Determining the role of choline dehydrogenase in sperm cell function

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ABSTRACT

AMY ROSE JOHNSON: Determining the role of choline dehydrogenase in sperm cell function
(Under the direction of Steven H. Zeisel)

Approximately 15% of couples suffer from infertility and male factor infertility is the suspected cause in about half of these couples. 40-60% these male factor infertility cases are idiopathic, but genetic aberrations are associated with infertility in as many as 30% of these. Although the relationship between nutrition and reproduction is established, the role of micronutrient metabolism in male fertility is understudied. Choline, an essential nutrient for humans, is important for maintaining a healthy pregnancy and normal fetal development. Choline is necessary for normal mating behavior and male fertility in *D. melanogaster* but the reason why remains unknown.

Choline dehydrogenase (CHDH) catalyzes the conversion of choline into betaine, a methyl group donor and osmolyte. Several single nucleotide polymorphisms (SNPs) within the *CHDH* gene may alter the function of the CHDH. A choline dehydrogenase knockout mouse ($Chdh^{-/-}$) was created to model loss-of-function mutations in humans. Mutation of *Chdh* reduced CHDH activity and decreased betaine concentration in all tissues that normally express this enzyme. Fetal viability, growth and one-year survival rates were not affected. $Chdh^{-/-}$ animals had 59% more plasma homocysteine, but hepatic

AdoMet and AdoHcy were unchanged. $Chdh^{-/-}$ males were infertile due to poor sperm motility. Abnormal mitochondrial morphology and function were observed in $Chdh^{-/-}$ sperm. ATP concentration was 55% lower in $Chdh^{-/-}$ sperm. Dietary betaine supplementation resulted in increased $Chdh^{-/-}$ sperm motility, and full restoration of ATP concentration.

CHDH SNP rs12676 (G233T; R→L) and *IL17βR* SNP rs1025689 (G126C; P→P) are associated with changes in mitochondrial function and sperm motility in men. Men who were TT for rs12676 produced sperm with dysmorphic mitochondria. Compared to GG subjects, sperm produced by GT subjects contained 40% less ATP; men who were TT for this SNP had 73% less ATP. Motility characteristics were changed in men with at least one minor allele of either rs12676 or rs1025689. CHDH protein was decreased in primary hepatocytes from individuals who were TT for rs12676, indicating this SNP marks a functional haplotype. We propose that aberrant choline metabolism stemming from decreased CHDH activity may be an underlying cause of idiopathic male factor infertility in men.

To my Grandmothers, Mary Ruth Langan and Vivian Notwick, for serving as incredible role models and teaching me what is really important in life.

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LIST OF ABBREVIATIONS

AA	arachidonic acid, 20:4n6
ACho	acetylcholine
AdoHcy	S-adenosylhomocysteine
AdoMet	S-andeosylmethionine
AI	adequate intake
ALT	alanine transaminase
ANOVA	analysis of variance
ATP	adenosine triphosphate
BADH	betaine aldehyde dehydrogenase
BGT-1	betaine γ-aminobutyric acid transporter -1
BHMT	betaine:homocysteine methyltransferase
BUN	blood urea nitrogen
CDP-choline	cytidine diphosphocholine
ChAT	choline acetyltransferase
Chdh	choline dehydrogenase, mouse
CHDH	choline dehydrogenase, human
CHT1	choline transporter -1, high affinity
CK	choline kinase
CPK	creatinine phosphokinase
CTL-1/SLC44A1	choline transporter-like 1, human
Ctl-1/Slc44a1	choline transporter-like 1, mouse

DHA	docosahexanoic acid, 22:6n3
DPA	docosapentanoic acid, 22:5n3
ECAR	extracellular acidification rate
FAD	flavin adenine nucleotide
FCCP	carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone
Gapdhs	glyceraldehyde 3-phosphate dehydrogenase, spematogenic, mouse
GPCho	Glycerophosphocholine
Hcy	Homocysteine
HTF	human tubular fluid media
JC-1	5,5',6,6'-tetrachloro- 1,1,3,3'-tetraethylbenzimidazolylcarbocyanine iodide
LC-ESI-IDMS	liquid chromatography ionization-isotope dilution mass spectrometry
Ldh-c	lactate dehydrogenase - c, mouse
lyso-PtdCho	lyso-phosphatidylcholine
MAT	methionine adenosyltransferase
MS	methionine synthase
MT	mean tortuosity
MTHFD	5,10-methylenetetrahydrofolate dehydrogenase
MTHFR	methylenetetrahydrofolate reductase
MTRR	methionine synthase reductase
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MVUS	mean velocity, micrometers
neo	Neomycin
NHANES	National Health and Nutritional Estimation Survey

OCR	oxygen consumption rate
OCT	organic cation transporter
OXPHOS	oxidative phosphorylation
PAF	platelet-activating factor
PAS	periodic acid-Schiff stain
PCho	Phosphocholine
Pcyt1a/Pcyt1b	CTP:phosphocholine cytidyltransferase
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine N-methyltransferase, human
Pemt	phosphatidylethanolamine N-methyltransferase, mouse
Pgk-2	phosphoglycerate kinase - 2, mouse
PLA_2	phospholipase A 2
PLB	phospholipase B
PtdCho	Phosphatidylcholine
SEM	standard error of the mean
SM	Sphingomyelin
SNP	single nucleotide polymorphism
TAG	Triacylglyceride
TEM	transmission electron microscopy
tHcy	plasma total homocysteine
TK	thymidine kinase
TPN	total parenteral nutrition
VCL	mean curvilinear velocity

VLDL   very-low-density lipoprotein

VSL   mean straight line velocity

Introduction

Significance

An estimated 15% of couples are affected by infertility [1] and infertility attributed to the male partner (male factor infertility) is the suspected cause in 30-50% of these couples. An exact cause of male factor infertility can be determined in 40-60% of these cases. Common causes include advanced age (>55 years) [2], endocrine disorders [3], congenital defects [4, 5], infection [6-14] , testicular cancer [15, 16] and erectile dysfunction [17]. The remaining 40-60% of cases are idiopathic; however genetic aberrations such as chromosomal deletions, translocations and single nucleotide polymorphisms (SNPs) are associated with infertility in as many as 30% of these men [18]. Depending on the nature of the variation and the gene in which they are found, genetic anomalies may affect reproductive cellular function by altering processes such as proliferation, apoptosis and differentiation, as well as signaling and metabolism.

Nutritional status is an environmental exposure that can have profound effects on cell function. Although the relationship between overall nutritional status and reproduction is well known [19-21], the relationship between micronutrient metabolism and male infertility is understudied. Of the micronutrients that have been studied in this context, researchers have found that vitamin A is necessary for maintenance of germinal cells populations [309] and nutrients with antioxidant properties such as α-tocopheral (vitamin E), ascorbic acid (vitamin C), and carotenoids are necessary for protecting

sperm from oxidative damage [23, 24]. Selenium deficiency is associated with decreased sperm motility and abnormal midpiece morphology in these cells [25]. Dietary zinc deficiency is associated with hypogonadism and decreased testosterone production, in addition to abnormal chromatin condensation, decreased oxygen consumption and acrosin activity in sperm [26-33]. Because of its notable role in nucleic acid biosynthesis, and its effectiveness in preventing neural tube defects, the role of folate availability and metabolism has been examined with regard to male infertility. However, there is no evidence that dietary folate status, per se, is associated with decreased fertility in men [34].

One study examining choline deficiency and male fertility has been published [35]. Geer reported that both normal mating behavior and sperm motility required dietary choline in Drosophila melanogaster and that carnitine, a proposed choline substitute, was unable to support male fertility. Phosphatidylcholine (PtdCho) concentrations decrease within tissues during times of dietary choline deficiency [36] and Geer proposed this as an explanation for his observation. Indeed, decreased PtdCho in mature sperm may be partially responsible for poor motility in human sperm due to changes in phospholipid concentrations within these cells [37]. Supporting this hypothesis is the fact that mice lacking *CTP:phosphocholine cytidylyltransferase* are infertile, though infertility is not restricted to males [38].

Depending on the nature of the genetic change, variations in gene sequence resulting from DNA deletions, insertions and SNPs may affect cellular function. For example, gene transcription rates can be altered by variations in transcription factor binding sites,

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enhancer element regions or components of epigenetic transcriptional control (as discussed in the review by Cookson, et al. [39]). Amino acid substitutions can result from variations in gene DNA sequence that may alter the function of the protein coded by that gene. Therefore, it is highly likely that differences in nutrient metabolism stemming from genetic variation contribute to male infertility and may also account for some of the causes of idiopathic male factor infertility. For example, although dietary folate deficiency is not associated with male factor infertility, SNPs in the folate metabolism-related genes, including methylenetetrahydrofolate reductase gene (*MTHFR*), methionine synthase (*MS*) and methionine synthase reductase (*MTRR*) have been associated with idiopathic male factor infertility in some populations [42, 43] and *Mthfr*$^{-/-}$ males are infertile [44, 45]. Other types of genetic abnormalities may cause male infertility. The presence of an additional X chromosome is the cause of Klinefelter syndrome, a disease characterized by male infertility [40]. Additionally, male factor infertility is also associated with Y-chromosome deletions [41].

The data presented in this dissertation demonstrate that aberrant choline metabolism, either due to deletion of the choline dehydrogenase (*Chdh*) gene in mice or to a polymorphism in human *CHDH* is correlated with decreased sperm motility (mouse), dysmorphic mitochondrial structure (mouse and human), impaired function of these organelles (mouse) and decreased ATP concentration (mouse and human). In mice, these changes result in male infertility [46]. In humans, the amount of hepatic CHDH protein is associated with *CHDH* SNP genotype. The association between CHDH function and male infertility was an unexpected discovery; there is little evidence in the literature

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linking choline metabolism to fertility in men.

Choline is an essential nutrient

In 1998, the Institute of Medicine of the National Academy of Sciences (USA) set Adequate Intake (AI) levels for daily choline consumption [47] (Table 1.1). 550 mg/day is currently recommended for men and 425 mg/day for non-pregnant, non-lactating women. The developing fetus and newborn require large amounts of choline and these needs are met by maternal transmission of choline to the fetus across the placenta and to the newborn in breast milk [48-51]. Because of this, pregnant and lactating women require higher amounts of dietary choline in order to avoid deficiency.

Table 1.1: Adequate intake levels of choline. From [52]

Population	Age	AI	UL
Infants	0 – 6 months 6 – 12 months	125 mg/d, 18 mg/kg 150 mg/d	Not possible to establish
Children	1 – 3 years 4 – 8 years 9 – 13 years	200 mg/d 250 mg/d 375 mg/d	1000 mg/d 2000 mg/d
Males	14 – 18 years >19 years	550 mg/d 550 mg/d	3000 mg/d 3500 mg/d
Females	14 – 18 years >19 years	400 mg/d 425 mg/d	3000 mg/d 3000 mg/d
Pregnant women	All ages	450 mg/d	Age – appropriate UL
Lactating women	All ages	550 mg/d	Age – appropriate UL

Choline and choline metabolites can be found in significant amounts in many foods regularly consumed by humans. Additionally, some foods, such as infant formula and children's vitamins, are fortified with choline. The United States Department of Agriculture (USDA) has recently constructed a database reporting the choline content of a wide variety of foods (http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.html). Foods highest in choline content per 100g include beef and chicken liver, eggs and wheat germ. Foods with high betaine content per 100g include wheat bran, wheat germ and spinach. It is important to take betaine content into consideration since the presence of betaine will spare choline from use as a methyl group donor, leaving more choline available for acetylcholine (ACho) or PtdCho biosynthesis. Neither dietary recommended intake levels nor upper tolerable limits have been established for betaine; however, betaine was found to be non-toxic at all levels administered to rodents [53].

Analysis of the 2003 – 2004 National Health and Nutritional Estimation Survey (NHANES) revealed that while most children were eating the recommended amount of choline in their diet, only 10% of adults achieved the AI [54]. This is significant because dietary choline deficiency is associated with liver and muscle dysfunction [55-59] as well as increased lymphocyte apoptosis [60], renal failure [61-64] and increased plasma homocysteine concentrations, a risk factor for cardiovascular disease [65, 66].

Clinical observations of individuals receiving total parenteral nutrition (TPN) demonstrated the role of dietary choline in normal liver function. Historically, hepatosteatosis was a complication of long-term TPN. Buchman, et al. reported that

administration of choline in the TPN diet completely resolved hepatosteatosis in these individuals [67]. With this finding, non-alcoholic hepatosteatosis became known as a hallmark of dietary choline deficiency (reviewed in [68]). Triacylglycerol (TAG) from the diet or synthesized within the liver is distributed to other organs in very-low-density lipoprotein particles (VLDL). PtdCho is necessary for construction and secretion of VLDL particles, but choline deficiency limits the amount of PtdCho available for this function. The result is accumulation of TAG within the liver, which can progress to fibrosis, cirrhosis and hepatocellular carcinoma in some model systems [69, 70]. Choline deficiency, even in the absence of additional carcinogen exposure, provokes spontaneous hepatocellular carcinoma formation in rodents [69]. This phenomenon is enhanced when animals are treated with known carcinogens. While the exact mechanisms responsible for choline deficiency-induced liver cancer onset are not fully understood, they likely involve epigenetic changes in gene expression, changes in cell cycling rates, and increased oxidative stress [69, 71-79].

da Costa, et al. reported that some individuals have increased plasma levels of creatinine phosphokinase (CPK) when fed a choline - deficient diet, indicating muscle cell membrane damage [58]. As with hepatosteatosis, reintroduction of choline into the diet reverses these effects. Epidemiological studies demonstrate a relationship between dietary c h o l i n e deficiency and breast cancer risk [80], neural tube defects in infants [81, 82] and increased production of pro-inflammatory markers [83-86]. Rodent models demonstrate a link between maternal dietary choline deficiency and the incidence of heart defects as well as brain development changes ([87-89] and decreased brain function later in life [90-100].

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In addition to dietary sources, choline can also be synthesized *de novo* by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT); this occurs primarily in the liver [101]. Using phosphatidylethanolamine (PE) and S-adenosylmethionine (AdoMet) as substrates, PEMT catalyzes the formation of PtdCho which can be incorporated into cell membranes or hydrolyzed, generating the choline moiety. An estimated 30% of daily choline requirements can be met through the activity of PEMT [102, 103].

Genetic polymorphisms affect dietary choline requirements

There is significant variation in individual dietary requirements for choline. Some individuals rapidly deplete when deprived of choline (days) while others take much longer to become depleted (weeks) [104]. Some people require as much as 850 mg/d to prevent organ dysfunction while others need less than 550 mg/d [104], and these differences are likely attributable to genetic variation among individuals. These differences are due, in part, to common SNPs in the genes associated with choline and folate metabolism [105, 106]. The Zeisel laboratory has extensively studied the influence of SNP rs1235817, located in the promoter region of the human *PEMT* gene, on susceptibility to dietary choline deficiency. Premenopausal women are relatively resistant to developing choline deficiency when fed a choline deficient diet; however, approximately 44% of premenopausal women do deplete, just as men and post-menopausal women do. Eighteen percent of the Raleigh-Durham-Chapel Hill, North Carolina, USA population is

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homozygous (70% have 1 allele) for rs1235817, which renders them more susceptible to dietary choline deficiency (odds ratio 25: risk of becoming choline deficient is increased 25x, p=0.002) than are those without the SNP, because endogenous production of choline molecules via PEMT is impaired [105, 107]. 17-

beta-estradiol increases *PEMT* transcription and activity in human hepatocytes via an estrogen response element near the transcription start sites in both human and murine *Pemt* genes [107]; the premenopausal women with the rs1235817 *PEMT* SNP are unresponsive to estrogen induction of the gene [107], explaining why they show clinical signs of dietary choline deficiency.

Two SNPs that have been identified in the coding region of the human *CHDH* gene may also influence an individual's requirement for choline. da Costa, et al. report a protective effect of rs9001 and increased susceptibility to dietary choline deficiency with rs12676 [105].

Variability in genes of metabolic pathways associated with choline metabolism can also influence daily choline requirements. Choline and folate metabolism intersect at the point of methionine regeneration; thus alteration of folate metabolism may elicit a compensatory response in choline metabolism leading to choline deficiency. For example, individuals with the folate-related SNP rs1750560, located in the 5,10-methylenetetrahydrofolate dehydrogenase gene (*MTHFD*), are 15 times more likely to develop liver or muscle dysfunction when fed a choline deficient diet [106]. Caudill, et al. have found that rs1801133, a common SNP found in the folate metabolism gene *MTHFR*, influences choline status in Mexican Americans [108].

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Choline metabolism

Digestion and absorption

Dietary free choline is absorbed in the jejunum of the small intestine [109-113]. Choline is a positively charged quaternary amine and thus requires a transporter to cross cell membranes, although passive diffusion occurs at choline concentrations higher than 4mM [111]. Intestinal choline transporters are not "active" transporters as they do not require energy, nor are they sodium dependent [111, 113, 114]. Once absorbed, choline is released into the portal circulation and taken up by other tissues [111, 115].
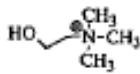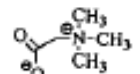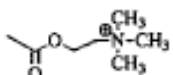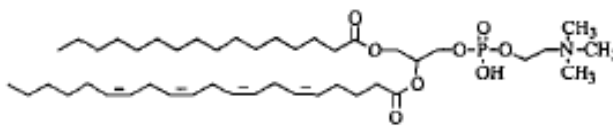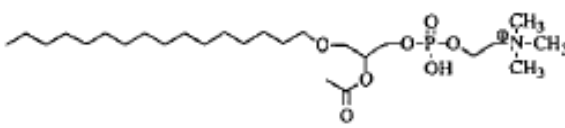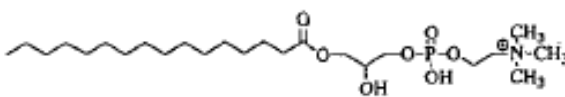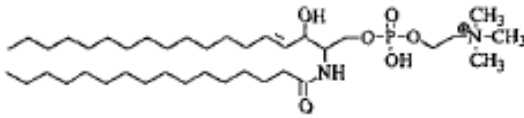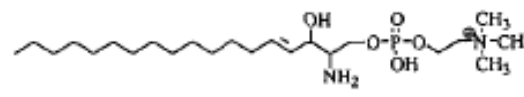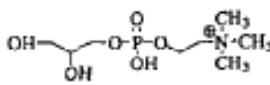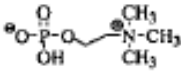
PtdCho (also called lecithin, though lecithin is not pure PtdCho) is the major form of choline in the diet. PtdCho must be hydrolyzed to lysophosphatidylcholine (lyso-PtdCho) by pancreatic phospholipase A_2 (PLA_2) before it is absorbed by enterocytes [115-119]. Once inside the enterocyte, lyso-PtdCho may be re-acylated, thus regenerating PtdCho. Alternatively, lyso-PtdCho can be de-acylated by phospholipase B (PLB) resulting in the formation of glycerophosphocholine (GPCho). Chylomicron particles, of which PtdCho is a component, are assembled in the endoplasmic reticulum of the enterocyte and are subsequently released into the lymphatic circulation where their contents are cleared from the systemic circulation via uptake by other organs. Within cells, the metabolism of PtdCho leads to the generation of multiple signaling molecules that have important roles in cellular function. For example, platelet-activating factor (PAF), a choline-containing

phospholipid derived from PtdCho, imparts hormone-like activity and has been demonstrated to regulate inflammation [85, 120], blood pressure, and glycogenolysis among other physiological aspects (reviewed in [83-86, 120]).

GPCho, phosphocholine (PCho) and sphingomyelin (SM) are also present in small quantities in the diet. SM is taken up into enterocytes intact; however, it is completely degraded in the enterocyte [118, 121]. GPCho is degraded by 3-glycerylphosphocholine glycerophosphohydrolase releasing glycerophosphate and free choline that is subsequently transported into intestinal cells and released into the portal circulation [118, 119]. PCho is likewise degraded by alkaline phosphatase producing free choline and phosphate [122].

Dietary betaine is absorbed in the duodenum of the small intestine [123, 124]. Betaine uptake into cells occurs either by active Na+ or Cl- coupled transport or by a Na+-independent passive transport mechanism via amino acid transport system A or the activity of γ-aminobutyric acid transporter protein (BGT-1) [125-128]. Table 1.2 shows the structure of biologically active choline metabolites.

Table 1.2: Structures of choline - containing molecules. From [68]

Molecule	Chemical structure	Biological function
Choline		
Betaine		Methyl group donor; renal osmolyte
Acetylcholine		Neurotransmitter
Phosphatidylcholine		Necessary building block of biomembranes; VLDL component necessary for hepatic VLDL secretion
Platelet-activating factor		Hormone
Lysophosphatidylcholine		Putative second messenger modulating PKC activity
Sphingomyelin		Necessary building block of biomembranes
Lysosphingomyelin		Putative second messenger mediating growth-factor actions, mitogen
Glycerophosphocholine		Renal osmolyte
Phosphocholine		Intracellular storage pool of choline

Transport

There are three choline transport mechanisms described in tissues: a high affinity, sodium-dependent active transport system (CHT1), a facilitated diffusion system (polyspecific organic cation transporters; OCT), and a lower affinity active transport system (choline transporter-like; CTL) [129]. CHT1 is localized to cells that primarily use choline for ACho generation, i.e. cholinergic neurons. Thus, the forebrain, brain stem, striatum and spinal cord are the areas within the central nervous system with highest CHT1 expression [130-134]. OCTs are a family of non-specific organic cation transporters primarily expressed in liver, kidney, brain, and intestine [129]. Expression of mouse CTL transporters have been detected in many mouse tissues; they are the only choline-specific transporters detected in testis to date [129, 135]. CTL1 (also known as SLC44A1) is localized primarily to the mitochondrial membrane of cells [136] and deficiency of this transporter results in decreased PtdCho [136]. In mouse, *Ctl-1/Slc44a1* messenger RNA is detected in the epididymis, and male germinal cells [137].

Acetylcholine

Figure 1.1 illustrates the major pathways of choline metabolism. A small portion of dietary choline is converted to ACho by the enzyme choline acetyltransferase (ChAT). This enzyme is found in high concentrations in cholinergic neurons, ensuring that ACho is available for release by these cells. Since it is unlikely that choline and/or acetyl-CoA are present at levels that would saturate the ChAT enzyme, choline

availability determines the rate of ACho synthesis [138] and modulates the amount of this neurotransmitter released into the synapse [139-141]. Choline-containing phospholipids, including PtdCho and SM, incorporated into cholinergic cell membranes serve as a reserve of choline available for ACho synthesis in cells with a high demand for this molecule [142, 143].

Phosphatidylcholine and sphingomyelin

An important role for choline is for biosynthesis of cellular membrane phospholipids, such as PtdCho and SM. PtdCho represents more than 50% of the total phospholipid content in cell membranes [144]. PtdCho synthesis is highly regulated and can occur through two pathways [101]. In the first step of the cytidine diphosphocholine (CDP-choline/ Kennedy Pathway) pathway, choline is phosphorylated by choline kinase (CK), generating phosphocholine (PCho).

PCho is converted to cytidine diphosphocholine in a reaction catalyzed by the enzyme CTP:phosphocholine cytidylyltransferase (PCYT1A/PCYT1B); this is the rate limiting step in the CDP-choline pathway [145]. The rate of this reaction is regulated by the phosphorylation status, and thus intracellular localization, of the PCYT1A/PCYT1B enzyme. Specifically, phosphorylated PCYT1A/PCYT1B is inactive and primarily found in the cytosol while dephosphorylated PCYT1A/PCYT1B is active and localized to cellular membranes [146-148]. CTP:phosphocholine cytidylyltransferase phosphorylation status is regulated by protein kinase A via the second messenger cAMP [147, 149].

The combination of CDP-choline with diacylglycerol results in the formation of PtdCho; this reaction is catalyzed by the enzyme CDP-choline:1,2-diacylglycerol choline phosphotransferase [150]. As discussed above, an alternative pathway synthesizes PtdCho from AdoMet and PE through the enzymatic activity of PEMT. The activity of this enzyme is regulated primarily by PE availability and AdoMet/S-adenosylhomocysteine (AdoHcy) ratio [151].

Sphingomyelin is derived from the combination of ceramide with the phosphocholine provided by PtdCho in a reaction catalyzed by phosphatidylcholine:ceramide choline phosphotransferase [152].

Betaine

Betaine is formed from choline by two successive, irreversible oxidation reactions via the formation of an aldehyde intermediate [153-155]. In mammals, this process involves two enzymes: CHDH and betaine aldehyde dehydrogenase (BADH). This conversion takes place in the mitochondrial matrix following choline transport into these organelles [156]. Betaine is a zwitterionic quaternary amine that freely diffuses down its concentration gradient across plasma membranes from the mitochondrial matrix into the cytosol [157]. Once in the cytosol, labile methyl groups on the betaine molecule can be used for methylation of homocysteine in a reaction catalyzed by betaine:homocysteine methyltransferase (BHMT) [158]. The product of the BHMT reaction is methionine and dimethylglycine; therefore, betaine availability may play a role in protein turnover

within cells [159]. Methionine adenosyltransferase (MAT) converts methionine to AdoMet, the substrate necessary for nucleic acid and protein methylation as well as biosynthesis of molecules such as phospholipids and carnitine [160].

During times of osmotic stress, cells use betaine that is not degraded by BHMT as an organic osmolyte to maintain hydration and volume [161, 162]. Hyper-osmotic stress, induced by high salt and/ or urea exposure stimulates betaine accumulation in cells by upregulating expression of the betaine γ-aminobutyric acid transporter (BGT-1), particularly in the glomerulus of the kidney [125-128]. In addition, betaine has been reported to modulate immune function [163-165], red blood cell ATPase activity [166], protect skeletal muscle myosin ATPases from urea-induced damage [167], protect rodent heart mitochondrial function in models of myocardial infarction [168-170] and is effective in preserving protein synthesis mechanisms in osmotically stressed preimplantation embryos *in vitro* [171, 172].

Figure 1.1: Choline metabolism [68].
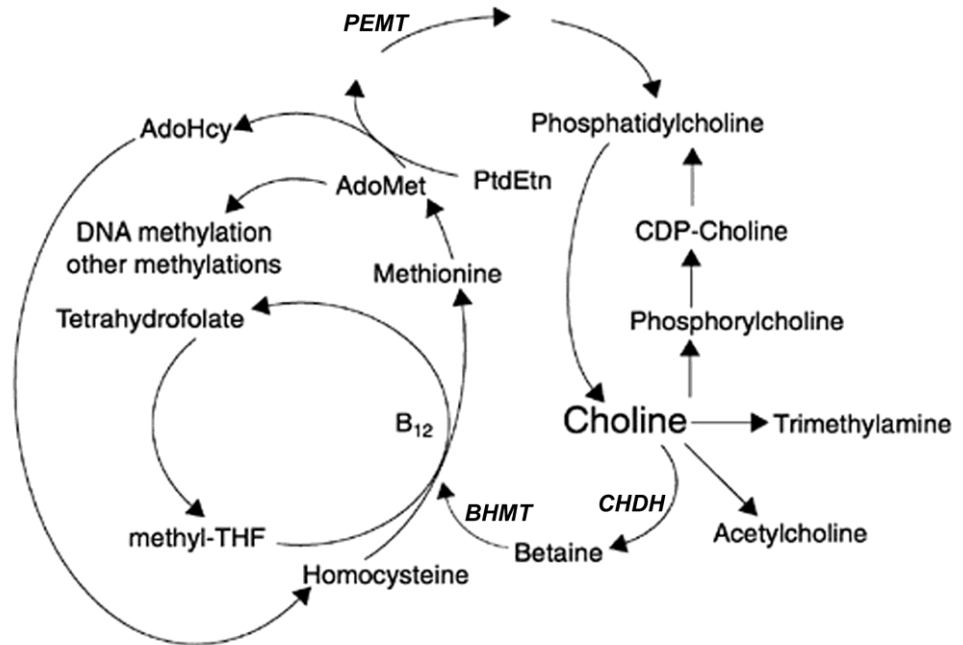
Choline dehydrogenase

Gene

In humans, *CHDH* is a 30,000 base pair gene located on chromosome 3 (3p21.1). It is composed of 7 exons and is coded on the negative/ minus DNA strand [173]. To date, only one full-length *CHDH* transcript has been characterized in humans [172]. Murine *Chdh* is also composed of 7 exons [173]. It is located on chromosome 14 and is approximately 31,000 base pairs in length. There are 3 transcripts reported for *Chdh*, differing in the length of the 5'-untranslated region. The same protein is translated

from each of the three transcripts [174].

Enzyme

CHDH is highly conserved; in addition to humans this enzyme is expressed in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, C. elegans, and A. thaliana, among others [173]. The human and mouse CHDH protein are similar in size, 594 and 596 amino acids, respectively.

The active site of CHDH shares homology with the glucose-methanol-choline oxidoreductase family of proteins. This protein also contains a NADB_Rossman fold in which flavin adenine nucleotide (FAD) is predicted to bind [173, 174]. In humans, *CHDH* is expressed in liver and kidney as well as parathyroid, brain, adipose tissue, testis, cervix, embryonic tissue, lung, mammary gland, ovary, eye, intestine, lymph, muscle, pancreas, placenta, prostate, stomach and fertilized ovum [175]. Mouse *Chdh* expression has been detected in the same tissues as human with the exceptions of brain, adipose tissue, eye, pancreas [176]. Depending on the species, the apparent K_m for CHDH ranges from 0.27mM to 7mM when using choline as a substrate in the presence of the electron acceptor phenazine methosulfate [177-179]. Rat CHDH has a Vmax of 6.3nmole betaine formed/mg protein/minute [180]. CHDH may also use betaine aldehyde as a substrate, again in the presence of phenazine methosulfate indicating that BADH may not be absolutely necessary for the complete oxidation of choline to betaine. In this reaction the K_m of CHDH is reduced (3.1mM) [178]. Interestingly, betaine aldehyde may also inhibit CHDH activity at

a slightly higher concentration (3.3mM) [178]. CHDH is most active at a pH of 7.6 and within a temperature range from 37°C to 45°C [177]. CHDH requires the association of an electron acceptor for activity. *In vivo* this is FAD while *in vitro* assays are performed most often using phenazine methosulfate as the acceptor [177, 178, 181]. DNA sequence variation in the human *CHDH* gene may affect the activity of the enzyme translated from this gene. Rs12676 is one such variation, a non-synonymous SNP located in the coding region of the *CHDH* gene. The presence of the minor allele results in replacement of arginine with a leucine 6 amino acids from the FAD binding site of this enzyme (Figure 1.2). The frequency in which rs12676 is detected changes with ethnicity. For example, the *T* allele is found in 58% of Caucasians, 23% of individuals of African descent, 4% of Mexican Americans and is indictable in Asian Americans (Ziesel laboratory, unpublished data).

Figure 1.2: Schematic of CHDH protein
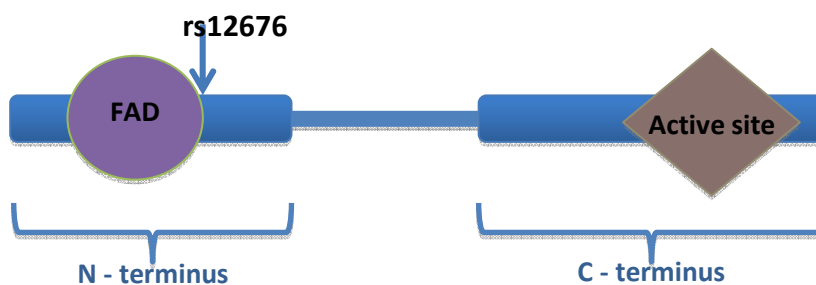
Sperm development and choline dehydrogenase

In the seminiferous tubules of the testis, sperm cells are formed through the process of spermatogenesis [182]. Spermatogenesis begins with the onset of puberty in mammals and continues throughout life resulting in continuous production of sperm available for egg fertilization. Seminiferous tubules are populated with Sertoli cells; these somatic cells form tight junctions with neighboring Sertoli cells and by doing so, separate the seminiferous tubule into basal and adluminal compartments. In addition, Sertoli-Sertoli cell junctions effectively create a testis-blood barrier that serves to protect developing sperm from environmental exposure and immune defense mechanisms [183]. Another important function of these cells is to provide the components necessary and, thus create, the environment necessary to support spermatogenesis (reviewed in [310]). Sertoli cells secrete glycoproteins that have an array of functions. These glycoproteins may act as signaling molecules that regulate spermatogenesis, may have hormonal function, and may regulate metabolism in the developing spermotgenic cells. Sertoli cells also provide nutrition for spermotgenic cells. Developing sperm are in close contact with, and develop in such as way as they are surrounded by, Sertoli cells.

Spermatogenesis can be divided into two major stages: a proliferative phase called spermatocytogenesis, followed by spermiogenesis during which extensive differentiation and remodeling of spermatids occurs, culminating in the formation of a sperm (Figure 1.3).

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Spermatogenic stem cells and spermatogonia are located along the basal edge of the seminiferous tubule. These are mitotic, diploid cells. Spermatogonial daughter cells can either remain spermatogonia (Type A spermatogonium), thus maintaining the population of these cells, or they may can become Type B spermatogonium which will eventually give rise to a primary spermatocyte. Next, primary spermatocytes enter meiosis I, during which DNA replication and chromosomal recombinations occur [182]. This stage ends with the production of two haploid secondary spermatocytes from each primary spermatocyte. Secondary spermatocytes then enter meiosis II. Meiosis II goes to completion rapidly and ends with the generation of two haploid round spermatids from each secondary spermatocyte. Round spermatids begin to elongate and enter the second stage, spermiogenesis. It is during spermiogenesis that spermatogenic cells acquire their characteristic morphology as the principal piece forms and mitochondria are arranged in a helical gyrus localized to the newly formed midpiece section. Nuclear chromatin becomes highly condensed during spermiogenesis as the majority of histones are replaced by transition proteins and finally by small, basic protamines; gene transcription is silenced at the onset of spermiogenesis, largely due to chromatin condensation [184]. The Golgi apparatus in sperm releases vesicles that eventually form the acrosome, a specialized region of the sperm head containing high concentrations of hydrolytic enzymes used by sperm to penetrate the zona pellucida of the egg. Fully formed sperm are released from the Sertoli cells into the lumen of the seminiferous tubule and begin migration through the efferent ducts to the caput epididymis. Sperm migrate from the caput epididymis to the corpus epididymis and are finally stored in the cauda epididymis. Sperm entering the caput epididymis are not capable of motility, but as sperm transit through the regions of the

epididymis, they undergo further maturation, a process that includes the addition of epididymal proteins to sperm, changes in membrane lipid composition of these cells and accumulation of low molecular weight ions and organic osmolytes, presumably for use in regulating cell's volume [185, 183]. Figure 1.4 shows the relative concentrations of osmolytes identified in epididymal fluid. Note that epididymal fluid betaine concentrations have not been measured, though because of its role as an osmolyte, it is expected to be present. Indeed, we have found betaine concentrations in the epididymis to be 10 times higher than levels measured in liver (See page 63). Some of this betaine may be generated within the male reproductive tract. *Ctl-1/Slc44a1* and *Chdh* mRNA expression have been reported in mouse male reproductive tissues (Table 1.3). Chdh mRNA has been detected in pachytene spermatocytes and Sertoli cells [137]. At the same time, we have found that CHDH protein is expressed in mouse testicular and epididymal tissues (Figure 5.2) Therefore, a mechanism is in place to not only transport choline into these cells, but also oxidize that same choline to generate betaine.

Figure 1.3: Spermatogenesis in humans. From [186]
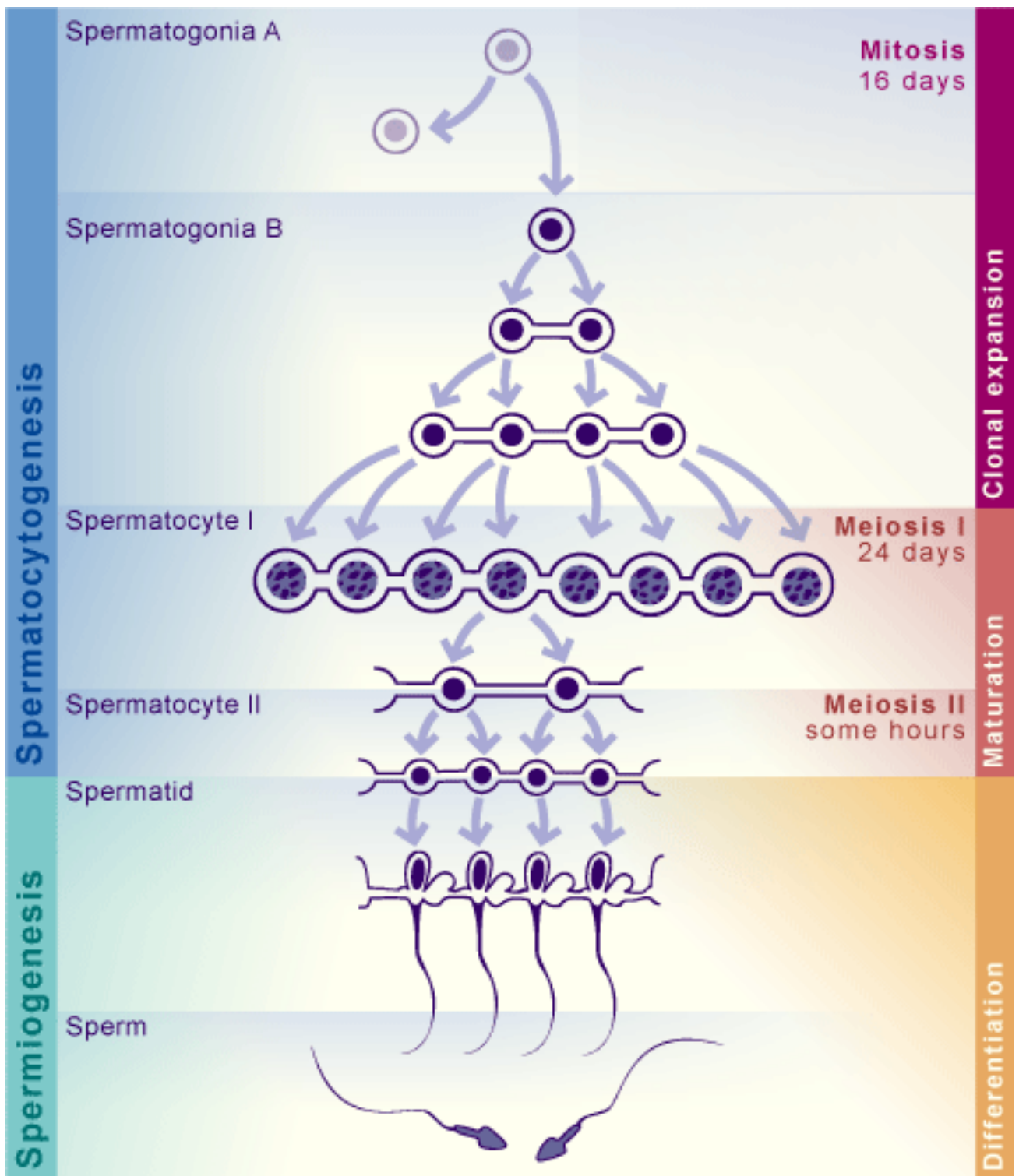
Figure 1.4: Concentrations of putative sperm osmolytes in rodent epididymal fluid. Adapted from [187]
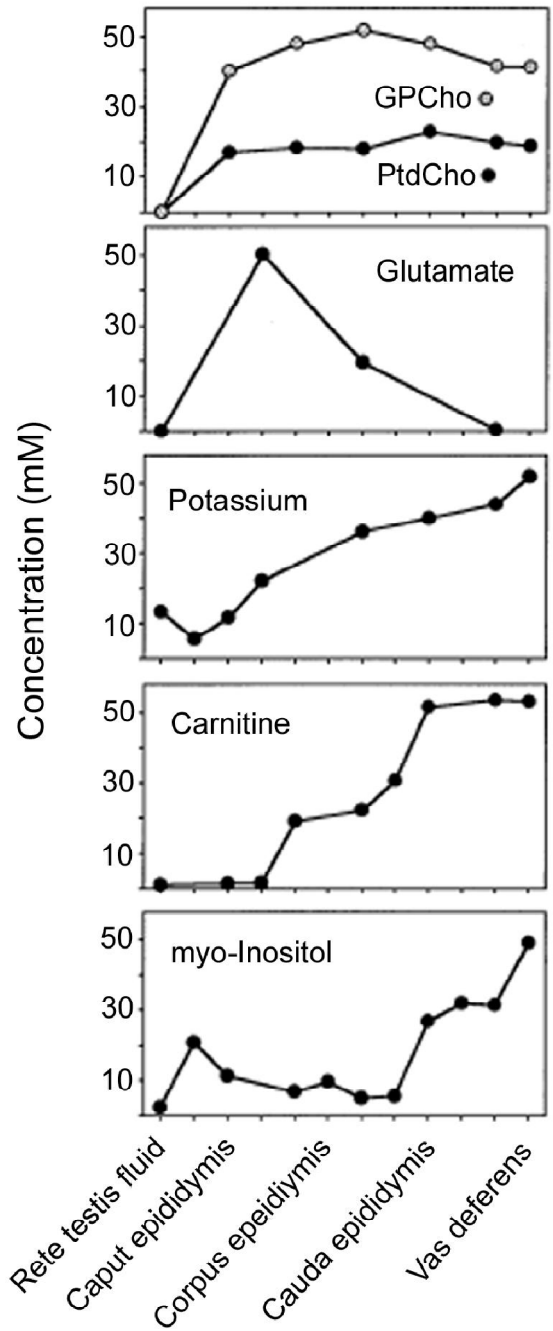
Table 1.3: Expression of choline transporter and *Chdh* in male reproductive organs. From [137]

Gene symbol	Gene name	Species	Tissue in which gene is expressed	Specific cell type in which gene is expressed
Clt-1/ Slc44al	Choline-like transporter-1/ solute carrier 44, subtype a1	Mouse	Epididymis	NA
			Spermatogenic cells	Type A spermatocytes, Type B spermatocytes, pachytene spermatocytes, round spermatids
Chdh	Choline dehydrogenase	Mouse	Epididymis	NA
			Spermatogenic cells	Type A spermatocytes, Type B spermatocytes, pachytene spermatocytes, round spermatids
			Somatic cells	Sertoli cells

Expressions of the *Clt-1/Sla44a1* and the *Chdh* gene has been reported in spermatogenic cells and supportive, somatic cells in the testis. This offers a mechanism by which betaine concentration can reach the high levels measured in testis and epididymis

Sperm motility and energy metabolism

The sperm tail is comprised of four major structures: the connecting piece, the midpiece (where mitochondria are localized), the principal piece (where the fibrous sheath and glycolytic enzymes are localized) and the end piece [188] (Figure 1.5). The axoneme, composed of nine microtubule doublets that surround a centrally located doublet, is connected to the sperm head and spans the length of the sperm tail. Radial spokes extend inward from the nine doublets to the central doublets of the axoneme and serve to supply structural support for the sperm tail. Dynein arms extend outward from the nine doublets and are responsible for generating the force necessary for sperm tail movement. Sperm become motile when dynein ATPase hydrolyses ATP, causing sliding of the outer microtubule doublets and bending of the tail.

ATP is required for sperm to be motile, but considerable controversy surrounds the source of the ATP. Because mitochondria are only found in the midpiece there is some question as to whether ATP generated by oxidative phosphorylation (OXPHOS) can diffuse the length of the tail to supply substrate for the dynein ATPases [189]. A creatine phosphate shuttle system has been proposed that may traffic ATP from mitochondria through the tail, but experimental evidence supporting the existence of this mechanism is still lacking [190]. Alternatively, glycolytic enzymes are localized to the principal piece of the sperm tail, thus providing a source of ATP at the site in which it will be used [191-195]. In most species there is evidence that both pathways are active in sperm cells; however, the relative importance of each pathway differs among species. For example,

OXPHOS-derived ATP supports bull and ram sperm motility [196], while mouse sperm has a definitive need for glycolytic generation of ATP for motility [191-195]. The energy substrates available in the female reproductive tract are probably the factor dictating which pathway sperm of a particular species will use for ATP production.

Some evidence exists that links betaine to sperm motility. Preserving normal motility characteristics in sperm that have been frozen and thawed is an active area of research both in the fields of human andrology and veterinary animal husbandry. Kroskinen, et al [197] and Sanchez-Partida et al [198] reported increased sperm motility in thawed sperm when betaine was added to the cryopreservation media. It is hypothesized that betaine may directly interact with membrane lipids and proteins, altering the hydration status of these molecules, and thus protecting them through the freeze/thaw cycle [199]. In addition, there is some evidence that boar sperm have the capacity to use choline-containing phospholipids for ATP production [200], but this likely changes depending on animal species.

Animal model nutrition

The *Chdh* mouse colony, including breeding pairs, was maintained on AIN76A purified diet at all times. This diet was formulated to contain 1.1g/kg choline chloride, which represents an adequate level of dietary choline for rodents [201]. The formulation of AIN76A, including the concentration of choline metabolites, is presented

on Table 1.4. Because the *Chdh* animals were always ingesting adequate amounts of choline, any differences observed in the *Chdh*$^{-/-}$ mice were a result of deletion of the gene and not dietary deficiency.

Summary

Approximately half of cases of male factor infertility are idiopathic; genetic aberrations are the suspected cause in as many as many as 30% of these. There is evidence that altered micronutrient metabolism, stemming either from dietary deficiency or genetic variation in metabolism-related genes, may play a role in sperm cell development and function. This dissertation details evidence that choline metabolism is required for normal sperm morphology, energy homeostasis and motility patterns, both in mice and humans.

Figure 1.5: Human sperm cell structure. *(Figure legend from reference 201)* Illustration of a mammalian sperm cell with the cell membrane removed, revealing the underlying structure including the details of the axoneme. (*a*) Regions of the sperm flagellum, with representative approximate human sperm length scales indicated (Cummins & Woodall 1985, Curry & Watson 1995). The acrosomal cap of the sperm head and connecting piece are also highlighted. (*b*) A cross section of the mid-piece flagellum (looking from the sperm head to the distal, rear, tip), featuring the presence of nine outer dense fibers immediately exterior to the microtubule doublets, a characteristic feature of internal fertilizers. The doublets and their associated fibers are labeled with the standard convention, clockwise from 1 to 9, with the first doublet and fiber defined by the radial vector in the direction perpendicular to both the centerline and the separation of the two central microtubules. This structure is also illustrated in panel *e*. The detailed geometry of the outer dense fibers is species specific although fibers 1, 5, and 6 usually have larger cross sections, whereas fibers 3 and 8 are typically the shortest. All fibers taper and end prior to the distal tip of the sperm, are embedded in the connecting piece, and are also bound to their associated doublet at their distal end along the flagellum. In the principal piece of the sperm flagellum (*a*), there is a fibrous sheath with circumferential ribs, and two longitudinal columns initially attached to fibers 3 and 8. On moving distally, the ribs of the fibrous sheath become thinner and the columns taper; in addition, the columns appear to be attached to microtubule doublets 3 and 8 distally beyond the termination of the outer dense fibers 3 and 8, as depicted in panels *c* and *f*. The end of the fibrous sheath delimits the principal piece from the end piece, where only the axoneme structure persists, as depicted in panels *d* and *g*. The axoneme structure is further schematically illustrated in panels *h* and *i*; in the former the microtubules are depicted with their nexin bridges and radial links, whereas the latter illustrates the inner and outer dynein arms, which exert a shearing force between the microtubule doublets. This force bends the microtubules and their associated accessory structures, driving the flagellar waveform. Figure adapted from Fawcett (1975), copyright 1975, and Olson & Linck (1977), copyright 1977, with permission from Elsevier (2283601155396 and 2370901182457, respectively).

Figure 1.5: Human sperm cell structure. From [202]
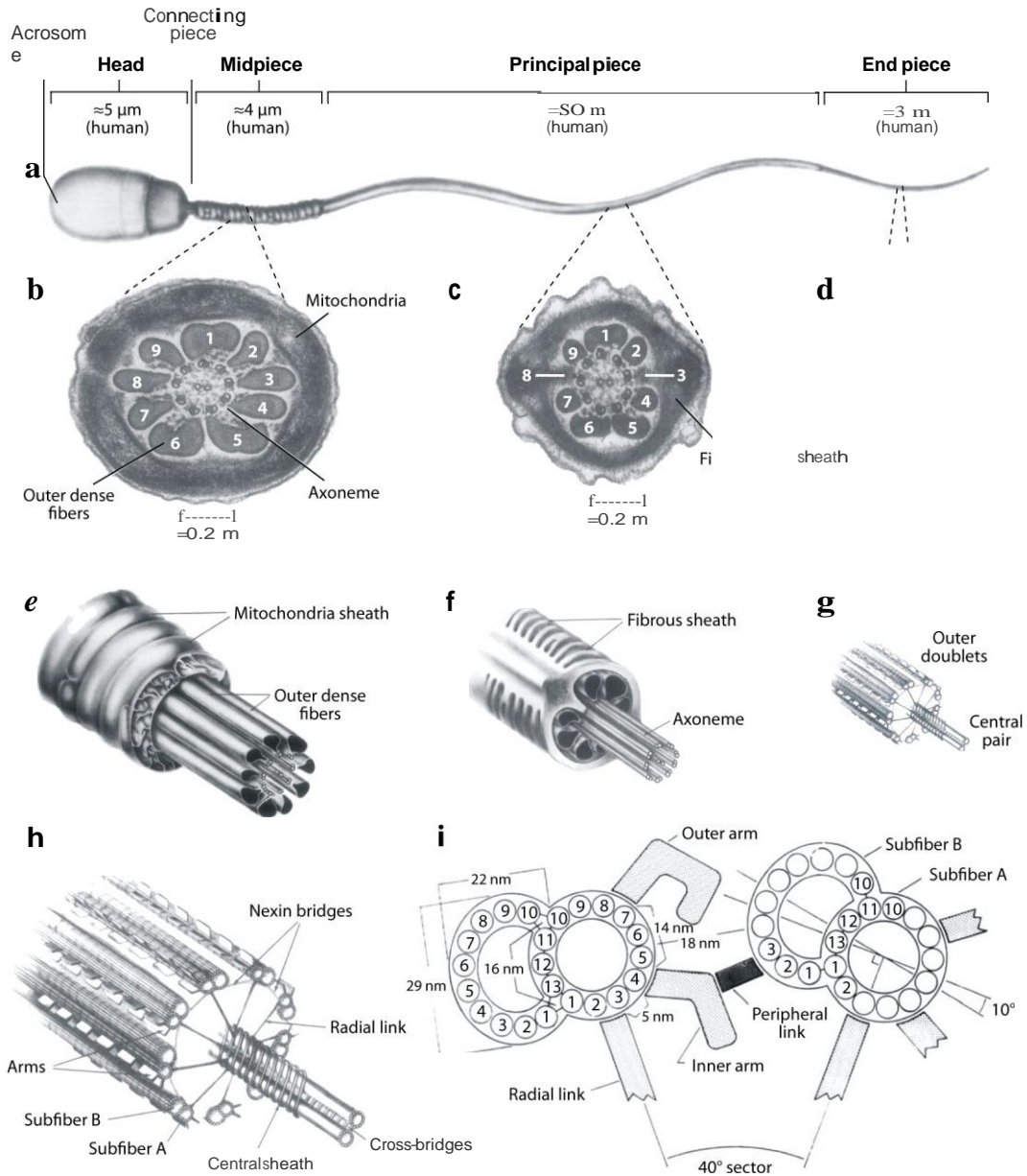
Table 1.4. Formulation of AIN76A purified diet. From [201]

Ingredient	% of total diet
Casein	20.0
DL-methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fiber	5.0
Corn oil	5.0
AIN mineral mix	3.5
AIN vitamin mix	1.0
Choline chloride*	0.2
AIN76 Vitamin Mix	**Per kg mixture**
Thiamine-HCl	600mg
Riboflavin	600mg
Pyridoxine-HCl	700mg
Nitcotinic acid	3g
D-calcium pantothenate	1.6g
Folic acid	200mg
D-biotin	20mg
Cyanocobalamine (B12)	1mg
Retinyl palmitate or acetate (vitamin A	400,00 international units (IU)
DL-α-tocopheryl acetate (vitamin E)	5000 international units (IU)
Cholecalciferol (vitamin D3)	2.5mg
Menaquinone (vitamin K)	5.0mg
Sucrose, finely powdered	Up to 1kg

Continued on next page

*original formulation contains choline bitartrate. Our laboratory routinely substitutes and equimolar amount of choline chloride

AIN76 Mineral Mix	g/kg mixture
Calcium phosphate dibasic ($CaHPO_4$)	500.0
Sodium chloride (NaCl)	74.0
Potassium citrate, monohydrate ($K_2C_4H_5O_7$-H_2O)	220.0
Potassium sulfate (K_2SO_4)	52.0
Magnesium oxide (MgO)	24.0
Manganous carbonate (43-48% Mn)	3.5
Ferric citrate (16-17% Fe)	6.0
Zinc carbonate (70% ZnO)	1.6
Cupric carbonate (53-55% Cu)	0.3
Potassium iodate (KIO_2)	0.01
Sodium selenite (Na_2SeO_2-$5H_2O$)	0.01
Chromium potassium sulfate [$CrK(SO_4)_2$-$12H_2O$]	0.55
Sucrose, finely powdered	Up to 1kg
Choline metabolites	**nmol/g (Total choline 1368mg/kg)**
Betaine	0
Choline	12915
GPCho	6
PCho	0
PtdCho	84
SM	149

CHAPTER II

DELETION OF MURINE CHOLINE DEHYDROGENASE RESULTS IN DIMINISHED SPERM MOTILITY

Amy R. Johnson, Corneliu N. Craciunescu, Zhong Guo , Ya-Wen Teng , Randy J. Thresher, Jan K. Blusztajn, and Steven H. Zeisel

Abstract:

Choline dehydrogenase (CHDH) catalyzes the conversion of choline to betaine, an important methyl donor and organic osmolyte.   We have previously identified single nucleotide polymorphisms (SNPs) in the human *CHDH* gene that, when present, seem to alter the activity of the CHDH enzyme. These SNPs occur frequently in humans. We created a *Chdh$^{-/-}$* mouse to determine the functional effects of mutations that result in decreased CHDH activity. *Chdh* deletion did not affect fetal viability or alter growth or survival of these mice. Only one of eleven *Chdh$^{-/-}$* males was able to reproduce. Loss of CHDH activity resulted in decreased testicular betaine and increased choline and PCho concentrations. *Chdh$^{+/+}$* and *Chdh$^{-/-}$* mice produced comparable amounts of sperm; the impaired fertility was due to diminished sperm motility in the *Chdh$^{-/-}$* males. Transmission electron microscopy revealed abnormal mitochondrial morphology in *Chdh$^{-/-}$* sperm.   ATP content, total mitochondrial dehydrogenase activity and inner mitochondrial membrane polarization were all significantly reduced in sperm from *Chdh$^{-/-}$* animals. Mitochondrial changes were also detected in liver, kidney, heart and testis tissues. We suggest that men who have SNPs in *CHDH* that decrease the activity of the CHDH enzyme could have decreased sperm motility and fertility.

Introduction:

Betaine is an important methyl donor required for the conversion of homocysteine to methionine [203, 204], and it is an organic osmolyte that is needed for normal kidney glomerular function [205, 206]. Betaine can be obtained from the diet (from wheat, shellfish, spinach, and sugar beets, for example) [158, 207, 208] and it is formed, in mammals and some microorganisms, via the oxidation of choline in 2 steps catalyzed by choline dehydrogenase (E.C. 1.1.99.1; CHDH) in the inner mitochondrial membrane [153, 154, 181, 209, 210] and betaine aldehyde dehydrogenase (EC 1.2.1.8; BADH) in the mitochondria and cytosol [155]. Choline oxidase (EC 1.1.3.17; CO), also found microorganisms and in some plants, is capable of catalyzing both oxidation reactions [211-214]. The oxidation of choline is irreversible, committing the choline moiety to the methyl-donation pathway for use in one- carbon metabolism [101]. Choline that is not used to form betaine can be acetylated to form acetylcholine, or phosphorylated to form phosphatidylcholine and sphingomyelin [101].

CHDH activity can influence tissue homocysteine (Hcy) concentrations because betaine donates a methyl group to homocysteine in a reaction catalyzed by betaine:homocysteine methyl transferase (EC 2.1.1.5; BHMT). The product of this reaction is methionine, which is the precursor for S-adenosylmethionine (AdoMet), the most important methyl donor in biochemical reactions (including DNA, RNA, protein and phospholipid methylations). Dietary betaine supplementation is effective in lowering plasma total Hcy (tHcy) concentrations in humans [215]; elevated plasma tHcy concentration is associated with

increased risk of cardiovascular disease [216, 217].

Several single nucleotide polymorphisms (SNPs) in *CHDH* occur frequently in humans. One such SNP, rs12676, is located in the coding region of the *CHDH* gene with 42 - 47% of the population having one allele and 9% being homozygous [80, 105]. Eighty-three percent of pre-menopausal women who were heterozygous for the rs12676 allele developed organ dysfunction (liver or muscle) when fed a choline deficient diet compared to only 20% of women who were wild type [105], suggesting that this SNP alters CHDH function.

The functional effects of null mutations of the *CHDH* gene have not been further investigated, but it would be reasonable to predict that they would decrease betaine concentrations and increase choline and homocysteine concentrations in tissues, alter osmolyte-dependent functions of the renal glomerulus, and might perturb mitochondrial function (as the enzyme is located on the inner mitochondrial membrane and concentrations of betaine in the mitochondria are μmole/L [218]). In order to better characterize the role of CHDH, we created a *Chdh*$^{-/-}$ mouse and now present evidence that *Chdh* mutation

contributes to impaired mitochondrial function in several tissues with the most striking effects observed in sperm.

Materials and Methods:

Generation of _Chdh^{-/-}_ Mice: _Chdh_ chimeric mice were generated using a gene targeting vector that removed exons 1 though 3 of the gene. The 5' arm of homology was derived from the region of the gene immediately 5' of exon 1 (approximately 5.5 kb) and the 3' arm was derived from the region 3' of exon 3 (approximately 1.5 kb) and encompassed exons 4 through 7 (Figure 2.1). The vector, containing positive and negative selection cassettes (neo and TK, respectively), was electroporated into E14TG2a ES cells. PCR-positive clones were confirmed for homologous recombination by Southern hybridization. Targeted cells were injected into blastocysts derived from mouse strain C57Bl/6 (B6) to create transmitting chimeras.

Chdh chimeric mice were bred to B6 mice. _Chdh^{+/-}_ breeding pairs were used to generate litters composed of _Chdh^{+/+}_, _Chdh^{+/-}_ and _Chdh^{-/-}_ mice, which were used in these experiments. These animals are on a mixed 129/B6 genetic background. Mice were maintained on AIN-76A diet containing 1.1 g/kg choline chloride (Dyets, Bethlehem, PA). Genomic DNA was isolated from tail biopsies collected at weaning using phenol:chloroform:isoamylalcohol purification. Animals were genotyped by multiplexing PCR using TaKaRa Ex Taq DNA polymerase (TaKaRa Bio USA, Madison, WI, USA) and the following primer sequences: _Chdh^{+/+}_ 5' - AGGGCCACAAGTGTGGGCTGGCTGAAACTG-3', _Chdh_ common 5' – GCTAGCTTGAACCCTTTGAAGGGTCTTCTCAGACTC – 3' and _Chdh_ neo 5' – ACGCGTCACCTTAATATGC – 3'. The primer locations are illustrated in Figure 2.1A. PCR conditions were as follows: 95°C for 3 minutes, 94°C for 30 seconds, 56°C for

30 seconds, 72°C for 3 minutes (repeated 35 times), and 72°C for 10 minutes. *Chdh*$^{+/+}$ reactions produced a product 2.3kb in size. The *Chdh* neo product was 1.6 kb in size (Figure 2.1B). The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all experimental protocols.

Body length, total body weight, fetal viability and survival: Body length and total body weight of *Chdh*$^{+/+}$, *Chdh*$^{+/-}$ and *Chdh*$^{-/-}$ mice were measured at weaning (postnatal day 21; P21), P42 and P63. Mice were weighed on a scale and a ruler was used to measure "Body length" (the distance between the tip of the nose to the end of the buttock when the mouse is lying on its stomach). The genotype distribution of litters produced by 20 *Chdh*$^{+/-}$ mating pairs over a 3 year period was recorded. 10 *Chdh*$^{-/-}$ mice were maintained over the course of one year to determine whether or not *Chdh* mutation affected the one-year survival rates.

Measures of liver, kidney and muscle function: Plasma alanine transaminase (ALT) activity, plasma total bilirubin concentration, blood urea nitrogen (BUN) concentration and plasma creatinine concentration were measured by the Animal Clinical Chemistry and Gene Expression Facility located at the UNC - Chapel Hill using an automatic chemical analyzer (Johnson and Johnson VT250, Rochester, NY, USA). Plasma creatinine phosphokinase (CPK) activity was determined using a Creatine Kinase-SL assay kit according to manufacturer's instructions (Diagnostic Chemicals Limited, Oxford, CT, USA). Urine collection and specific gravity measurement study was performed as described [219]. Urine specific gravity was measured using a refractometer (AO Instrument Company, Buffalo,

NY, USA) by the UNC - Chapel Hill Department of Laboratory Animal Medicine Veterinary and Technical Services Facility.

CHDH enzymatic assay: *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice were anesthetized with halothane (Henry Schein, Inc, Melville, NY, USA) until they no longer responded to a hard pinch of their tail or foot. Tissues were harvested from animals and immediately snap frozen in liquid nitrogen. Liver, kidney, testis, brain, skeletal muscle (vastus medialis) and heart were then pulverized in liquid nitrogen and stored at -80° C until CHDH activity was assayed.

200 mg of liver or kidney was homogenized in 500 µL cold homogenization buffer (250 mM sucrose, 50 mM Tris, 0.1 mM EDTA, pH 7.8) using a motorized tissue homogenizer (Talboys Engineering Corporation, Montrose, PA, USA). The entire brain, skeletal muscle or heart was homogenized in 300µL cold homogenization buffer. Both testes from one animal were homogenized together in 300uL of homogenization buffer. Protein concentrations were measured by Lowry assay [220]. CHDH activity was measured in *Chdh*$^{+/+}$ sperm that were treated with betaine *in vitro*. CHDH activity assay was measured as previously described [221]. The amount of betaine formed was determined by HPLC as described previously [222] using a Varian ProStar solvent delivery system (PS-210, Varian Inc., Palo Alto, CA, USA), and a Pecosphere Silica column (3 µM, 4.6 x 83mm) (Perkin Elmer, Norwalk, CT, USA) with a Pelliguard LC-Si guard column (Supleco, Bellefonte, PA). Radiolabeled choline and betaine peaks were detected using a Berthold LB506 C-1 radiodetector (Oak Ridge, TN, USA).

Targeted metabolomics:

Choline metabolites: Liver, brain, kidney, skeletal muscle, heart and testis tissues were collected from 7.5-week-old *Chdh*^{+/+} and *Chdh*^{-/-} mice, quick frozen in liquid nitrogen and then pulverized under liquid nitrogen. Mitochondria were isolated using a Percoll gradient following previously described methods [223-225]. All procedures for mitochondria isolation were performed at 4ºC. The concentration of choline metabolites was measured by liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC–ESI– IDMS) as previously described [226].

Plasma Total Homocysteine (tHcy): Blood from *Chdh*^{+/+} and *Chdh*^{-/-} anesthetized animals was collected via cardiac puncture. Plasma was isolated from other blood components by centrifugation at 400 x *g* for 5 min at room temperature. Samples were derivatized using 7- fluorobenzofurazan-4-sulfonic acid (SBD-F) as described previously [227]. tHcy concentration was measured by HPLC using a ProStar solvent delivery system (PS-210, Varian Inc., Palo Alto, CA), a Microsorb-MV C18 (5μm, 100 Å, 25cm, Varian) and a fluorescence spectrophotometric detector (Varian Prostar model 360) with an excitation wavelength of 385 nm and an emission wavelength of 515 nm. Ten μM cysteamine was used as an internal standard.

S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy): AdoMet and AdoHcy concentrations were measured in 50 mg of pulverized liver tissue using HPLC [228, 229]. The assay was performed on a Varian ProStar HPLC system (PS-210) using a Beckman Ultrasphere ODS 5μm C18 column, 4.6mm x 25cm (Fullerton, CA, cat# 235329) at 55°C with an online Gilson 118 UV/VIS detector (Middleton, WI).

Tissue histology: 7.5-week-old $Chdh^{+/+}$ and $Chdh^{-/-}$ mice were anesthetized and liver, kidney, skeletal muscle, heart and brain were collected. The tissues were fixed in 4% paraformaldehyde/ 0.2% gluteraldehyde for 72 hours, processed, paraffin embedded and sectioned for hemotoxylin and eosin staining using standard techniques. Brain sections were stained with Luxol blue. Reproductive organs from adult (14 - 17 weeks old) $Chdh^{+/+}$ and $Chdh^{-/-}$ male mice were fixed for 24 hours in modified Davidson's fixative. Testis and epididymal sections were stained with hemotoxylin and eosin as well as with periodic acid- Schiff stain (PAS) for histological analysis. A UNC - Chapel Hill Department of Laboratory Animal Medicine veterinary pathologist examined all tissues except brain. An expert in neuroanatomy examined brain sections.

Transmission electron microscopy: Epididymal tissue was harvested from 8 to 10 week old $Chdh^{+/+}$ and $Chdh^{-/-}$ mice. The tissue was fixed in 2% paraformaldehyde, 2.5% gluteraldehyde, 0.2% picric acid in 0.1M sodium cacodylate, pH 7.2. Liver, kidney, brain, skeletal muscle, heart and testis tissues were harvested from animals perfused via cardiac puncture of the left ventricle using a gravity perfusion system (IV-140, Braintree Scientific, Braintree, MA, USA) with the same fixative. The UNC-Chapel Hill Microscopy Services Laboratory processed tissues for TEM according to standard techniques. TEM grids were observed and photographed using a Zeiss EM-10A transmission electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY) with an accelerating voltage of 60kV.

Sperm count and motility: Sperm from the cauda epididymis were collected from 8-10 week old $Chdh^{+/+}$, $Chdh^{+/-}$ and $Chdh^{-/-}$ male mice into M16 media (Sigma Aldrich, St.

Louis, MO, USA). Sperm count was determined by counting cells with a hemocytometer. Percent progressive motility was determined by counting the number of progressively motile sperm per total number sperm present using a hemocytometer. "Progressively motile sperm" refers to sperm that swim forward in a somewhat straight line.

MTT assay: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) acts as a hydrogen acceptor in oxidation reactions and its conversion from a yellow substrate to a blue product measures total mitochondrial dehydrogenase activity. We assayed this conversion using a commercially available kit (CellTiter 96 Non-radioactive Cell Proliferation Assay, Promega, Madison, WI) with some modifications. To assay MTT conversion in sperm, 100

µL of sperm/media suspension were incubated in F10 media (Gibco, Carlsbad, CA) in a 96 well plate with 15 µL of MTT solution at 37ºC/ 5% CO_2 for 2 h [230]. Following solubilization, the absorbance of each sample was read at a wavelength of 562nm using a BioTek plate reader (Winooski, VT). Absorbance was normalized to the number of sperm assayed.

Mitochondria from fresh liver, kidney, brain and testis were isolated at 4ºC using a Percoll gradient following previously described methods [223-225]. An aliquot of each sample (approximately 10-20 µg mitochondrial protein) was incubated in 100 µL of assay buffer (AB; 110mM KCl, 10mM ATP, 10mM $MgCl_2$, 10mM sodium succinate, 1mM EGTA in 20mM MOPS, pH 7.5) and 15 µL MTT solution (from kit) for 15 min at 37ºC with 5% CO_2. Absorbance was normalized to the amount of mitochondrial protein assayed.

ATP assay: ATP concentration in sperm and isolated mitochondria was measured using an ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. ATP concentration was normalized to either number of sperm or amount of mitochondrial protein assayed.

JC-1 assay (inner mitochondrial membrane potential,Ψm): Membrane potential across the inner mitochondrial membrane was estimated using the fluorescent indicator dye JC-1 (Sigma Aldrich, St. Louis, MO, USA). Sperm were incubated with JC-1 dissolved in DMSO at a final concentration of 10 µg/mL in F10 media (Invitrogen, Carlsbad, CA, USA) at 37º C for 10 min. Sperm were pelleted at 800 x *g* for 1 min. The supernatant was discarded and sperm were resuspended in 37º C PBS. 20 µL of each stained sample was examined with a fluorescence microscope (Olympus BX50, Center Valley, PA, USA) and large band epifluorescence filters. Cells that fluoresced red in the sperm midpiece were counted in 5 random fields of vision for each sample and results are expressed as a percent of total cells in those fields.

Mitochondria (approximately 100µg protein) were incubated with 1.72 mL of assay buffer (AB) and 18 µL of 0.2mg/mL JC-1 dissolved in DMSO for 10 min at room temperature protected from light. A mitochondrial sample from *Chdh*$^{+/+}$ liver, treated with valinomycin (1:200 final dilution, Sigma Aldrich, St. Louis, MO, USA) for 20 min, was used as a negative control. Fluorescence was measured using an excitation wavelength of 490nm and an emission wavelength scan from 500nm to 700nm on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi HTA, Pleasanton, CA, USA). A peak at 595nm corresponds to red fluorescence of J-aggregates. Fluorescent units (FLU) were

normalized to the amount of mitochondrial protein assayed.

Oral betaine supplementation: For betaine supplementation experiments, 8 – 10 week old *Chdh*^{+/+}, *Chdh*^{+/-} and *Chdh*^{-/-} mice were given free access to drinking water supplemented with betaine (Sigma Aldrich, St. Louis, MO, USA) to a final concentration of 2% for 42 days. 2% betaine was chosen as it was previously shown to be an optimal concentration for treating animals with other fertility problems [44, 45, 231].

Statistics: Statistical differences were determined using JMP software, version 6.0 (SAS Institute, Cary, NC, USA) using ANOVA, Tukey-Kramer HSD, and Student's t-test assuming equal variances. All tests were performed at $\alpha = 0.05$.

Results:

***Chdh* deletion has no effect on fetal viability, growth or liver, kidney or muscle function**

The genotype distribution for litters born to *Chdh*^{+/-} mating pairs (total 604 pups) was 23% *Chdh*^{+/+}, 51% *Chdh*^{+/-} and 27% *Chdh*^{-/-}. Litter size for *Chdh*^{+/-} mated pairs was 6 – 11 pups. Wild type C57 mating pairs typically produce litters of similar size. Mating a *Chdh*^{-/-} female with a male *Chdh*^{+/-} mouse resulted in normal size litters (6 – 9 pups). However, ten of eleven pairs of *Chdh*^{-/-} males mated with female *Chdh*^{-/-} mice had no litters over the course of eight months; during this time one mating pair had a litter of 2 pups, both of which showed impaired growth.

There were no differences among $Chdh^{+/+}$, $Chdh^{+/-}$ and $Chdh^{-/-}$ mice in growth either in terms of body length or total body weight (data not shown). Selected tests of liver, kidney and muscle function showed no changes in the $Chdh^{-/-}$ animals (Table 2.1). Mean plasma alanine aminotransferase (ALT) activity, a measure of hepatic damage, was unchanged (16.7±2.6 U/I in $Chdh^{-/-}$ vs. 19.1±4.0 U/I in $Chdh^{+/+}$ mice; p = 0.64, N= 10/group). Plasma total bilirubin concentrations, a measure of hepatic function, was unchanged (0.23 ± 0.02 mg/dL in $Chdh^{-/-}$ vs. 0.24 ± 0.03 mg/dL in $Chdh^{+/+}$ mice; p = 0.71, N= 10/group). Blood urea nitrogen (BUN) concentrations, a measure of renal function, was unchanged (13.0 ± 0.8 mg/dL in $Chdh^{-/-}$ vs. 13.2 ± 1.2 mg/dL in $Chdh^{+/+}$ mice; p = 0.88, n = 10/group). Plasma creatinine concentrations, a measure of renal function, were normal (less than 0.1 mg/dL) in both $Chdh^{+/+}$ and $Chdh^{-/-}$ mice. Additionally, urine specific gravity following water deprivation and vasopressin injection was unchanged in $Chdh^{-/-}$ mice (1.081 ± 0.004, n = 14) compared to $Chdh^{+/+}$ animals (1.080 ± 0.008, n = 12). Plasma creatine phosphokinase (CPK) activity, a measure of muscle damage, was unchanged in $Chdh^{-/-}$ (235 ± 28 U/L) vs. $Chdh^{+/+}$ mice (165 ± 25 U/L); p = 0.07, N= 12/group); these values were higher than CPK activity ranges previously reported for C57 mice (102 – 139 U/I). All other measured values were within the published normal ranges for C57 mice. No differences were observed when data were analyzed for sex-specific effects. $Chdh^{-/-}$ mice lived for at least one year without any obvious serious health problems.

***Chdh* deletion results in loss of CHDH enzyme activity:**   In wild type mice, kidney, liver and testis had the highest activity of CHDH, with almost no activity detected in brain and skeletal muscle; CHDH activity was undetected in heart. Reduced CHDH activity was

detected in tissues of *Chdh*$^{-/-}$ mice compared to *Chdh*$^{+/+}$ mice (Figure 2.2). Liver samples from *Chdh*$^{+/-}$ mice had 37% of the CHDH activity measured in *Chdh*$^{+/+}$ samples (data not shown).

Choline metabolite profiles are changed in *Chdh*$^{-/-}$ in whole tissue and isolated mitochondria: *Chdh*$^{-/-}$ mice had greatly diminished hepatic betaine concentrations as compared to *Chdh*$^{+/+}$ mice (by 85%; p< 0.001), while having more than doubled hepatic choline concentrations (p<0.01) (Table 2.2 and Table 2.3). Hepatic phosphatidylcholine (PtdCho) concentrations were decreased in *Chdh*$^{-/-}$ mice, but this was a sex-specific effect. There were no differences in hepatic PtdCho between male *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice; however, female *Chdh*$^{-/-}$ mice had significantly less hepatic PtdCho (17,226 ± 86 nmol/g) then did their female *Chdh*$^{+/+}$ littermates (20,108 ± 540 nmol/g, p< 0.05; n = 6/group). *Chdh*$^{--}$ mice had significantly lower hepatic sphingomyelin (SM) concentrations compared to *Chdh*$^{+/+}$ mice (p< 0.05). This change was not sex-specific. There were no sex-related changes in hepatic glycerophosphocholine (GPCho) or phosphocholine (PCho) concentrations.

  Chdh$^{-/-}$ mice had less than 2% of renal betaine concentrations compared to *Chdh*$^{+/+}$ mice (p< 0.001), increased renal choline concentrations (p< 0.05) and more than doubled renal PCho concentrations (p< 0.0005) as did *Chdh*$^{+/+}$ littermates. There were no changes in renal GPCho or SM concentrations. While there were no significant differences in PtdCho concentrations between *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice compared grouped or by sex, male *Chdh*$^{+/+}$ animals had significantly higher renal PtdCho concentrations (25,983 ± 1558

nmol/g) than did *Chdh*$^{+/+}$ females (13,276 ± 1102 nmol/g; p< 0.001, N = 9/group).

In brain, betaine concentrations were very low in both wildtype and knockout mice. Brain PCho concentrations were increased in *Chdh*$^{-/-}$ mice (p< 0.05) compared to *Chdh*$^{+/+}$ littermates. Brain choline, PtdCho, SM and GPCho concentrations were not different between groups.

In skeletal muscle, *Chdh*$^{-/-}$ animals had 1% (p < 0.001) of the amount of betaine and369% (p < 0.001) of the amount of choline found in *Chdh*$^{+/+}$ animals. PCho concentrations were significantly increased in *Chdh*$^{-/-}$ skeletal muscle tissue (p < 0.01). There were no changes in GPCho, PtdCho or SM concentrations.

Betaine concentrations in *Chdh*$^{-/-}$ heart tissue were significantly lower than *Chdh*$^{+/+}$ heart tissue (p < 0.001) and choline concentrations significantly higher (p < 0.01). No changes were measured in GPCho, PCho, PtdCho, or SM in this tissue.

Among the tissues examined in the wild type mice, testis had the highest betaine concentrations (3.5 fold higher than the kidney). Testicular betaine concentrations were diminished by more than 99% in *Chdh*$^{-/-}$ mice as compared to *Chdh*$^{+/+}$ mice (p < 0.001). Also, testicular choline concentrations were nearly twice as high in *Chdh*$^{-/-}$ mice (p < 0.001) and testicular PCho concentrations were increased (p < 0.05). There were no changes in testicular GPCho, PtdCho or SM concentrations.

Betaine concentrations were undetectable in mitochondria purified from liver, kidney, brain, skeletal muscle, heart and testis of *Chdh*$^{-/-}$ mice, while there were nmol/mg protein concentrations of betaine in mitochondria from liver, kidney, heart and testis of *Chdh*$^{+/+}$ mice (Table 2). Betaine was not detected in mitochondria from brain and skeletal muscle of *Chdh*$^{+/+}$ mice. Choline concentrations were significantly increased in isolated mitochondria from liver (p <0.05) and kidney (p <0.001) and testis (p< 0.001) in *Chdh*$^{-/-}$ animals as compared to *Chdh*$^{+/+}$ mice. There were no changes in choline concentrations in mitochondria from brain, skeletal or heart due to genotype. Additionally, no changes due to genotype were noted in any tissue's mitochondria for GPCho, PCho, PtdCho or SM concentrations with the exception of PCho in testis mitochondria where concentrations were increased by 85% in *Chdh*$^{-/-}$ mice (p< 0.001). There was no sex-effect.

***Chdh*$^{-/-}$ mice have increased tHcy:** Deletion of *Chdh* resulted in a significant increase in tHcy concentrations (from 6.1μmol/L ± 0.9 in wild type to 10.4μmol/L ± 0.9 in knockouts) (p <0.01). Hepatic AdoMet concentrations were the same in *Chdh*$^{+/+}$ mice (73.4 ± 6.2 pmole/mg liver), *Chdh*$^{+/-}$ mice (70.0 ± 4.2 pmole/mg liver) and *Chdh*$^{-/-}$ mice (57.8 ± 5.4 pmole/mg liver) (p = 0.11, N = 6/ genotype). Hepatic AdoHcy concentrations did not change (*Chdh*$^{+/+}$, 51.2 ± 6.9 pmole/ mg liver; *Chdh*$^{+/-}$, 57.0 ± 14 pmole/mg liver; *Chdh*$^{-/-}$, 54.0 ± 11.7 pmole/mg liver; p=0.95, N=6/genotype). The AdoMet/AdoHcy ratio was not different among genotypes (p=0.49, N=6/genotype).

Mitochondrial morphology is altered in *Chdh*$^{-/-}$ sperm: Gross anatomic and histopathologic examination at the light microscopy level of testis revealed no difference between *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice (Figure 2.3). Transmission electron microscopic

examination of sperm ultrastructure revealed that mitochondria in the midpiece of the $Chdh^{-/-}$ sperm had malformed cristae and were enlarged/ swollen compared to $Chdh^{+/+}$ mitochondria (Fig 3B; *top*, cross-sectional view; *bottom*, longitudinal view). Skeletal muscle was the only other tissues examined to show similar morphological changes (Figure 2.4).

***Chdh* deletion resulted in decreased sperm motility and ATP content which was partially reversed by dietary betaine supplementation :** Although they display normal mating behavior, $Chdh^{-/-}$ males were largely unable to sire litters (as noted earlier, one out of eleven $Chdh^{-/-}$ males fathered a litter of two pups). Female $Chdh^{-/-}$ mice had no reproductive impairment. There were no differences in sperm counts among $Chdh^{+/+}$, Chdh$^{+/-}$ and $Chdh^{-/-}$ males (Figure 2.5A). Normal sperm counts for C57/129 mixed mice are 21 x 10^6 per mL [232]. $Chdh^{-/-}$ males had significantly decreased sperm motility, with only 16% of sperm classified as being progressively motile (Figure 2.5B). In contrast, wild type and heterozygous males had 58% progressively motile sperm (p <0.01 different from $Chdh^{-/-}$) and bred successfully. Betaine supplementation did not have any effect on sperm concentration in $Chdh^{+/+}$, Chdh$^{+/-}$ or $Chdh^{-/-}$ mice. Sperm motility, however, was increased to 30% progressively motile sperm in treated $Chdh^{-/-}$ males compared to untreated $Chdh^{-/-}$ mice, but this was still significantly lower than the motility of treated and untreated $Chdh^{+/+}$ and Chdh$^{+/-}$ males (Figure 2.5B). There was an interaction between genotype and treatment (p = 0.004).

MTT conversion (a measure of total dehydrogenase activity in mitochondria) was

diminished in sperm from $Chdh^{-/-}$ mice compared to sperm from wild type and heterozygous animals (p<0.05) (Figure 2.5C). Betaine treatment decreased MTT conversion in $Chdh^{+/+}$ sperm by 67%, but did not alter MTT conversion in $Chdh^{+/-}$ or $Chdh^{-/-}$; mice. There was an interaction between genotype and treatment (p = 0.02) (Figure 2.5C).

Sperm from $Chdh^{-/-}$ males had 55% less ATP than did $Chdh^{+/+}$ mice (p<0.05); there were no differences in ATP content between $Chdh^{+/+}$ and $Chdh^{+/-}$ sperm (Figure 2.4D). Betaine supplementation restored ATP content of $Chdh^{-/-}$ sperm, but had no effect on $Chdh^{+/+}$ or $Chdh^{+/-}$ sperm. There was no interaction between genotype and treatment. Significantly fewer sperm from $Chdh^{+/-}$ and $Chdh^{-/-}$ mice were able to maintain an Ψm of at least -160mV (Figure 2.5E) and betaine treatment did not correct these differences.

Mitochondrial function is altered in $Chdh^{-/-}$ non-testicular tissues: Mitochondria from $Chdh^{-/-}$ liver had 62% lower ATP concentration than $Chdh^{+/+}$ liver mitochondria (p< 0.01) and $Chdh^{-/-}$ heart mitochondria had double the ATP concentration than did mitochondria from $Chdh^{+/+}$ heart (p<0.05) (Figure 2.6A). Ψm was increased in mitochondria samples from $Chdh^{-/-}$ testis as compared to $Chdh^{+/+}$ testis (Figure 2.6B) (p <0.05). There were no genotype-specific differences in Ψm in the other tissues tested. Deletion of $Chdh$ resulted in a decrease in the total mitochondrial dehydrogenase activity in liver and kidney (p<0.05, Figure 2.6C). There were no differences in brain, skeletal muscle or heart mitochondria for MTT conversion.

Discussion:

We report, for the first time, the successful creation of a mouse in which the *Chdh* gene has been deleted. Fetuses from heterozygous mating were viable, grew normally, and survived to at least one year of age without presenting any obvious health problems. The most striking phenotype observed in these animals was severely impaired sperm motility in *Chdh*^{-/-} males.

Deletion of *Chdh* greatly reduced CHDH activity in all tissues which normally express this gene (Fig 2). The small residual activity detected probably reflects activity of other dehydrogenases that can use choline as a substrate. These were probably cytosolic enzymes, as mitochondrial betaine was not detectable in knockout mice (Table 2.3). Liver samples from *Chdh*^{+/-} mice had 37% of the CHDH activity measured in *Chdh*^{+/+} samples, suggesting that there is one copy of the *Chdh* gene present in mice and that it is biallelically expressed.

Chdh^{-/-} mice had significantly higher plasma tHcy concentrations than did their *Chdh*^{+/+} littermates, again suggesting that *Chdh* deletion limited methyl-group availability for the methylation of homocysteine which is removed by three pathways: it can be methylated using betaine as a methyl donor, or methylated using methyltetrahydrofolate as a methyl donor, or condensed with serine to form cystathionine [233]. An accumulation of tHcy has been associated with an increased risk of cardiovascular disease [216, 217] and betaine has been employed as a treatment for elevated tHcy [204, 234-236]. Based on the *Chdh*^{-/-} mouse model, we would predict that individuals who harbor SNPs that decrease

CHDH activity will have higher plasma tHcy concentrations and may be at greater risk for developing cardiovascular disease.

In all tissues studied, deletion of the gene resulted in extremely low concentrations of betaine; the small remaining amounts likely were derived from dietary betaine (AIN76A diet contains 70.4 nmole betaine/g diet) or from activity of cytosolic dehydrogenases. We note that testis, liver and kidney are the tissues where CHDH activity was highest and where betaine and choline concentrations were most changed by the gene deletion (Figure 2.2. Table 2.2 and Table 2.3). Betaine concentrations were nmole/mg protein in mitochondria from liver, kidney and testes in wild type animals (in agreement with previously published results for liver mitochondria [218]), but in $Chdh^{-/-}$ mice almost no betaine was detected in mitochondria (Table 2.3). Assuming approximately 10% of a cell is protein; we estimate that betaine concentrations in the mitochondria are between 2 and 7 times more concentrated than concentrations measured in the whole cell, depending on the tissue. The failure to convert choline to betaine resulted in the accumulation of choline in some tissues (in testis and liver, choline concentrations nearly doubled in $Chdh^{-/-}$ animals), while in others PCho accumulated due to the activity of choline kinase (in kidney, brain and testis whole tissue). Testicular betaine concentrations were the highest of all tissues studied - almost 10 times higher than concentrations found in the liver, the organ thought of as the primary site of choline metabolism. This data, in conjunction with the asthenospermic phenotype of the male $Chdh^{-/-}$ mice suggest that betaine plays a critical role in testicular function.

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In females, deletion of *Chdh* was associated with diminished PtdCho concentrations in liver and, in females and males, with diminished SM concentrations in liver (SM is formed from PtdCho). A major pathway for PtdCho synthesis is the methylation of phosphatidylethanolamine catalyzed by phosphatidylethanolamine-*N*-methyltransferase (PEMT); this gene is induced by estrogen [107]. We speculate that *Chdh* deletion limited methyl-group availability for this pathway. However, we observed no decrease in hepatic AdoMet concentrations in *Chdh*$^{-/-}$ mice.

Sperm function, though not viability (ARJ, unpublished data), was severely compromised in *Chdh*$^{-/-}$ males. Homozygous males were largely infertile due to decreased motility of their sperm. We present evidence that a decrease in spermatic ATP content contributes to the poor motility of these cell. *Chdh*$^{+/-}$ males did not demonstrate any impairment in sperm motility, likely because developing sperm share cytosolic components during spermatogenesis [237, 238] and the products of choline oxidation (reducing equivalents, betaine) could be shared between cells. Indeed, we routinely use *Chdh*$^{+/-}$ mice to generate animals used in our studies.

A constant supply of ATP, both from mitochondrial oxidative phosphorylation and glycolysis, is required for sperm motility [239-241]. It is important to note that in sperm, the processes of oxidative phosphorylation and glycolysis are separated by permanent compartmentalization. Mitochondria are localized to the sperm midpiece while glycolytic enzymes are found in the sperm principal piece. When CHDH, an enzyme localized to the inner mitochondrial membrane, is absent, mitochondria in the sperm midpiece appeared

grossly abnormal when examined by electron microscopy (Figure 2.3B). Additionally, these cells showed a decrease in mitochondrial dehydrogenase activity and ATP content and, $Chdh^{-/-}$ sperm mitochondria were unable to maintain an electrochemical gradient across the inner mitochondrial membrane of at least -160mV. Measuring the ability of mitochondria to reduce MTT is a measurement of the activity of the sum total of mitochondrial dehydrogenases; therefore, a decrease in the amount of MTT reduction in the $Chdh^{-/-}$ sperm reinforces the idea that CHDH activity represents a significant portion of the total mitochondrial reductive capacity. The JC-1 assay measures the polarity across the mitochondrial inner membrane (Ψm), a mitochondrial characteristic not necessarily related to the activity of mitochondrial dehydrogenases. For example, inhibiting the electron transport chain will reduce the Ψm [242, 243]but may not have an effect on mitochondrial dehydrogenase activity. Ψm is directly related to oxidation of NADH [244]; therefore, a lack of NADH could result in a collapse of the inner membrane polarity. In this case, a smaller number of protons will be transported into the inner membrane space and Ψm may be compromised. A decrease in either mitochondrial dehydrogenase activity or Ψm – or both – can ultimately result in a decrease in ATP production by the mitochondria. These results suggest that $Chdh^{-/-}$ sperm did not generate sufficient ATP through oxidative phosphorylation and that ATP generated by substrate-level phosphorylation during anaerobic glycolysis was inadequate to support sperm motility, although all necessary substrates were provided in the media (M16 media contains 1.0 g/L glucose, 0.036 g/L pyruvic acid and 4.35 g/L lactic acid).sperm motility, although all necessary substrates were provided in the media (M16 media contains 1.0 g/L glucose, 0.036 g/L pyruvic acid and 4.35 g/L lactic acid).

The oxidation of choline to form betaine produces electron transport chain substrates. The addition of choline to isolated rat hepatic mitochondria increased resting state respiration (State II), accelerated ADP-stimulated respiration (State III) and slightly increased respiration in the presence of oligomycin (State IV) *(Bruce Kristal, personal communication).* Functional CHDH enzyme contains a flavin adenine dinucleotide (FAD) prosthetic group, which acts as an electron acceptor during the oxidation of choline to betaine aldehyde, producing $FADH_2$. Further oxidation of the betaine aldehyde intermediate to betaine, catalyzed by mitochondrial betaine aldehyde dehydrogenase, produces NADH [180]. Therefore, oxidation of one choline molecule to betaine results in the generation of 5 ATP molecules by the mitochondrial electron transport chain [245]. We present data showing that choline and betaine are highly concentrated in mitochondria, suggesting that choline flux through CHDH is high in this organelle and, therefore, choline is likely an important source of energy for the sperm.

When $Chdh^{+/+}$, $Chdh^{+/-}$ and $Chdh^{-/-}$ males were supplemented with betaine via drinking water for 42 days, the asthenospermic phenotype of $Chdh^{-/-}$ males was improved, but not fully rescued, by betaine supplementation. In addition, the ATP content of the $Chdh^{-/-}$ sperm was increased with betaine supplementation. Though the proposed defect in mitochondrial ATP production mechanism described above is appealing, it does not explain why betaine supplementation restored normal sperm ATP concentrations (as no new oxidation of choline occurs due to this treatment). The conversion of choline to betaine cannot be the only defect in production of ATP; there must be another mechanism responsible for this effect.

As noted earlier, betaine is an important osmolyte, and we suggest that perturbation of betaine production in mitochondria resulted in fluid shifts that distorted the 3-dimensional architecture of the mitochondria in $Chdh^{-/-}$ mice. Treatment with betaine may allow mitochondria to accumulate betaine as they should, establish osmotic balance, and restore function. It is possible that ionic effects of reduced betaine alter ATP-dependent ion pumps and increase utilization of ATP. ATP from the mitochondria may not diffuse out fast enough to sustain activity at the distal end of the sperm flagellum [241]. Glycolytic enzymes are concentrated in the principal piece, and some are bound to the fibrous sheath of the flagellum [193]. Glycolysis produces ATP adjacent to the site where it is required to support active sliding of the flagellar filaments [241]. It is possible that betaine, via osmotic effects, alters glycolysis. When the maturing sperm leave the testis, they are non-motile and their full maturation, including potential to display motility, takes place during transit through the epididymis [246]. The maturational changes in sperm are caused by changes in the luminal ion concentration and the proteins secreted into the lumen by the epididymal epithelium [246]. It is possible that betaine, via osmotic effects, alters sperm maturation. The volume of the entire sperm is modulated by intracellular osmolarity, and this too influences sperm motility and flagellar movement [247].

No improvement in MTT conversion was achieved because this measurement is a reflection of the mitochondrial dehydrogenases in these cells, as discussed above. Since CHDH was still absent from the $Chdh^{-/-}$ sperm we would not expect a restoration of MTT conversion with betaine supplementation.

Unexpectedly, MTT conversion in sperm was decreased in betaine – supplemented $Chdh^{+/+}$ mice compared to untreated $Chdh^{+/+}$ mice. The simplest explanation would be that CHDH activity (which can catalyze MTT conversion to formazan) was inhibited by the reaction's product, betaine; however, we found that this is not the case when this hypothesis was tested *in vitro* (data not shown). Perhaps betaine suppresses the activity of other mitochondrial dehydrogenases or somehow interferes with uptake of the MTT by the mitochondria. Taken together, these results indicate that while sperm motility is partially dependent on the presence of betaine, full motility may require intact choline oxidation.

Mitochondrial abnormalities were not limited to sperm in $Chdh^{-/-}$ mice. MTT assays performed in isolated mitochondria from liver and kidney show that deletion of CHDH compromised mitochondrial dehydrogenase activity (Figure 2.6C); however, unlike in sperm, Ψm was maintained in mitochondria from these tissues (Figure 2.6B). Interestingly, compared to mitochondria from $Chdh^{+/+}$ testis, more mitochondria from $Chdh^{-/-}$ testis maintain Ψm. The testis contains 12 times more betaine than liver and about 3 times more than kidney (Table 2.2 and Table 2.3). Therefore, a loss of betaine via *Chdh* deletion would significantly reduce the amount of organic osmolyte available to the cells of the testis. This would create a hypo-osmotic cytosol with regard to the mitochondrial matrix. Devin, et al. have reported that when mitochondria are studied in a hypo-osmotic media there is an increase in State III respiration and in the Ψm across the inner mitochondrial membrane [248, 249]. The JC-1 assay only indicates whether the electrochemical gradient across the inner mitochondrial membrane is at least -160mV and does not measure hyperpolarization; it is possible that all of the testis mitochondria are in fact hyperpolarized.

The decrease in liver mitochondrial dehydrogenase activity is associated with a decrease in ATP content in mitochondrial from this tissue; however, this was not true in kidney mitochondria. Interestingly, $Chdh^{-/-}$ heart mitochondria had double the ATP content of $Chdh^{+/+}$ mitochondria. Because CHDH activity was undetectable in this organ the change in ATP content must result from a mechanisms other than a direct effect of CHDH, perhaps metabolic changes in other tissues that affect energy metabolism in heart. Skeletal muscle mitochondria appeared abnormal in $Chdh^{-/-}$ mice when examined by TEM (Figure 2.4) although indicators of mitochondrial function in this tissue were not different than in $Chdh^{+/+}$ animals. It is possible that more extensive studies of mitochondrial function in this tissue will reveal more subtle abnormalities not detected by the assays performed thus far.

Our studies in the $Chdh^{-/-}$ mouse suggest that functional *CHDH* gene polymorphisms in humans may have important consequences, including elevated tHcy concentrations and male infertility. The rs12676 SNP, located in the coding region of the human *CHDH* gene, occurs at a high frequency in the human population (9% have two variant alleles, [80]), and is associated with an increased risk of breast cancer and renders pre-menopausal women more susceptible to developing fatty liver when they are ingesting a choline-deficient diet [105]. Several other *CHDH* SNPs have been identified in humans but have unknown functional consequences. It is estimated that approximately 20% of human couples worldwide are infertile; in 50% of these couples the infertility was attributed to male factor infertility [250]. Of these, asthenospermia was diagnosed in 15% - 17% of these men [251, 252]. It would be interesting to explore whether these men have mutations

in *CHDH*.

Figure 2.1: Mutation of the *Chdh* gene. *A*. Targeting vector used to generate *Chdh*$^{-/-}$ mice. *1*. Wild type *Chdh* allele. *2*. Targeting vector. *3*. Targeted *Chdh* allele with exons 1 through 3 replaced with *neo*^{r} cassette. *B*. *Chdh* mouse genotyping. Tail biopsies were collected from mice at weaning. Tail tissue was digested in buffer containing proteinase K and genomic DNA purified by phenol:choloroform extraction. DNA was used for PCR-mediated genotyping of all animals. *neo*^{r} , neomycin resistance cassette; *TK*, thymidine kinase

Figure 2.1: Mutation of the *Chdh* gene
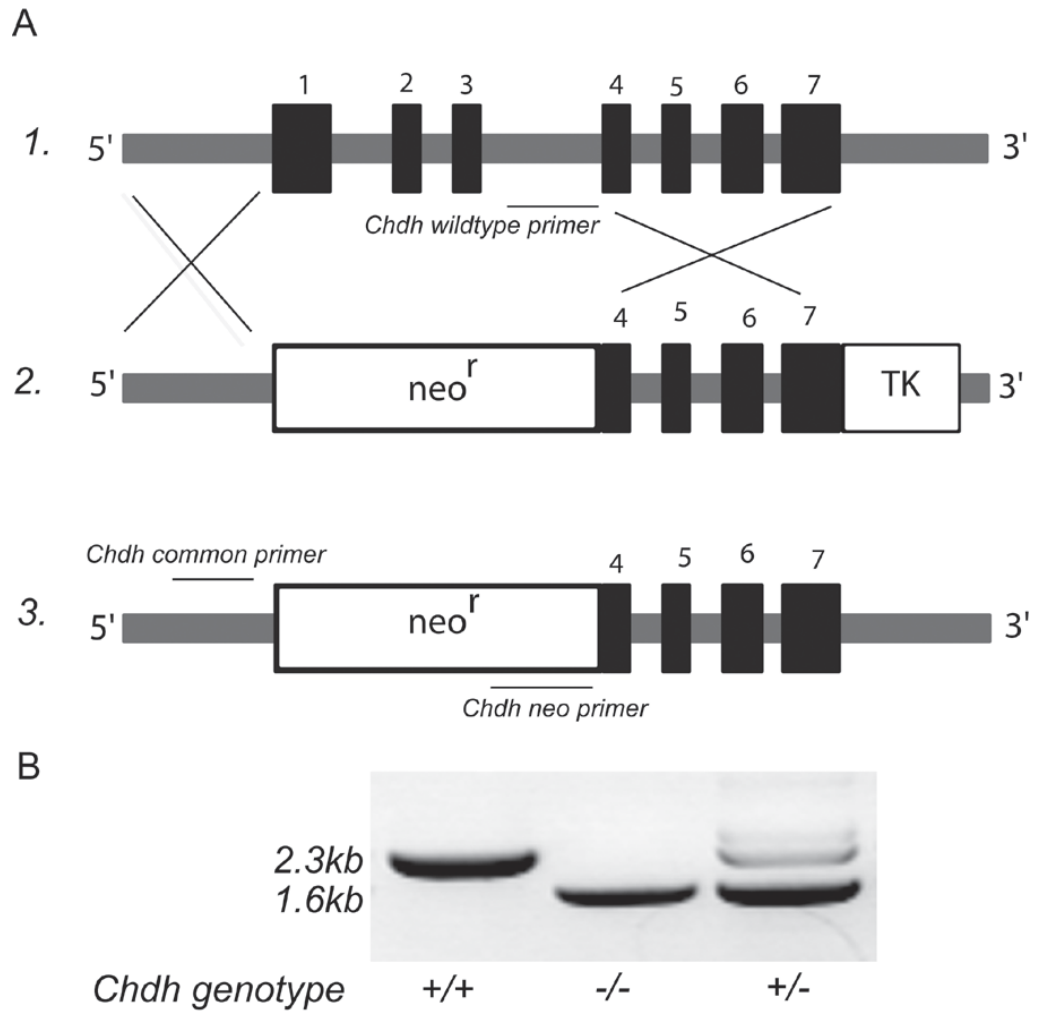
Table 2.1: Indicators of Organ Function Were Normal in *Chdh*^{-/-} Mice. Selected tests of liver, kidney and muscle function were performed. Mean plasma alanine aminotransferase (ALT) activity, a measure of hepatic damage, was unchanged (16.7±2.6 U/I in *Chdh*$^{-/-}$ vs. 19.1±4.0 U/I in *Chdh*$^{+/+}$ mice; p = 0.64, N= 10/group). Plasma total bilirubin concentrations, a measure of hepatic function, was unchanged (0.23 ± 0.02 mg/dL in *Chdh*$^{-/-}$ vs. 0.24 ± 0.03 mg/dL in *Chdh*$^{+/+}$ mice; p = 0.71, N= 10/group). Blood urea nitrogen (BUN) concentrations, a measure of renal function, was unchanged (13.0 ± 0.8 mg/dL in *Chdh*$^{-/-}$ vs. 13.2 ± 1.2 mg/dL in *Chdh*$^{+/+}$ mice; p = 0.88, n = 10/group). Plasma creatinine concentrations, a measure of renal function, were normal (less than 0.1 mg/dL) in both *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice. Additionally, urine specific gravity following water deprivation and vasopressin injection was unchanged in *Chdh*$^{-/-}$ mice (1.081 ± 0.004, n = 14) compared to *Chdh*$^{+/+}$ animals (1.080 ± 0.008, n = 12) Plasma creatine phosphokinase (CPK) activity, a measure of muscle damage, was unchanged in *Chdh*$^{-/-}$ (235 ± 28 U/L) vs. *Chdh*$^{+/+}$ mice (165 ± 25 U/L); p = 0.07, N= 12/group); these values were higher than CPK activity ranges previously reported for C57 mice (102 – 139 U/I). All other measured values were within the published normal ranges for C57 mice. No differences were observed when data were analyzed for sex-specific effects.

Table 2.1: Measures of liver, kidney and muscle function

Organ	Measure	*Chdh*^{+/+}	*Chdh*^{-/-}	Normal range [253]
Liver	Plasma alanine aminotransferase (U/L)	19.14 ± 3.95	16.60 ± 2.58	~ 10 U/L
	Plasma total billirubin (mg/dL)	0.24 ± 0.03	0.23 ± 0.02	< 1.0 mg/dL
Kidney	Plasma blood urea nitrogen (mg/dL)	13.22 ± 1.21	13.00 ± 0.80	~10 mg/dL
	Plasma creatinine (mg/dL)	<0.01	< 0.01	<1.0 mg/dL
	Urine specific gravity			
Muscle	Plasma creatine phosphokinase activity (U/L)	165 ± 25	235 ± 28	102 – 129 U/L

Figure 2.2: *Chdh$^{-/-}$* **animals have reduced CHDH enzyme activity.** Liver, kidney, brain, skeletal muscle, heart and testis were collected from *Chdh$^{+/+}$* and *Chdh$^{-/-}$* mice. CHDH activity was measured using a radioenzymatic assay. Black bars are *Chdh$^{+/+}$* and white bars are *Chdh$^{-/-}$*. Data are presented as mean ± SEM. ***p<0.001 different from *Chdh$^{+/+}$* by ANOVA and Tukey-Kramer HSD test. N=6 animals per group. ND, not detected.

Figure 2.2: *Chdh$^{-/-}$* animals have reduced CHDH enzyme activity
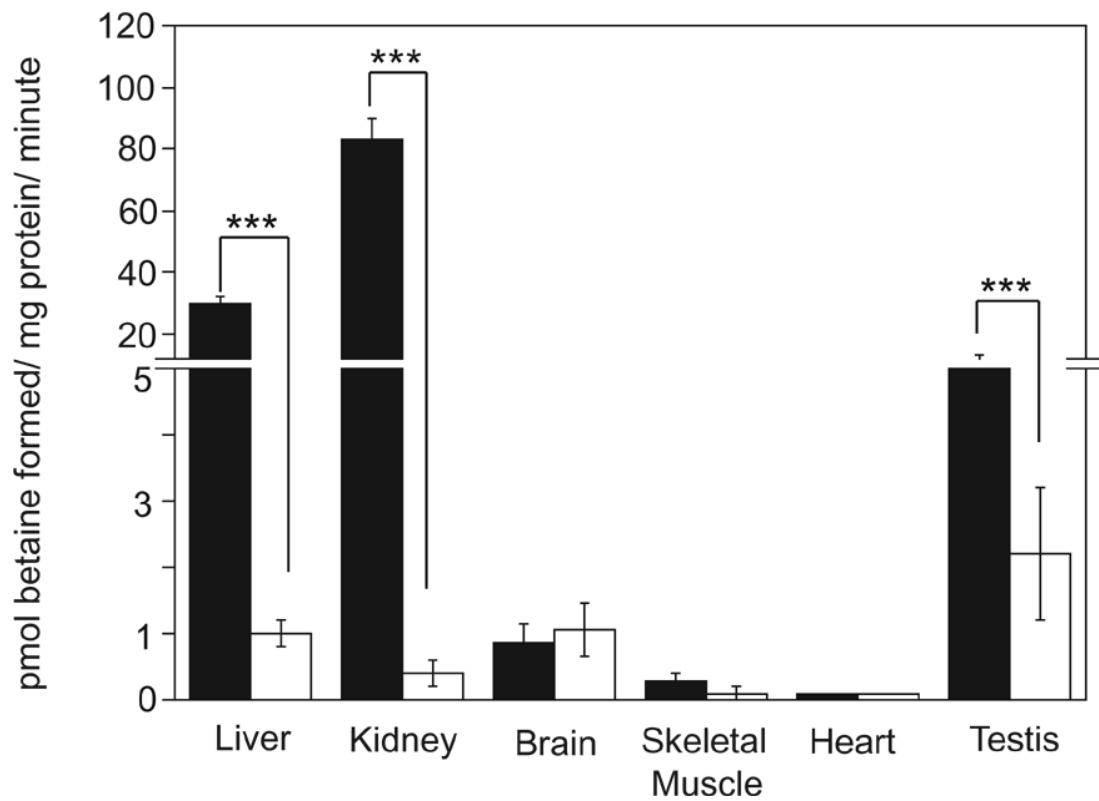
Table 2.2: Choline metabolites in liver, kidney, brain, testis, skeletal muscle and heart tissue. Liver, kidney, brain, testis, skeletal muscle and heart were collected from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice and choline metabolites were measured using LC-ESI-IDMS. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 different from *Chdh*$^{+/+}$ by Student's *t* test. Interaction between genotype and gender tested by two-factor ANOVA. For liver, N= 6 animals/group; kidney, n = 9 animals/group; brain, N= 11 (*Chdh*$^{+/+}$) and 19 (*Chdh*$^{-/-}$); testis, N= 5 animals/group; skeletal muscle, N= 9 (*Chdh*$^{+/+}$) and 7 (*Chdh*$^{-/-}$); heart, N= 9 (*Chdh*$^{+/+}$) and 7 (*Chdh*$^{-/-}$). GPCho, glycerophosphocholine; PCho, phosphocholine; PtdCho, phosphatidylcholine; SM, sphingomyelin.

Table 2.2: Choline metabolites in liver, kidney, brain, testis, skeletal muscle and heart tissue

		Betaine (nmol/g)	Choline (nmol/g)	GPCho (nmol/g)	PCho (nmol/g)	PtdCho (nmol/g)
Liver						
	$Chdh^{+/+}$	358±37	202±54	741±196	248±68	19226±337
	$Chdh^{-/-}$	56±23***	432±82**	664±124	305±70	17913±376*
Kidney						
	$Chdh^{+/+}$	1388±207	3028±184	11608±2196	615±60	17512±2283
	$Chdh^{-/-}$	25±8***	4226±389*	8214±1440	1221±46***	18068±1358
Brain						
	$Chdh^{+/+}$	20±5	238±20	781±30	361±21	22322±951
	$Chdh^{-/-}$	10±3	208±11	794±26	437±14**	22518±692
Testis						
	$Chdh^{+/+}$	4589±401	367±30	816±45	4030±111	9176±773
	$Chdh^{-/-}$	31±11***	722±32***	836±26	4861±229*	9599±819
Skeletal Muscle	$Chdh^{+/+}$	20± 2	79±15	43±6	47±5	6232±220
	$Chdh^{-/-}$	ND***	292± 17***	43±4	74±8**	5917± 307

Table 2.3: Choline metabolites in purified mitochondria from liver, kidney, brain, testis, skeletal muscle and heart. Mitochondria were purified from fresh liver, kidney, brain, testis, skeletal muscle and heart tissues and choline metabolites were measured using LC-ESI- IDMS. Results are presented as mean ± SEM per mg mitochondrial protein. *p<0.05, **p<0.01, ***p<0.001 different from $Chdh^{+/+}$ by Student's *t* test. Interaction between genotype and gender tested by two-factor ANOVA. For all tissues N= 4 animals/group, except testis where N= 4 ($Chdh^{+/+}$) and 5 ($Chdh^{-/-}$). ND indicates metabolite concentration was below the detectable limit of the assay. GPCho, glycerophosphocholine; PCho, phosphocholine; PtdCho, phosphatidylcholine; SM, sphingomyelin. Data is expressed as nmol per mg mitochondrial protein.

Table 2.3: Choline metabolite concentrations in isolated mitochondria
(from liver, kidney, brain, testis, skeletal muscle and heart)

		Betaine	Choline	GPCho	PCho	PtdCho	SM
Liver	$Chdh^{+/+}$	20±6	1±0.3	49±37	14±9	204±30	9±1
Mitochondria	$Chdh^{-/-}$	ND*	4±1*	30±13	6±1	178±42	8±2
Kidney	$Chdh^{+/+}$	26±5	0.6±0.4	40±18	2±1	270±34	11±2
Mitochondria	$Chdh^{-/-}$	ND**	21±2***	40±3	1±0.0	222±7	11±1
Brain	$Chdh^{+/+}$	ND	4±2	23±12	4±2	327±74	9±3
Mitochondria	$Chdh^{-/-}$	ND	4±2	10±2	4±1	211±24	3±1
Testis	$Chdh^{+/+}$	296±33	4±4	101±35	332±36	4452±364	92±20
Mitochondria	$Chdh^{-/-}$	ND***	119±10***	103±44	617±23***	5266±346	89±14
Skeletal Muscle	$Chdh^{+/+}$	ND	3±1	4±2	2±1	103±40	5±1
Mitochondria	$Chdh^{-/-}$	ND	1±0.3	4±2	1±0.5	69±10	3±0.4
Heart	$Chdh^{+/+}$	7±11	10±2	28±37	2±1	172±13	16±2
Mitochondria	$Chdh^{-/-}$	ND	6±2	16±17	1±0.5	167±32	13±3

Figure 2.3: Testis histology and sperm transmission electron microscopy in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ mice. *A.* Testes were harvested from 14 -17 week old *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ males, fixed, and 5 µm sections were stained with hematoxylin and eosin. (Periodic acid-Schiff stain showed similar results). Images shown are 20X and are representative of N=4 animals/group. *B.* Electron microscopy of sperm cross-sectional view (top two panels) and longitudinal view (bottom two panels). For transmission electron microscopy, cauda epididymides was harvested and processed as described in methods. 70nm ultrathin sections were analyzed with an electron microscope with an accelerating voltage of 60kV. Images shown are 50,000X and are representative of N=2 animals/group. Arrows indicate typical mitochondria in sperm midpiece.

Figure 2.3: Testis histology and sperm transmission electron microscopy in *Chdh*^{+/+} and *Chdh*^{-/-} mice

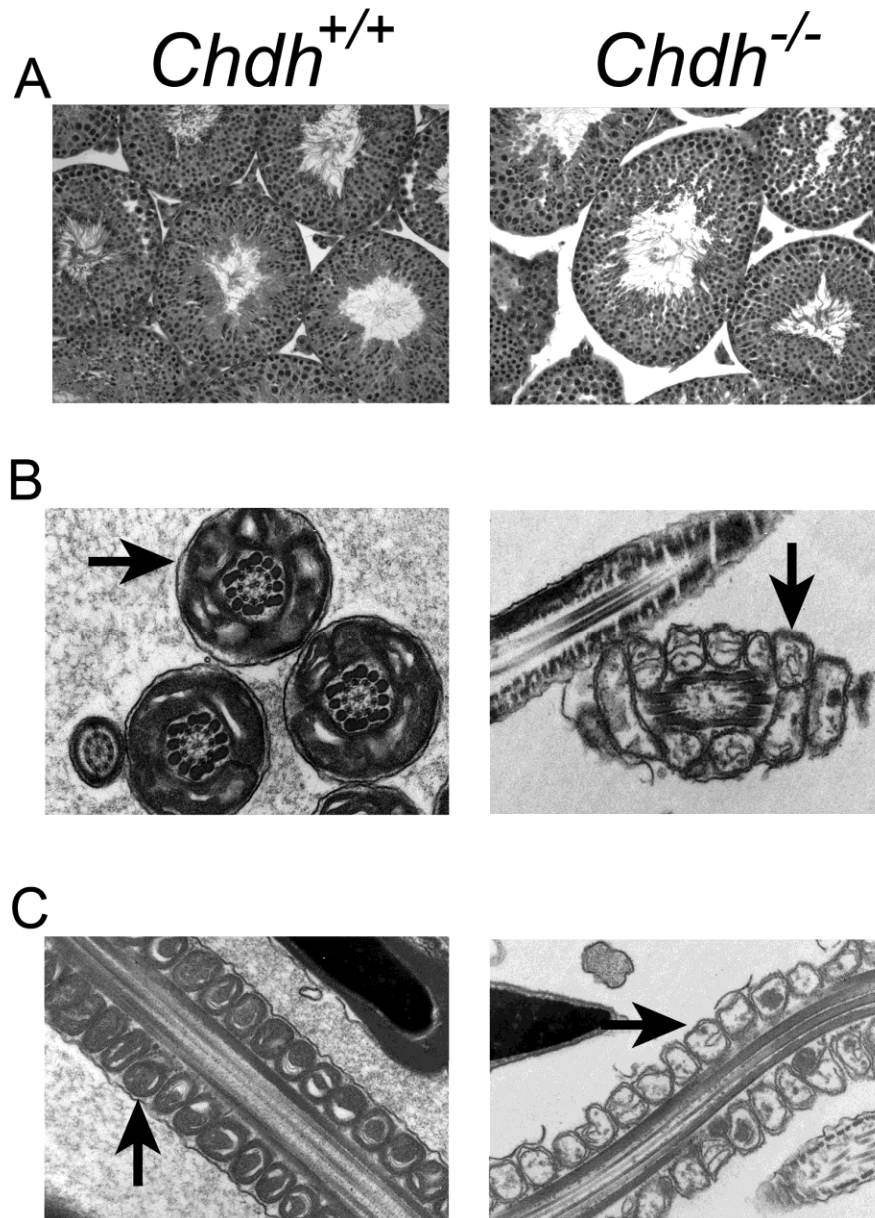**Figure 2.4: Skeletal muscle mitochondria morphology.** Animals were fixed by gravity perfusion via cardiac puncture. Following fixation, tissues were collected and examined by transmission electron microscopy for mitochondrial morphology. Representative images shown, N= 2 animals/ group. Images are 50,000X. Arrows indicate typical mitochondria.

Figure 2.4: Transmission electron microscopy of skeletal muscle mitochondria
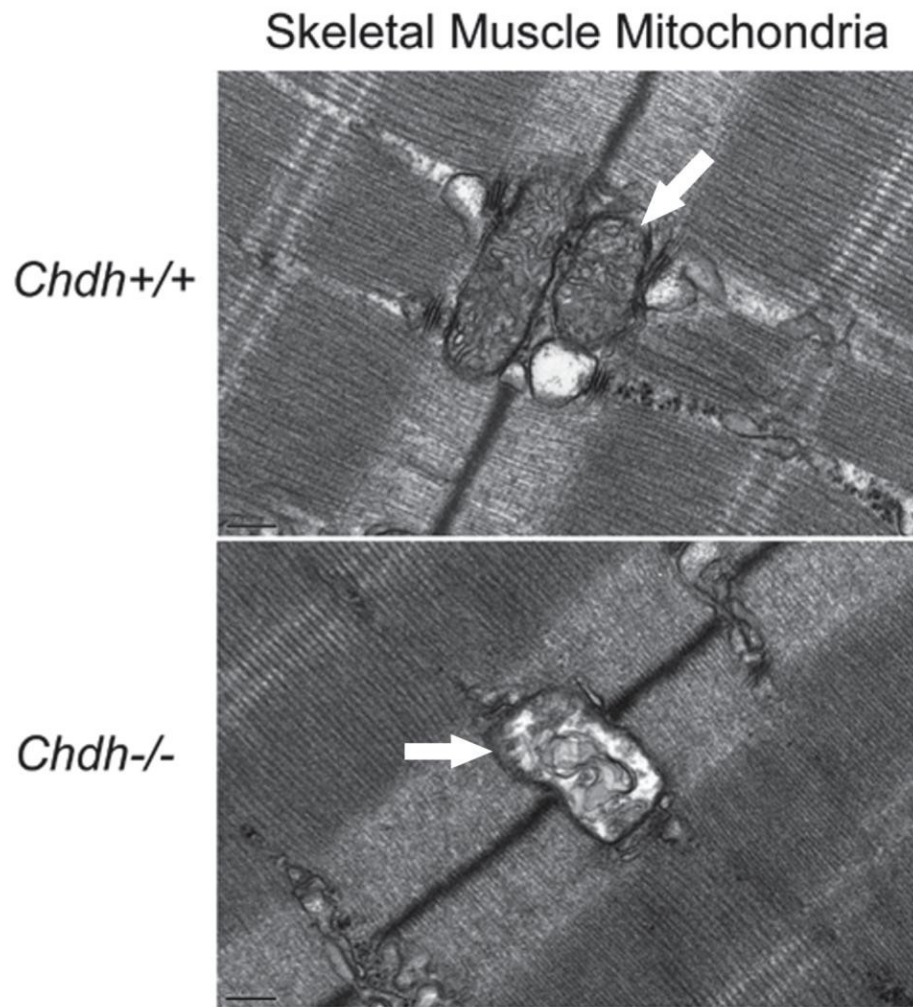
Figure 2.5: Sperm counts, motility and mitochondria function. *A.* Counts. Sperm were harvested from 8 – 10 week old *CHDH*$^{+/+}$ ($^{+/+}$), *Chdh*$^{+/-}$ (+/-) and *Chdh*$^{-/-}$ ($^{-/-}$) mice. Sperm count was determined by counting cells using a hemocytometer. Black bars represent untreated animals, gray bars represent betaine-supplemented animals. Results are presented as mean ± SEM. N=11 (*Chdh*$^{+/+}$, untreated), 5 (*Chdh*$^{+/-}$, untreated) and 8 (*Chdh*$^{-/-}$, untreated) mice per group. For betaine supplementation study, N=11 (*Chdh*$^{+/+}$), 5 (*Chdh*$^{+/-}$), 7 (*Chdh*$^{-/-}$) mice per group. *B.* Motility. Sperm were harvested from 8 – 10 week old *Chdh*$^{+/+}$ *Chdh*$^{+/-}$ and *Chdh*$^{-/-}$ mice. Motility assays were performed by counting the number of progressively motile sperm per total number of sperm in 5 squares of a hemocytometer. Results are presented as mean ± SEM. N=11 (*Chdh*$^{+/+}$, untreated), 5 (*Chdh*$^{+/-}$, untreated) and 8 (*Chdh*$^{-/-}$, untreated) mice per group. For betaine supplementation study, N=11 (*Chdh*$^{+/+}$), 5 (*Chdh*$^{+/-}$), 7 (*Chdh*$^{-/-}$) mice per group. **p<0.01 by two-factor ANOVA and Tukey-Kramer HSD, *** p< 0.001. There is an interaction between genotype and treatment, p = 0.0004. *C.* MTT reduction. MTT reduction determined as described in methods. Absorbance values were normalized to the number of sperm assayed. All assays were performed in duplicate. The value for each animal is the mean of 4 assays (2 cauda epididymides, each assayed in duplicate per animal). Results are presented as the mean ± SEM. *p<0.05 by two-factor ANOVA and Tukey-Kramer HSD. There is an interaction between genotype and treatment, p = 0.02. N=5 mice per group. *D.* ATP content in sperm. Results are presented as the mean % of untreated *Chdh*$^{+/+}$ mean ± SEM. * p<0.05 by Student's *t* test. N=5 mice per group. *E.* Membrane potential. JC-1 staining determined as described in methods. The number of cells that fluoresced red in the sperm midpiece was counted in 5 random fields of vision for each sample and results are expressed as a percent of the total cells in those fields. Results are presented as mean ± SEM. *p<0.05, **p<0.01 by two-factor ANOVA and Tukey-Kramer HSD. There is no interaction between genotype and treatment. N=5 mice per group.

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Figure 2.5: Sperm counts, motility and mitochondrial function
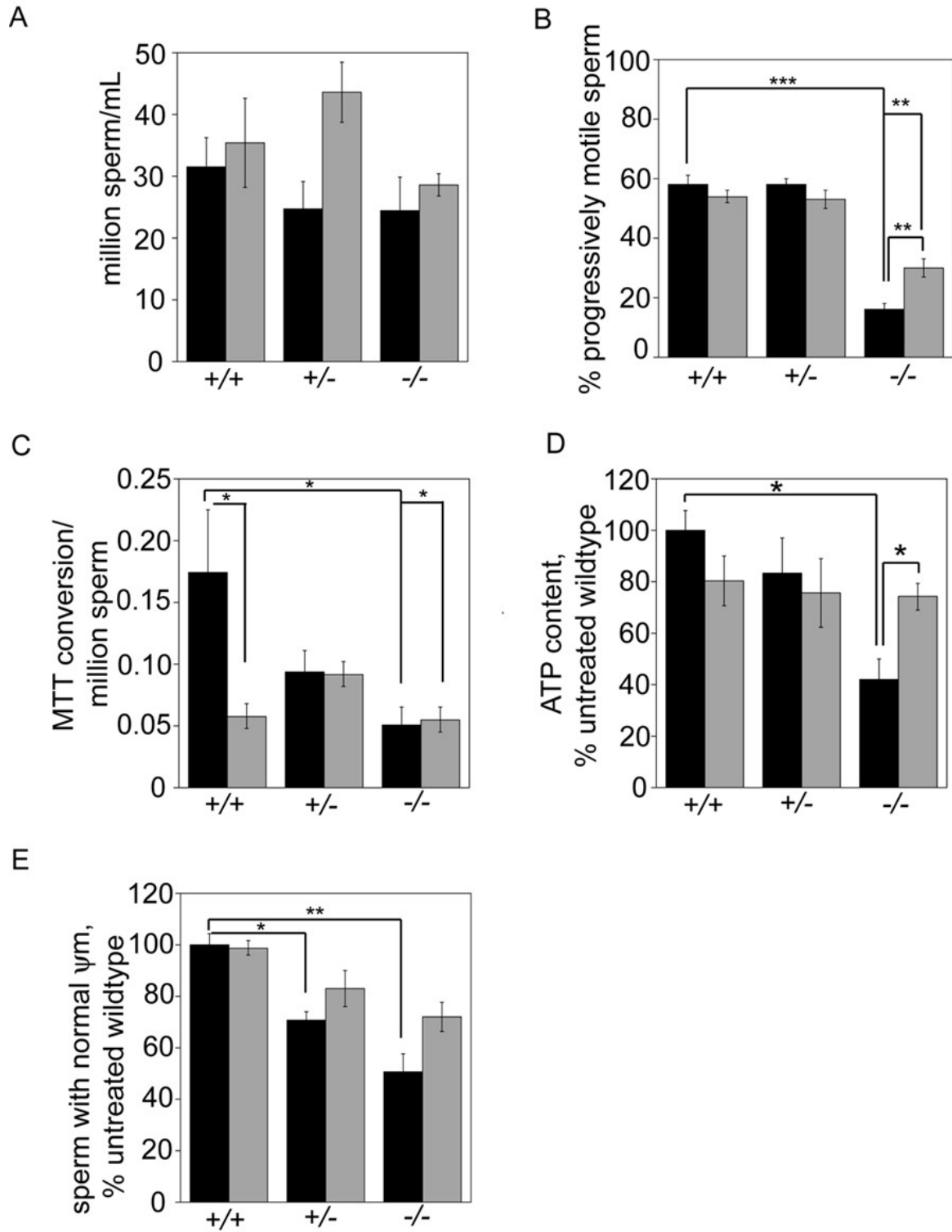
Figure 2.6: Functional assays of mitochondria isolated from liver, kidney, brain, skeletal muscle, heart and testis. *A*. ATP content. Mitochondria were purified from fresh tissue as described. Black bars are *Chdh*$^{+/+}$ and white bars are *Chdh*$^{-/-}$. Values were normalized to the amount of mitochondrial protein assayed. Results presented as are the mean % of *Chdh*$^{+/+}$ mean $\pm$ SEM, * different from *Chdh*$^{+/+}$, p<0.05 by Student's *t* test N= 5 animals/group. *B*. Membrane Potential. Mitochondria were purified from fresh tissues and JC-1 staining determined as described in methods. Values were normalized to the amount of mitochondrial protein assayed. Results presented as are mean $\pm$ SEM, *different from *Chdh*$^{+/+}$, p<0.05 by Student's *t* test N=5 mice per group. *C*. MTT reduction. Mitochondria were purified from fresh liver, kidney, brain, skeletal muscle, heart and testis tissues and MTT reduction determined as described in methods. Black bars are *Chdh*$^{+/+}$ and white bars are *Chdh*$^{-/-}$. Values were normalized to the amount of mitochondrial protein assayed. Results are presented as mean $\pm$ SEM, *p<0.05 different from *Chdh*$^{+/+}$ by Student's *t* test. N= 9-11 animals/group.

Figure 2.6: Functional assays of mitochondria isolated from liver, kidney, brain, skeletal muscle, heart and testis
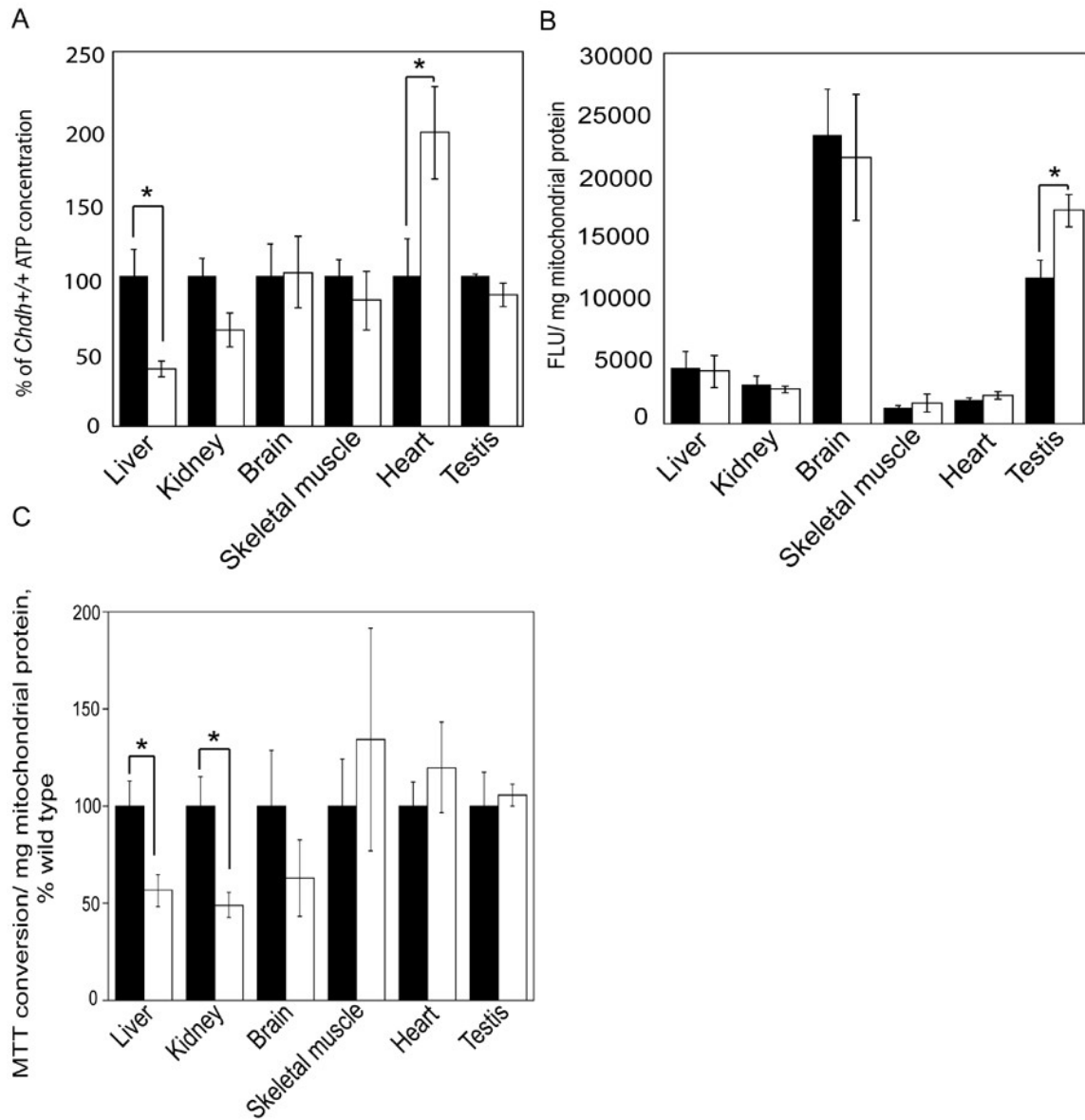
CHAPTER III

CHOLINE DEHYDROGENASE POLYMORPHISM RS12676 IS A FUNCTIONAL VARIATION AND IS ASSOCIATED WITH CHANGES IN HUMAN SPERM FUNCTION

Amy R. Johnson, Sai Lao, Tongwen Wang, Joseph A. Galanko
and Steven H. Zeisel

Abstract:

Approximately 15% of couples are affected by infertility and up to half of these cases arise from male factor infertility. Unidentified genetic aberrations such as chromosomal deletions, translocations and single nucleotide polymorphisms (SNPs) may be the underlying cause of many cases of idiopathic male infertility. Deletion of the choline dehydrogenase (*Chdh*) gene in mice results in decreased male fertility due to diminished sperm motility; sperm from *Chdh*$^{-/-}$ males have decreased ATP concentrations likely stemming from abnormal sperm mitochondrial morphology and function in these cells. Several single nucleotide polymorphisms (SNPs) have been identified in the human *CHDH* gene that may result in altered CHDH enzymatic activity. rs12676 (G233T), a non-synonymous SNP located in the *CHDH* coding region, is associated with increased susceptibility to dietary choline deficiency and risk of breast cancer. We now report evidence that this SNP is also associated with altered sperm motility patterns and dysmorphic mitochondrial structure in sperm. Sperm produced by men who are GT or TT for rs12676 have 40% and 73% lower ATP concentrations,

respectively, in their sperm. rs12676 is associated with decreased CHDH protein in human hepatocytes. A second SNP located in the coding region of an adjacent gene *IL17BR*, rs1025689, is linked to altered sperm motility characteristics.

Introduction:

An estimated 15% of couples are affected by infertility [1] and male factor infertility is the suspected cause in 30-50% of these couples. Although the exact cause of many of these cases are unknown, chromosomal deletions, translocations and SNPs are associated with infertility in as many as 30% of these men [18]. Identifying and understanding how genetic anomalies affect spermatogenesis and fertilization will improve the likelihood of overcoming male infertility. Conversely, effective male contraception may be developed based on data showing associations between genetic variation, nutrient metabolism and male factor infertility.

Although the relationship between overall nutritional status and reproduction is well documented [20, 21, 254], the relationship between micronutrient metabolism and reproduction is understudied. There is some evidence that aberrant micronutrient metabolism may play a causative role in male factor infertility. Dietary deficiencies of vitamins A, C and E as well as trace metals such as zinc and selenium are associated with male infertility in animals and humans [22-33, 309]. Choline is an essential nutrient for humans [255] and is important for normal fetal development [256]. A link between

choline metabolism and male fertility has been demonstrated in only one paper [35]. Geer reported both normal mating behavior and sperm motility required adequate choline availability in Drosophila *melanogaster* and that carnitine, a proposed choline substitute, was unable to support male fertility. We discovered that choline dehydrogenase (CHDH) is necessary for normal male fertility in mice [46]. Male choline dehydrogenase knockout mice (*Chdh*$^{-/-}$) are infertile due to severely compromised sperm motility; decreased motility is the result of abnormal mitochondrial structure and function in sperm cells.

Betaine (*N,N,N*-trimethylglycine), a metabolite of choline, donates methyl groups for the formation of methionine from homocysteine and is an organic osmolyte used by cells to regulate cell volume [257]. Dietary sources of betaine include wheat, shellfish, spinach and sugar beets [158, 207]. In addition, betaine can be made *de novo* via the oxidation of choline in a series of reactions catalyzed by CHDH and betaine aldehyde dehydrogenase [153-155, 181, 209, 210]. Conversion of choline to betaine takes place in the mitochondrial matrix following the transport of choline across mitochondrial membranes [136, 258, 259]. The betaine formed is a zwitterion at neutral pH and diffuses out of mitochondria for use in one-carbon metabolism [157].

SNPs have been identified in the human *CHDH* gene. Rs12676 (G233T) is a non-synonymous SNP located in exon 3 of the *CHDH* gene. Occurrence of the variant T allele results in the replacement of arginine, a polar, hydrophilic amino acid, with leucine, a hydrophobic amino acid. 38 - 40% of individuals are heterozygous and 2 – 9% are

homozygous for rs12676 [80, 105]. The *CHDH* minor T allele is associated with increased susceptibility to developing clinical symptoms of dietary choline deficiency (steatosis and muscle cell damage) [105] as well as increased risk of breast cancer [80]. Although not in the *CHDH* gene, a SNP in the interleukin 17 beta receptor (*IL17βR*), rs1025689, is associated with increased susceptibility to developing choline deficiency specifically in men (Zeisel Laboratory, unpublished data). We now present data indicating that the rs12676 genotype is also associated with dysmorphic mitochondrial structure, changes in sperm motility patterns and decreased energy status in human sperm. Hepatocytes harboring the TT rs12676 genotype have less CHDH protein when compared to hepatocytes from humans with GG ro GT genotypes, suggesting that this SNP is functional. In addition, rs1025689 is linked to changes in sperm motility patterns, suggesting that these SNPs may be contributing factors in the occurrence of idiopathic male infertility.

Materials and Methods:

Chemicals and Reagents: All chemicals and reagents used were obtained from Sigma Aldrich (St. Louis, MO), unless otherwise noted.

Study design and recruitment: This study was conducted at the University of North Carolina at Chapel Hill Nutrition Research Institute (Kannapolis, North Carolina) and was implemented in two phases – screening subjects for genotypes of interest and

analysis of semen and sperm specimens collected from subjects with SNPs of interest. All procedures were approved by the University of North Carolina at Chapel Hill Office of Human Research Ethics Institutional Review Board. Subjects were at least 18 years of age and were recruited from the Charlotte – Kannapolis, North Carolina region. Informed consent was obtained at the initial screening visit. Subjects were compensated up to $125 for their participation in the study.

Blood collection and DNA isolation: In order to screen subjects for the SNPs of interest, blood was collected into Vacutainer Cell Preparation Tubes (CPT tubes; BD Diagnostics, Franklin Lakes, NJ) containing sodium citrate; lymphocytes were separated from other blood components for subsequent genomic DNA extraction. All CPT tubes were stored on ice if not processed immediately; all CPT tubes were processed within 1 hour of sample collection. Briefly, CPT tubes were centrifuged in a Sorvall RC-3B centrifuge equipped with an H-2000B rotor (Thermo Fisher Scientific, Waltham, MA) at 1500 x g for 30 minutes at room temperature. Plasma was aliquoted into 2mL microfuge tubes and stored at -80˚ C for choline metabolite analysis. The lymphocyte layer was washed with PBS, transferred to a 15mL conical tube and was pelleted by centrifugation at 1000 x g for 5 minutes at room temperature. Pellets were again washed with PBS, transferred to 1.5mL microfuge tubes and pelleted by centrifugation in an Eppendorf 5415D microcentrifuge at 800 x g for 5 minutes at room temperature.

Genomic DNA was purified from lymphocyte pellets using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions with some

modification. Specifically, lymphocyte pellets were equilibrated to room temperature and resuspended in 500µL PBS. The amounts of Qiagen Protease, Buffer AL and ethanol (96%-100%) were adjusted proportionally as indicated by the manufacturer's instructions. Two separate elutions of 100µL with Qiagen Buffer AE were performed. Samples were incubated at room temperature for 5 minutes prior to each elution. Both elutions were collected in the same 1.5mL microfuge tube for a final volume of 200µL. DNA quality and concentration was determined using a Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

rs12676 genotyping: rs12676 genotype was determined by direct sequencing. A 260 base pair region of the *CHDH* gene containing rs12676 was PCR amplified using Deep Vent$_R$ ^{TM} (exo-) DNA polymerase (New England Biolabs, Ipswich, MA) according to manufacturer's recommendations. The primers used for amplification were: *CHDH forward* 5'- ATTCCCCTCCGTGGATCAG-3' and *CHDH reverse* 5'-TGTCGTCGCACAGGTTGG-3'. Each 50µL reaction contained 600ng of genomic DNA, primers at a final concentration of 200nM, and 4 units of Deep Vent$_R$ ^{TM} (exo-) DNA polymerase. The PCR conditions were: an initial denaturing step at 98˚C for 10 minutes followed by 30 cycles of denaturing at 98˚C for 1 minute and annealing/extension at 72˚C for 5 minutes. PCR products were purified from other reaction components using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and the resulting DNA concentration was determined using Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Purified *CHDH* fragments were sequenced using BigDye® Terminator chemistries (Applied Biosystems, Carlsbad, CA) by Eton Bioscience, Inc (Research Triangle Park, NC) using the *CHDH forward* primer. rs12676 genotype was

determined by examining sequencing chromatograms using Sequence Scanner software (version 1.0, Applied Biosystems, Carlsbad, CA).

rs1025689 genotyping: rs1025689 genotype was determined using a TaqMan® SNP genotyping assay (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions. PCR reactions were performed using a StepOne™ Real Time PCR System and 2X TaqMan® Genotyping Master Mix (Applied Biosystems, Carlsbad, CA).

Semen collection and processing: Subjects were asked to refrain from sexual activity for 48 hours prior to semen donation. Semen was produced by masturbation and collected into 50mL sterile sample cups. Olive oil was provided as a lubricant to use as necessary. Samples were incubated at room temperature for 30 minutes to allow for liquefaction. Semen volume was measured with a pipette. Sperm were separated from other seminal fluid components by layering the sample over a 45% ISolate®/ human tubular fluid (HTF; 190mM NaCl, 9mM KCl, 0.7mM KH_2PO_4, 0.3mM $MgSO_4$-7$H_2$0, 4mM $CaCl_2$ – 2$H_2$0, 0.025mM $NaHCO_3$, 2.78mM D-glucose, 21.4mM lactate, 0.33mM pyruvate and 5mg/mL BSA (Fraction V), 5M NaCl was added as necessary to adjust osmolality) media gradient followed by centrifugation at 300 x g for 20 minutes at room temperature using a Beckman-Coulter Allegra X-15R Centrifuge and SX4750A rotor. ISolate® was purchased from Irvine Scientific (Santa Ana, CA). The supernatant was discarded and the resulting sperm pellet was washed twice in 3mL HTF followed by centrifugation at 300 x g for 10

minutes at room temperature. Sperm were resuspended in 4 mL HTF and used for subsequent analyses.

Sperm counts: The total number of sperm per ejaculate and sperm concentration were determined by counting cells in a hemocytometer.

Sperm motility: Sperm were diluted 1:10 – 1:15 in HTF for motility measurements. 200µL of diluted sperm were placed into a 35mm glass bottom dish and covered with a coverslip. For each sample, video of 10 random, unique microscope frames were recorded using a Zeiss Axio Observer (Carl Zeiss, Inc, Thornwood, NY) inverted microscope equipped with a temperature controlled incubation chamber equilibrated to 37˚C. Sperm were viewed under phase contrast conditions with a 20X objective lens.

Motility parameters including mean velocity (MVUS), curvilinear velocity (VCL), straight distance velocity (VSL) and mean tortuosity (MT)) were determined using Zeiss AxioVision (release 4.7) image tracking software (Carl Zeiss, Inc, Thornwood, NY) as previously described [260].

Electron microscopy: Approximately 500µL of washed sperm were transferred to 1.5mL microfuge tubes; sperm were pelleted by centrifugation at 16,000 x g for 5 minutes at room temperature. The supernatant was discarded and sperm pellets were fixed for transmission electron microscopy in 2% paraformaldehyde, 2.5% gluteraldehyde, 0.2% picric acid in 0.1M sodium cacodylate, pH 7.2. The pellet was encapsulated in 2% agarose buffered with

0.1M sodium cacodylate buffer, pH 7.2. The encapsulated pellet was post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour. Samples were washed in deionized water, dehydrated through an ethanol series, transferred to propylene oxide, infiltrated and embedded in Polybed 812 resin (Polysciences, Inc., Warrington, PA). 70nm ultrathin sections were post-stained in 4% aqueous uranyl acetate and 0.4% lead citrate. Samples were examined and photographed using a Zeiss EM-10A transmission electron microscope (LEO Electron Microscopy, Thornwood, NY) with an accelerating voltage of 60kV.

ATP assay: ATP concentration in sperm was measured using an ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Luminescence was measured using a 1420 VICTOR^2 microplate reader (Perkin Elmer, Waltham, MA). ATP concentration was normalized to number of sperm analyzed.

CHDH expression: Primary human hepatocyte protein lysates were made in radioimmunoprecpitation assay (RIPA) buffer and protein concentration measured using BCA assay (Pierce/ Thermo Scientific, Rockford, IL). Equal volumes of lysate (80 – 200μg protein) were resolved by SDS-PAGE and transferred to PVDF as described above. The membrane was incubated with anti-CHDH antibody (1:1000 dilution in 5% BSA/ PBS-T; ProteinTech Group, Inc., Chicago, IL) at 4°C overnight. A 1:10,000 dilution of HRP- conjugated anti-rabbit secondary antibody (Millipore, Billerica, MA) was incubated with the membrane and bands detected with enhanced chemiluminescence (SuperSignal® West Pico ECL, Thermo Scientific, Rockford, IL). The number of pixels in the CHDH

band was quantitated using the lasso tool in Adobe Photoshop (Adobe Photoshop CS3 Extended v.10.0.1) and the number of pixels per microgram of total protein loaded was calculated. As an external control for protein integrity, the abundance of beta-actin protein was determined using a mouse monoclonal anti-beta-actin antibody (1:10,000 dilution in 5% BSA/PBS-T; Abcam, Cambridge, MA) and an HRP-conjugated goat anti-mouse secondary antibody (1:10,000 dilution in 5% BSA/PBS-T; Abcam, Cambridge, MA)

Plasma choline metabolite analyses: The concentrations of choline and its metabolites [choline, betaine, phosphatidylcholine (PtdCho), and sphingomyelin (SM)] in plasma were measured by liquid-chromatography ionization-isotope dilution mass spectrometry (LC-ESI- IDMS) as previously described [226]

Statistical analyses: Statistical differences among genotypes were determined using JMP 9.0 software (SAS Institute, Cary, NC) using ANOVA and Tukey-Kramer HSD. Statistical tests were performed on log 10 transformed data for semen volume, total sperm per ejaculate and sperm concentration as these data failed test of normality. Only data from sperm recorded for at least 3 seconds were included in statistical analyses. In order to address the intra-individual variation in the sperm motility data the following method was used to determine statistical differences. For each continuous measure (MT, MVUS, VCL and VSL) cutpoints for quartiles were determined from all observations having the wild type/wild type (WW) genotype for that SNP. Then using those cutpoints, all observations were placed into a quartile and an association between SNP level and the most extreme quartiles (1 and 4) was assessed via a repeated measures logistic regression with quartile (1

or 4) as the response and SNP as the predictor. The repeated measures on subject were taken into account by using a compound symmetric correlation matrix for observations within the same subject. P-values less than 0.05 were considered statistically significant.

Results:

rs12676 and rs1025689 distribution frequencies: rs12676 and rs1025689 distribution frequencies were calculated for the population of men screened for inclusion in this study. For rs12676, 52% of subjects were GG, 41% were GT and 7% were TT (Table 3.1). For rs102689, 22% were GG, 48% were GC and 30% were CC. These results are in agreement with published data regarding these two SNPs [80, 105] and K. da Costa, unpublished data). It is important to note that 100% of men who were homozygous for rs12676 had at least one minor C allele of rs1025689; 83% of men with the TT rs12676 genotype were also CC for rs1025689. In addition, 91% of men who were GT for rs12676 had at least 1 C allele for rs1025689.

Study population: Average age, average number of biological children per subject and occurrence of abnormal semen characteristic or infertility were calculated from self-reported information provided by the health questionnaire form completed on Day 1 (Figure 3.1). The average age of the entire screen population was 33.5 years and subject age ranged from 18 to76 years. The average number of biological children per subject was calculated by dividing the total number of children per genotype by the number of subjects who answered this question. Men who were TT for rs12676 reported the lowest number of biological

children per subject (0.33) while men who were wild type for rs1025689 reported the highest (0.94). The average number of biological children for the other genotypes was: rs12676 GG, 0.80; GT, 0.78; rs1025689 GC 0.70; CC, 0.61. 33% (2 of 6) of men with rs12676 TT genotype reported semen abnormalities or infertility; these were reported by 9.7% (4 of 41) of men who were wild type for rs12676. Men who were GT for this SNP did not report any abnormalities. For rs1025689, 11.7% (2 of 17) of GG, 5.2% (2 of 38) of GC and 8.3% (2 of 24) of CC subjects reported semen abnormalities or infertility.

Semen parameters: The rs12676 genotype was not associated with changes in semen volume, number of sperm per ejaculate or sperm concentration (Table 3.2). Men who were homozygous for rs1025689 had decreased sperm concentration compared to men who were heterozygous for this SNP. Mean values for these parameters were all within the normal range expected of the general human population [261].

Sperm motility characteristics: Sperm from men who were homozygous for rs12676 had increased curvilinear velocity and tortuosity when compared to sperm from men who were wild type for this SNP (Figure 3.2, Table 3.3). Sperm produced by men with the GT genotype for rs12676 traveled greater distances at a faster rate and had more tortuous paths than sperm collected from men who were GG for this SNP. No differences between sperm from heterozygous and homozygous subjects were detected.

Sperm from men who were CC for rs1025689 had increased average velocity as well as curvilinear and straight line velocity when compared to sperm collected from

men who were wild type for this SNP (Figure 3.3, Table 3.3). Men who were homozygous for rs1025689 produced sperm that traveled in more tortuous paths as compared to sperm from men who were heterozygous for this SNP. Subjects who were GC for rs1205689 produced sperm with higher measures of average velocity, curvilinear and straight line velocity compared to sperm from men with the GG genotype. Tortuosity was decreased in sperm from heterozygous men compared to sperm from men who were CC for rs1025689.

Sperm mitochondrial ultrastructure and energy levels: Abnormal mitochondrial structure was observed in sperm collected from men who had two copies of the rs12676 minor allele (Figure 3.4). Mitochondria in the midpiece of these sperm appeared swollen with disordered cristae structure. Sperm from men with one variant allele of rs12676 (GT) had 40% less ATP than sperm produced by men who were GG for this SNP (Figure 3.5). Men carrying two variant alleles (TT) produced sperm with 73% less ATP than men who were GG. 83% of subjects who were homozygous for rs12676 were also homozygous for rs1025689, but when mitochondrial morphology and ATP concentrations were analyzed in men who were homozygous for rs1025689 only, we found there was no relationship between rs1025689 genotype and these measures (Figure 3.6).

CHDH protein expression: Individuals who harbored the TT rs12676 genotype had a lower CHDH:β-ACTIN due to decreased amounts of CHDH protein in their hepatocytes compared to individuals who were wild type for this SNP (Figure 3.7). The CHDH proteins levels in heterozygous hepatocytes were not significantly changed from either GG or TT

hepatocytes. β-ACTIN protein expression was not changed among the genotypic groups (data not shown).

Plasma choline metabolite concentrations: Choline metabolite concentrations in plasma and sperm were measured using LC-ESI-IDMS. Men who were homozygous for rs12676 minor allele had a small, but significant increase in plasma free choline concentrations (Table 3.5). There were no differences in plasma betaine, PtdCho or SM among the other rs12676 or rs1025689 genotypes.

Discussion:

We previously reported that *Chdh* mutation in mice results in male infertility due to compromised sperm motility. We now present evidence that rs12676, a common SNP in the human *CHDH* gene, is associated with altered sperm motility patterns, dysmorphic mitochondrial ultrastructure and decreased ATP concentrations in sperm. Humans who were homozygous for this SNP had less CHDH protein in their liver than those who were wild type or heterozygous. Further studies are required to determine whether this is a result of decreased *CHDH* mRNA translation or increased CHDH protein degradation. Either way, these data suggest that rs12676 is a functional SNP, or that it is a tag SNP that marks a functional haplotype of the *CHDH* gene. In addition, rs1025689, a SNP located in the *IL17βR* gene that is highly associated with increased susceptibility to dietary choline deficiency particularly in men, is correlated with changes in sperm motility

patterns.

The allele frequency distributions of the screened population for both rs12676 and rs1025689 were in agreement with previously reported frequencies ([80, 105] and Zeisel laboratory unpublished data). The average age of the genotypic groups was not significantly different; therefore, we conclude that any changes detected in semen characteristics or sperm cell function were not due to differences in age among the groups. The rs12676 TT genotype group reported the lowest number of biological children per subject (0.33) and the highest rate of semen abnormality/infertility diagnosis (33%) of all groups. Unfortunately, the health questionnaire items were not worded in such a way that we would be able to accurately and specifically calculate decreased fertility. Interestingly, however, subjects who were homozygous for rs12676 had higher free choline concentrations in their plasma. This would be expected with a decrease in CHDH activity, as less choline would be converted to betaine. We did not detect a decrease in betaine in these men probably because we did not require the subjects to fast prior to their blood draw, and betaine can be obtained from the diet.

Sperm from men who harbor at least one variant allele of rs12676 were less progressively motile, as indicated by an increase in curvilinear velocity and tortuosity (Figure 3.2, Table 3.3). However, we do not know whether these data indicate that these sperm were less progressively motile from the time of ejaculation, or if they achieve hyperactivation earlier than sperm from men who are wild type for this SNP, as hyperactivated motility could also be described by similar changes. Mean velocity was

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significantly increased in sperm from heterozygous and homozygous subjects. The association between rs12676 genotype and mean velocity was stronger in sperm from men of the GT genotype; the association was weak in sperm from men who were TT for rs12676. It is possible that more sperm from rs12676 heterozygous men were hyperactivated at the time of measurement based on the vigorous, non-linear path in which these cells are moving. Hyperactivated sperm are those displaying motility patterns characterized by vigorous, non-linear trajectories [262] and are in contrast to progressively motile sperm which move forward in a somewhat linear path [263]. Sperm released from the cauda epididymis typically are progressively motile at first and their transition to hyperactivated motility when incubated in media formulated to support this process [262]. Sperm collected from the female reproductive tract are generally hyperactivated [262]. Similar changes were observed in sperm from men with at least one minor C allele of rs1025689. On the other hand, sperm from men who were TT for rs12676 may be less progressively motile from the time of ejaculation as suggested by the increased curvature of their path and relative lack of change in speed from wild type sperm.

rs12676, but not rs1025689, was associated with dysmorphic mitochondrial structure (Figure 3.4 and Figure 3.6). ATP concentration was inversely correlated in a dose-dependent manner with the number of rs12676 minor alleles (Figure 3.5) Although the exact mechanism causing these changes remains unknown, these results are very similar to those we observed in the *Chdh*$^{-/-}$ mice [46].

As noted before, humans who are homozygous for the minor T allele of rs12676 are more likely to develop clinical symptoms of dietary choline deficiency (e.g. liver or muscle

dysfunction) when fed a choline-deficient diet [105]. Since we have demonstrated this genotype is associated with decreased CHDH protein, we hypothesize that aspects of choline deficiency are a direct result of a lack of betaine; perhaps stemming from a reduction of S- adenosylmethionine (AdoMet) available for methylation reactions.   This change may be reflected in sperm membrane phospholipid composition. AdoMet is required for the *de novo* generation of phosphatidylcholine (PtdCho) from phosphatidylethanolamine (PE) in a reaction catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT).   Sperm contain high levels of PtdCho in which polyunsaturated fatty acids, such as docosahexanoic (DHA, 22:6n3) or arachidonic acid (AA, 24:4n6), represent the majority of the fatty acid species incorporated into this molecule [264, 265]. This type of PtdCho is generated primarily via the PEMT pathway [266-269] and Pemt expression has been reported in spermatogenic cells, Sertoli cells and epididymal tissue [137]. *Chdh*^{-/-} sperm have 56% less PtdCho than sperm produced by *Chdh*^{+/+} males (ARJ, unpublished data) and changes in sperm membrane composition can impair sperm motility [264, 270]. Although this is an attractive explanation it is unlikely to be the only cause of impaired sperm function as there are no reports of male infertility in *Pemt*^{-/-} mice. Additional evidence arguing against lack of methyl groups from betaine as the root cause of poor sperm function include the fact that betaine:homocysteine methyltransferase knockout (*Bhmt*^{-/-}) males are fertile [271]. These animals are unable to use methyl groups from betaine to remethylate homocysteine, thus forming methionine that can be converted to AdoMet [272]. The *Bhmt*^{-/-} males have very high concentrations of betaine in all tissues except testis, suggesting the presence of a mechanism for controlling betaine accumulation in this tissue and implying that

betaine concentration is possibly controlled to protect testicular function. At the same time, dietary folate may provide labile methyl groups to compensate for a lack of betaine and serve as an alternate means for providing methyl groups (reviewed in [273]).

It is possible that the betaine molecule itself plays an important role in maintaining testicular and sperm cell function. Betaine is an organic osmolyte used by cells for protection during times of osmotic stress [161, 162]. Sperm mature as they move from the lumen of the testis and through the caput, corpus and cauda regions of the epididymis [187]. During transit, sperm accumulate molecules found within the epididymal environment including organic osmolytes such as glycerophosphocholine and carnitine [187]. We measured betaine concentrations in the mouse epididymis and found levels 10 times greater than those measured in liver (ARJ, unpublished data). The epididymal environment is relatively hyperosmotic (~340mmol/kg, [183]). In comparison, the osmolality of unliquified whole semen and of the fluid in the female reproductive tract is approximately 276 – 302mmol/kg [274, 275] suggesting that epididymal sperm experience an osmotic "challenge" within the male urethra [183]. An inability to regulate volume in response to the varied osmotic environments would render sperm susceptible to swelling which can impair motility [275]. In addition increased osmotic stress due to a reduction of betaine can also account for decreased PtdCho concentrations observed in $Chdh^{-/-}$ sperm. Phospholipase A_2 (PLA$_2$), which is highly expressed in sperm [276], catalyzes the hydrolysis of the fatty acid in the sn-2 position of PtdCho, resulting in the release of the fatty acid and the generation of lyso-PtdCho [264]. PLA$_2$ activity is enhanced by osmotic stress [277, 278]. Increased osmotic stress may increase hydrolysis of sperm

PtdCho, resulting in the release of free DHA and AA and increasing concentrations of lyso-PtdCho. All of these molecules have been shown to inhibit sperm motility [264].

rs1025689 is a synonomous SNP located in the coding region of human *IL17βR* [279]. Individuals, particularly men, who were homozygous for the minor C allele, were more likely to develop signs of liver or muscle dysfunction when ingesting a choline- deficient diet (Zeisel laboratory, unpublished data). Because the presence of this SNP does not result in an amino acid change, it most likely tags a functional haplotype within this gene. *CHDH* and *IL1βBR* are situated in a head-to-head orientation on opposite strands on human chromosome 3 and mouse chromosome 14 [280, 281]. According to available data, rs12676 and rs1025689 are not in linkage disequilibrium; however, we noted a high degree of concurrence of these SNPs within our study population. Because they share a promoter region, it is likely that transcriptional regulation of *CHDH* and *IL17βR* are similar. For example, transcription of these genes is enhanced by estrogen; an estrogen response element is located within the shared promoter region [280]. Aberrant expression of *CHDH* and *IL17βR* has been associated with breast cancer survival prognosis [280, 281]. Ours is the first report linking the function of this chromosomal region to male sperm cell function.

Our data suggest that rs12676 is the primary predictor of abnormal sperm mitochondrial morphology and ATP concentration, and this hypothesis is strengthened by the finding that CHDH protein abundance is decreased in hepatocytes from individuals who were homozygous for this variant. No changes in mitochondrial ultrastructure or ATP

level were detected in sperm from individuals who were CC for rs1205689, but not TT for rs12676 (Figure 3.6). Together, this evidence indicates that altered CHDH activity due to rs12676 genotype may be an underlying cause of iodiopathic male factor infertility in men. This is an especially interesting finding because deficits in CHDH function could potentially be overcome by dietary supplementation with betaine. Indeed, sperm motility and ATP concentration were improved in $Chdh^{-/-}$ male mice that ingested betaine-supplemented drinking water [46].

Figure 3.1: Study design. On Day 1, all subjects gave informed consent, completed a health questionnaire form and blood samples were collected. Plasma was separated from other blood components and reserved for measures of choline metabolites. Genomic DNA was isolated from purified lymphocytes and used to genotype subjects for rs12676 and rs1025689 SNPs. Individuals with the genotypes of interest were invited to complete Day 2 of the study which entailed leaving a semen sample for measures of semen characteristics, sperm motility, ATP concentration and mitochondrial morphology.

Figure 3.1: Study design.
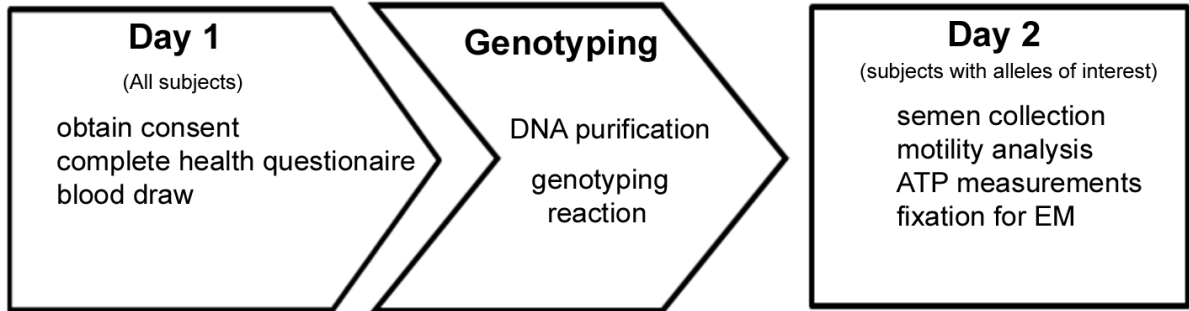
Table 3.1: rs12676 and rs1025689 SNP distribution frequencies in the screened population. Allele distribution frequency was calculated as a percentage of the total screened population following genotyping by direct sequencing (rs12676) or TaqMan assay (rs1025689). N = 79.

Table 3.1: rs12676 and rs1025689 SNP distribution frequencies in the screened population.

rs12676 $G{\rightarrow}T$	
GG	41 (52%)
GT	32 (41%)
TT	6 (7%)
rs1025689 $G{\rightarrow}C$	
GG	17 (22%)
GC	38 (48%)
CC	24 (30%)

Table 3.2: Semen parameters by rs12676 and rs1025689 genotype. Semen volume, total number of sperm per ejaculate and sperm concentration was determined by measuring the volume of the ejaculate before washing and counting the number of sperm using a hemocytometer. For rs12676 $N = 19$ (GG), 22 (GT) or 5 (TT). For rs1025689 $N = 12$ (GG), 22 (GT), 11 (CC). Values are mean ± SEM. Statistical differences among groups were tested on log 10 transformed data using ANOVA and Tukey-Kramer HSD. * indicates P <0.05.

[†]World Health Organization reference values for human semen characteristics, 2010.

Table 3.2: Semen parameters by rs12676 and rs1025689 genotype

Parameter	Normal Range[†]	rs12676			rs1025689		
		GG	GT	TT	GG	GC	CC
Semen volume (mL)	0.8 – 7.0	1.9 ± 0.3	2.5 ± 0.4	2.4 ± 0.8	1.6 ± 2.2	2.8 ± 0.4	1.9 ± 0.3
Total sperm per ejaculate (million sperm)	11 – 772	21.9 ± 6.9	48.9 ± 16.7	30.6 ± 24.5	24.9 ± 8.8	50.7 ± 15.9	16.4 ± 11.3
Sperm concentration (million sperm per mL)	4 – 237	9.7 ± 2.6	22.0 ± 6.6	11.2 ± 8.0	14.4 ± 4.0	20.9 ± 6.3	6.7 ± 3.7*

Figure 3.2: Motility characteristics of sperm change with rs12676 genotype. Mean velocity (MVUS, *A*), curvilinear velocity (VCL, *B*), straight line velocity (VSL, *C*) and mean tortuosity (MT, *D*) were determined for sperm. Sperm were prepared and motility characteristics measured as described in the Methods section. N = 2756 (GG), 2553 (GT), 827 (TT) sperm. Statistical analyses were performed as described in the Methods section; refer to Table 3.3 for p-values. X-axis values indicate the upper limit of each quartile; the line at 25% represents values for sperm from GG subjects.

Figure 3.2: Motility characteristics of sperm change with rs12676 genotype
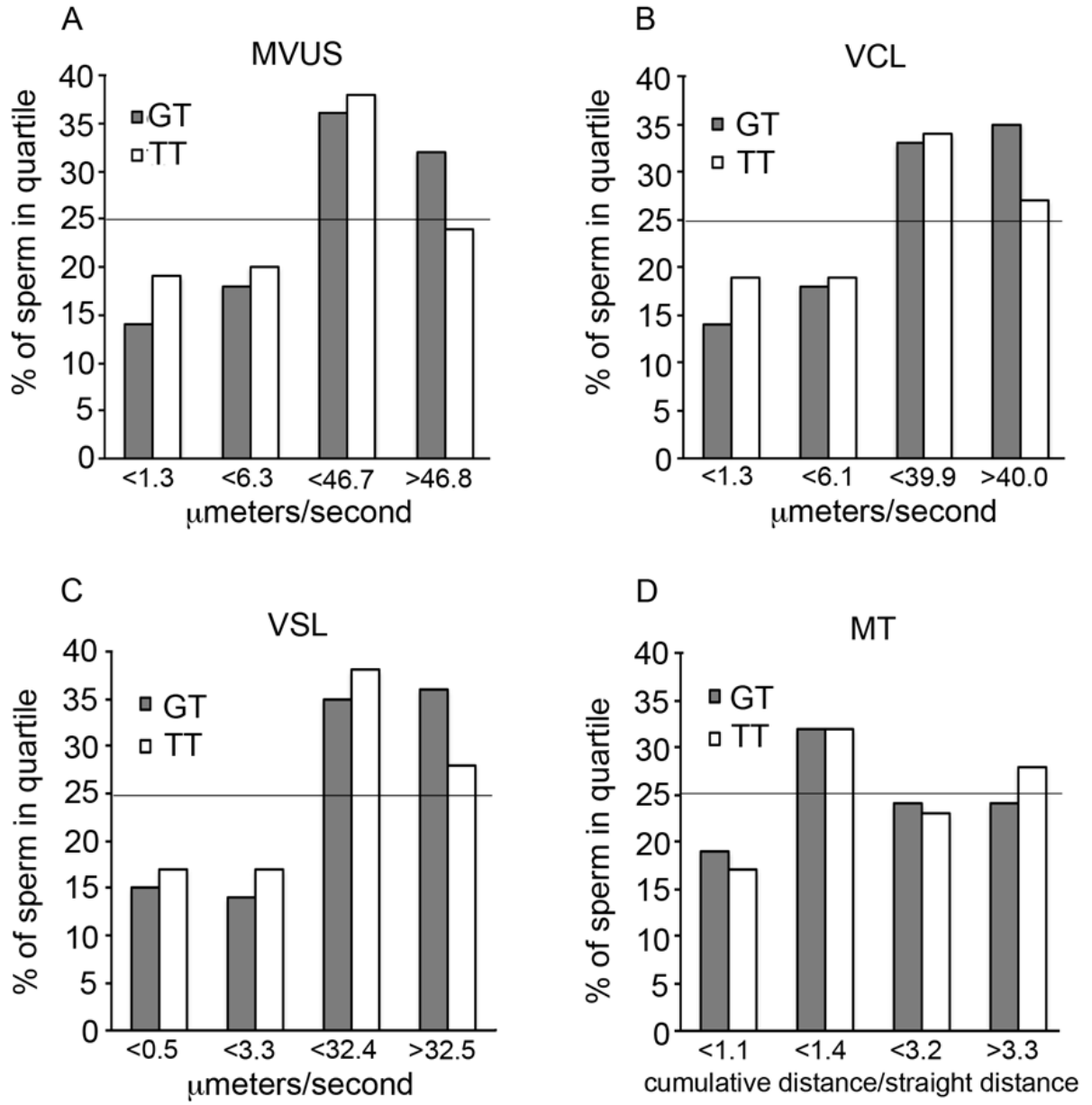
Figure 3.3: Motility characteristics change with rs1025689 genotype. Mean velocity (MVUS, *A*), curvilinear velocity (VCL, *B*), straight-line velocity (VSL, *C*) and mean tortuosity (MT, *D*) were determined for sperm. Sperm were prepared and motility characteristics measured as described in the Methods section. N = 1708 (GG), 2709 (GC), 1719 (CC) sperm. Statistical analyses were performed as described in the Methods section; refer to Table 3.3 for p-values. X-axis values indicate the upper limit of each quartile except for last label which indicates the lower limit of the fourth quartile; the line at 25% represents values for sperm from GG subjects.

Figure 3.3: Motility characteristics change with rs1025689 genotype

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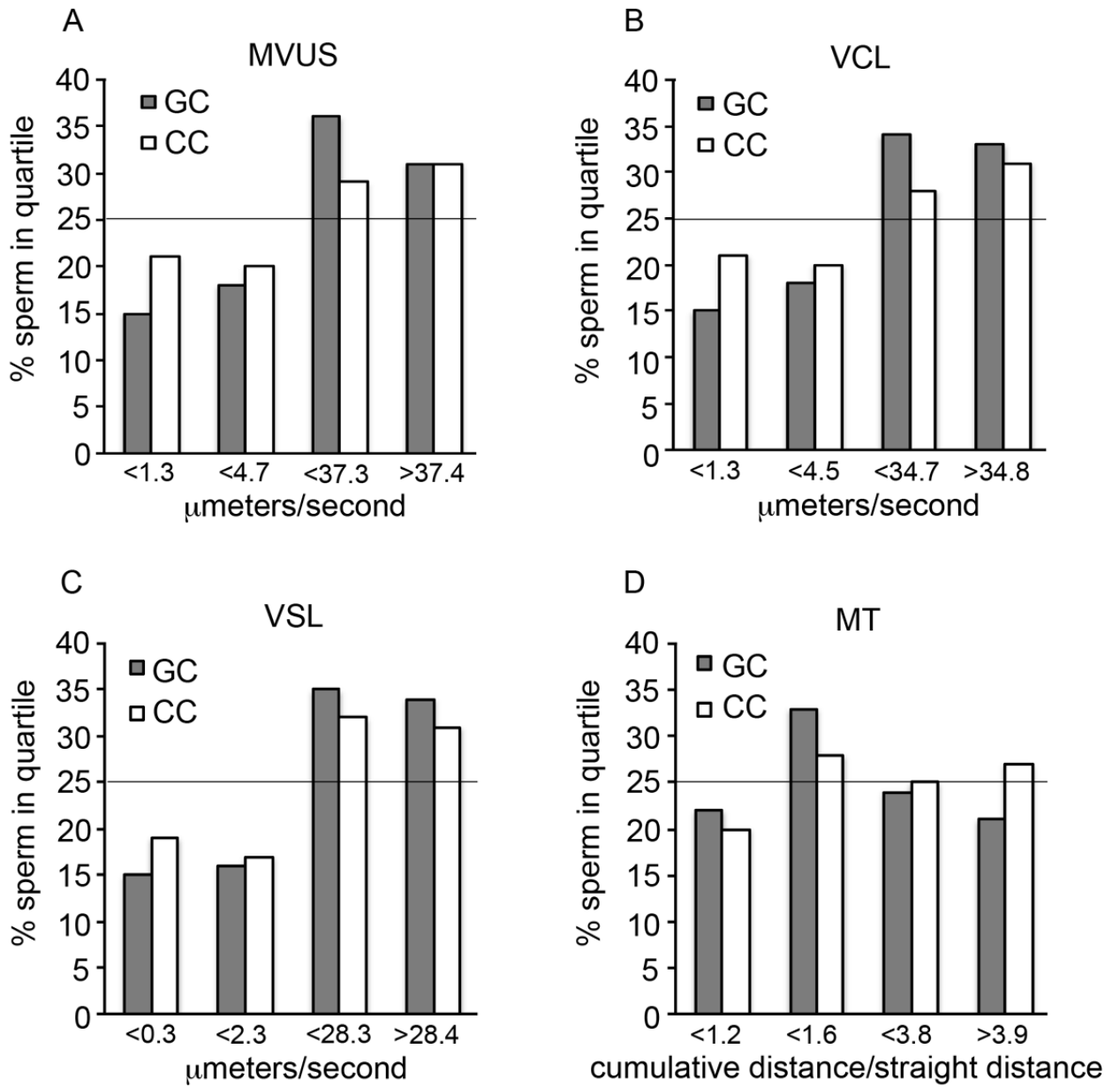
Table 3.3: Significance values for sperm motility analyses. Statistical analyses were conducted as described in the Methods section. N.S, not significant.

Table 3.3: Significance values for sperm motility analyses

SNP	Motility measure	Overall p-value	Specific comparison		
rs12676			**GG vs. GT**	**GG vs. TT**	**GT vs. TT**
	MVUS	0.003	0.001 (GT > GG)	0.047 (TT > GG)	N.S.
	VCL	0.002	0.0006 (GT > GG)	0.02 (TT > GG)	N.S.
	VSL	N.S.	N.S.	N.S.	N.S.
	MT	<0.0001	0.0001 (GT > GG)	0.0009 (TT > GG)	N.S.
rs1025689			**GG vs. GC**	**GG vs. CC**	**GC vs. CC**
	MVUS	<0.0001	<0.0001 (GC > GG)	<0.0001 (CC > GG)	N.S.
	VCL	<0.0001	<0.0001 (GC > GG)	<0.0001 (CC > GG)	N.S
	VSL	<0.0001	<0.0001 (GC > GG)	0.005 (CC > GG)	N.S
	MT	0.02	N.S.	0.69	0.01 (CC > GC)

Table 3.4: Range of values for motility measures. Mean velocity, micrometers per second (MVU), curvilinear velocity, micrometers per second (VCL), straight line velocity, micrometers per second (VSL), mean tortuosity, VCL/VSL (MT).

Table 3.4: Range of values for motility measures

SNP	Motility measure	GG	GT	TT
rs12676		**GG**	**GT**	**TT**
	MVUS	0 - 237	0 - 212	0 - 169
	VCL	0 – 236	0 - 117	0 - 157
	VSL	0 - 181	0 - 116	0 - 155
	MT	0 – 746	0 – 1082	0 - 759
rs1025689		**GG**	**GC**	**CC**
	MVUS	0 – 95	0 – 212	0 - 237
	VCL	0 – 96	0 – 117	0 - 236
	VSL	0 - 91	0 - 116	0 - 181
	MT	0 - 746	0 - 1082	0 -1759

Figure 3.4: rs12676 TT genotype is associated with dysmorphic mitochondrial structure in sperm. Sperm were fixed and processed for transmission electron microscopy as described in the Methods section. Longitudinal and cross-sectional sections of sperm were examined for mitochondria structure anomalies. Representative images for rs12676 genotypes (GG, panel *A* and *D*; GT, panel *B* and *E*; TT, panel *C* and *F*) are shown. N = 5 per genotype. Micrographs shown are at 80,000X magnification and arrows indicate mitochondria.

Figure 3.4: rs12676 genotype TT was associated with dysmorphic mitochondrial structure in sperm.
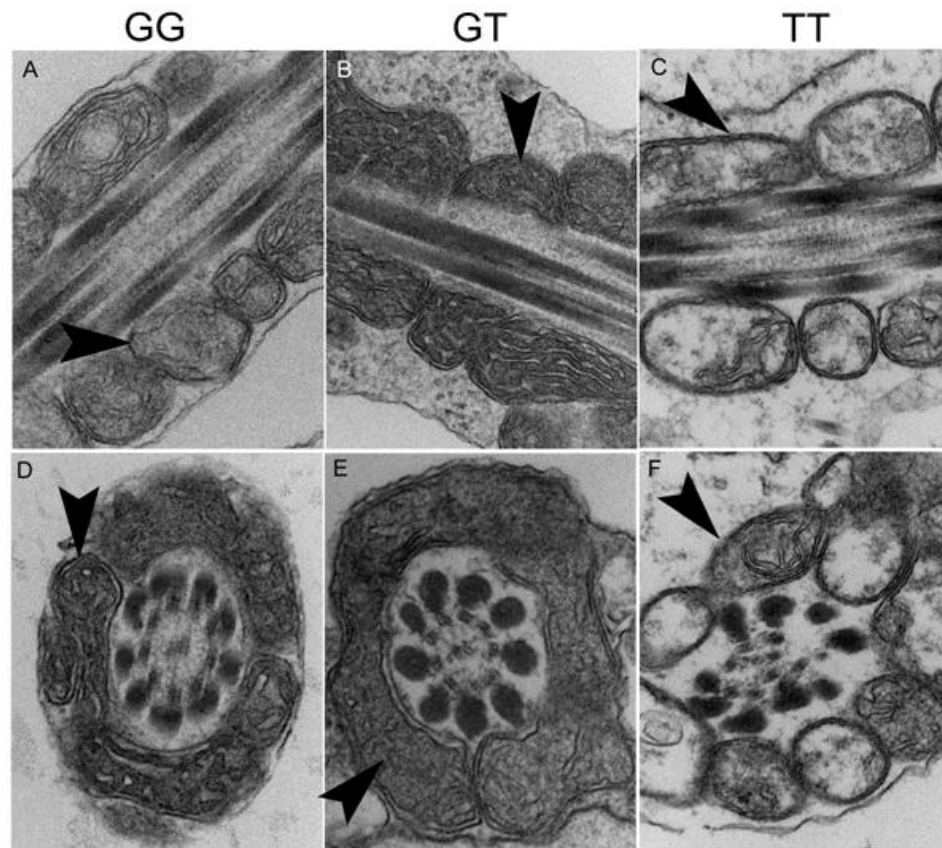
Figure 3.5: The rs12676 minor T allele is associated with decreased ATP concentrations in sperm. ATP concentrations were determined in sperm using a commercially available bioluminescent assay kit and ATP concentration was normalized to number of sperm assayed. *A*, Men who were heterozygous or homozygous for the rs12676 variant T allele have reduced ATP concentrations in their sperm. N = 17 (GG), 18 (GT) and 5 (TT). * indicates difference from GG by ANOVA and Tukey-Kramer HSD, p-value<0.05. *B*, ATP concentrations are not different with rs1025689 genotype. N = 10 (GG), 21 (GC) and 9 (CC). Data presented are mean ± SEM.

Figure 3.5: The rs12676 minor T allele is associated with decreased ATP concentrations in sperm.
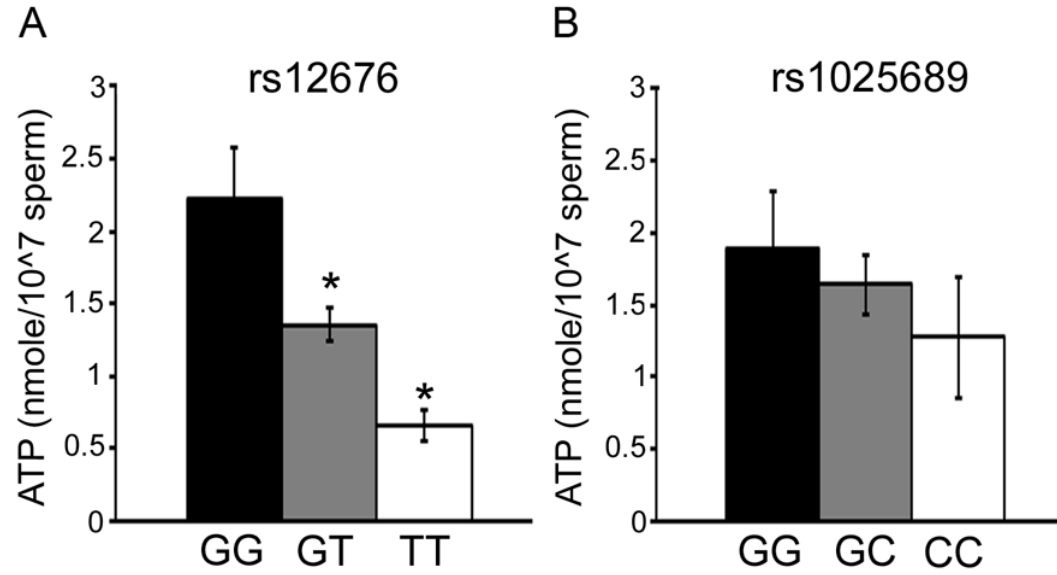
Figure 3.6: Sperm mitochondrial morphology and ATP concentrations are not changed in samples from men who are homozygous for rs1025689, but not rs12676. *A.* Representative longitudinal section of sperm midpiece. *B.* Representative cross-sectional section of sperm midpiece. Arrows indicate mitochondria. *C.* ATP concentrations as measured previously; "CC" group only contains data from men who are CC for rs1025689 and not TT for rs12676. Data presented are mean ± SEM. N = 9 (GG), 22 (GC) and 5 (CC).

Figure 3.6: Sperm mitochondrial morphology and ATP concentrations are not changed in samples from men who are homozygous for rs1025689, but not rs12676.
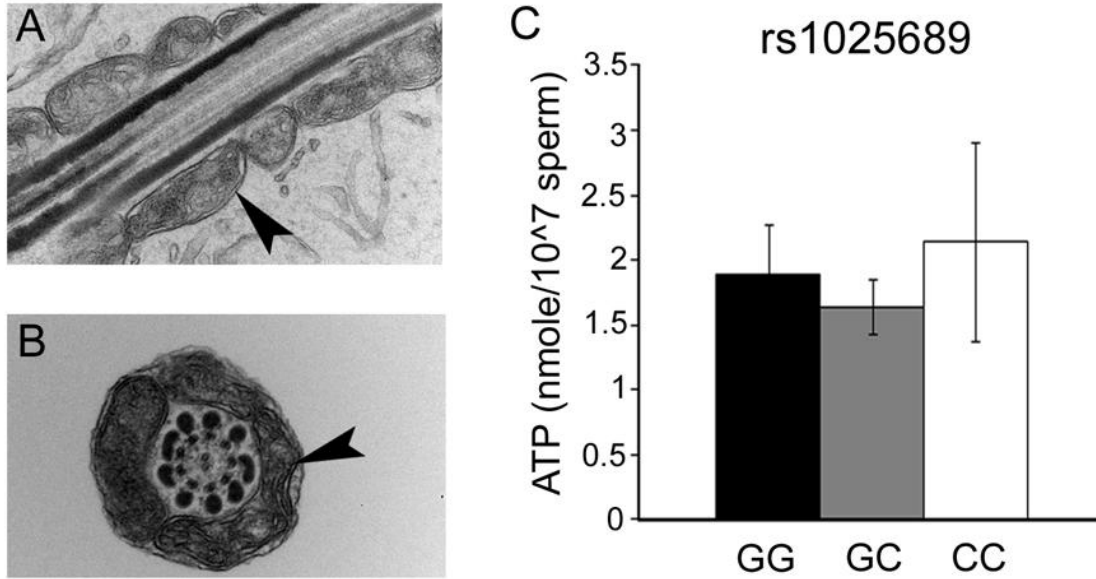
Figure 3.7: Expression of CHDH protein is decreased in primary hepatocytes from humans who are homozygous for the rs12676 SNP. The abundance of CHDH protein in hepatocyte lysates was measured by blot and expressed as the number of pixels per μg of protein. Blots were probed for β-ACTIN and data presented are the mean ± SEM of the ratio of CHDH:β-ACTIN protein. Statistical differences were tested by ANOVA and Student's t test. * indicates different from GG, p-value < 0.05. N = 3 per genotype.

Figure 3.7: Expression of CHDH protein is decreased in primary hepatocytes from humans who are homozygous for the rs12676 SNP
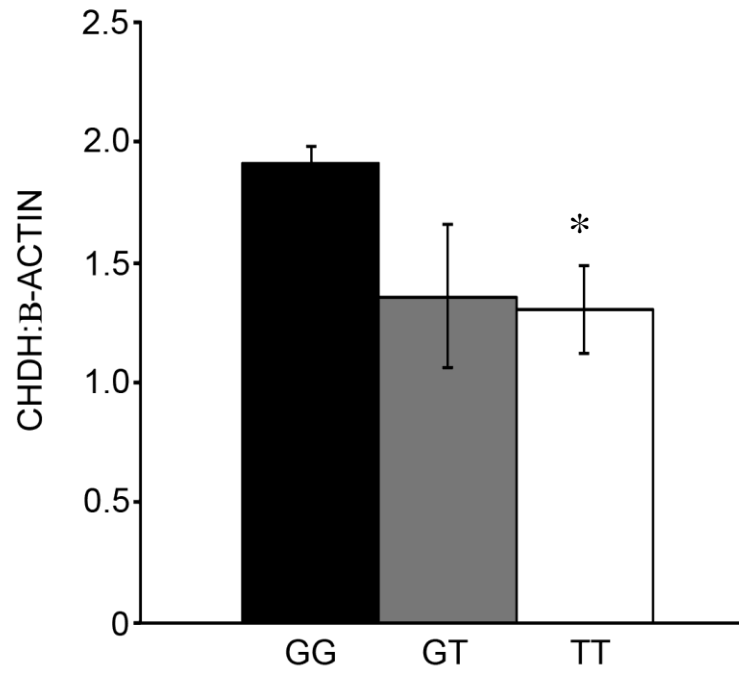
Table 3.4: Choline metabolite concentrations in plasma. Concentrations of choline and its metabolites were measured in plasma collected during the Day 1 clinic visit as described in Methods. For rs12676 $N = 40$ (GG), 30 (GT) and 6 (TT). For rs1025689 $N = 15$ (GG), 37 (GC) and 24 (CC). Data are mean $\pm$ SEM. ANOVA and Tukey-Kramer HSD were used to detect statistical differences among groups. * indicates $p < 0.05$.

Table 3.5: Choline metabolite concentrations in plasma

rs12676		GG	GT	TT
	Betaine (nmol/L)	62.2 ± 2.5	65.1 ± 2.8	75.3 ± 6.4
	Choline (nmol/L)	11 ± 0.4	10.5 ± 0.5	13.0 ± 1.0*
	PtdCho (nmol/L)	1802.4 ± 60.1	1938.9 ± 70.3	1964.6 ± 157.2
	SM (nmol/L)	727.8 ± 26.0	780.0 ± 30.0	759.6 ± 67.2
rs1025689		**GG**	**GC**	**CC**
	Betaine (nmol/L)	57.4 ± 4.0	66.0 ± 2.6	66.3 ± 3.2
	Choline (nmol/L)	10.4 ± 0.7	18.9 ± 0.4	10.8 ± 0.6
	PtdCho (nmol/L)	1872.3 ± 99.8	1924.6 ± 63.5	1781.5 ± 78.9
	SM (nmol/L)	728.4 ± 42.8	766.4 ± 27.2	741.1 ± 33.8

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

The data presented in this dissertation illustrate that normal sperm cell function, in both mice and men, relies, in part, on CHDH activity; however, the exact mechanism responsible for changes in sperm function accompanying decreased CHDH activity remains unknown. Because the altered sperm function observed is involved in many aspects of cellular function, it is likely to be the result of a complex array of cellular changes stemming from decreased CHDH activity, including shifts in cell membrane composition and epigenetic regulation of gene transcription. Further studies are necessary to understand CHDH activity and betaine support male fertility and suggested future research directions are presented below.

Future research directions

Finding the intersection between choline and carbohydrate metabolism:
detailed characterization of energy producing pathways and substrate utilization in sperm

In order to successfully migrate to and fertilize an egg, sperm cells must have a sufficient supply of ATP for use by dynenin motors responsible for bending of the sperm principal piece and, thus, motility of these cells (reviewed in [202]). As described earlier in this dissertation, there is some controversy as to the source of the ATP used for

this purpose.

We found that $Chdh^{-/-}$ sperm have lower ATP concentration than sperm from $Chdh^{+/+}$ males, but were surprised by this finding because HTF media contains glycolytic substrates and we are unaware of how deletion of CHDH could perturb glycolytic activity in cells. Glycolysis is necessary for normal motility in mouse sperm [191-193, 282, 283], while mitochondrial function may not be [195, 284].

Measurements of mitochondrial respiration (oxygen consumption rates, OCR) and glycolytic activity (extracellular acidification, ECAR) were made in $Chdh^{+/+}$ and $Chdh^{-/-}$ sperm and the results are shown in Figure 4.1. As glycolysis continues any pyruvate that is not taken into mitochondria is converted to lactate; as it accumulates, lactate is extruded from the cells, thus acidifying the media. Deletion of $Chdh$ results in a reduction of both OCR and ECAR in $Chdh^{-/-}$ sperm. As there are several possible explanations for this finding, a series of studies should be conducted to characterize and determine the causes of differences in mitochondrial respiration and glycolysis in $Chdh^{-/-}$ sperm.

Assessment of mitochondrial respiration in $Chdh^{-/-}$ sperm should begin with measuring the relative abundance of electron transport chain proteins as well as the activity of each of the individual electron transport chain complexes. Functional deficits in the electron transport chain, not stemming from decreased protein abundance, can be pinpointed by measuring OCR in the presence of electron transport chain modulators such as oligomycin, rotenone, antimycin A and carbonyl cyanide p-[trifluoromethoxy]-phenyl- hydrazone (FCCP).

Because ECAR is not a direct measure of glycolytic rate, the decreased activity of this pathway inferred from the ECAR data should be confirmed by measuring the concentration of lactate in the HTF media in which $Chdh^{+/+}$ and $Chdh^{-/-}$ sperm have been incubated. As glycolysis proceeds in sperm, lactate formed by the conversion of pyruvate to lactate is extruded from the cells into the culture media. Certain glycolytic enzymes have been shown to be necessary for normal sperm motility in mice. They include, glyceraldehyde 3-phosphate dehydrogenase, spermatogenic (GAPDHS), phosphoglycerate kinase -2 (PGK- 2) and lactate dehydrogenase – c (LDH-c) [191, 192, 283]. The activity of each of these enzymes should be measured once it is determined that lactate production is decreased in the $Chdh^{-/-}$ sperm. In addition, expression of glucose transporters (Glut 8 and Glut 9) and uptake of 2-deoxyglucose should be compared in $Chdh^{+/+}$ and $Chdh^{-/-}$ sperm to determine if an impairment in glycolytic rate is due to decreased glucose transport into the $Chdh^{-/-}$ sperm.

Epigenetic control of gene transcription may offer another means by which aberrant choline metabolism may affect sperm energy metabolism. For example, expression of genes that encode proteins necessary for oxidative phosphorylation, glucose uptake, and glycolysis may be altered by methylation patterns associated with these genes. Choline availability and, specifically, CHDH activity can influence the epigenetic status of a cell buy modulating betaine concentrations. Betaine availability can modulate AdoMet concentrations, which can modulate methylation patterns on genes [285-289]. During spermatogenesis, the process of epigenetic mark placement on DNA is dynamic. For example, Pgk-2, one of the glycolytic enzymes necessary for normal sperm motility, is

methylated at one specific restriction site in spermatogenic stem cells. That same site is unmethylated in spermatogia, spermatocytes and spermatids [290] suggesting the presence of a de-methylation mechanism. Interestingly, this site becomes re-methylated, and remains so, at some stage before sperm reach the corpus epididymis. The expression of many genes involved in sperm motility, including genes that code for structural proteins and enzymes required for ATP production, may be regulated by DNA methylation [192, 291-297]. The relationship between *Chdh* genotype, epigenetic status and gene expression should involve the following studies: 1.) quantification of the concentrations of AdoMet and AdoHcy in testis tissue to determine if deletion of *Chdh* corresponds to a decrease in AdoMet in this tissue; 2.) transcriptomic assessment in isolated spermatocytes, either by gene expression array or next generation high-throughput sequencing; 3.) determination of the methylation status of genes found to be differentially expressed between *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ spermatocytes using methylation-specific polymerase chain reaction and pyrosequencing techniques.

Chromatin condensation is another epigenetic mechanism by which gene transcription can be regulated. Gene transcription is silenced at the spermiogenesis stage largely due to extensive chromatin condensation that occurs as histones are replaced with highly basic protamines (reviewed in [184]). Defects in chromatin condensation are associated with a failure to initiate progressively motility [298], poor semen quality [299], and negative outcomes with the use of assisted reproductive technology [300]. A fluorescence-based assay using acridine orange can be used to evaluate the degree of chromosomal condensation in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$

sperm [300]. In a d d i t i o n , t h e inappropriate presence of histone in mature sperm chromatin can be detected by staining with analine blue [301].

Changes in testicular energy metabolism should also be investigated in the $Chdh^{-/-}$ male mice. CHDH is expressed in testis and betaine concentrations in this tissue are 10 times higher than concentrations found in liver [46]. In addition, inner mitochondrial membrane potential is increased in mitochondrial isolated form testis which, together, may indicate hyperpolarization of the mitochondria. ATP concentrations are not decreased in $Chdh^{-/-}$ testis; however measurements comparing $Chdh^{+/+}$ and $Chdh^{-/-}$ testis metabolic profiles indicate changes in carnitine and branched-chain amino acid metabolism as a result of $Chdh$ deletion (Table 4.1). Characterization of these two pathways, including gene expression, protein abundance and enzymatic activities are a necessary starting point in determining the cause of these aberrations.

Figure 4.1: Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in *Chdh$^{+/+}$* and *Chdh$^{-/-}$* sperm. Sperm were released from the cauda epididymides from *Chdh$^{+/+}$* and *Chdh$^{-/-}$* male mice into modified HFT media as previously described [46]. Modified HTF did not contain sodium bicarbonate, but did contain 1mM Sp-5,6-dichloro-1- beta-D- ribofuranosylbenzimidazole-3',5'-monophosphorothioate (Sp-5,6-DCl-cBiMPS), a cell permeable cAMP analog, and 1mM 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. Together, these additives are a substitute for sodium bicarbonate in the HTF media. Sodium bicarbonate signaling increases cAMP levels in sperm which is a signal necessary for achieving capacitation. 4 million sperm were aliquoted into each well of a 24 well Seahorse Bioscience tissue culture plate. Modified HTF media was added so that the final volume in each well was 500μL. The Seahorse analyzer was calibrated and equilibrated according to manufacturer's instructions. OCR and ECAR measurements were recorded over the course of ~ 100 minutes following a protocol of mixing for 2 minutes, waiting for 3 minutes and measuring for 4 minutes. N = 5 (*Chdh$^{+/+}$*) and 3 (*Chdh$^{-/-}$*); data are mean ± SEM for each genotype group.

Figure 4.1: Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ sperm
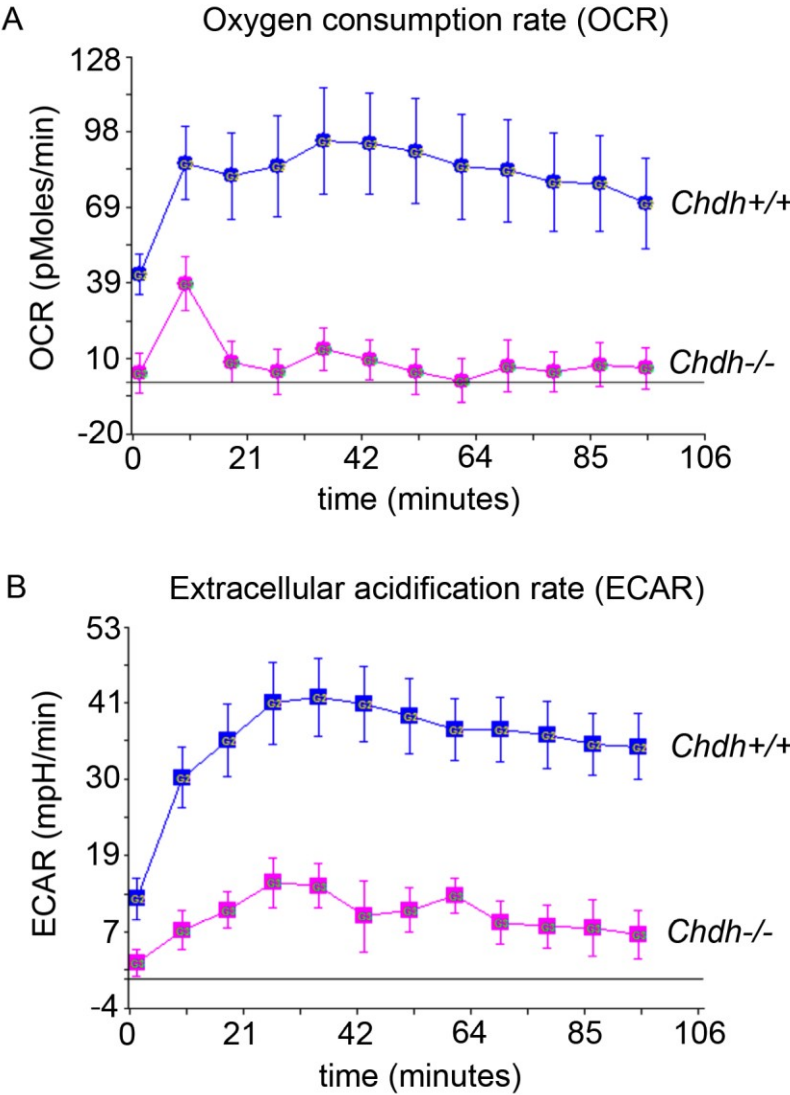
Table 4.1: Relative changes in carnitine and branched-chain amino acid metabolites in *Chdh*^{+/+} and *Chdh*^{-/-} testis. Testis was collected from 5 week-old male *Chdh*^{-/-} or wildtype littermate control animals. All animals were fed AIN76A purified diet containing 1.1gm/kg choline (Dyets, Inc., Bethlehem, PA) chloride *ad libitum* and housed in a climate-controlled facility with a 12-hour light:dark cycle. Animals were fasted for 4 hours, anesthetized with Isofluorane until they no longer responded to pain stimuli at which time the testes were dissected from the animal, immediately snap frozen and pulverized in liquid nitrogen. The relative abundance of testis metabolites was measured by liquid and gas chromatography mass spectrometry by Metabolon (Research Triangle Park, NC). Metabolon performed statistical analyses; data presented are a ratio of *Chdh*^{+/+} to *Chdh*^{-/-}. N = 5 animals per genotype. Red indicates a relative increase and green a relative decrease in metabolite concentration.

Table 4.1: Relative changes in carnitine, creatine and branched-chain amino acid metabolites in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ testis.

SUB PATHWAY	BIOCHEMICAL NAME	CHDH KO / CHDH WT
Valine, leucine and isoleucine metabolism	3-methyl-2-oxovalerate	0.80
	alpha-hydroxyisocaproate	0.94
	isoleucine	1.00
	leucine	1.00
	N-acetylleucine	1.10
	valine	1.04
	2-hydroxyisobutyrate	0.99
	4-methyl-2-oxopentanoate	0.74
	alpha-hydroxyisovalerate	0.82
	isobutyrylcarnitine	0.36
	2-hydroxy-3-methylvalerate	0.89
	2-methylbutyroylcarnitine	0.40
	isovalerylcarnitine	0.24
	hydroxyisovaleroyl carnitine	0.17
Creatine metabolism	creatine	1.22
	creatinine	1.32
Fatty acid metabolism (also BCAA metabolism)	propionylcarnitine	0.38
	butyrylcarnitine	0.27
Fatty acid metabolism	valerylcarnitine	0.36
Carnitine metabolism	deoxycarnitine	3.27
	carnitine	0.35
	3-dehydrocarnitine	0.46
	acetylcarnitine	0.48
	hexanoylcarnitine	0.38

Dysmorphic mitochondrial structure is apparent in sperm from $Chdh^{-/-}$ mice and men who are homozygous for the rs12676 *CHDH* SNP. Whether this is a result of alterations in sperm development or a response to changes in the environment in which they develop and mature remains unknown.

As discussed in the introduction section, sperm undergo extensive molecular changes as they move from the lumen of testis into the caput, and through the corpus and cauda epididymis. Sperm mature as they exchange and accumulate molecules found within the epididymal environment, for example organic osmolytes such as GPCho and carnitine [187]. Although there are no reports in the literature, we would expect – and have found (Table 4.2) betaine to be present, possibly due to its function as an organic osmolyte. The epididymal environment is relatively hyperosmotic (~340mmol/kg, [183]). In comparison, the osmolality of unliquified whole semen and of the fluid in the female reproductive tract is approximately 276 – 302mmol/kg ([274, 275]), suggesting that epididymal sperm experience an osmotic "challenge" within the male urethra [183]. An inability to regulate volume in response to the varied osmotic environments would render sperm susceptible to swelling which can impair motility [275].

The activity of some enzymes can be enhanced by osmotic stress. Phospholipase A_2 (PLA_2) is one such enzyme [277, 278]. PLA_2 catalyzes the hydrolysis of the fatty acid in the sn-2 position of PtdCho resulting in the release of the free fatty acid and formation of lysophosphatidylcholine (lysoPC) [302]. PLA_2 is highly expressed in sperm [276]. There

are many isoforms of PLA$_2$ that range in size from small secretory isoforms of 13-18kDa to large, cytosolic forms that can be as large as 85kDa [303]. Secretory PLA$_2$ protein has been detected in bull, hamster and human sperm [304, 305]. Sperm phospholipids most often contain docosahexanoic acid (DHA, 22:6n3), docosapentanoic acid (DPA, 22:5n3) or arachidonic acid (AA, 20:4n6) in this position; therefore, increased PLA$_2$ activity in response to osmotic stress should result in increased free DHA, DPA and AA and lysoPC - all of which can inhibit sperm motility [265, 302]. Although the mechanistic cause of this inhibition is unclear, it is presumed that poor motility results from changes in plasma membrane composition [270, 302, 306, 307]. It is possible that this is the mechanism by which *Chdh* deletion (and subsequent lack of betaine) causes diminished sperm motility. *Chdh*$^{-/-}$ sperm have significantly decreased concentrations of PtdCho (Figure 4.2); an attempt was made at measuring PLA2 activity in epididymal homogenates and sperm, but the assay requires further optimization , and no data was collected to date.

Table 4.2: Choline metabolite concentrations in *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ epididymis. *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ male mice, at least 10 weeks of age were anesthetized using Isofluorane until they no longer respond to a pain stimulus. Caput, corpus and cauda epididymides were collected; both whole epididymides were pooled together for each animal. Tissues were snap frozen in liquid nitrogen, sonicated for 1 minute and processed for choline metabolite measurements as described [226]. N = 6 (*Chdh*$^{+/+}$) and 3 (*Chdh*$^{-/-}$). Data are presented as mean ± SEM. Student's t test was used to test for statistical differences between genotypic groups. * indicate p-value > 0.05, ** indicate p-value > 0.01.

Table 4.2: Choline metabolite concentrations in *Chdh*^{+/+} and *Chdh*^{-/-} epididymis

		Betaine (nmol/g)	Choline (nmol/g)	GPCho (nmol/g)	PCho (nmol/g)	PtdCho (nmol/g)	SM (nmol/g)
Epididymis							
	Chdh^{+/+}	2602 ± 688	1442 ± 459	20582 ± 1389	952 ± 60	14085 ± 917	2556 ± 104
	Chdh^{-/-}	2 ± 2*	2235 ± 497*	32405 ± 6208**	798 ± 172	12233 ± 2203	2525 ± 168

Figure 4.2: PtdCho and GPCho concentrations in *Chdh*^{+/+} and *Chdh*^{-/-} sperm.
Chdh^{+/+} and *Chdh*^{-/-} male mice, at least 10 weeks of age, were anesthetized using Isofluorane until they no longer responded to a pain stimulus. Sperm were released into HTF media from the cauda epididymis as described previously [46]. After a 2 hour incubation in HTF, 4 million sperm from each animal were pelleted in a 1.5mL microcentrifuge tube and processed for choline metabolite analysis as described previously [226]. N = 5 animals per genotype. Data are presented as mean ± SEM. Student's *t* test was used to test for statistical differences between genotypic groups. ** indicate p-value > 0.01. Only metabolites in which there were significant changes are shown.

Figure 4.2: PtdCho and GPCho concentrations in *Chdh*^{+/+} and *Chdh*^{-/-} sperm
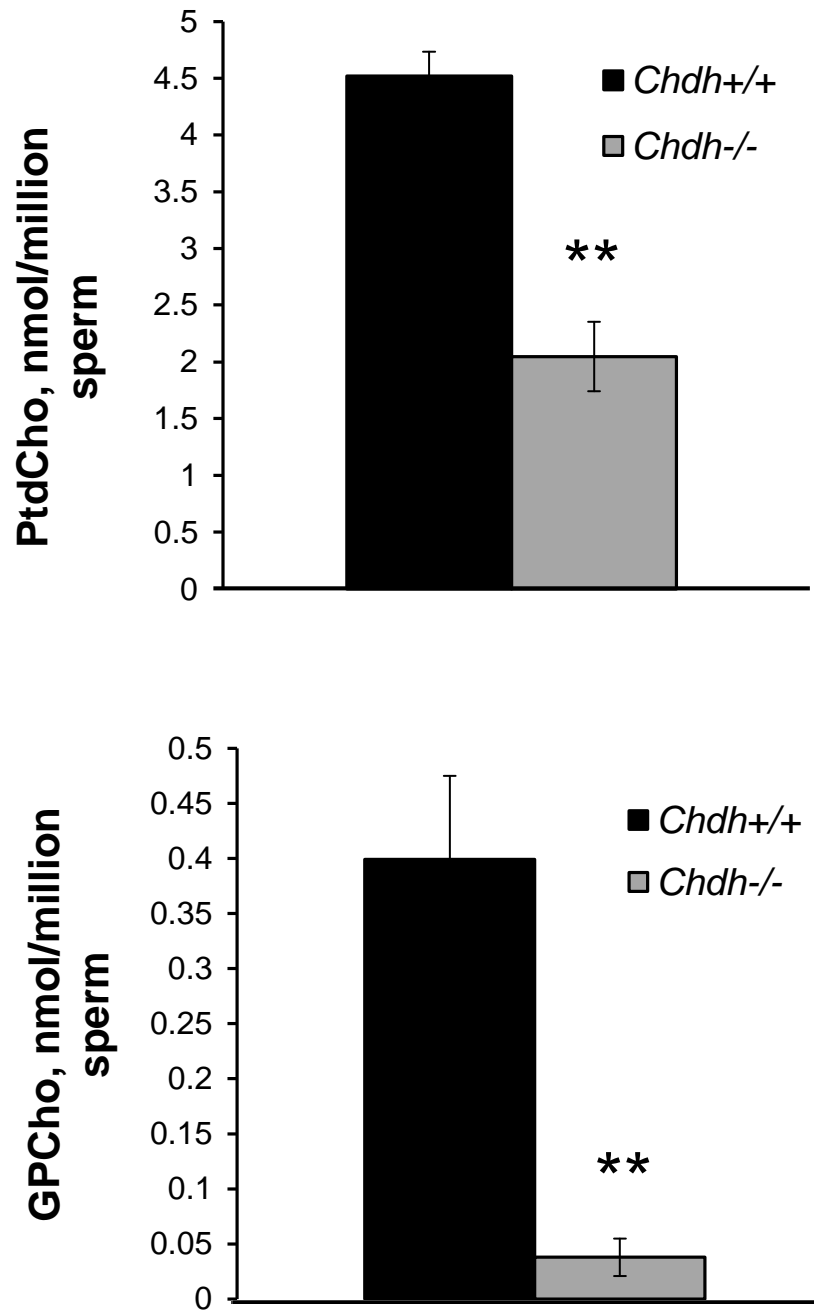
Dietary betaine intervention study with human subjects

Betaine administration to *Chdh*^{-/-} mice improves, but does not fully rescue the male infertility phenotype observed in these animals [46]. While the exact dose of betaine should be optimized in future experiments, this study provides compelling evidence that human male factor infertility may be improved either by increasing dietary betaine intake or ingestion of betaine supplements. A cross-over study design would be appropriate for investigating whether increased betaine intake was associated with improvement in measures of sperm motility and morphology. The subject population would include a case cohort comprised of men suffering from idiopathic infertility and an equal number of age-matched controls. Typical choline and betaine intakes should be determined for each subject using food frequency questionnaires or 3 day food records. Baseline measurements of sperm concentration, motility, morphology and plasma choline metabolites would be made after which betaine supplementation would be initiated and continued for approximately 85 days (the human spermatogenic cycle takes 64 days). Periodic urine or blood samples would be collected throughout the treatment period to measure betaine concentrations and monitor compliance. At the end of the treatment period sperm concentration, motility, sperm morphology and choline metabolite measures would be repeated. The next phase would be a washout period of 85 days followed by a no-treatment period of 85 days. Again, periodic urine or blood choline metabolite measurements will be analyzed to test for compliance (to ensure subjects are not taking betaine supplements) and measures of sperm concentration, motility, morphology and choline metabolite concentrations will be repeated. Statistical analyses of the data collected would be used to evaluate the effect of betaine supplementation on fertility characteristics.

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Special consideration would have to be made in the study design regarding SNPs in the choline dehydrogenase gene.

The effect of decreased CHDH activity on Sertoli cell function

Chdh mRNA has been detected in Sertoli cells, somatic cells that support spermatogenesis in a variety of ways [310]. Altered function of these cells could possibly have detrimental effects on the spermatogenic process and may account for some of the structural and biochemical changes observed in the Chdh-/- sperm or sperm produced by men who were homozygous variant for the rs12676 SNP. Further animal studies can differentiate the relative importance of decreased CHDH activity in Sertoli cells versus spermatogenic cells. A conditional Chdh knockout mouse could be generated and be used to selectively delete Chdh in Sertoli cells or spermatogenic germ cells. Alternatively, spermatogenic cells could be isolated from *Chdh*$^{-/-}$ males and transplanted into recipient *Chdh*$^{+/+}$ males, and vice versa.

CHAPTER V

SUPPLEMENTAL DATA AND EXPERIMENTS SUGGESTED
BY COMMITTEE MEMBERS

1. *Expression of Chdh with mutation of the gene*

Purpose: To determine whether any Chdh mRNA is expressed in the $Chdh^{-/-}$ model system

Methods: $Chdh^{+/+}$ and $Chdh^{-/-}$ liver tissue was collected from animals that had been anesthetized using Isofluorane as described previously. Liver was snap frozen and then pulverized in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy kit (Valencia, CA) according to manufacturer's instructions. RNA concentration and purity was determined using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was reverse transcribed to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Calsbad, CA). Relative expression levels of Chdh were determined by semi- quantitative real-time PCR using a *Chdh*-specific TaqMan gene expression assay (Applied Biosystems, Carlsbad, CA). Changes in relative expression of Chdh were calculated suing the $\Delta\Delta C_t$ method. See Figure 5.1 for results.

Figure 5.1: *Chdh* **mRNA expression in** *Chdh*$^{+/+}$ **and** *Chdh*$^{-/-}$ **liver.** RNA was isolated from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ liver tissues. Expression of Chdh was measured using a *Chdh*-specific TaqMan gene expression assay and calculated by the $\Delta\Delta C_t$ method. Data are mean $\pm$ SEM. N = 5 animals per group. *** indicates different from *Chdh*$^{+/+}$ p $-$ value <0.001 by Student's *t* test.

Figure 5.1: *Chdh* **mRNA expression in** *Chdh*$^{+/+}$ **and** *Chdh*$^{-/-}$ **liver**
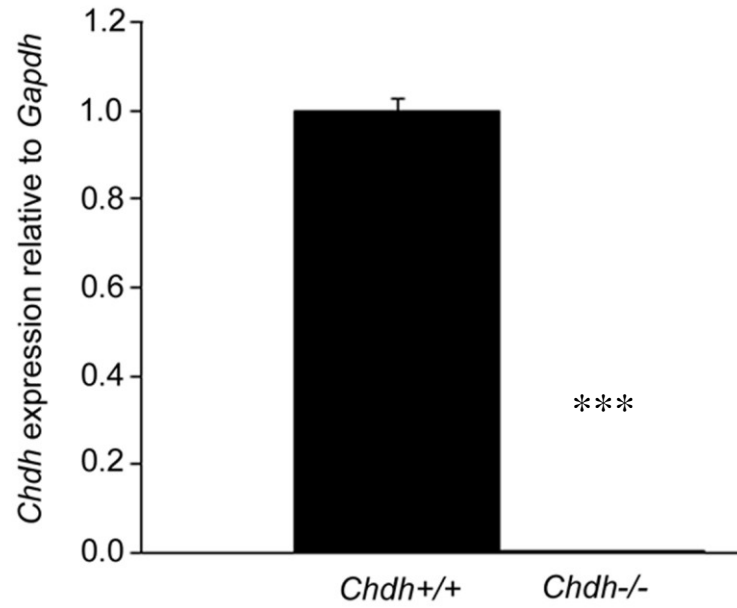
Conclusions: Removal of *Chdh* gene exons 1 through 3 results in decreased mRNA abundance of *Chdh*.

2.   *CHDH mRNA and protein expression in mouse epididymis/ CHDH*

      protein expression in sperm

Purpose: To confirm that CHDH protein is expressed in epididymal tissue and mature sperm cells.

Methods:

Epididymis – 10 week old *Chdh$^{+/+}$* and *Chdh$^{-/-}$* males were anesthetized using Isofluorane as described above. Epididymides were microdissected from other structures and snap frozen in liquid nitrogen. Epididymal tissue is difficult to homogenize; therefore, epididymides were homogenized in RLT buffer (from Qiagen RNA extraction kit) or RIPA buffer (for blot) using a Bullet Blender and 0.5mm zirconium oxide beads (Next Advance, Averill Park, NY). Samples were homogenized on speed 9 for 3 minutes followed by speed 10 for 3 minutes; samples were cooled on ice between.

For real-time PCR, total RNA was extracted using a Qiagen RNeasy kit (Valencia, CA) according to manufacturer's instructions. RNA concentration and purity was determined using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was reverse transcribed to cDNA using a High Capacity Reverse Transcription Kit (Applied Biosystems, Calsbad, CA). Relative expression levels of Chdh were determined by semi- quantitative real-time PCR using a Chdh-specific TaqMan gene expression assay (Applied Biosystems, Carlsbad, CA).

For   blotting, protein concentration was determined by BCA protein assy. 20µg of protein lysate were resolved by SDS-PAGE and transferred to nitrocellulose membrane. CHDH blot was conducted as described in Methods, Chapter 3.

Sperm - week old *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ males were anesthetized using Isofluorane as described previously. Sperm were released into HTF media, pelleted by centrifugation and washed twice with 1X PBS. Sperm pellets were lysed in a buffer of 2% w/v SDS, 0.375M Tris, pH 6.8, 10% sucrose [308]. Lysates were boiled at 100˚ C for 5 minutes and clarified by centrifugation at 16.1 x *g* for 10 minutes at 4˚ C. Protein concentration was determined by BCA protein assy. 20µg of protein lysate were resolved by SDS-PAGE and transferred to nitrocellulose membrane. CHDH blot was conducted as described in Methods, Chapter 3. See Figure 5.2 for results.

Figure 5.2: *Chdh* mRNA and protein expression in epididymis, protein expression in sperm. RNA was isolated from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ epididymal tissue. Expression of *Chdh* was measured using a *Chdh*-specific TaqMan gene expression assay and calculated by the $\Delta\Delta C_t$ method. Data are mean $\pm$ SEM. N = 5 animals per group. *** indicates different from *Chdh*$^{+/+}$ p – value <0.001 by Student's *t* test. CHDH blot of epididymal tissue and sperm cells. N = 3 per genotype.

Figure 5.2: *Chdh* mRNA and protein expression in epididymis, protein expression in sperm

A.
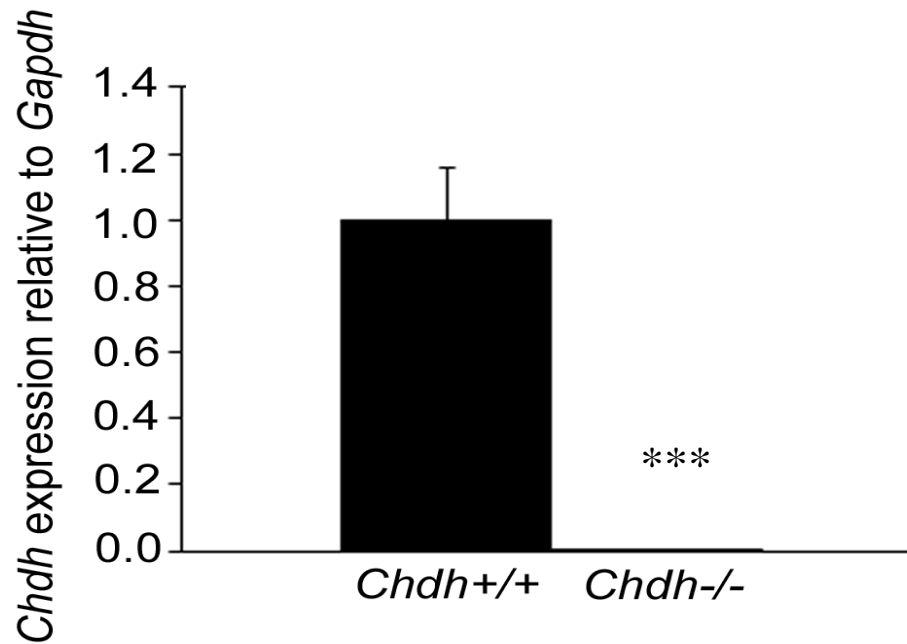
B.
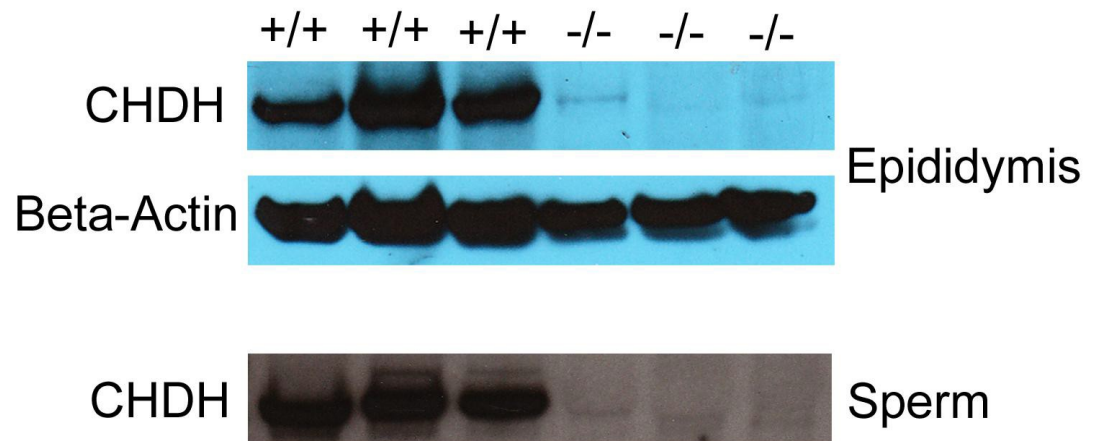
Conclusions: *Chdh* mRNA is expressed in epididymis and protein is found in both epididymis and mature sperm.

3. Localization of CHDH in sperm cells and testis tissue

Purpose: The purpose of this study was to confirm that CHDH protein is localized to the midpiece of sperm cells and pachytene spermatocytes and Sertoli cells in the testis, as expected based on gene expression array data [137].

Methods:

Sperm – Chdh$^{+/+}$ and *Chdh*$^{-/-}$ males were anesthetized using Isofluorane. Sperm were released from the cauda epididymis into 1 X PBS, sperm concentration was determined and adjusted to 5 x 10^6 sperm per mL An aliquot of sperm were spread onto SuperFrost Plus slides (Fisher Scientific) and air dried for 15 minutes. Slides were fixed in freshly prepared 4% paraformaldehyde for 15 minutes, washed with PBS then permeabalized in 0.2% Triton X-100 for 5 minutes. Autoflourescence was quenched by incubating the slides in 10mg/mL sodium borohydride at room temperature for 40 minutes. Slides were washed in PBS-T and blocked in 4% normal goat serum for 30 minutes at room temperature. Slides were incubated in a 1:500 dilution of anti-CHDH primary antibody (ProteinTech Group, Inc., Chicago, IL) in PBS-T overnight at 4˚ in a humid chamber. The next day slides were warmed to room temperature and washed in PBS-T. Goat anti-rabbit-Alex488 secondary antibody (Molecular Probes, Carlsbad, CA), diluted 1:2000 in PBT-T, was incubated with the slides for 1 hour. Slides were washed again and sperm were counter-stained with 4',6-diamidino-2- phenylindole (DAPI), washed and coverslipped. Staining was examined using an Olympus BX61 microscope and Velocity imaging software available in the UNC Microscopy Services Core Facility. Sperm were examined with a 40X oil objective lens.

Testis – Testes were collected from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ males at least 10 weeks of age and were fixed overnight in Bouin's fixative. Testes were embedded in paraffin and 5µm sections cut for immunohistochemistry. CHDH staining was performed by the UNC Laboratories for Reproductive Biology Core Facility using anti-CHDH primary antibody. Three primary antibody dilutions were tested for this staining protocol; however, a high degree of non- specific staining is observed and this protocol requires additional optimization. No results are presented.

Figure 5.3: CHDH Localization in mouse sperm. $Chdh^{+/+}$ and $Chdh^{-/-}$ sperm were released from the cauda epididymis into 1X PBS and fixed onto Super Frost Plus glass slides. CHDH protein was detected as described above. A. $Chdh^{+/+}$ sperm, DIC; B. $Chdh^{+/+}$ sperm stained for CHDH (green) and DNA (blue). C. $Chdh^{-/-}$ sperm, DIC; D. $Chdh^{-/-}$ sperm stained for CHDH (not detected) and DNA (blue). Arrows indicate areas of positive staining in the sperm midpiece.

Figure 5.3: CHDH localization in mouse sperm
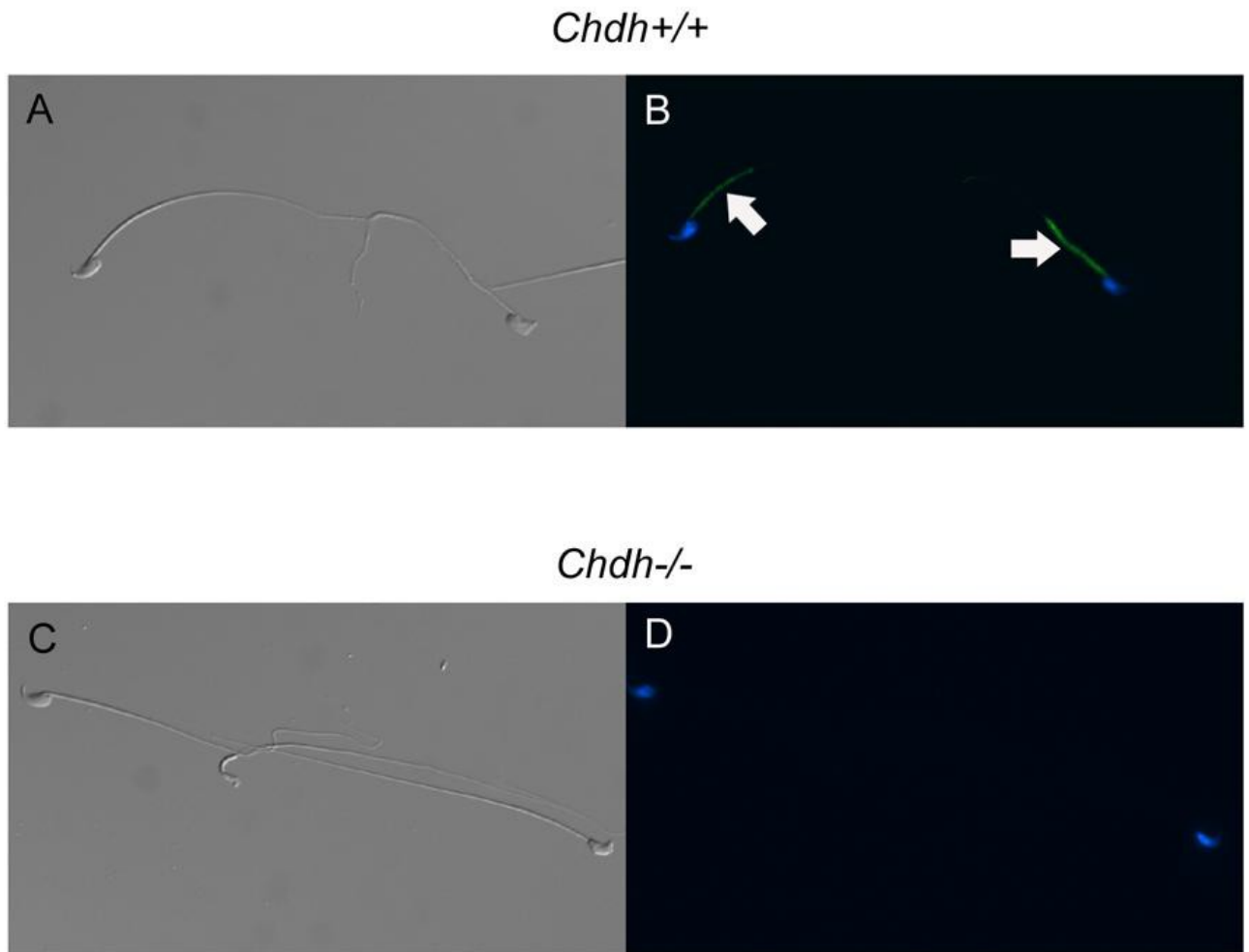
Chdh+/+

Chdh-/-

Conclusion: CHDH protein is localized to the midpiece region and is detected in *Chdh$^{+/+}$,* but not *Chdh$^{-/-}$,* sperm.

4. Testicular and epididymal choline metabolite concentrations with betaine supplementation

Purpose: To determine the extent to which a 2% solution of betaine in drinking water increases betaine concentration in testis and epididymis in *Chdh*$^{-/-}$ males.

Methods: Testis and epididymides were collected from animals at the end of the betaine feeding study described in Chapter 2. Choline metabolite concentrations were measured as described [226]. See Table 5.1 and Table 5.2 for results.

Table 5.1: Testis choline metabolite concentrations with and without betaine supplementation. Measures of choline metabolite concentrations in testis from un-supplemented *Chdh*$^{-/-}$ males and males who were supplemented with betaine in their drinking water were performed as described in Chapter 2. For un-supplemented animals, N = 5 per genotype. For betaine-supplemented animals, N = 6 (*Chdh*$^{+/+}$, *un-supplemented*), 3 (*Chdh*$^{-/-}$, *un-supplemented*), 5 (*Chdh*$^{+/+}$, *supplemented*) and 3 (*Chdh*$^{-/-}$, *supplemented*). Data presented are mean ± SEM. ND, not detected. Statistical differences were determined by ANOVA and Tukey-Kramer HSD. *p<0.05 different from *Chdh*$^{+/+}$ (un-supplemented). †indicates different from un-supplemented group of the same genotype, p<0.05. ζ indicates different from un- supplemented of the opposite genotype, p<0.05. ∞ indicates different from *Chdh*$^{+/+}$, supplemented, p<0.05. Values are expressed in nmol/g tissue.

Table 5.1: Testis choline metabolite concentrations with and without betaine supplementation

	Betaine supplementation	Betaine	Choline	GPCho	PCho	PtdCho	SM
Testis	**No**						
Chdh$^{+/+}$		3539±810	847±120	456±46	4840±537	14396±1313	1953±190
Chdh$^{-/-}$		ND	1219±144	446±45	5342±292	15690±735	2033±96
	Yes						
Chdh$^{+/+}$		4838±156	859±22	416±45	4493±131	14608±691	2049±65
Chdh$^{-/-}$		642±31†ζ∞	1202±136	573±59	5960±254	15686±600	2210±60

Conclusions: Betaine concentrations are not detectable in *Chdh*$^{-/-}$ testis; betaine supplementation via drinking water increases betaine concentrations in *Chdh*$^{-/-}$ testis, but is inadequate for completely restoring to *Chdh*$^{+/+}$ levels. Choline concentrations tend to be increased in *Chdh*$^{-/-}$ tissues, but differences do not reach statistical significance. There are no significant changes in GPCho, PCho,PtdCho or SM among genotype and treatment groups.

Table 5.2: Epididymal choline metabolite concentrations with and without betaine supplementation. Measures of choline metabolite concentrations in epididymides from un-supplemented $Chdh^{-/-}$ males and males who were supplemented with betaine in their drinking water were performed as described in Chapter 2. For un-supplemented animals, N = 5 per genotype. For betaine-supplemented animals, N = 6 ($Chdh^{+/+}$, *un-supplemented*), 3 ($Chdh^{-/-}$, *un-supplemented*), 5 ($Chdh^{+/+}$, *supplemented*) and 3 ($Chdh^{-/-}$, *supplemented*). Data presented are mean ± SEM. Statistical differences were determined by ANOVA and Tukey-Kramer HSD. *$p < 0.05$ different from $Chdh^{+/+}$ (un-supplemented). † indicates different from un- supplemented group of the same genotype, $p < 0.05$. ζ indicates different from un-supplemented of the opposite genotype, $p < 0.05$. ∞ indicates different from $Chdh^{+/+}$, *supplemented*, $p < 0.05$.

Table 5.2: Epididymal choline metabolite concentrations with and without betaine supplementation

	Betaine supplementation	Betaine	Choline	GPCho	PCho	PtdCho	SM
Testis	**No**						
Chdh$^{+/+}$		2607±688	1442±188	20582±1389	952±60	14085±917	2256±105
Chdh$^{-/-}$		1±2*	2235±287	32406±3584*	799±172	12234±2203	2525±168
	Yes						
Chdh$^{+/+}$		4860±288†	1231±53	19106±140	1090±48	14111±36	2585±52
Chdh$^{-/-}$		2655±186†	1930±160ζ	30474±1508ζ∞	1161±60	13563±500	2531±64

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Conclusion: *Chdh*$^{-/-}$ epididymal tissue has less betaine than *Chdh*$^{+/+}$ and supplementation of *Chdh*$^{-/-}$ animals with drinking water containing 2% betaine. Choline concentrations were increased in *Chdh*$^{-/-}$ epididymis, but differences did not reach statistical significance likely due to small numbers of individual samples in some groups. GPCho concentrations were increased in *Chdh*$^{-/-}$ epididymal tissue; there was no effect of betaine supplementation. No significant changes were detected in PCho, PtdCho or SM.

5. Analysis of sperm ultrastructure by TEM in the caput, corpus and cauda epididymis

Purpose: The purpose of this study was to determine if sperm mitochondrial structure becomes abnormal in a specific region of the epididymis, or if these structures are dysmorphic in sperm released from the testis.

Methods: Epididymides were collected from *Chdh*$^{+/+}$ and *Chdh*$^{-/-}$ male mice, all at least 10 weeks of age, and cut into caput, corpus and cauda regions. The three regions from one epididymis were fixed for TEM as described in Chapter 2. The remaining segments from the second epididymis were snap frozen in liquid nitrogen for choline metabolite analysis. N = 6 per genotype.

Results: No results have been obtained for this study to date.

6. Test fatty acid oxidation capacity in Chdh$^{+/+}$ and Chdh$^{-/-}$ sperm

Purpose: The purpose of this study was to determine whether sperm, in particular *Chdh$^{-/-}$* sperm, are capable of oxidizing fatty acid for ATP production.

Methods: *Chdh$^{+/+}$* and *Chdh$^{-/-}$* male mice were anesthetized using Isofluorane. The cauda epididymides were dissected from the males and placed into a dish containing modified HTF media as described on page 121. Sperm concentration was determined by counting cells with a hemocytometer and 4 million sperm were plated into a 24 well Seahorse Biosciences tissue culture plate in HTF. Measurements of OCR and ECAR were made at baseline and repeated following treatment with fatty acid-free bovine serum albumin - conjugated oleate. Final oleate concentration in each well was 200μM. See Figure 5.4 for results.

Figure 5.4: Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in *Chdh*^{+/+} and *Chdh*^{-/-} sperm treated with oleate. Sperm were released from the cauda epididymides from *Chdh*^{+/+} and *Chdh*^{-/-} male mice into modified HFT media as previously described [46]. 4 million sperm were aliquoted into each well of a 24 well Seahorse Bioscience tissue culture plate. Modified HTF media was added so that the final volume in each well was 500μL. The Seahorse analyzer was calibrated and equilibrated according to manufacturer's instructions. OCR and ECAR measurements were recorded over the course of ~ 70 minutes following a protocol of mixing for 2 minutes, waiting for 3 minutes and measuring for 4 minutes. N = 3 for each genotype; data are mean ± SEM for each genotype group.

Figure 5.4: Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in *Chdh*^{+/+} and *Chdh*^{-/-} sperm treated with oleate.

Oxygen consumption rate (OCR)

Extracellular acidification (ECAR)
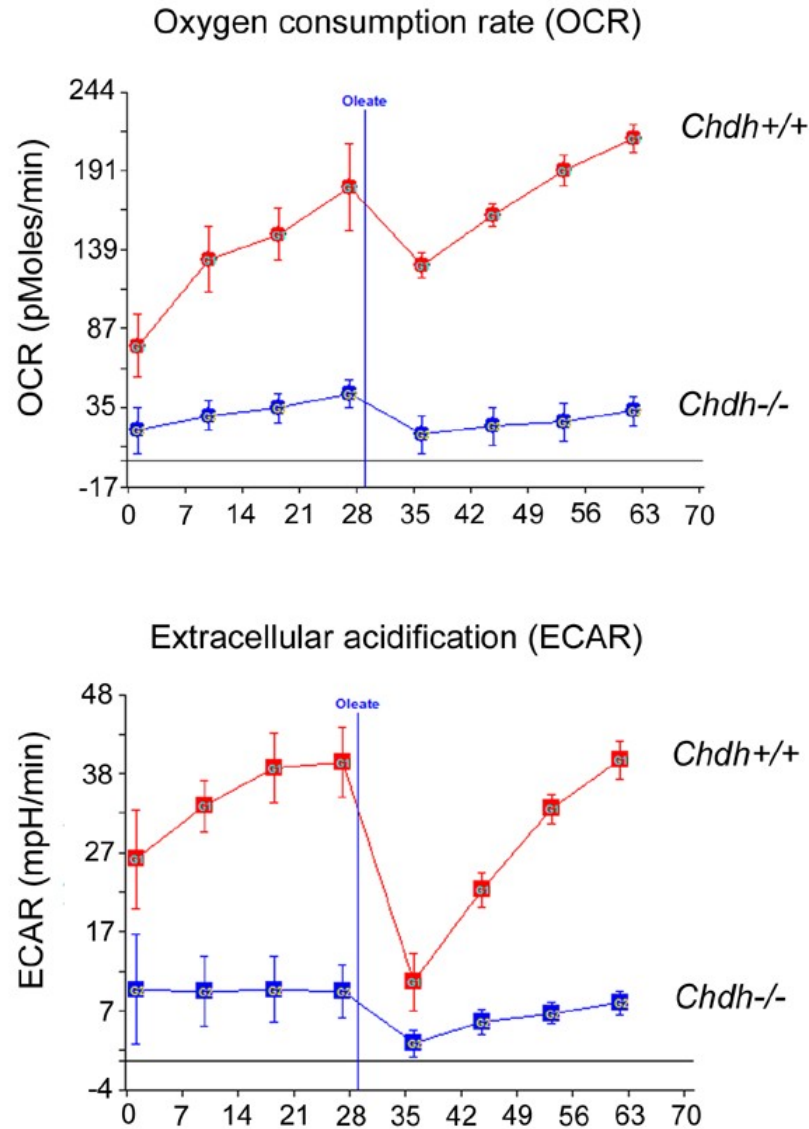
Conclusion: Both OCR and ECAR are reduced in *Chdh*^{-/-} sperm, as previously observed. 200µM oleate transiently decreased OCR and ECAR of *Chdh*^{+/+} and *Chdh*^{-/-} sperm, suggesting that the oleate treatment might by somewhat toxic to the sperm. Notably, ECAR returned to baseline within 35 minutes after oleate treatment indicating that, even if the sperm were able to use oleate for energy production, mouse sperm have an obligate need for glycolysis.

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