of circulating insulin may influence testis size resulting in reduced sperm production in DM-2. As this is often the case in DM-2, especially when the disease is not monitored closely, insulin insensitivity may also cause spermatogenesis to be impaired such that premature germ cells are released in the seminiferous tubules, or that there is full germ cell degeneration (Shirneshan *et al.*, 2008).

4.2.2. Hormone Levels

Since insulin is involved in spermatogenesis, it is also likely that it is involved in steroidogenesis. In patients with metabolic syndrome experiencing infertility, decreased insulin sensitivity was strongly correlated to a decrease in circulating SHBG levels (Niskanen *et al*., 2004). Furthermore, in streptozotocin-induced diabetic rats, plasma testosterone levels were significantly decreased (Hassan *et al.,* 1993; Scarano *et al*., 2006). Weight loss increased circulating SHBG and total testosterone levels, indicating that a decrease in circulating insulin and an increase in insulin sensitivity was able to increase the SHBG and total testosterone levels (Niskanen *et al.*, 2004).

4.2.3. Semen Profile

Studies show that DM influences sperm quality. The sperm number in the testis, caput and corpus and the cauda of the epididymis were significantly reduced in streptozotocin-induced rats (Shirneshan *et al.*, 2008; Hassan *et al.*, 1993; Scarano *et al.*, 2006). Sperm motility was also significantly decreased in diabetic rats and mice (Shirneshan *et al.*, 2008; Hassan *et al.*, 1993; Scarano *et al.*, 2006), as well as a decrease in progressive ability of the sperm (Shirneshan *et al.*, 2008), but sperm morphology was

not affected (Scarano *et al.*, 2006). On the other hand, a human study found that the sperm count was in fact higher in diabetic men than in the control group, whereas the number of abnormal sperm increased in men with DM, although the increase in sperm abnormalities was not statistically significant (Vignon *et al*., 1991). The increase in round spermatid concentration indicates the release of premature germ cells (Vignon *et al.*, 1991), meaning that spermatids did not complete spermiogenesis to become functional spermatozoa (Kvist and Björndahl, 2002).

The studies discussed above are inconclusive, and very few human studies have been done to elucidate the relationship between the semen profile and DM. Based on these studies, however, it appears that low sperm counts and decreased sperm motility contribute to reduced fertility in diabetic males.

4.2.4. Oxidative stress

DM induces a greater amount of oxidative stress in the body (Baynes and Thorpe, 1999). Therefore, the spermatids in men with DM are especially susceptible to this oxidative stress because the spermatids require an extra twenty days in the epididymis to mature (Jones and Lopez, 2006). In addition, spermatids are susceptible to ROS because they have high unsaturated fatty acid content (Ben Abdallah *et al.,* 2009), and they do not contain repair mechanisms for damaged DNA (Mallidis *et al*., 2009). When the PUFAs are attacked by ROS, membrane fluidity decreases, and this impairs normal sperm motility (Agbaje *et al*., 2007). This reduces the sperm's ability to swim to the egg, or if it reaches and fertilizes the egg, the DNA might be damaged to the extent that it impairs DNA replication, leading to decreased zygote quality (Lewis and Aitken, 2005). Asthenozoospermic men had an increased malondialdehyde (MDA) level 1.5 times

higher in the semen than normozoospermic males (Tavilani *et al.*, 2007). As MDA is a measurement of lipid peroxidation, and the PUFA with the highest concentration in the sperm is DHA, it is likely that the DHA is being oxidized, leading to decreased sperm motility (Tavilani *et al.*, 2007). Sperm motility was decreased in men with DM (Scarano *et al.*, 2006), and this is perhaps indicative of asthenozoospermia that is present in diabetic men that is propagated by the oxidation of PUFA in the sperm tail.

There is a cause for concern that the oxidative stress associated with DM will in turn impair the functions of the sperm tail. This could be one mechanism that explains the infertility that is caused by DM. Studies in the past have looked at how antioxidants affect semen quality, and have determined that antioxidants such as selenium (Shalini and Bansal, 2008), or vitamin D (Hamden *et al.,* 2008) significantly reduced oxidative stress in the semen parameters due to free radical scavenging.

4.3. Environmental Toxins

Lead has been shown to have detrimental effects on male reproductive function. Lead exposure is directly correlated to the amount of lead found in the blood plasma, but seminal plasma was only slightly increased (Kasperczyk *et al*., 2008). Furthermore, the plasma lead and cadmium concentrations are directly correlated to circulating MDA and 8-hydroxydeoxyguanosine (8-OHdG) concentrations (Kasperczyk *et al.*, 2008; Xu *et al*., 2003). As MDA and 8-OHdG are markers for oxidative stress, increased circulating lead and cadmium levels result in increased oxidative stress. While lead did not enter the seminal plasma to directly affect the sperm, there was a strong negative correlation between sperm motility and MDA (Kasperczyk *et al.*, 2008). Therefore, the increase in oxidative stress produced by the lead might have been the cause for the decreased

motility. However, with low doses of lead exposure, 8-OHdG in sperm DNA increased, but there was no effect on sperm concentration, morphology or motility (Xu *et al.*, 2003). Seminal plasma cadmium, on the other hand, was negatively correlated with total sperm count and sperm motility (Xu *et al.*, 2003). Therefore, environmental toxins such as lead and cadmium cause increased oxidative stress that may lead to oligozoospermia and asthenozoospermia.

4.4. Lifestyle and Dietary Influences

Although attributed to different molecules, recreational drugs are associated with reduced fertility. People who are current or former smokers are more likely to experience ED, than those who do not smoke (Selvin *et al.*, 2007). This is most likely due to the vasoconstrictive effects of nicotine (Widmaier *et al.*, 2006). As vasodilation is crucial for erection, a substance that induces the opposite effect would lead, at least temporarily, to ED. Furthermore, smokers are more likely to have a lower intake of ascorbic acid, which strongly correlates to the seminal plasma levels of ascorbic acid (Fraga *et al*., 1991). This would potentially decrease the antioxidant capacity of the seminal plasma to fight ROS (Michael *et al*., 2008).

Ethanol consumption has produced mixed results in regard to semen analysis (Anderson *et al*., 1983). Any dose of ethanol showed a decrease in sperm motility (Anderson *et al.*, 1983), and perhaps to the extent of asthenozoospermia. While low doses of ethanol over a short time frame improved sperm concentration, the longer ingestion period of 20 weeks in mice showed that there was a drastic decrease in sperm concentration (Anderson *et al.*, 1983). Furthermore, by increasing the dose over a short time frame, the sperm concentration also declined drastically, and the number of head

and tail abnormalities increased significantly (Anderson *et al.*, 1983). After a single dose of methamphetamine, DNA laddering, seminiferous tubule degradation and apoptosis occurred in the testis, signifying the detrimental effects this drug can have on reproductive function (Yamamoto *et al*., 1999).

Exercise is another factor important for male fertility. Frequency and extent of physical activity is inversely correlated to ED, and is 'dose-dependent' (Selvin *et al.*, 2007). Furthermore, the longer amount of time spent in front of a computer or television was strongly correlated to the increase in ED (Selvin *et al.*, 2007). However, too much exercise can also pose a risk to male fertility. While the decrease in total sperm count and sperm concentration after moderate intensity exercise was negligible, the decrease in these parameters after high intensity exercise was considered to be statistically significant (Safarinejad *et al*., 2009). Testosterone levels are statistically lower at baseline and for the duration of the high intensity exercise and moderate intensity exercise, but were found to normalize during the recovery period (Safarinejad *et al.*, 2009). This shows that a moderate level of daily exercise is likely beneficial for the reduction of ED, and for ensuring the maintenance of normal semen parameters.

Finally, dietary factors play a large role in fertility. Zn has been shown to be important in male virility. While the percentage of body weight of the testis and epididymis was statistically higher in Zn deficient rats, the actual weight of the organs were statistically lower than the control rats, which would still indicate reproductive problems (Merrells *et al.*, 2009). Zn deficient rats showed a decrease in morphologically normal sperm, and an increase in the number of head abnormalities (Merrells *et al.*, 2009). Head abnormalities are an issue for fertilization potential, as a specific head

conformation is required (Jones and Lopez, 2006). Furthermore, DPA was significantly decreased in Zn deficient rats in comparison to their control counterparts. However, Zn supplementation greatly improved the quantity of DPA found in the testis (Merrells *et al.*, 2009).

Other nutrients have also been found to be beneficial to semen parameters. Selenium (Se) concentration in seminal plasma showed a positive correlation between total sperm count, motility and sperm viability (Xu *et al.*, 2003). Mice fed a Se deficient diet for 4 or 8 weeks have a decrease in sperm motility, and sperm concentration sperm and an increase in MDA, indicating increased lipid peroxidation in the testis (Shalini and Bansal, 2008). However, Se fed in excess of that required also showed to have reduced sperm motility and increased MDA levels (Shalini and Bansal, 2008). This could indicate that Se in excess of what is required may act as a pro-oxidant (Shalini and Bansal, 2008).

It was previously mentioned that the testis has a unique lipid environement. As such, the use of dietary lipids as a functional food is another way in which to modulate the testis environment. Male rats fed rapeseed oil for 3 weeks were found to have a significant increase in the activity of 17β-hydroxysteroid dehydrogenase in comparison to the being fed the rapeseed oil for 1 week (Gromadzka-Ostrowska *et al*., 2002). The intake of saturated fatty acids is also negatively correlated with 17β-hydroxysteroid dehydrogenase activity (Gromadzka-Ostrowska *et al.*, 2002), which indicates that testosterone production is inversely associated with saturated fatty acid intake. Furthermore, when male boars were fed a diet rich in eicosapentaenoic acid (EPA) and DHA, the levels of sex hormones fluctuate, indicating a dependence on diet (Castellano

et al., 2011). Tuna oil was found to decrease both testosterone and estradiol, whereas menhaden oil was found to increase both testosterone and estradiol (Castellano *et al.*, 2011). However, dietary means cannot always improve fertility. One study showed that despite increased DHA incorporation into the sperm of DHA-supplemented humans, there was no improvement in sperm parameters, including sperm concentration, total sperm count and sperm motility (Conquer *et al*., 2000).

5. Conjugated Linoleic Acid (CLA)

5.1. Structure, Metabolism and Absorption

Conjugated linoleic acid (CLA), synthesized by the *Butyrivibrio fibrosolvens* bacteria present in ruminants, is a conjugated diene of LA (Churruca *et al*., 2009). This means that one single bond separates the two double bonds in CLA instead of the two single bond separation in LA. There are two major forms of CLA: *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, the former being synthesized naturally by the bacteria, and the latter synthesized synthetically (**Figure I-4**).

CLA and LA have similar metabolism; however, the metabolites of CLA are generally conjugated (Banni *et al*., 2001). CLA may produce γ-linolenic acid (GLA, C18:3n-6), dihomo-γ-linolenic acid (DGLA, C20:3n-6), arachidonic acid (AA, C20:4n-6) and docosatetraenoic acid (DTA, C22:4n-6) (**Figure I-5**). CLA may also be elongated to form very long chain fatty acids, although this has not been experimentally demonstrated.

CLA can be incorporated into triacylglycerols (TAG), phospholipids (PL), and other compounds in the body's tissues. Therefore, some digestion might be required prior to absorption. After ingestion, CLA is incorporated into micelles that help the absorption process, and is subsequently absorbed into the enterocytes by diffusion

(Widmaier *et al.*, 2006). Once inside the enterocyte, CLA and other fatty acids are re-esterified into TAG and integrated into chylomicrons for transport throughout the body (Widmaier *et al.*, 2006). Analysis of absorption showed that CLA in the form of TAG was 93% absorbed, and absorption did not differ from LA over a time period of 24 hours (Tsuzuki and Ikeda, 2007). However, CLA was absorbed more slowly than LA during the initial stages of absorption (Tsuzuki and Ikeda, 2007).

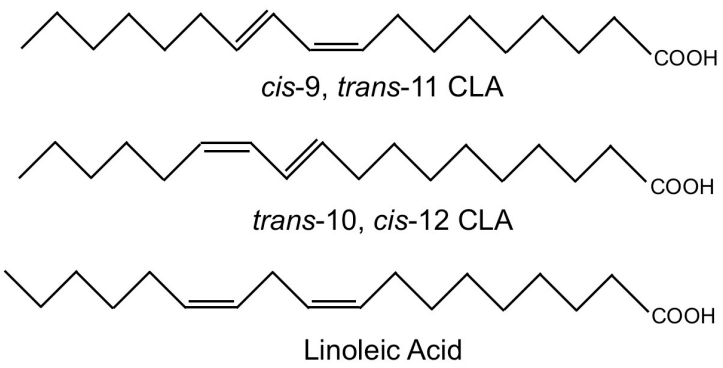

Figure I-4A: Linoleic acid and conjugated linoleic acid isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12.

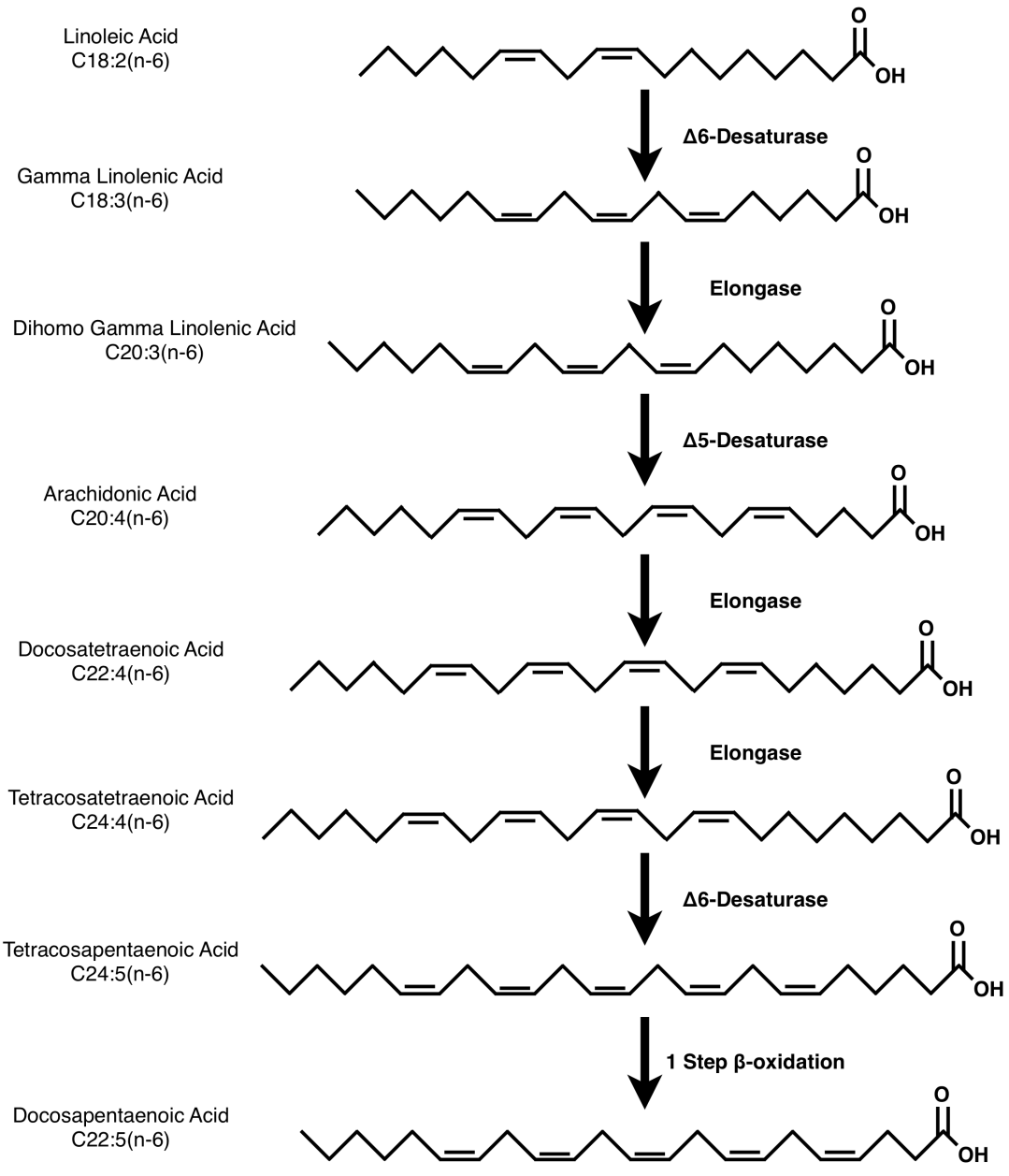

Figure I-4B: Linoleic Acid Metabolism

5.2. Transport and Tissue Distribution

Chylomicrons act as vessels to carry all types of lipids, including CLA. They are released from the enterocyte into the lymph system. The lymph system connects with the circulatory system at the thoracic duct, which then acts as the transport mechanism for chylomicrons to transport CLA to the tissues (Widmaier *et al.*, 2006). CLA transportation after initial delivery to tissues occurs through the use of lipoproteins (Widmaier *et al.*, 2006). Lipids are likely sent to the testis specifically by high-density lipoprotein (HDL) rather than low-density lipoprotein (LDL) or very low-density lipoprotein (VLDL), as the testis has an HDL receptor (Coniglio, 1994).

CLA can be found throughout the body. In human plasma, CLA is found to be 0.19% and 0.14% of total fatty acids for *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, respectively (Zlatanos *et al*., 2008). This level was found to increase if the subjects were supplemented with *trans*-10, *cis*-12 CLA capsules (Zlatanos *et al.*, 2008). In Atlantic salmon, supplementation with CLA showed a dose dependent effect of CLA incorporation into the liver, as well as the flesh (Leaver *et al*., 2006). CLA is normally found in the liver of Sprague-Dawley rats at approximately 2.5 μg/mg lipid, but that with CLA supplementation, this value increased to 15.7 μg/mg lipid (Banni *et al.*, 2001). Moreover, obese *fa/fa* Zucker rats showed that incorporation of CLA was significantly reduced in comparison to their lean counterpart in the PL fraction by 1/2, and in the TAG fraction at approximately 2/3 (Noto *et al*., 2006). CLA is mostly (~86%) incorporated into neutral lipids of the tissues with a small proportion in phospholipids (Banni *et al.*, 2001). CLA supplementation slightly decreases the proportion of CLA in the neutral lipid fraction, and increases the CLA content of the phospholipid fraction (Banni *et al.*,

2001). More importantly, CLA exists in human testes as well as the testis sub-cellular fractions in an amount of 0.2-0.35% (w/w) (Hoffmann *et al*., 2005). While studies have not been done to show how this concentration changes with respect to dietary influence, previous studies showing increased incorporation into other tissues and plasma with supplementation would suggest that CLA supplementation would also increase the amount of CLA found in the testis.

5.3. CLA and Diabetes

The studies that have investigated the effects of CLA on insulin sensitivity have shown mixed results. In *fa/fa* Zucker rats, a 1.5% (w/w) CLA mixture diet was shown to significantly improve insulin sensitivity, though it was not increased to the level of the lean controls (Noto *et al*., 2007). Furthermore, CLA has shown to reduce hyperinsulinemia associated with DM-2 in the Zucker Diabetic Fatty rat model (Nagao *et al*., 2003). In humans, the insulin sensitization effect of CLA was not found. In a study where a CLA mixture and *trans*-10, *cis*-12 CLA were studied independently to determine their effects on men with metabolic syndrome, *trans*-10, *cis*-12 CLA decreased insulin sensitivity and increased plasma glucose compared to the placebo, and increased plasma insulin and HbA_{1c} compared to baseline (Riserus *et al*., 2002). This indicates that instead of anti-diabetic effects, CLA may enhance the insulin resistance in metabolic syndrome and possibly promote progression to diabetes.

When compared to other PUFA, CLA was the only fatty acid that resisted oxidative stress 48 hours and 7 days after a tert-butylhydroperoxide (t-BOOH) cytotoxicity test (Arab et al., 2006). The theory behind CLA resistance to oxidative stress was that it was shown to interact with PPARγ, causing an increase in glutathione

production, which subsequently attenuated oxidative stress (Arab *et al.*, 2006). If this is the case in human fibroblasts, it is very likely that CLA can also have an effect in the testis, attenuating oxidative stress associated with diabetes. While CLA is not a free radical scavenger, providing the DM male with CLA may prove beneficial, as it is more resistant to lipid peroxidation due to its conjugated double bond system (Schmidt, 2000). If CLA has the ability to be incorporated into sperm tails, then perhaps it may be able to attenuate the lipid oxidation occurring in the sperm during its transit through the epididymis.

5.4. CLA and Obesity

The dose effect of CLA on mice was determined by feeding 0.15g or 0.20g CLA per kilogram body weight (Parra *et al*., 2009). The CLA contained an equal mixture of both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. The group fed 0.20g CLA/kg body weight had a 35% decrease in adipose tissue weight with respect to the control group (Parra *et al.*, 2009).

CLA maintained lean body mass during energy restriction or weight loss (Park *et al*., 2007). One of the treatment groups received a CLA mixture-*ad libitum* (0.5% CLA) and the other a CLA-energy restricted (0.7% CLA) diet (Park *et al.*, 2007). These two groups, along with the control-energy restricted diet group had statistically similar weights at the end of the experiment, but they was statistically different from the control-*ad libitum* group (Park *et al.*, 2007). The CLA-*ad libitum* group had a reduced food intake in comparison to the control-*ad libitum* group (Park *et al.*, 2007). As CLA takes longer to absorb than LA, this perhaps is one mechanism for the reduced food intake, and

subsequently the weight loss. Furthermore, lean mass was maintained, so the weight loss correlated to reduced fat accumulation and reduced existing fat (Park *et al.*, 2007).

Triacylglyceride (TAG) accumulation was reduced in adipocytes, but the correlation to reduced TAG accumulation was only found with the *trans*-10, *cis*-12 CLA isomer *in vitro* (Brown and McIntosh, 2003). In hamsters, although there was a reduction in the number of adipocytes, no statistical significance was found with the *trans*-10, *cis*-12 CLA treatment group, indicating that no body fat reduction or reduction in body fat accumulation occurred (Lasa *et al*., 2008). However, CLA may require a longer time frame to take effect, as those studies that have stated that CLA is incorporated into tissues have lasted for anywhere from 4 weeks to 6 months, and therefore may require more time to show biochemical results (Banni *et al.*, 2001; Zlatanos *et al.*, 2008; Leaver *et al.*, 2006; Noto *et al.*, 2006). As the study was 3 weeks in duration, it may be appropriate to increase the time allotted to see an effect (Lasa *et al.*, 2008).

In a randomized controlled trial with obese human males ingesting a CLA mixture, sagittal abdominal diameter (SAD) was significantly reduced in the CLA treatment group compared to the placebo group after four weeks of treatment (Riserus *et al*., 2001). Moreover, the CLA treated group had a significant decrease in SAD from the baseline values (Riserus *et al.*, 2001). The waist circumference in the CLA-fed group was also significantly reduced in comparison to the baseline levels, but not in comparison to the placebo group (Riserus *et al.*, 2001). In this study, neither group experienced significant weight loss; therefore, CLA may not help in weight loss, but perhaps CLA decreased the abdominal fat (Riserus *et al.*, 2001). As abdominal fat is risk factor for many diseases such as diabetes and coronary heart disease, any decrease in abdominal fat

is considered to be beneficial (Anonymous). As adiposity is a factor in hypogonadotropic hypogonadism, perhaps loss of adipose tissue will decrease the estradiol levels, and increase total testosterone levels associated with obesity. This, in turn, should increase spermatogenesis and improve oligozoospermia associated with obesity.

A randomized, double-blind placebo controlled trial in humans showed that over a supplementation period of 12 weeks, CLA increased lean muscle mass (Steck *et al*., 2007). The 6.4g/day and 3.2g/day CLA fed groups showed trends toward weight loss in comparison with the placebo-controlled group (Steck *et al.*, 2007). While the 6.4g/day group had a lower weight at the end of the study than did the 3.2g/day, neither group was found to be statistically significant in comparison to the control (Steck *et al.*, 2007). In addition to the weight loss, the increase in lean muscle mass of the 6.4g/day CLA-fed group was statistically significant. Furthermore, the 6.4g/day CLA-fed group trended toward an increase in resting energy expenditure in comparison to baseline, though found to be statistically insignificant, appeared to be significantly higher than the placebo group after treatment (Steck *et al.*, 2007). This increase in lean body mass may also help with insulin sensitivity that can occur in obesity, and improve spermatogenesis through that mechanism.

5.4.1. CLA Effect on the Testes of Obese Rats

The effect of dietary CLA on seminolipid production was studied in obese *fa/fa* Zucker rats. These rats have altered sperm morphology and reduced seminolipid in comparison to the lean control rats (Kim *et al.*, unpublished data). However, when fed with 0.4% (w/w) of CLA isomers, *cis*-9, *trans*-11 CLA and *trans-10, cis-12* CLA Zucker *fa/fa* rats showed an increase in seminolipids, which positively affected spermatogenesis.

It appears that *trans*-10, *cis*-12 CLA increased spermatogenesis to a greater extent than the *cis*-9, *trans*-11 CLA isomer (Kim *et al.*, unpublished data).

In underdeveloped testes, the mRNA levels of *cst* were significantly reduced in all groups, lean control, *fa/fa* control, and both CLA treated groups, in comparison to the developed testes (Kim *et al.*, unpublished data). However, the *cis*-9, *trans*-11 CLA group had increased *cst* expression in the testes compared to the *fa/fa* control group, and *trans*-10, *cis*-12 CLA increased *cst* expression to be statistically similar to the lean control. In the developed testes, only the *trans*-10, *cis*-12 CLA group was able to increase the *cst* expression from the fat control group to levels statistically similar to the lean control (Kim *et al.*, unpublished data).

The *fa/fa* control group that had underdeveloped testes had a significantly decreased expression of *cgt* compared to the lean control; however, the level of *cgt* gene expression in the developed testis did not vary between groups (Kim *et al.*, unpublished data). Furthermore, in the underdeveloped testes, both CLA groups were able to significantly increase the expression of *cgt* compared to the *fa/fa* control, so that it was statistically similar to the lean control. In the normal testes, *trans*-10, *cis*-12 CLA significantly increased the *cgt* gene expression in comparison to the lean control (Kim *et al.*, unpublished data). Although the CLA fed treatment groups were able to increase *cst* and *cgt* gene expression in the underdeveloped testes, and *cst* gene expression in the developed testes, seminolipid production was only increased in the developed testes.

The underdeveloped testes had significantly lower seminolipid content than the normal testes (Kim *et al.*, unpublished data). Furthermore, within the developed testes, the amount of seminolipid increased significantly in the CLA treated groups, an effect

that was more pronounced in *trans*-10, *cis*-12 CLA treated group than in the *cis*-9, *trans*-11 CLA treated group (Kim *et al.*, unpublished data). This shows that CLA is directly correlated to the amount of seminolipid present in the developed testis. Therefore, by feeding the rats a diet supplemented with CLA, seminolipid production was increased, and subsequently, so was spermatogenesis. As a result, it is reasonable to believe that CLA fed to diabetic and obese mice will also have this increase in seminolipid and spermatogenesis.

CHAPTER II

RESEARCH PLAN

Study Rationale

Infertility affects approximately 15% of couples. Among infertile couples, 40% of cases can be attributed to male infertility (Smith *et al.*, 2007). Environmental and lifestyle factors, including diet, can negatively influence male fertility. Additionally, several chronic disease states can influence fertility, in particular diabetes and obesity. In most developed countries, the majority (~60-70%) of adult males are either overweight or obese, and these rates are continuously rising (Tjepkema, 2005). Obese males, particularly those with DM-2 often have reduced semen quality, defined as low sperm count, poor sperm morphology or low sperm motility. In obese males, sperm number is typically reduced (Jensen *et al.*, 2004), while in those with DM-2, sperm motility is reduced (Shirneshan *et al.*, 2008; Hassan *et al.*, 1993; Scarano *et al.*, 2006). Studies of sperm morphology in obese males with and without DM-2 have yielded mixed results (Scarano *et al.*, 2006; Vignon *et al.*, 1991). One potential mechanism to explain poor semen quality in the states of DM-2 is an increase in oxidative stress secondary to hyperglycemia (Baynes and Thorpe, 1999). Sperm are particularly susceptible to ROS because of their high unsaturated fatty acid content (Ben Abdallah *et al.*, 2009), lack of repair mechanisms for DNA (Mallidis *et al.*, 2009), and the length of time that they remain in the body (Jones and Lopez, 2006). In obesity, poor semen quality may be related to an increase in aromatase activity that would increase the ratio of estradiol-to-testosterone (Cohen, 1999), that tends to increase with increasing adiposity (Schneider *et*

al., 1979). Since testosterone is required for the initiation of spermatogenesis (McPhee *et al.*, 2007), an increase in estradiol-testosterone could reduce sperm production.

A few animal models of obesity and DM-2 have demonstrated smaller testes relative to lean controls (Suh *et al.*, 2011; Hassan *et al.*, 1993), suggesting impaired spermatogenesis (Bhasin and Jameson, 2008). Additionally, obese animal models also display shortened or lower weight epididymides (Suh *et al.*, 2011; Hassan *et al.*, 1993), which can affect sperm concentration, maturation and motility (Jones and Lopez, 2006; Widmaier *et al.*, 2006). Furthermore, DPA content is significantly lower in the underdeveloped testes of obese rats (Suh *et al.*, 2011), indicating a direct involvement of lipid in testis development. Although these negative effects on testicular development may be associated with reductions in SHBG and testosterone that are frequently observed in both obesity and DM-2 (Chavarro *et al.*, 2009; Niskanen *et al.*, 2004), spermatogenesis in these animals have not been studied.

As male fertility is most frequently quantified by sperm concentration and morphology, disruptions in spermatogenesis may be at the root cause of obesity-related infertility. Spermatogenesis requires form 65-75 days in humans and ⅓ of that time exists in prophase I (Jones and Lopez, 2006). Within each seminiferous tubule, there is continual sperm production, but different sections will occupy a different phase of the spermatogenic wave. A new spermatogenic wave begins every 16 days in humans (Jones and Lopez, 2006). The Sertoli cells support the developing spermatozoa as they change from spermatogonia to primary and secondary spermatocytes, and finally to spermatids (Jones and Lopez, 2006). Spermiogenesis then converts the spermatids to spermatozoa, which are released from the Sertoli cells (Jones and Lopez, 2006). Spermatozoa travel to

the epididymis, where they mature to become fully functional spermatozoa and become more concentrated for approximately 20 days (Jones and Lopez, 2006). Any interruption in this process can greatly hinder sperm parameters that are measured when men are being assessed for infertility. There is no information on how this process is affected by dietary treatment or by obesity and DM-2.

Seminolipid, or 3-sulfogalactosyl-1-alkyl-2-acyl-*sn*-glycerol, a unique lipid found only in the testis and sperm, has been shown to be important in spermatogenesis (Honke *et al.*, 2002; Kim *et al.*, unpublished data). Synthesis of seminolipid occurs using the precursor alkylacylglycerol, which is converted to galactosylalkylacylglycerol then seminolipid by the enzymes, ceramide galactosyltransferase (*cgt*) and cerebroside-3 sulfotransferase (*cst*), respectively. A deficiency in seminolipids reduces the expression of genes involved in spermatogenesis, in particular *Ccna*1, *Cdc*25*c* and *Prm*1 (Honke *et al.*, 2002). These genes are involved different stages of cellular division, including chromatin arrangement, condensation and stabilization (Honke *et al.*, 2002; Manipalviratn *et al.*, 2007; Petronczki *et al.*, 2003) and induction of cellular division (Honke *et al.*, 2002; Liu *et al*., 1998). Only a handful of researchers have measured the genes that form these proteins and enzymes, and it remains unclear how obesity and DM-2 alter genes involved in seminolipid synthesis, seminolipid production and spermatogenesis. Moreover, it remains unclear if defects in seminolipid gene expression, as well as gene expression related to spermatogenesis play a role in poor semen quality often seen in obesity and DM-2.

DHA and DPA are the major PUFAs of the human and rodent spermatozoa and testes, respectively. These lipids are altered when different dietary fats are provided (Suh

et al., 2011; Castellano *et al.*, 2011). Diet has proven to have a role in male fertility (Merrells *et al.*, 2009; Shalini and Bansal, 2008; Xu *et al.*, 2003; Gromadzka-Ostrowska *et al.*, 2002), but the direct impact of diet on parameters regulating spermatogenesis has not been studied.

CLA has been studied in many different areas, including body fat reduction (Parra *et al.*, 2009; Park *et al.*, 2007; Brown and McIntosh, 2003), increased immune function (Ramirez-Santana *et al.*, 2009; Ruth *et al*., 2008), anti-hypertension (Zhao *et al*., 2009), inflammation (Nakamura and Omaye, 2009), bone mineral content (Roy *et al*., 2008; Park *et al*., 2008), enrichment into breast milk (Moutsioulis *et al*., 2008) and insulin sensitivity (Riserus *et al.*, 2002). Whether this anti-obesity effect of CLA also influences male fertility in obesity and DM-2 has not been explored. Although CLA has shown mixed results in obese human and animals, the general consensus is that CLA has the ability to decrease fat accumulation. CLA has been studied in female reproduction of dairy cows, which demonstrated the ability of CLA to increase the likelihood of becoming pregnant (de Veth *et al*., 2009). Our lab found that both CLA isomers were able to improve sperm morphology, and seminolipid content in the testes of obese *fa/fa* Zucker rats (Kim *et al.*, unpublished data). It is possible that CLA is also incorporated into the testis and may affect spermatogenesis.

The *db/db* mice have a defect in the leptin receptor (Chen *et al.*, 1996). As leptin is a hormone that controls hunger signals, the receptor mutation for leptin causes the mice to become hyperphagic, and subsequently obese, hyperinsulinemic and hyperglycemic, the defining characteristics of DM-2. These mice have been used for numerous studies on insulin sensitivity (Hamura *et al*., 2001) and dyslipidemia (Kobayashi *et al.*, 1999). By

using this animal model, the present study investigated if CLA supplementation influences the determinants of male fertility in obesity and DM-2. CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 were provided as a part of a diet to measure sperm quality, lipids in the testis and genes involved in spermatogenesis.

Objective

The overall objective of this study was to determine if dietary CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 affect spermatogenesis in *db/db* mice.

More specifically,

1. To understand the characteristics of the testis and sperm of *db/db* mice in comparison to lean mice.
2. To study if CLA influences seminolipid levels by altering gene expression of the enzymes involved in seminolipid synthesis.
3. To study if CLA is involved in spermatogenesis by increasing selected genes involved in sperm differentiation.
4. To determine if CLA is incorporated into testicular tissue lipids.
5. To compare if CLA isomers have similar effects on spermatogenesis.

Hypothesis

The working hypothesis is that dietary CLA will modulate spermatogenesis. Specifically *trans*-10, *cis*-12 CLA, compared to the *cis*-9, *trans*-11 CLA, will improve parameters involved in spermatogenesis problems associated with diabetes and obesity

CLA will

1. Increase seminolipid production through increased gene expression of *cst* and *cgt*.
2. Increase the gene expression of genes involved in prophase I of spermatogenesis: *Hlf*3, *Ccna*1, *Sprm*-1, *Prm*1, *Cdc25c*.
3. Be incorporated into the testicular tissues in mice.

CHAPTER III
MATERIALS AND METHODS

The study was originally designed to test for the effects of CLA supplementation on adiposity and hepatic steatosis in the setting of obesity and DM-2 (Hunt, 2009, data not shown). Male sex organs were taken to test the hypotheses for this thesis study.

1. Animal and Diets

Six-week-old male *db/db* C57BLKS/J mice (*Lepr^{db} db, n*=40) homozygous for deficiency in the leptin receptor and C57BLKS/J mice (lean, *n*=10) were purchased from the Jackson Laboratory (JAX Mice and Services, Bar Harbor, Maine). They were housed in stainless steel hanging cages with a 12:12 hr dark-light cycle in a temperature and humidity controlled room, and allowed to acclimatize for 5-9 days prior to study onset. The *db/db* mice were randomly assigned to one of four experimental diet groups with ten mice in each group. The mice were fed a semi-synthetic diet that had different sources of oil (8.5%, w/w of total diet) for 6 weeks (**Table III-1**). The control diet contained soy oil (db-C, 8.5%, w/w) and the CLA supplemented diets were formulated by replacing soy oil with *cis*-9, *trans*-11 CLA (0.4%, w/w, db-9,11 CLA) or *trans*-10, *cis*-12 CLA (0.4%, w/w, db-10,12 CLA). The lean mice were only fed soy oil and served as a control group (LC). A paired-weight *db/db* group (db-PW) was fed the control diet in restricted amounts to match the *db/db* group with the lowest body weight during the study period. It was used to determine whether it is solely weight loss that is beneficial, or whether CLA is actually able to further improve male reproduction, regardless of the weight loss. Considering each cycle of spermatogenesis requires approximately 8-9 days in mice (Oakberg, 1956), the feeding period allowed almost 5 cycles of spermatogenesis to take place. CLA was obtained from Larodan AB (Limhamns Gardens, Malmo, Sweden). The

cis-9, *trans*-11 CLA (Cat #: 10-1823-90 Lot G438:12) was 91.9% pure, and *trans*-10, *cis*-12 CLA (Cat #: 10-1826-90 Lot 1261:8) was 92% pure, with impurities including oleic acid, and other CLA isomers. The CLA isomers were fed in the form of free fatty acids.

After the 6-week feeding period, animals were euthanized by carbon dioxide asphyxiation. Trunk blood was collected after decapitation. Testis, epididymis and prostate were excised and weighed. Canadian Council on Animal Care Guidelines (Olfert *et al*., 1993) were followed, and the study was approved by the Fort Gary Campus Protocol Management and Review Committee.

1.1. Sperm morphology and sperm count

To assess sperm morphology and numbers, the caudal epididymis was minced to release the spermatozoa in 2 mL 0.9% NaCl. An aliquot of sperm emersion was fixed in formalin and stained with eosin Y. The sperm were first counted using 11 μL of sample in a hemocytometer. The eight outer corners on the hemocytometer were counted and an average was taken. To assess sperm morphology 100 μL of the sperm-formalin solution was smeared on a microscope slide and allowed to dry. A total of 200 sperm were assessed at 400x magnification on a compound light microscope (Olympus BH2 RCFA, Markham, Canada). Abnormalities were determined using the guidelines set out for rat spermatozoa, measuring head, tail as well as head and tail abnormalities (Industrial Reproductive Toxicology Discussion Group, Computer Assisted Sperm Analysis (CASA) Group, 2000).

2. *Lipid Analysis*

2.1. Lipid Extraction

The right testis of each animal was used to analyze seminolipid and fatty acid composition. Total lipid was extracted with chloroform:methanol:water (30:60:8, v/v/v), after homogenization, using an Ultra-Turrax Polytron homogenizer. Samples were vortexed and then centrifuged for 20 min at 2000 rpm at 25˚C. The organic layer was transferred to a previously weighed 30 mL test tubes. The remnant was re-extracted with chloroform:methanol (2:1, v/v) and filtered. The combined organic phase was dried down with a nitrogen evaporator (N-Evap 111 + OA-SYS heating system, Berlin, USA) until it was dry, and the weight of the test tube was subsequently measured to determine the total lipid in the testis. The sample was dissolved in 2 mL of chloroform:methanol (2:1, v/v) and stored in the -20˚C freezer.

2.2. DEAE-Sephadex A-25 column for neutral lipids and seminolipids

The lipid extract was loaded onto a DEAE-Sephadex A-25 column (acetate form, 1x10 cm) three times to ensure the sample adhered to the column. To make the DEAE-Sephadex gel (Sigma Aldrich, Oakville, Canada), a slurry of the gel was made with ~800 mL of 0.2 N HCl, mixed and left for 30 min. It was then washed with 7 L of water so that the pH of the gel became neutral. This was repeated with 0.2N NaOH, and subsequently 0.1 N acetic acid. The gel was washed with 10 volumes (~950 mL) of methanol to remove the water, and finally stored in chloroform:methanol:water (30:60:8, v/v/v). To prepare the column, it was loaded with chloroform:methanol:water (30:60:8, v/v/v) until almost full. The DEAE-Sephadex was added continuously until the gel reached the bottom of the crown located on the column. Just prior to adding the sample,

about 6 mL of chloroform:methanol:water (30:60:8, v/v/v) was run through the column to wash it, and the stopper was replaced. All solutions, including the DEAE-Sephadex gel, were sonicated (Branson 3510) for 30 min to remove any gaseous bubble from forming in the column.

The neutral lipid faction, containing triglycerides, phospholipids, cholesterol, and other lipids was eluted with 20 mL chloroform:methanol:water (30:60:8, v/v/v). The acidic lipid fraction was eluted with 20 mL chloroform:methanol:0.2 M sodium acetate (30:60:8, v/v/v). The acidic fraction containing the seminolipid was eluted with 20 mL chloroform:methanol:0.4 M sodium acetate, followed by chloroform:methanol:0.8 M sodium acetate (30:60:8, v/v/v). The 0.4 M and 0.8 M sodium acetate fractions were pooled, and the sample was evaporated under nitrogen. They were reconstituted in 30 mL of chloroform:methanol:water (30:60:8, v/v/v).

Since the sodium acetate was also co-eluted, a Sep-Pak Plus C18 cartridge (Waters, Tauton, USA) was used to remove the salts. Each sample was loaded 3 times onto the Sep-Pak C18 cartridge. After drying for 2 hours, the column was washed with 40 mL of deionized water (Millipore filtration system), to remove the salts. The seminolipid, which remained in the column, was eluted with 10 mL of methanol, and subsequently with 20 mL of chloroform:methanol (2:1, v/v). These samples were dried down and reconstituted in 500 µL of chloroform:methanol (1:1, v/v).

2.3. Quantitation of Seminolipids

The seminolipids were analyzed by measuring the sulfur content of seminolipid by the Azure A assay (Tadano-Aritomi and Ishizuka, 1983). In the Azure A assay, 200 µL of the sample was dried down, and 5 mL of chloroform:methanol (1:1, v/v), 5 mL of 0.05 N

H_2SO_4 and 1 mL of dye solution containing Azure A was added. After centrifuging for 10 min at 2000 rpm (Beckman Coulter – Allegra 6 Centrifuge, Indianapolis, USA), the organic phase was used for measurement on the spectrophotometer (General Electrics, Ultrospec 4300 Pro UV/Visible Spectrophotometer, Baie d'Urfe, Canada) at a wavelength of 645 nm. A standard curve using sulfatide (from bovine brain, Sigma Aldrich, Oakville, Canada) as the standard was made at the amounts of 0, 20, 85, 150, 215, 280, 350 nmol to ensure the whole range was covered. All samples in both measurements of seminolipid were analyzed in one session.

2.4. Fatty Acid Analysis

The neutral lipid eluted from the DEAE-Sephadex column was dried down with the nitrogen and dissolved in 100 µL or 200 µL chloroform:methanol (1:1, v/v). Approximately 1.5mg of each sample was loaded onto a pre-activated 20x20, 250 micron Uniplate Silica Gel G plate (Analtech, Newark, USA). These plates were developed using a mobile phase of petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v). The lipid band was visualized with 0.1% (w/v) 4-amino-3-hydroxy-1-naphthalenesulfonic acid (ANSA, Sigma Aldrich, Oakville, Canada). The phospholipid and triacylglyceride bands were then scraped off into 15mL screw cap test tubes.

Methylation Procedure

The PL and TAG were methylated with 1.5 mL of 3.0 M methanolic HCl (Supelco, Oakville, Canada) by placing them in a sand bath for 1 hour at 80˚C. Two mL of hexane and 2mL of H_20 were added to each tube, capped and vortexed for 30 seconds. Each sample was then centrifuged for 10 min at 2000 rpm. The top hexane layer was used for fatty acid analysis.

Fatty Acid Methyl Esters

Fatty acid methyl esters were analyzed by gas chromatography (Varian CP-3800 GC, Mississauga, Ontario, Canada) equipped with a WCOT fused silica capillary column CP-7400 (100mx0.25mm, id, 0.25). The temperature program was as follows: 50˚C for 3 min; 18˚C/min to 180˚C, held for 10 min; 2˚C/min to 220˚C, held for 3 min; 20˚C/min to 240˚C, held for 20 min. Injection and detection temperatures were 250˚C and 300˚C, respectively. Hydrogen (99.9%) was used as a carrier gas with a flow rate of 1.5ml/min. All fatty acids were compared with a standard (NuChek, Prep 463, Elysian, USA) and CLA-Me isomers (NuChek Standard UC59M, Elysian, USA). These samples were then run through the gas chromatograph to obtain important fatty acid composition in both PL and TAG samples that are involved in spermatogenesis. Furthermore, the extent of incorporation of CLA into these tissues was also determined.

3. Gene Expression of cgt, cst and other genes involved in spermatogenesis

3.1. RNA extraction and cDNA synthesis

To determine the gene expression, total RNA was extracted from the testis using the Qiagen RNeasy extraction kit (Qiagen, Toronto, Canada). The procedure provided by the kit was followed. A portion of the testis was homogenized in 600 μL RLT buffer (composed of guanidine thiocyanate and β-mercaptoethanol). The lysate was centrifuged for 3 min at maximum speed; the supernatant was subsequently transferred to a gDNA eliminator spin column and centrifuged for 30 sec at 10,000 rpm. The liquid that flowed through the column was then combined with 600 μL of 70% ethanol, transferred to an RNeasy spin column and centrifuged for 15 sec at 10,000 rpm. Seven-hundred μL of RW1 buffer was added to the column and centrifuged again. Finally, 500 μL RPE buffer

was added twice to the column and centrifuged for 2 min at 10,000 rpm. The RNeasy spin column was then transferred to a new 1.5 mL collection tube, and RNA was eluted with RNase-free water and centrifuged for 1 min at 10,000 rpm. Extracted RNA was read with a spectrophotometer at a wavelength of 260 nm and 280 nm. RNA samples were considered to be pure with a 260/280 ratio of 1.8-2.0. Samples were stored in the -80˚C freezer.

3.2. Real Time quantitative RT-PCR (q-RT-PCR)

q-RT-PCR was performed using the qScript 1-Step SYBR Green q-RT-PCR kit for iQ (Quanta Biosciences, Gaithersburg, USA). The MyiQ2 Two-Colour Real-Time PCR Detection System was used for PCR and quantification (BioRad, Mississauga, Canada). The primers used for q-RT-PCR work in the conditions indicated in the Appendix (**Table A-3**). Analysis of gene expression was carried out using the $\Delta^{\Delta Ct}$ where Ct is the threshold cycle. The samples were done in duplicate, and their values averaged. The PCR contained (final vol, 25 μL), 25 ng of reverse-transcribed RNA, 12.5 μL of SYBR Green Master Mix and 0.75 μL of each primer. β-actin served as a control gene.

After q-RT-PCR was carried out, the cDNA samples were visualized using gel electrophoresis (Model# HU13 Serial #8038). To make the 2% agarose gel, 4 g of agarose was mixed with 200 mL of 0.5x TBE buffer. The mixture was heated in the microwave until melted. Three μL of SYBR Green was added, and the solution poured into an electrophoresis tray with a comb to set. TBE buffer (0.5x) was poured in the tray to cover the gel. The DNA standard was loaded in addition to samples that were mixed with 4μL loading buffer.

Primers were obtained from Invitrogen, and were designed by our lab using an online program that is commercially available. *Ccna1*, (F: 5'-agt ggg gcc aac taa cgt c-3' and R: 5'-caa aca gca agt tgt tta tt-3'), *Prm1*, (F: 5'-cag acg gag gag gcg atg c-3' and R: 5'-ctc ctg ttt ttt cat cgg cgg t- 3'), *Cdc25c*, (F: 5'-agc tga acc tcg ggc cac gta-3' and R: 5'-aga ccg act tga att ttg tga g-3'), *Sprm-1*, (F: 5'-tgc tgg ggg tcc cat cca cc-3' and R: 5'-tgg ctg cct ggc tgc tca ac-3'), *Hlf3*, (F: 5'-agt tgc cac cat gcc cag gc-3' and R: 5'-agg agc aga cac ggt gcc ga-3'), *cst* (F: 5'-tca ccg tca tcc gcg acc ct-3', R: 5'-cct gca gga act cgg cca gc-3'), cgt (F: 5'-ccg ctt acc gcg tcc ttc gg-3' R: 5'-tgc cct aga gcg ccc gtc tt-3'), and β-*actin* as the house gene (F: 5'-caa gta ctc tgt gtg gat tgg tgg-3' and R: 5'-aga tta ctg ccc tgg ctc cta-3').

3.3. Statistical Analysis

To see the effect of dietary treatment, one-way analysis of variance was used in the SAS program. Duncan's multiple comparison was used to identify the differences among dietary treatments. A $p<0.05$ was considered to be statistically significant.

RESULTS

1. Body Weight and Characteristics of Testis and Accessory Sex organs in db/db *mice*

The body weight of *db/db* mice was significantly higher than the lean control mice (**Table III-2**). The *db/db* mice fed a *trans*-10, *cis*-12 CLA and the restricted diet were only 69% or 75%, respectively, of the weight of *db/db* mice fed a control diet, at the end of the study.

The db/db mice fed a control diet were found to have significantly decreased testis, epididymis and seminal vesicle weights and increased prostate weights when compared to the lean control mice (**Table III-2**). The *db/db* mice fed a *trans*-10, *cis*-12 CLA diet significantly increased the testis weight in comparison to the db-C group in both absolute weights and in percentage of body weight. The *trans*-10, *cis*-12 CLA group also had a significant reduction in their prostate weight from the db-C group. Part of this decrease in prostate weight can be attributed to weight loss; however, the diet restricted db-PW group had a prostate size that was 3 times larger when compared to the *trans*-10, *cis*-12 CLA group. Therefore, *trans*-10, *cis*-12 CLA played an important role in the reduction of the prostate weight. The mice fed a *cis*-9*, trans*-11 CLA diet showed no differences in the organ weights of the testes, epididymides and prostate when compared to the db-C group.

The left and right testis size in each animal was measured to show how many unbalanced testes (**Table A-4**) were found in each group. The db-C group had 8 out of 9 mice that had unbalanced testes, ranging from 10% to greater than 60% difference between the right and left testes, whereas, the LC group had only 2 out of 10 animals that had unbalanced testes with a difference of 10-19.9%. The *db/db* mice treated with the

cis-9, *trans*-11 CLA diet had only 3 out of 10 mice with a percent difference between the testes greater than 10%. Interestingly, no animals fed the *trans*-10, *cis*-12 CLA diet had unbalanced testes.

Testis weight was correlated to body weight (**Figure III-1A**) and showed genotypic differences between *db/db* mice and the LC mice that as body weight increased, the testis weight decreased. Although the *cis*-9, *trans*-11 CLA isomer only showed a trend toward improving the testis weight proportional to the body weight, the *trans*-10, *cis*-12 CLA isomer improved the proportional weight of the testis to the body weight of the animal to LC values (**Table III-2**). The mice fed the *trans*-10, *cis*-12 CLA isomer had a lower body weight, and although the proportional change could have been the result of weight difference alone, the db-PW mice had no change in the proportional of testis weight to body weight, despite having similar body weight loss as the *trans*-10, *cis*-12 CLA group.

The testis weight was also compared to the epididymis weight. There was no statistical significance between any of the groups when total epididymis was calculated as a percentage of the testis; however, there was a significant correlation between testis weight and epididymis weight (**Figure III-1B**). This is because a change in weight of the epididymis is likely directly proportional to the increase or decrease in weight of the testis.

2. Sperm Counts

The *db/db* mice had a significant reduction in sperm concentration compared to their lean counterparts (**Figure III-2A**). Although the *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA groups trended to have higher sperm concentration, they were not

statistically different from the db-C group, and they did not reach levels that were similar to the LC mice. The db-PW group in this case had statistically lower sperm concentration compared to all other groups.

3. Sperm Morphology

The *db/db* mice had significantly higher sperm abnormalities in comparison to the lean control mice (**Figure III-2B**, **Figure A-2**). Both CLA treated groups had a significant increase in the morphologically normal sperm compared to the db-C group, although these values were still lower than the LC group.

The db-C group had significantly more head, tail and head and tail abnormalities than the LC group. The CLA isomers were equally able to significantly reduce the numbers of head and tail abnormalities compared to the db-C group. Furthermore, both CLA isomers trended to decrease the head abnormalities from the db-C group. The CLA isomers had no effect on the tail abnormalities found db-C mice. The db-PW group had similar numbers of head abnormalities to the db-C group, and the highest amount of head and tail abnormalities amongst all of the groups.

4. Lipid Profiles

Triacylglycerols

The fatty acid composition of testis triacylglycerols (TAG) was determined (**Table III-3**). The major difference between the lean and *db/db* mice was total saturated and total n-6 fatty acids. Compared to lean mice, the *db/db* mice had more total saturated fatty acids (especially C16:0) and less total n-6 fatty acids. Mice fed a *trans*-10, *cis*-12 CLA diet showed the highest level of C20:4(n-6) and C22:5(n-6) in the TAG, but they were not significantly different from all other groups. The incorporation of CLA was

measured in the testis and neither *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA were detected in this lipid fraction.

Phospholipids

The fatty acid composition of testis PL was determined (**Table III-4**). Unlike the TAG, there were no obvious differences in fatty acid composition between lean and *db/db* mice. Only C20:4(n-6) was significantly lower in the db-C mice in comparison to their lean counterparts. Both CLA isomers increased the level C18:2(n-6) from the LC group; however, only the *trans*-10, *cis*-12 CLA isomer was able to significantly increase the level of C18:2(n-6) from the db-C group. Both CLA isomers were able to increase the percentage of C20:4(n-6) from the db-C group; however, only the *cis*-9, *trans*-11 CLA isomer was significantly increased from the db-C group. Finally, both CLA isomers trended to increase the proportion of C22:5(n-6); however, these levels were not statistically different from the control diets. The incorporation of CLA was also measured and no CLA isomers were detected in the phospholipid fraction.

Seminolipid

The effect of CLA on the level of seminolipids was measured in the testis (**Figure III-3**). The level of seminolipid (μg/mg lipid) was significantly higher in *db/db* mice fed the control diet in comparison to the lean mice. A similar trend was also identified when the seminolipid concentration was expressed as μg/mg testis. Animals fed a *trans*-10, *cis*-12 CLA diet had the lowest concentration of seminolipids among the *db/db* mice, which was comparable to the lean control level. Furthermore, there was a significant increase in the seminolipid μg/mg testis in the db-PW group.

5. Gene Expression of cgt and cst Involved in Seminolipid Synthesis

The gene expression of *cgt* and *cst* was significantly lower in the *db/db* mice fed the control diet in comparison to the lean control mice (**Figure III-4**). The *trans*-10, *cis*-12 CLA significantly increased the mRNA levels of *cgt*, but not *cst*. The db-PW group also had a significant increase in *cgt* and *cst* expression to levels similar to the LC mice.

6. Gene Expression of Genes Involved in Spermatogenesis

Five genes related to prophase I in spermatogenesis were measured (**Figure III-5**). In comparison to the lean control mice, *Ccna*1, *Cdc*25*c* and *Hlf*-3 gene expression was significantly reduced in the db-C mice, indicating genotypic differences in spermatogenesis. The other genes, *Sprm* and *Prm*, were also decreased in *db/db* mice, but not statistically different from the LC group. Both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA significantly increased the gene expression of the *Ccna*1 gene, but no other genes were affected by the CLA treatment in *db/db* mice.

DISCUSSION

The *db/db* mice used in the study have a genetic mutation that occurs in the leptin receptor (Chen *et al.*, 1996). This mutation leads to the development of obesity and diabetes in the mice. As expected the db-C mice were significantly heavier at the end of the study, in comparison to the LC mice. Surprisingly, mice fed a *trans*-10, *cis*-12 CLA diet had significant weight loss in comparison to all of the other groups, even less than that of the LC mice, despite a greater intake of food (Hunt, 2009, data not shown). On the other hand, mice fed a *cis*-9, *trans*-11 CLA diet did not show any weight loss in comparison to the db-C group.

The serum glucose and the homeostasis-model assessment of insulin resistance (HOMA-IR) analyzed by Hunt (2009, data not shown), and were significantly increased

in all *db/db* mice compared to the lean mice, which indicate that the animals are hyperglycemic and are insulin resistance. However, the serum insulin levels were significantly high in the db-C group in comparison to the LC group. Though the *cis*-9, *trans*-11 CLA was not able to reduce the serum insulin levels compared to the db-C group, both the *trans*-10, *cis*-12 CLA and the db-PW groups were able to significantly reduce the serum insulin levels.

While not knowing the exact mechanism of *trans*-10, *cis*-12 CLA-induced weight loss, a few studies have related the weight loss to downstream targets that increase energy utilization. For instance, in 3T3-L1 cell culture, the *trans*-10, *cis*-12 CLA isomer has been shown to increase phosphorylase kinase A (PKA) gene expression, which acts to phosphorylate hormone sensitive lipase (HSL) (Zhai *et al*., 2010). At the level of the adipocyte, HSL causes lipolysis, resulting in the reduction of fat mass (Zhai *et al.*, 2010). The fatty acids released from the adipocytes are then used for β-oxidation in the mitochondria. CLA is a PPARα agonist, and as such has been shown to upregulate mRNA and protein levels of carnitine palmitoyl transferase (CPT) (Zhai *et al.*, 2010) and uncoupling proteins (UCP) 1 and 3 (Zhai *et al.*, 2010; Takahashi *et al*., 2003). CPT is used to transport fatty acids into the mitochondria, and the UCPs are responsible for the efflux of protons generated from the electron transport chain with the production of ATP. The uncoupling of β-oxidation with ATP production (Zhai *et al.*, 2010), results in wasting an energy source, which can lead to weight loss. The same effect is not observed with the *cis*-9, *trans*-11 CLA isomer (Zhai *et al.*, 2010).

The characteristics of male sex and accessory organs of *db/db* mice were observed in the present study. In comparison to lean animals, the *db/db* mice fed a

control diet had significantly smaller testes, epididymides and seminal vesicles, but larger prostate glands than LC mice. A similar pattern was found in obese *fa/fa* Zucker rats (Suh *et al.*, 2011). Dietary deprivation in the weight matched db-PW mice significantly decreased sexual organ size, for example, testes, epididymides, prostate glands and seminal vesicles were 63%, 45%, 60% and 65%, respectively, the weight of the reproductive organs in db-C mice.

Despite considerable weight loss, mice fed a *trans*-10, *cis*-12 CLA diet had the largest testes among all of the *db/db* mice. This phenomenon was also found in obese Zucker rats fed a *trans*-12, *cis*-12 CLA diet for 8 weeks in comparison to obese rats fed non-CLA diet (Kim *et al.*, unpublished data). Similarly, the testes weight was increased in the *cis*-9, *trans*-11 CLA mice, but it was not significantly different from the db-C group. As the size of the testes is strongly related to spermatogenesis and testicular function (Schorge and Williams, 2008; Scarano *et al.*, 2006), the increase in weight is important to the fertility potential in these mice. In fact, this study showed that the weight of the testis was correlated with sperm counts. LC mice had the largest testis and the highest sperm concentration. The *db/db* mice fed the CLA isomers also showed a trend toward increased sperm numbers, although the numbers were not significantly different form the db-C mice. As previously discussed in Chapter I, the lower cut-off point for sperm concentration to be diagnosed with a oligozoospermia is $15x10^6$/ml in humans (Cooper *et al.*, 2009). Compared to lean healthy control mice ($15x10^6$/mL), sperm concentration in *db/db* mice was reduced by approximately 74% ($4.0x10^6$/mL). Treating *db/db* mice with CLA increased sperm concentration by 50-75% relative to *db/db* controls ($6\text{-}8x10^6$/mL). While this was not statistically significant, it suggests that

CLA may increase fertility by increasing sperm concentration. Importantly, treatment with *cis*-9, *trans*-11 CLA increased sperm concentration by 75% without any changes in body weight, suggesting there is a direct effect of this functional food on sperm count/fertility.

While sperm number is an important fertility index, sperm integrity must also be considered. The WHO generally quotes percentages of normal sperm (Cooper *et al.*, 2009). The numbers of normal sperm were significantly reduced in the db-C group in comparison to the LC group with 5.5% and 46% normal sperm, respectively. Fewer normal sperm combined with a decreased sperm count ultimately results in reduced fertility. It is clear that the CLA-fed *db/db* mice had significantly improved levels of normal sperm in comparison to the db-C group. Values were not normalized compared to healthy controls, therefore it is possible that factors such as blood glucose and ROS may adversely affect sperm morphology. The *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 CLA groups had improvements in sperm morphology with 17.5% and 23.0% of normal sperm forms, respectively. On the other hand, the db-PW group did not have improved sperm morphology or sperm counts, even though they had a similar body weight to the *trans*-10, *cis*-12 CLA group. This indicates that both CLA isomers are able to decrease sperm abnormalities.

Collectively, the significant improvements in sperm morphology and subtle increase in sperm concentration suggest that CLA treatment may improve fertility rates among *db/db* mice. The improvements in sperm count and morphology were observed following treatment with *cis*-9, *trans*-11 CLA isomer, independent of body weight, suggests that this functional food may directly affect spermotogenesis. Furthermore,

these improvements in sperm parameters occurred despite hyperglycemia and insulin resistance in all of the *db/db* mice, indicating that CLA isomers may be making changes at the level of the testis. Therefore it is important to determine a mechanism by which CLA functions to improve the sperm parameters.

Effect of CLA Isomers on Triacylglycerols and Phospholipids

Based on the sperm counts and the sperm morphology in *db/db* mice, it is clear that obesity and DM-2 greatly affects spermatogenesis. The present study identified that obese diabetic *db/db* mice have higher saturated fatty acids (especially C16:0), but lower total n-6 fatty acids in the testis TAG. Otherwise, there was no major difference in fatty acids between the LC and the *db/db* groups in TAG and PL. To understand if CLA is incorporated into lipid classes, thereby influencing sperm production in obesity and DM-2, the fatty acid profile of the testes was measured. CLA was not detected in either the TAG or the PL fractions. CLA has been previously detected in the testis of humans (Hoffmann *et al.*, 2005); therefore, it could be that the amount of lipid present in the sample was insufficient for the CLA to be detected (Hoffmann *et al.*, 2005), or that the methylation procedure with methanolic HCl prevented detection. CLA fed to Zucker *fa/fa* obese rats was incorporated into the TAG fraction, but not into the PL fraction when 1,1,3,3-tetramethylguanidine was used as the methylating agent (Kim *et al.*, unpublished data). The *trans*-10, *cis*-12 CLA fed mice had significantly increased levels of LA, but there was no further increase in the elongated and desaturated longer chain fatty acids in PL. This indicates that there is minimal effect of CLA in the testis fatty acid composition.

DPA has been shown to be important in spermatogenesis in rodent testes, and is particularly important at the end of the sperm maturation process (Beckman *et al.*, 1978). This study found that the DPA content showed a trend to increase in both CLA groups. Although this increase was not significant from the db-C group, it could be that the slight increase along with other changes in the testicular environment may be responsible for the positive improvement in sperm function.

Synthesis of DPA is the greater in the Sertoli cell than the germ cells, although the germ cells do have all of the components to synthesize DPA (Coniglio, 1994). Interestingly, early in DPA synthesis, it is found in TAG, and is largely in the Sertoli cells; however, as the germ cells become more differentiated, the DPA moves from the TAG to PL by crossing from the Sertoli cell into the germ cell (Coniglio, 1994). This could indicate that DPA is particularly important for sperm movement and for sperm morphology. Furthermore, there would be constant DPA turnover in the testis, as the DPA that is formed in the Sertoli cell is subsequently relinquished to the spermatozoa. This could be a driving force for DPA synthesis, because when a product, such as DPA, is removed from a pathway, there would be an increase in formation of the product. LA and AA supplementation has also been shown to increase DPA production, their elongated and desaturated product, in the testis and is correlated with the concentration of the LA or AA intake (Retterstol et al., 2000).

Effects of CLA on Seminolipids and Genes Involved in Seminolipid Synthesis

Despite having a significant amount of seminolipid in the testis, it is largely unknown if this testis specific lipid is affected by obesity and DM-2, and/or the diet treatment. The present study found that obese *db/db* mice have a significant increase in

the amount of seminolipid with respect to lipid in the testis when compared to lean mice. Supplementation with the *cis*-9, *trans*-11 CLA isomer in *db/db* mice was found to slightly decrease, though not significantly, the levels of seminolipid per lipid compared to the db-C group. The *trans*-10, *cis*-12 CLA isomer significantly decreased the seminolipid levels compared to the db-C group, and the seminolipid levels were statistically similar to the LC group. In contrast, obese *fa/fa* Zucker rats fed *trans*-10, *cis*-12 CLA had significantly increased seminolipids in the testes when compared to obese rats not fed CLA (Kim *et al.*, unpublished data). The differences could be attributed to a difference in species, age (25 week old Zucker rats v.s. 6 week old *db/db* mice), and/or seminolipid purification steps.

The genes regulating seminolipid synthesis were also measured in order to better understand the influence of CLA on testes in *db/db mice*. Both *cgt* and *cst* gene expression were significantly decreased in obese and diabetic mice fed control diet in comparison to the lean mice, indicating that seminolipid production was decreased. The *db/db* mice fed *trans*-10, ci*s*-*1*2 CLA had significantly increased *cgt* gene expression such that it was statistically similar to the LC group. There was a trend for increased *cgt* and *cst* expression in *cis*-9, *trans*-11 CLA fed *db/db* mice. Although the *cis*-*9*, tr*ans*-11 CLA isomer trended toward increasing the gene expression of *cst*, the level was not statistically different from the db-C group. These results were consistent with results from a previous study in our lab. They found that *cgt* expression significantly increased in both normal testes with *trans*-10, *cis*-12 CLA treatment, but the same was not true of the *cst* gene expression (Kim *et al.*, unpublished data).

Effects of CLA on Spermatogenic Genes

The genes involved in spermatogenesis were analyzed in this study. All of the genes measured, *Ccna*1, *Cdc*25*c*, *Hlf*3, *Sprm*-1 and *Prm*1, had decreased expression in the db-C group in comparison to the LC mice. In particular, *Ccna*1, *Cdc*25*c* and *Hlf*3 were significantly lower at only 57.6%, 45.6% and 42.4%, respectively, of the LC group. This indicates that there are genotypic differences in these genes, implying a lower rate of spermatogenesis in *db/b* mice. Considering the lower sperm counts in the db-C group, the decreased mRNA levels of *Ccna*1, *Cdc*25*c* and *Hlf*3 genes may reduce sperm production in the setting of DM-2 and obesity. In regards to dietary CLA treatment, there were no differences in mRNA levels, except for *Ccna*1. Both CLA isomers showed a significant increase in the *Ccna*1 expression.

Ccna1 encodes for the cyclin A1 protein, and is used to phosphorylate Cdc25 phosphatases (Liu *et al.*, 2000) and thereby play a role in regulating the cell cycle. Even though *Cdc25c* was not responsive to dietary treatment, the cyclin A1 proteins may have compensated for the lack in increase in *Cdc25c* gene expression by increasing the phosphorylation of the existing Cdc25c phosphatases. This would result in the activation of the Cdc25c protein (Clarke *et al.*, 1993), which subsequently allows the cell to undergo meiosis.

Spermatogenesis is a complicated process; therefore, other factors may also explain reduced sperm counts or normal morphology in DM-2 or obesity. For example, the testosterone-to-estradiol ratio may be altered (Cohen, 1999; Schneider *et al.*, 1979). ROS production may be increased, which would affect the lipid medium of the testis and spermatozoa (Ben Abdallah *et al.*, 2009; Mallidis *et al.*, 2009). DM-2 is also associated

with vascular problems that can cause ED (Selvin *et al.*, 2007). There may also be other genes involved in spermatogenesis that are affected by DM-2 that were not measured in this study.

In addition to the genotype difference, there may be another explanation for some of the results in this study. Leptin has a few mechanistic pathways, some more prominent than others. The well-known pathway is leptin activation of JAK2, followed by activation of the phosphatidylinositol-3-kinase (PI3K) pathway (Bjorbaek and Kahn, 2004). However, the other pathway through which leptin possibly works is the mitogen-activated protein kinase (MAPK) pathway (Perez-Perez *et al*., 2008). MAPK phosphorylates Cdc25 proteins, including Cdc25c (Wang *et al*., 2007). If this is the case for Cdc25c, perhaps the decrease in the stimulus, due to defective leptin signalling, would decrease the expression of *Cdc*25*c*. Furthermore, as was already discussed, cyclin A1 proteins play a role in the pathways of Cdc25c phosphatases. Although leptin signalling would be an issue for all of the *db/db* mice involved in the study, it clear that CLA affects the *Ccna1* gene regardless of any deficiencies in leptin signalling, potentially making it the possible route through which fertility potential is improved in the CLA treated mice.

Strengths and Weaknesses

A limitation in the current study relates to the model of DM-2 and obesity studied. *Db/db* mice develop extreme obesity and hyperglycemia as a result in a defect in the leptin receptor. Therefore, in this study it is unclear if the defects in sperm count and morphology are the result of DM-2 itself, or defective leptin signaling. Leptin receptors are found throughout the body, most notably on the adipocytes, and central to this study, in the testes, and in the hypothalamus. The hypothalamus is responsible for releasing

GnRH, which signals the anterior pituitary to produce and release LH and FSH (Widmaier *et al.*, 2006). The LH signals the Leydig cells to produce testosterone, and the Sertoli cells to stimulate spermatogenesis. Leptin is important in the release of GnRH (Quennell *et al*., 2009). As GnRH is important in spermatogenesis, the reduction in sperm count and normal sperm morphology could potentially be explained by leptin-receptor mediated reductions in GnRH.

One of the major strengths in the current study relates to its originality. This study is novel because it is the first to focus on reproductive function in obesity and DM-2, using the *db/db* mouse model. It also is the first study to look into the effects that CLA has on male fertility in obesity and DM-2. In addition, this study design used a paired-weight group, which was an important strength in the study. The paired-weight group ensures that if CLA is found to be effective, that the improvement in reproductive function cannot be attributed to weight loss alone. Finally, this study determined sperm parameters and organ weights, but also studied a potential mechanism regarding the cause of obesity and DM-2 related infertility.

Summary

Overall this study found that there are genotypic differences in sperm parameters such as decreased sperm counts and decreased morphologically normal sperm in *db/db* mice. Furthermore, this study showed that testis weight, epididymis weight and seminal vesicle weights were all significantly decreased, and prostate weight was significantly increased in *db/db* mice compared to lean mice. The *trans*-10, *cis*-12 CLA diet improved the testis and prostate sizes, and significantly increased testis *cgt* mRNA expression that is involved in seminolipid synthesis. On the other hand, the *cis*-9, *trans*-11 CLA isomer

did not improve testis and prostate sizes. However, both CLA isomers were able to slightly improve sperm counts and significantly improve the numbers of morphologically normal sperm in *db/db* mice. Moreover, CLA treatment likely improved the number of functioning sperm by elevating the DPA levels in the PL fraction of the testes, and by increasing *Ccna*1 gene expression, which regulates meiosis, and therefore spermatogenesis. Despite hindered leptin signaling, there were many results in this study that showed that CLA isomers might be able to improve fertility in DM-2 and obesity.

Future studies

If further studies were being done to determine whether or not CLA is beneficial for diabetic and obese males who have problems reproducing, prior to any clinical trial, the next step would be trying a similar experiment on a different mouse model. In that way, the potential for error of the leptin receptor mutation can be eliminated. Although no model is perfect, perhaps the spiny mouse would be an appropriate next step. This mouse becomes diabetic and obese when it undergoes a high-energy dietary intervention, and thus it would be similar to the DM-2 present in the human population (Shafrir *et al.,* 2006; Srinivasan and Ramarao, 2007).

Table III-1. The diet formulation of the control and CLA diets.

Ingredients (g/kg)	Control	Treatment	
	LC, db-C, db-PW	db-9,11 CLA	db-10,12 CLA
Cornstarch[1]	363	363	363
Maltodextrin[2]	132	132	132
Sucrose[2]	100	100	100
Egg white[2]	212.5	212.5	212.5
Cellulose[2]	50	50	50
AIN-93MXG-Mineral Mix[2]	35	35	35
AIN-93VX-Vitamin Mix[2]	10	10	10
Choline	2.5	2.5	2.5
Biotin Mix (200mg biotin/kg cornstarch)[2]	10	10	10
Tert-butylhydroquinone[3]	0.014	0.014	0.014
Soy oil[2]	85	80.57	80.57
cis-9, *trans*-11 CLA[4]	0	4.44	0
trans-10, *cis*-12 CLA[4]	0	0	4.44

[1]Casco Inc. Etobicoke, Ontario Canada
[2]Harlan Teklad Madison, WI, USA
[3]Sigma-Aldrich, St. Louis, MO
[4]Larodan AB, Malmo, Sweden

Table III-2. Effect of dietary CLA isomers on body weight and reproductive organ weights in *db/db* mice[1]

	LC (n=10)	db-C (n=9)	db-9,11 CLA (n=10)	db-10,12 CLA (n=11)	db-PW (n=10)	Significant Effects[2] (p value)
Body Weight (g)	27.76 ± 1.84^{b}	34.83 ± 1.57^{a}	36.74 ± 5.98^{a}	23.96 ± 2.49^{c}	26.11 ± 2.88^{bc}	<0.0001
Testes[3] (mg)	176.00 ± 23.24^{a}	117.97 ± 17.09^{c}	130.72 ± 28.19^{bc}	139.24 ± 15.67^{b}	74.22 ± 17.22^{d}	<0.0001
Epididymides[3] (mg)	20.20 ± 2.54^{a}	12.94 ± 1.94^{b}	14.47 ± 4.19^{b}	13.76 ± 4.99^{b}	5.76 ± 1.72^{c}	<0.0001
Prostate (mg)	56.48 ± 16.50^{c}	162.69 ± 24.85^{a}	162.03 ± 50.74^{a}	32.09 ± 11.53^{c}	97.36 ± 25.82^{b}	<0.0001
Seminal Vesicles[3] (mg)	276.33 ± 33.53^{a}	152.40 ± 14.73^{b}	146.64 ± 41.87^{b}	127.38 ± 25.31^{bc}	99.63 ± 34.87^{c}	<0.0001
Relative to Body Weight (%, w/w)						
Testes[3]	0.64 ± 0.10^{a}	0.34 ± 0.05^{bc}	0.36 ± 0.08^{b}	0.58 ± 0.06^{a}	0.29 ± 0.06^{c}	<0.0001
Epididymides[3]	0.07 ± 0.01^{a}	0.05 ± 0.01^{b}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.02 ± 0.01^{c}	<0.0001
Prostate	0.20 ± .05^{c}	0.47 ± 0.09^{a}	0.44 ± 0.09^{a}	0.13 ± 0.04^{d}	0.37 ± 0.06^{b}	<0.0001
Seminal Vesicles[3]	1.00 ± 0.12^{a}	0.44 ± 0.04^{bc}	0.40 ± 0.08^{c}	0.53 ± 0.07^{b}	0.38 ± 0.13^{c}	<0.0001

[1]Values are means ± SD.

[2]The p value and significance was identified by one-way analysis of variance. Values within a row having a different letter superscript are significantly
different by multiple comparison, $p<0.05$. NS, not significant

[3]Combined weights of the right and left testis and epididymis

Table III-3. Effect of dietary CLA isomers on fatty acid composition of triacylglycerols in the testes of *db/db* mice[1]

Fatty Acid (%, w/w)	LC (n=8)	db-C (n=7)	db-9,11-CLA (n=8)	db-10,12-CLA (n=9)	db-PW (n=8)	Significant Effects[2] (p value)
C14:0	0.29 ± 0.22^{d}	0.65 ± 0.33^{c}	0.99 ± 0.21^{b}	0.64 ± 0.16^{c}	1.27 ± 0.17^{a}	<0.0001
C15:0	0.07 ± 0.04^{b}	0.12 ± 0.02^{a}	0.10 ± 0.02^{a}	0.09 ± 0.01^{a}	0.12 ± 0.02^{a}	<0.0009
C15:1	0.26 ± 0.26	0.07 ± 0.06	0.05 ± 0.02	0.18 ± 0.32	0.07 ± 0.05	NS
C16:0	16.40 ± 2.03^{c}	24.00 ± 1.79^{b}	23.82 ± 1.65^{b}	21.81 ± 1.76^{b}	27.41 ± 3.08^{a}	<0.0001
C16:1(n-5+n-7)	5.10 ± 1.90^{a}	4.57 ± .76^{a}	5.78 ± 1.55^{a}	2.83 ± 0.82^{b}	5.00 ± 1.63^{a}	0.0004
C17:0	0.14 ± 0.01^{b}	0.16 ± 0.03^{b}	0.14 ± 0.02^{b}	0.19 ± 0.02^{a}	0.14 ± 0.02^{b}	<0.0001
C18:0	3.54 ± 1.61^{c}	4.78 ± 0.62^{abc}	4.17 ± 1.20^{bc}	5.59 ± 0.92^{a}	4.95 ± 1.22^{ab}	0.0123
C18:1(n-7+n-9)	25.89 ± 4.53	22.88 ± 3.03	24.84 ± 4.37	24.17 ± 5.71	23.43 ± 4.96	NS
C18:2(n-6)	28.07 ± 8.02	22.00 ± 4.22	21.74 ± 5.97	19.93 ± 3.98	20.20 ± 4.94	NS
C18:3(n-6)	0.16 ± 0.04^{bc}	0.19 ± 0.03^{ab}	0.16 ± 0.06^{bc}	0.22 ± 0.06^{a}	0.13 ± 0.04^{c}	0.0112
C18:3(n-3)	2.48 ± 0.83^{a}	1.66 ± 0.38^{b}	1.74 ± 0.69^{b}	0.75 ± 0.19^{c}	1.29 ± 0.46^{bc}	<0.0001
C20:0	0.11 ± 0.05	0.10 ± 0.00	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.04	NS
C20:1(n-9)	0.48 ± 0.09^{a}	0.35 ± 0.04^{b}	0.36 ± 0.04^{b}	0.46 ± 0.07^{a}	0.34 ± 0.06^{b}	<0.0001
C20:2	0.21 ± 0.11^{c}	0.34 ± 0.07^{ab}	0.26 ± 0.08^{bc}	0.46 ± 0.14^{a}	0.36 ± 0.15^{ab}	0.0007
C20:3(n-9)	0.13 ± 0.09^{a}	0.10 ± 0.04^{ab}	0.09 ± 0.04^{ab}	0.03 ± 0.04^{c}	0.04 ± 0.04^{bc}	0.0033
C20:3(n-6)	0.96 ± 0.83	1.03 ± 0.28	0.88 ± 0.46	1.28 ± 0.44	0.81 ± 0.41	NS
C20:4(n-6)	1.68 ± 1.13	2.52 ± 0.68	2.15 ± 1.29	3.24 ± 1.07	2.35 ± 1.24	NS
C20:3(n-3)	0.01 ± 0.01	0.00 ± 0.01	0.02 ± 0.03	0.00 ± 0.01	0.00 ± 0.01	NS
C20:5	0.33 ± 0.029	0.31 ± 0.12	0.17 ± 0.08	0.34 ± 0.36	0.16 ± 0.08	NS
C24:0	0.22 ± 0.20	0.18 ± 0.15	0.17 ± 0.17	0.23 ± 0.18	0.12 ± 0.06	NS
C22:4(n-6)	1.31 ± 1.24	1.49 ± 0.43	1.29 ± 0.75	1.93 ± 0.63	1.11 ± 0.56	NS
C22:5(n-6)	6.40 ± 5.11	6.02 ± 1.96	5.49 ± 2.97	7.55 ± 2.68	4.43 ± 2.59	NS
C24:1	0.10 ± 0.15	0.05 ± 0.03	0.06 ± 0.06	0.14 ± 0.31	0.04 ± 0.02	NS
C22:5(n-3)	0.65 ± 0.50	0.73 ± 0.19	0.69 ± 0.42	0.96 ± 0.27	0.68 ± 0.36	NS
C22:6(n-3)	2.82 ± 2.13	3.45 ± 1.02	3.18 ± 1.89	4.69 ± 1.52	3.84 ± 2.14	NS
C24:4(n-6)	0.36 ± 0.32	0.38 ± 0.13	0.26 ± 0.14	0.40 ± 0.13	0.31 ± 0.15	NS
C24:5(n-6)	1.01 ± 0.82	1.23 ± 0.43	0.91 ± 0.52	1.36 ± 0.49	0.92 ± 0.50	NS
∑ SAT[3]	20.61 ± 1.50^{c}	29.81 ± 2.29^{b}	29.35 ± 2.68^{b}	28.45 ± 2.41^{b}	34.00 ± 3.89^{a}	<0.0001
∑ MUFA[3]	32.39 ± 3.89	28.31 ± 2.94	31.37 ± 4.73	28.06 ± 5.76	29.15 ± 5.68	NS
∑ n-6[3]	39.95 ± 2.35^{a}	34.86 ± 1.35^{bc}	32.87 ± 1.60^{c}	35.90 ± 2.58^{b}	30.27 ± 2.82^{d}	<0.0001
∑ n-3[3]	5.96 ± 1.83	5.84 ± 0.86	5.63 ± 1.73	6.40 ± 1.65	5.81 ± 2.15	NS

[1]Values are means ± SD.

[2]Significant effects were identified by one-way analysis of variance. Values within a row having a different letter superscript are significantly different by multiple comparison, $p<0.05$. NS, not significant

[3]∑ SAT, sum of saturated fatty acids; ∑ Mono, sum of monounsaturated fatty acids; ∑ n-6, sum of n-6 fatty acids; ∑ n-3, sum of n-3 fatty acids.

Table III-4. Effect of dietary CLA isomers on fatty acid composition of phospholipids in the testes of *db/db* mice[1]

Fatty Acid (%, w/w)	LC (n=8)	db-C (n=7)	db-9,11-CLA (n=8)	db-10,12-CLA (n=9)	db-PW (n=8)	Significant Effects[2] (p value)
C14:0	0.17 ± 0.03^{c}	0.17 ± 0.02^{bc}	0.28 ± 0.11^{a}	0.19 ± 0.06^{bc}	0.25 ± 0.11^{ab}	0.0186
C15:0	0.17 ± 0.02	0.19 ± 0.04	0.49 ± 1.21	0.09 ± 0.06	0.15 ± 0.03	NS
C15:1	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.02	0.02 ± 0.02	0.04 ± 0.03	NS
C16:0	42.84 ± 4.12	45.72 ± 3.21	42.25 ± 3.71	43.21 ± 8.51	43.18 ± 4.47	NS
C16:1(n-5+n-7)	0.66 ± 0.03^{a}	0.62 ± 0.13^{ab}	0.68 ± 0.07^{a}	0.56 ± 0.13^{b}	0.70 ± 0.07^{a}	0.0194
C17:0	0.12 ± 0.02^{ab}	0.14 ± 0.03^{a}	0.11 ± 0.01^{b}	0.15 ± 0.04^{a}	0.12 ± 0.02^{ab}	0.0204
C18:0	9.05 ± 0.58^{a}	9.30 ± 1.25^{a}	7.50 ± 0.66^{b}	8.30 ± 1.35^{ab}	9.01 ± 1.37^{a}	0.0182
C18:1(n-7+n-9)	11.55 ± 0.75	11.95 ± 2.18	10.16 ± 0.72	11.86 ± 1.03	12.14 ± 1.61	NS
C18:2(n-6)	1.54 ± 0.11^{c}	1.94 ± 0.48^{bc}	2.16 ± 0.32^{ab}	2.57 ± 0.39^{a}	2.35 ± 0.57^{ab}	<0.0001
C18:3(n-6)	0.03 ± 0.02	0.03 ± 0.02	0.06 ± 0.06	0.03 ± 0.02	0.06 ± 0.06	NS
C20:0	0.14 ± 0.01^{a}	0.14 ± 0.02^{a}	0.10 ± 0.01^{b}	0.08 ± 0.03^{b}	0.15 ± 0.04^{a}	<0.0001
C20:1(n-9+n-12)	0.16 ± 0.06	0.21 ± 0.06	0.15 ± 0.08	0.16 ± 0.11	0.14 ± 0.06	NS
C20:2 (n-6)	0.12 ± 0.01	0.14 ± 0.02	0.14 ± 0.02	0.15 ± 0.06	0.13 ± 0.02	NS
C20:3(n-9)	0.09 ± 0.04^{a}	0.05 ± 0.05^{ab}	0.07 ± 0.04^{ab}	0.04 ± 0.05^{b}	0.03 ± 0.04^{b}	0.0376
C20:3(n-6)	1.09 ± 0.12^{ab}	1.04 ± 0.22^{ab}	1.25 ± 0.15^{a}	1.25 ± 0.28^{a}	0.90 ± 0.23^{b}	0.0085
C20:4(n-6)	10.27 ± 1.24^{a}	7.89 ± 1.44^{b}	10.43 ± 1.07^{a}	9.54 ± 2.85^{ab}	8.17 ± 1.71^{b}	0.0248
C22:0	0.31 ± 0.04^{ab}	0.36 ± 0.05^{a}	0.26 ± 0.06^{b}	0.24 ± 0.10^{b}	0.39 ± 0.11^{a}	0.0014
C22:1	0.06 ± 0.02	0.06 ± 0.07	0.03 ± 0.06	0.01 ± 0.02	0.08 ± 0.06	NS
C20:5	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.05	0.08 ± 0.05	0.04 ± 0.02	NS
C22:4(n-6)	0.97 ± 0.16	0.89 ± 0.16	1.10 ± 0.11	1.01 ± 0.36	1.15 ± 0.25	NS
C22:5(n-6)	8.52 ± 1.49^{ab}	7.67 ± 1.84^{ab}	10.07 ± 1.78^{a}	9.51 ± 3.72^{a}	6.62 ± 1.75^{b}	0.0378
C24:1	0.11 ± 0.04^{a}	0.11 ± 0.04^{a}	0.05 ± 0.05^{b}	0.05 ± 0.05^{b}	0.10 ± 0.08^{ab}	0.0412
C22:5(n-3)	0.14 ± 0.03	0.12 ± 0.03	0.18 ± 0.05	0.12 ± 0.10	0.15 ± 0.08	NS
C22:6(n-3)	4.53 ± 0.86	3.63 ± 1.19	5.49 ± 0.97	4.79 ± 2.16	3.90 ± 1.35	NS
C24:4(n-6)	0.41 ± 0.06	0.36 ± 0.06	0.22 ± 0.23	0.39 ± 0.23	0.45 ± 0.07	NS
C24:5(n-6)	0.86 ± 0.16	0.75 ± 0.15	0.46 ± 0.50	0.80 ± 0.51	0.82 ± 0.19	NS
∑SAT[3]	56.26 ± 4.25	59.56 ± 4.48	54.20 ± 4.26	55.51 ± 9.76	57.42 ± 4.72	NS
∑ MUFA[3]	12.70 ± 0.79	13.09 ± 2.36	13.14 ± 0.82	12.77 ± 1.27	13.41 ± 1.50	NS
∑ n-6[3]	13.41 ± 2.05	12.69 ± 2.68	15.32 ± 2.20	15.56 ± 5.00	12.35 ± 2.48	NS
∑ n-3[3]	4.67 ± 0.88	3.75 ± 1.21	5.66 ± 0.99	4.91 ± 2.24	4.05 ± 1.35	NS

[1]Values are means ± SD.

[2]Significant effects were identified by one-way analysis of variance. Values within a row having a different letter superscript are significantly different by multiple comparison, $p<0.05$. NS, not significant

[3]∑ SAT, sum of saturated fatty acids; ∑ Mono, sum of monounsaturated fatty acids; ∑ n-6, sum of n-6 fatty acids; ∑ n-3, sum of n-3 fatty acids.

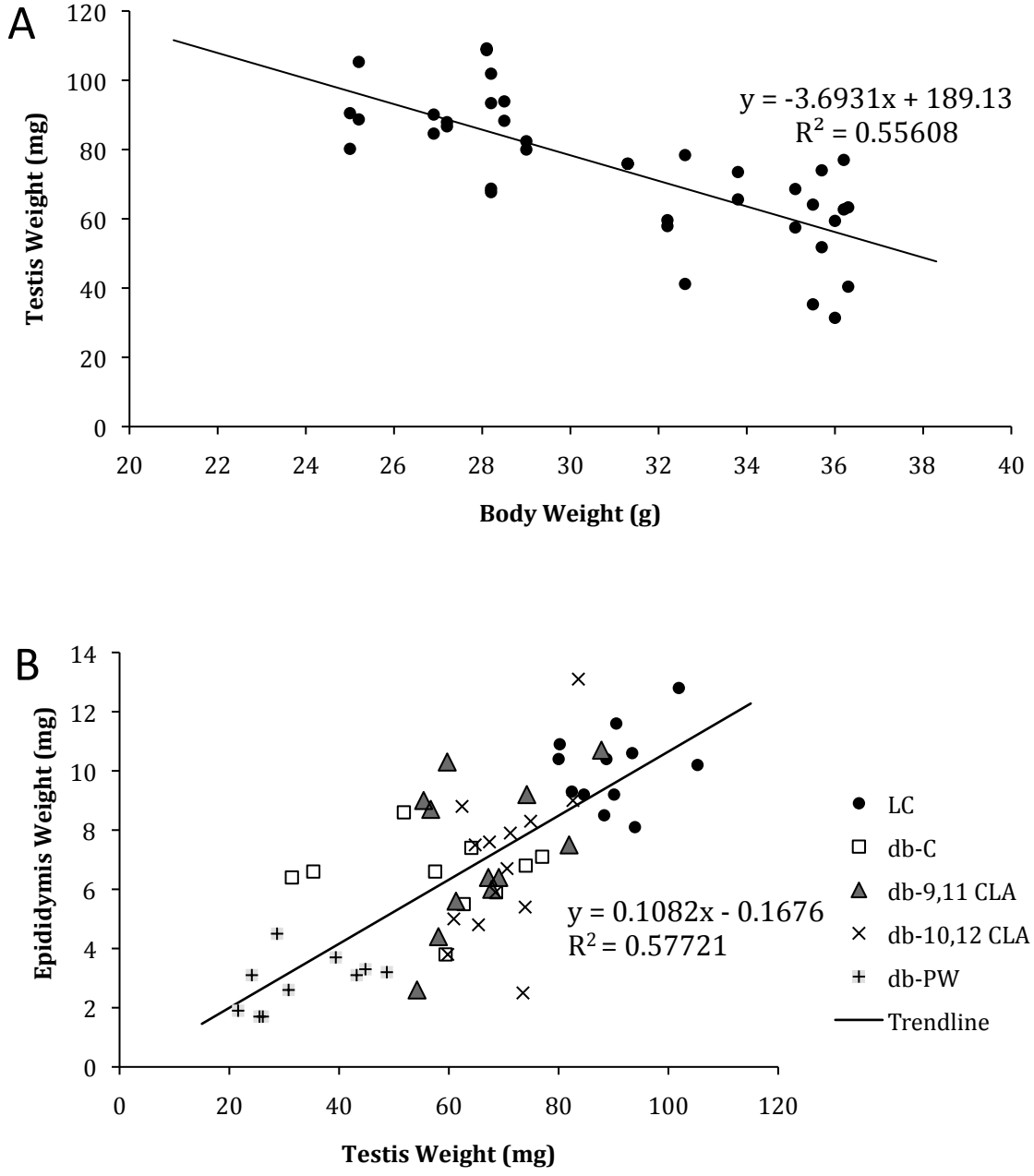

Figure III-1. Effects of CLA on testis weight in *db/db* mice.
A) Correlation of testis weight to body weight in LC versus db-C mice
B) Correlation of testis weight to its respective epididymis weight.

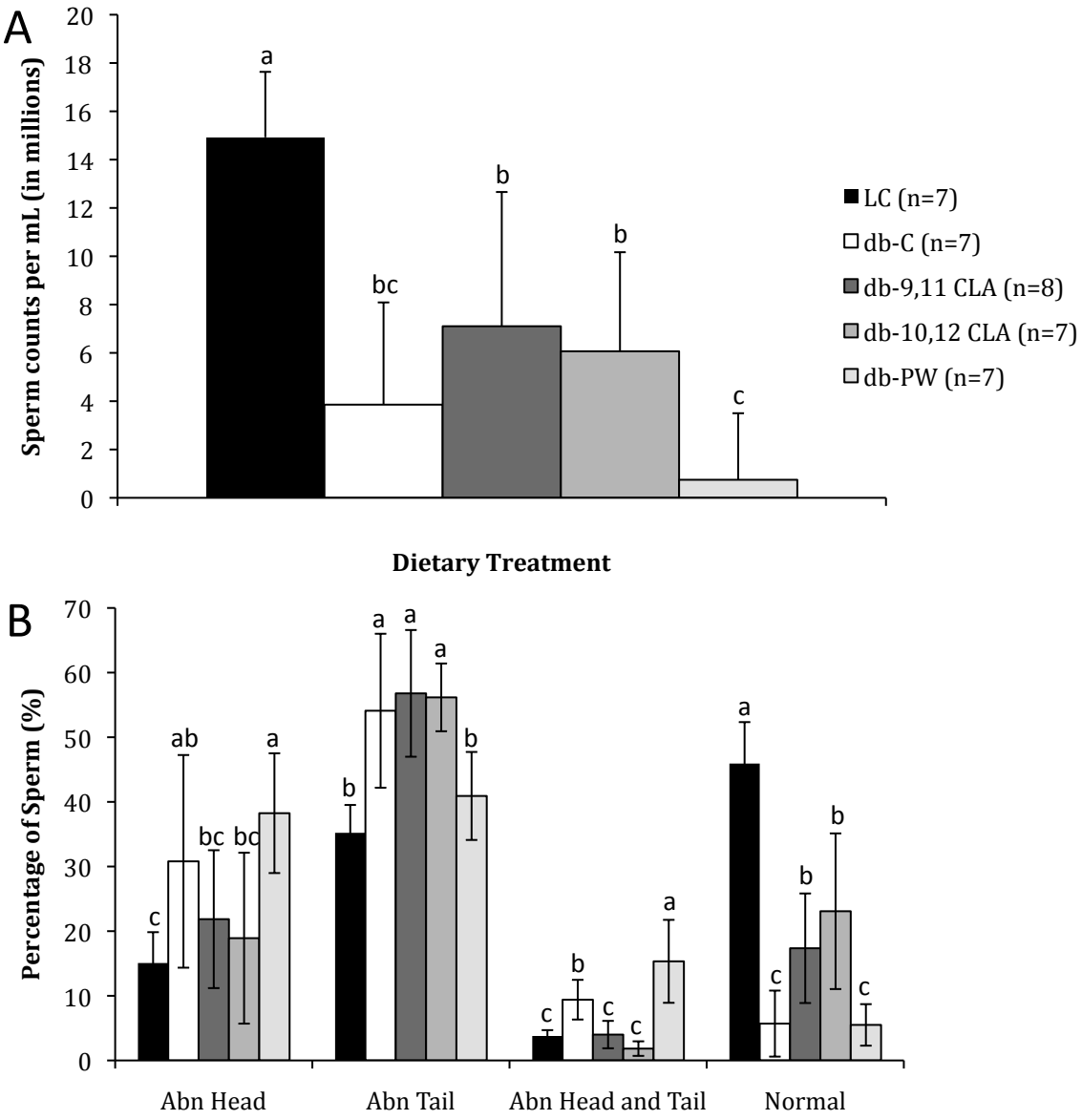

Figure III-2. Effect of dietary CLA on sperm parameters in *db/db* mice.
A) Effect of dietary CLA isomers on sperm counts, $p<0.0001$. Cauda epididymal spermatozoa were counted and expressed per millilitre.
B) Effect of dietary CLA isomers on sperm morphology, $p<0.0072$. Two hundred sperm were counted to identify head abnormalities, tail abnormalities as well as head and tail abnormalities. Bars represent the mean ± SD. Significant effects were identified by one-way analysis of variance. Different letters within a group of sperm parameters are significantly different by multiple comparison. Abn, abnormal.
Abn head and tail – $p<0.0001$ Abn head – $p=0.0072$ Abn tail – $p<0.0001$ Normal – $p<0.0001$

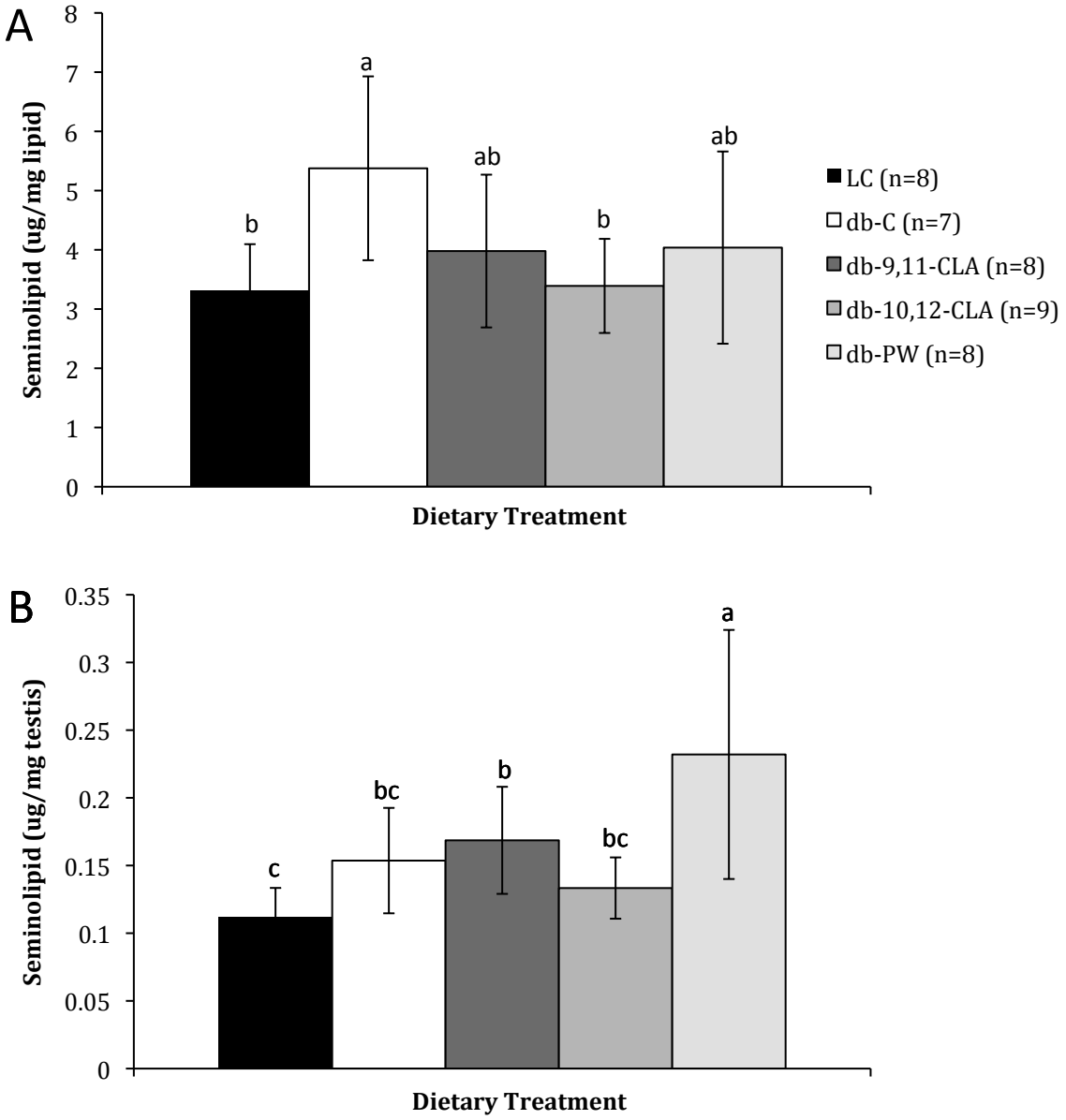

Figure III-3. Effect of dietary CLA on seminolipid in *db/db* mice.
A) Seminolipid in total lipid in the testes, p=0.0351
B) Seminolipid per mg testis, p=0.0004
Bars represent the mean ± SD. Significant effects were identified by one-way analysis of variance. Different letters within a group of seminolipid analysis are significantly different by multiple comparison.

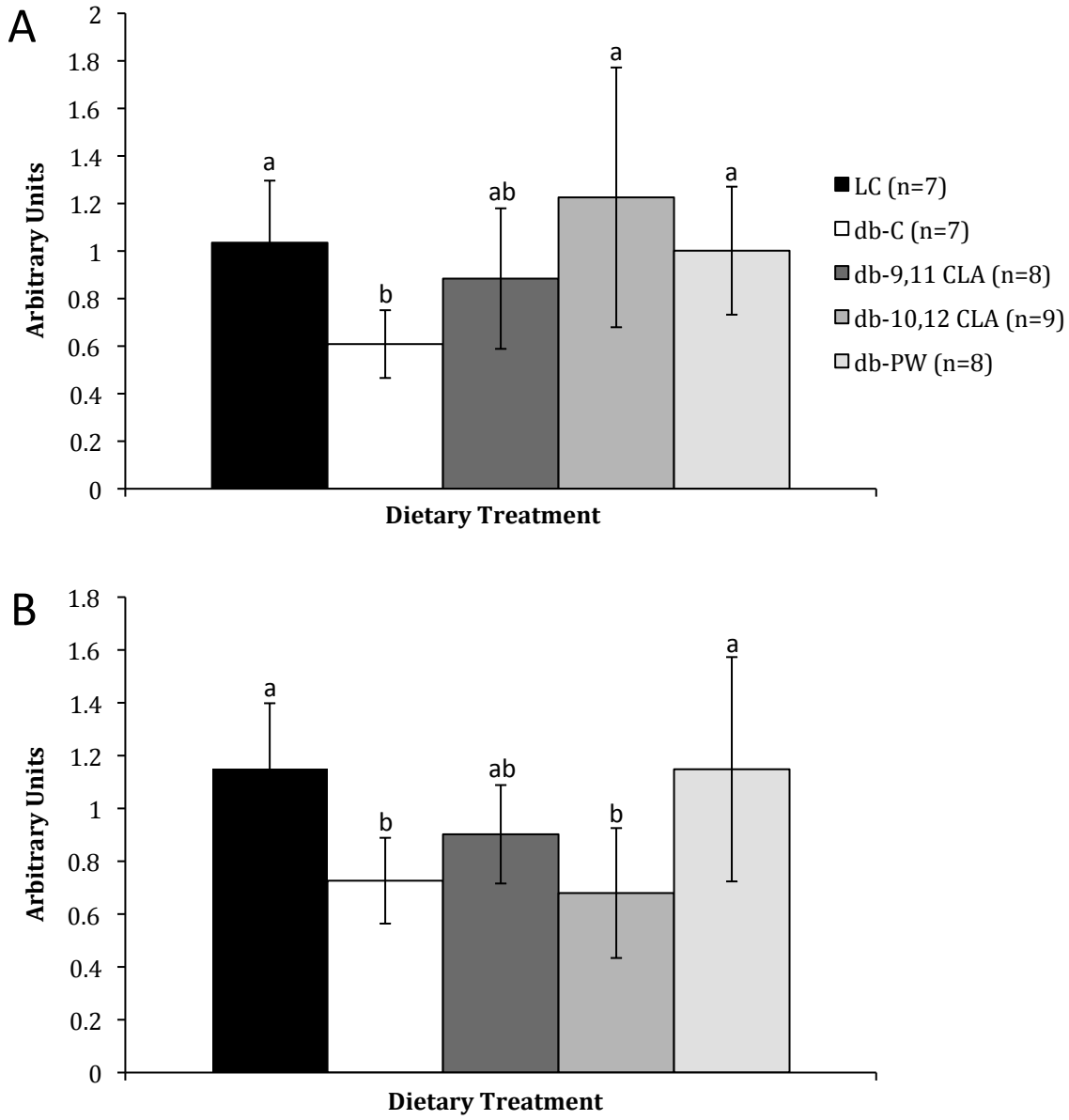

Figure III-4. Effect of dietary CLA on genes involved in seminolipid synthesis in *db/db* mice.

A) Ceramide galactosyl transferase (*cgt*), p=0.02

B) Cerebroside-3-sulfotransferase (*cst*), p=0.0026

Measured by $2^{\Delta\Delta Ct}$ using ß-actin as the housekeeping gene. Bars represent the mean ± SD. Significant effects were identified by one-way analysis of variance. Different letters within each gene are significantly different by multiple comparison.

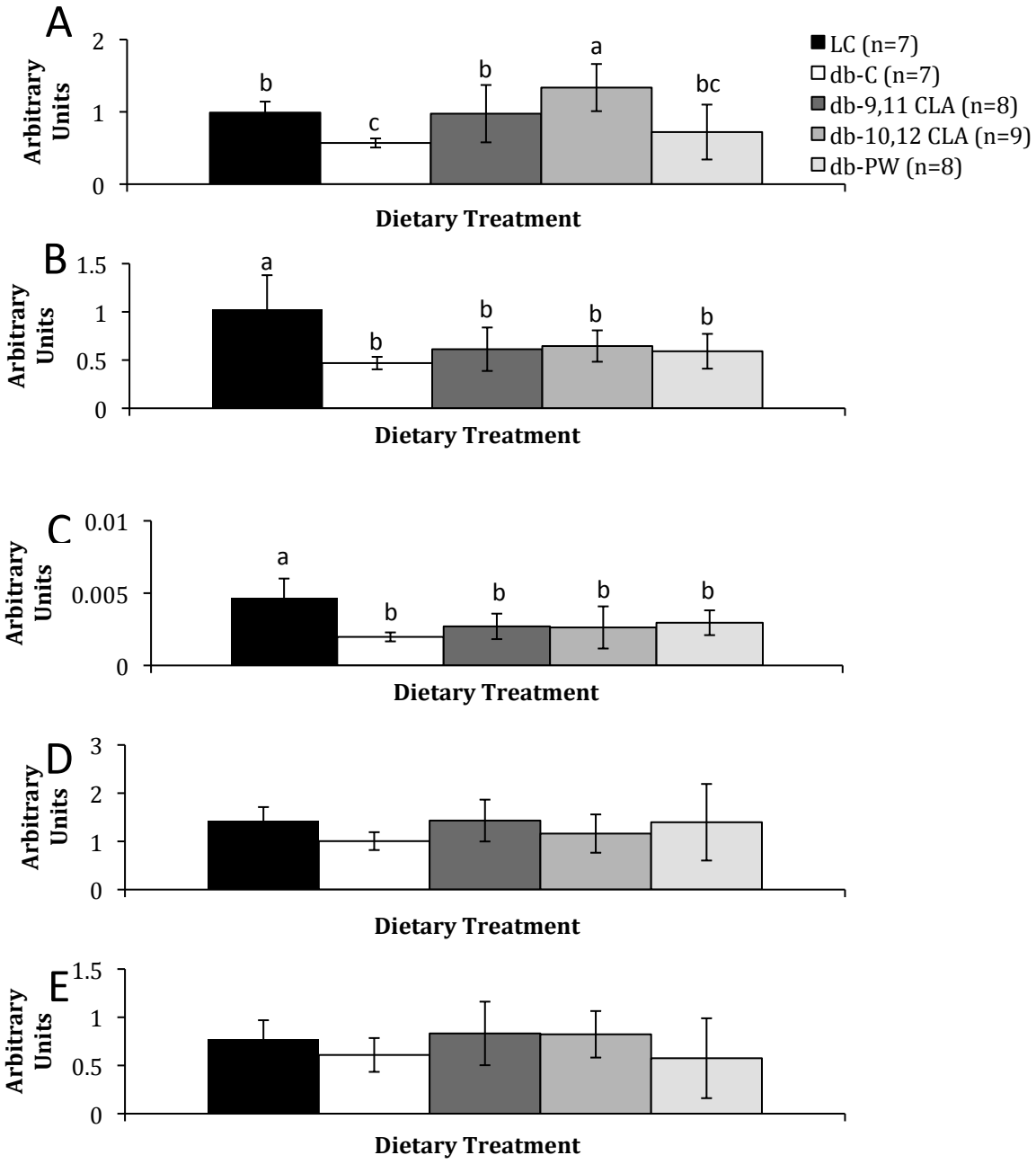

Figure III-5. Effects of dietary CLA on spermatogenic gene expression

A) *Ccna*1 gene expression, p=0.0002

B) *Cdc*25*c* gene expression, p=0.0004

C) *Hlf*3 gene expression, p=0.0007

D) *Sprm*-1 gene expression, NS

E) *Prm*1 gene expression, NS

Measured by $2^{\Delta\Delta Ct}$ using ß-actin as the housekeeping gene. Bars represent the mean ± SD. Significant effects were identified by one-way analysis of variance. Different letters within each gene are significantly different by multiple comparison. NS, not significant.

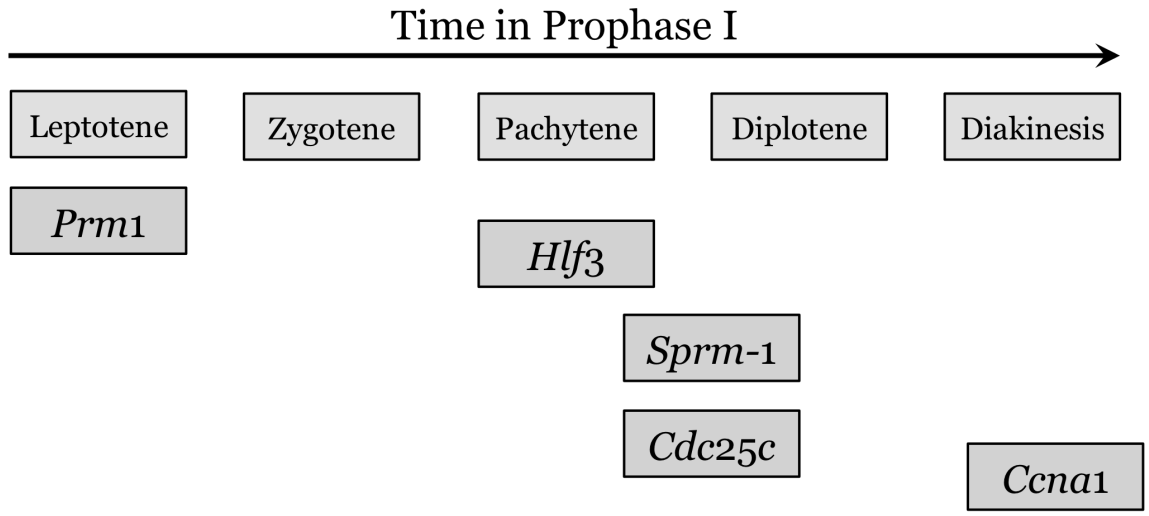

Figure III-6. Time course in the expression of genes associated with spermatogenesis.

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APPENDIX

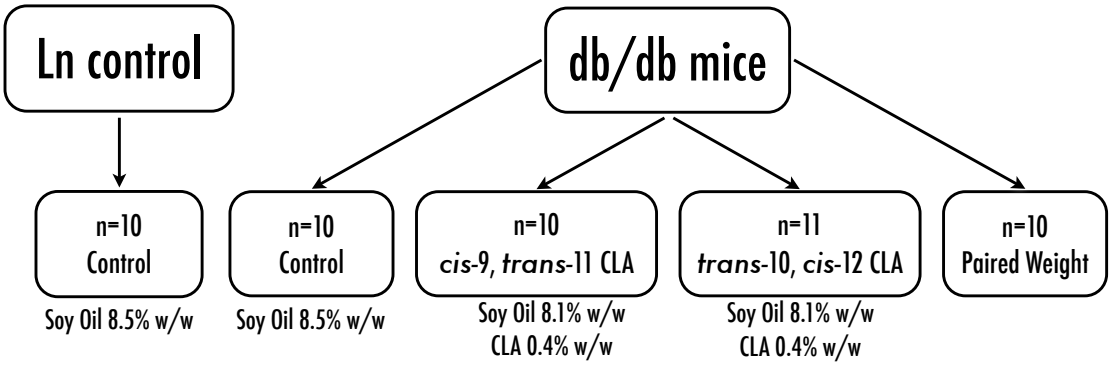

Figure A-1. Study Design

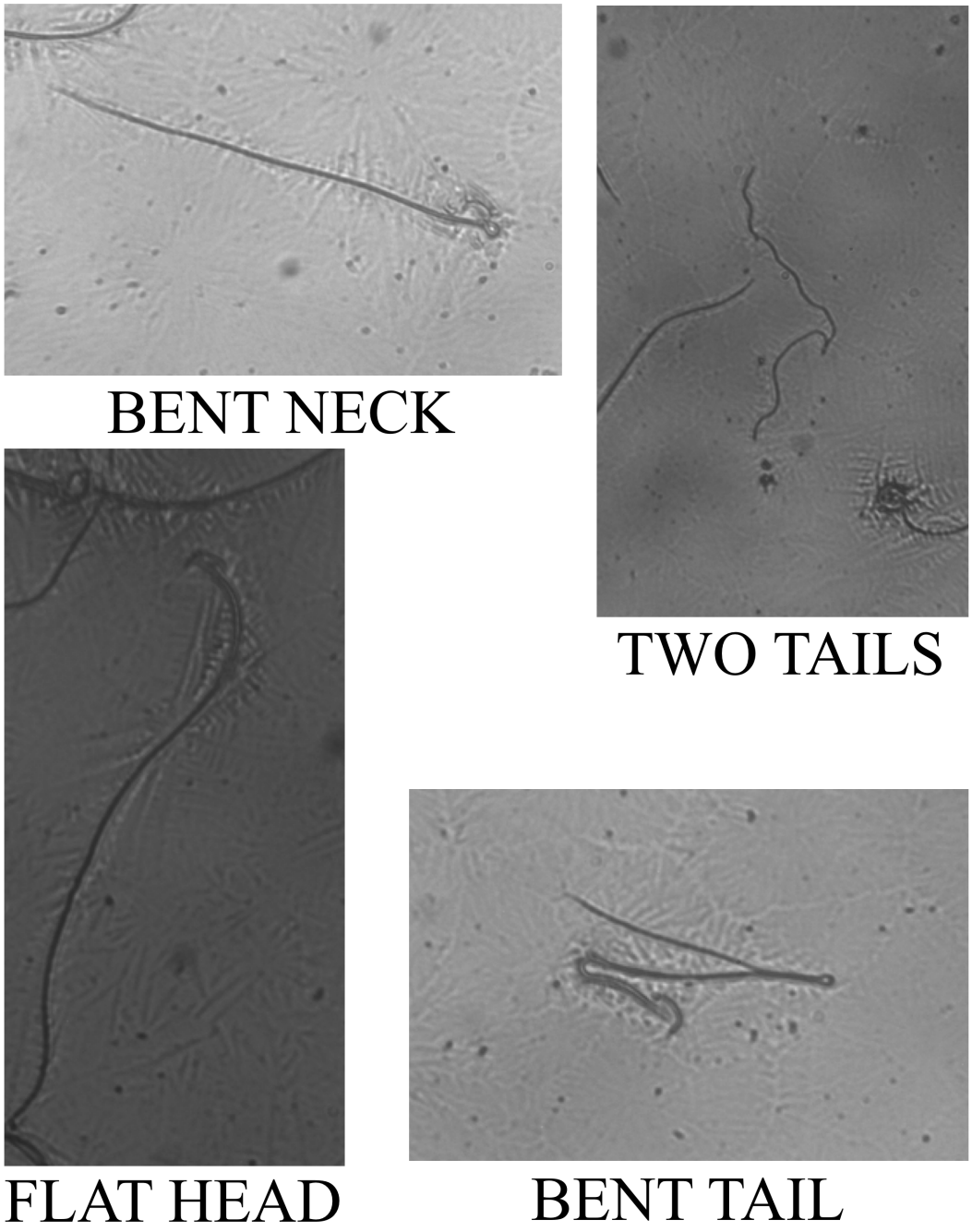

Figure A-2. Light microscopy of abnormal sperm found in *db/db* mice

Table A-3. Specifications for q RT-PCR conditions for respective genes.

Genes	***cst***		***cgt***		***Ccna1***		***Cdc25c***	
	T (˚C)	Time	T (˚C)	Time	T (˚C)	Time	T (˚C)	Time
Initial Temp.	50.0	10min	50.0	10min	50.0	10min	50.0	10min
	95.0	5min	95.0	5min	95.0	5min	95.0	5min
Denaturing	95.0	15s	95.0	15s	95.0	15s	95.0	15s
Annealing	66.0	30s	64.3	30s	57.1	30s	57.1	30s
	72.0	30s	72.0	30s	72.0	30s	72.0	30s
# of Cycles	40		40		40		40	

Genes	***Sprm-1***		***Hlf3***		***Prm1***		**β-*actin***	
	T (˚C)	Time	T (˚C)	Time	T (˚C)	Time	T (˚C)	Time
Initial Temp.	50.0	10min	50.0	10min	50.0	10min	50.0˚C	10min
	95.0	5min	95.0	5min	95.0	5min	95.0	5min
Denaturing	95.0	15s	95.0	15s	95.0	15s	95.0	15s
Annealing	63.3	30s	63.3	30s	61.4	30s	58.0	30s
	72.0	30s	72.0	30s	72.0	30s	72.0	30s
# of Cycles	40		40		40		40	

Table A-4. The number of unbalanced testes in each group and the percent difference between the testes.

	LC (n=10)	db-C (n=9)	db-9,11 CLA (n=10)	db-10,12 CLA (n=11)	db-PW (n=10)
<10%	8	1	7	11	3
10-19.9%	2	2	1	0	3
20-29.9%	0	1	1	0	0
30-39.9%	0	1	0	0	0
40-49.9%	0	2	0	0	0
50-59.9%	0	1	1	0	1
>60%	0	1	0	0	3