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Effects of Aging and Dietary Phytoestrogens on Sperm Production in Sprague Dawley Rats

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Effects of Aging and Dietary Phytoestrogens on Sperm Production in Sprague Dawley Rats

Estrogens, in addition to testosterone, are physiologically relevant to normal sperm production in the testis and sperm maturation in the epididymis. Previous studies from our lab demonstrated that daily sperm production declines from 15 to 18 months of age in Sprague Dawley rats, and treatment with estrogen during this period attenuated the age-associated decline. Phytoestrogens are present in standard rodent diets at high levels (350-650 mg/kg) and may be potential endocrine disruptors. The purpose of this study was to investigate the effect of removing dietary phytoestrogens during aging on sperm production. Retired breeder Sprague Dawley rats were obtained at 9 months of age, divided into three groups and further housed until 15 or 18 months of age. At 15 months of age, one group of animals was switched to a low phytoestrogen (0-20 mg/kg) rodent chow. A second group of animals was maintained on the high phytoestrogen diet. Groups one and two were maintained on their respective diets for three months until they were 18 months old. At 18 months of age, animals were euthanized and reproductive tissues were collected for analysis. The third group of animals was euthanized and tissues collected at 15 months of age. Results show that daily sperm production in both 18 month groups declined approximately 23% compared to animals 15 months of age, but was not different based on diet. The number of Sertoli cells decreased with age by about 21%, but the decrease was not affected by dietary phytoestrogens as cell numbers in both 18-month old groups were similar. Interestingly, concentrations of testosterone were not significantly different between ages or with dietary phytoestrogen content. However, there was a decrease in serum (~37%) and testicular (~42%) estradiol concentrations with age. Collectively these results further support the hypothesis that sperm production decreases with age, and the relationship between estradiol and Sertoli cells helps to maintain fertility. The findings also suggest that removal of dietary phytoestrogens does not affect the age-related decline in efficiency of spermatogenesis and daily sperm production.

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Chapter 1. Introduction

Given the potential effects of estrogen on spermatogenesis, dietary phytoestrogens have become a point of study in recent years. These phytoestrogens are often found in soy based products common to eastern cultures and becoming increasingly prevalent in western culture. Multiple studies have demonstrated a disruption to reproductive function due to phytoestrogen exposure. The majority of studies using rat models focus on the pre-pubertal developmental effects of phytoestrogens. However, the effects of manipulating phytoestrogen levels in adult males remains sparse and with variable results.

The effects of aging on spermatogenesis is also an area of interest given that human males experience a depletion of germ cells, reduced testosterone production, and lower motility of sperm with age. Previous studies in my lab investigated hormone concentrations and sperm production in aging rat models (Clark and Pearl, 2014). The results showed that testosterone levels did not significantly decline, but estradiol concentrations began dropping at 15 months. This age corresponded to an adult human roughly 47 years old. Alongside the falling estrogen levels, there was a significant drop in sperm production after 15 months. This decline in sperm production was prevented in animals given supplemental estrogen during aging. These results suggest a role for estrogen in maintaining spermatogenesis and male fertility. Given the changes observed with estrogen and spermatogenesis during aging, it is possible that this time period may be a target for disruption by phytoestrogens. However, no studies to our knowledge have investigated the effects of dietary phytoestrogens during aging.

Purpose

The purpose of this study was to determine the effects of aging and dietary phytoestrogens on testicular function.

Scope

Testicular function includes both the production of hormones and the production of sperm via spermatogenesis. Loss of function in either of these parameters can have significant effects on fertility. We will determine the extent that testicular function is altered by age or diet by focusing on testosterone and estradiol levels, as well as measuring total sperm production. We will also consider how both aspects of testicular function can interact to impact fertility.

Assumptions

1. Estradiol has a role in maintaining spermatogenesis and male fertility
2. Rats between the ages of 15 and 18 months appear sensitive to estrogen treatment indicating a possible target for endocrine disruption

Hypothesis

We hypothesize that dietary phytoestrogens negatively affect testicular function during aging, and removal of the endocrine disruptor will improve the age-related decline of spermatogenesis.

Significance

Because males are the contributing factor in up to 50% of infertility cases, a greater understanding of male reproductive physiology is essential. The effects of aging on testicular function are becoming increasingly relevant as couples are delaying the time they attempt to conceive. Phytoestrogens are a known endocrine disruptor commonly found in soy-based food products. Additional knowledge regarding the extent of this disruption could prove useful as soy-based diets are becoming increasingly prevalent in western cultures. This study is to our knowledge the first to examine the effects of dietary phytoestrogens during aging.

Chapter 2: Review of Literature

Testicular Anatomy and Function

Normal testicular function includes both the production of hormones and the production of sperm by process of spermatogenesis. These two primary functions of the testis occur within different compartments: the seminiferous tubules and the area between the tubules or the interstitial space. The coordination of these two components enables proper testicular function and fertility in mammalian males. A human testis contains between 200-300 lobules of extensively folded seminiferous tubules. Spermatogenesis occurs within this seminiferous tubule compartment. The interstitial compartment contains Leydig cells which produce testosterone and estradiol in response to luteinizing hormone (LH). Peritubular myoid cells rest just outside the basement membrane of the tubules, thus surrounding the seminiferous tubule. These flat contractile cells provide a level of support to mammalian seminiferous tubules.

The tubule can be thought of in terms of two distinct layers: the peripheral basement membrane and the internal epithelium. Germ cells progress from the basement membrane through the epithelial layer as they undergo maturation. Development of the germ cells occurs due to a predetermined program and via communication with neighboring Sertoli cells (Jégou, 1993). Sertoli cells, located within the epithelium, are activated upon encountering follicle-stimulating hormone (FSH) and testosterone. Despite resting on the basement membrane, Sertoli cells send cytoplasmic projections throughout the entire epithelium which support germ cell development. The Sertoli cells form tight junctions with each other that contribute to the blood-testis barrier, which protects the germ cells from the male immune system and other potential blood-borne factors (Lie et al., 2013; Mruk and Cheng, 2015). Maturing spermatid heads create

deep indentations into the Sertoli cell and are gradually transported towards the luminal portion of the seminiferous tubule (Hess, 1990).

Once spermatogenesis begins in the seminiferous epithelia, there are three phases of sperm development: mitosis, meiosis, and remodeling. Near the basement membrane, germ cells undergo waves of mitosis, thus increasing germ cell numbers. These mitotic cells are spermatogonia. Type A spermatogonia are the most basal cells and are responsible for maintaining stem cell populations, but they also divide to form type B spermatogonia. Type B cells will eventually divide and give rise to spermatocytes that progress through a process called spermatocytogenesis. The primary spermatocytes undergo the first meiotic cell division (meiosis I) to form secondary spermatocytes, followed by a second division (meiosis II) to form round spermatids. Spermatids undergo remodeling to eventually become elongated spermatozoa. This remodeling involves production of flagellum and acrosome, shaping of the head, condensation of nuclear chromatin, and formation of a mitochondrial sheath (Clermont, 1972). Spermiogenesis is complete when the cells are released into the lumen of the seminiferous tubule to proceed through the epididymis for further modification (Leblond and Clermont, 1952). Any given cross section of the seminiferous tubule will show one stage of developing germ cells (Fig. 1). In humans, up to six stages may be observed depending on the position in the tubule; whereas fourteen stages may be visible in a rat testis. This spermatogenic cycle qualitatively describes a seminiferous tubule based on the developmental states of the germ cells present in the epithelium. Endocrine changes within the testis can have distinct effects depending on what stage a given seminiferous tubule is in (Toyama et al., 2001).

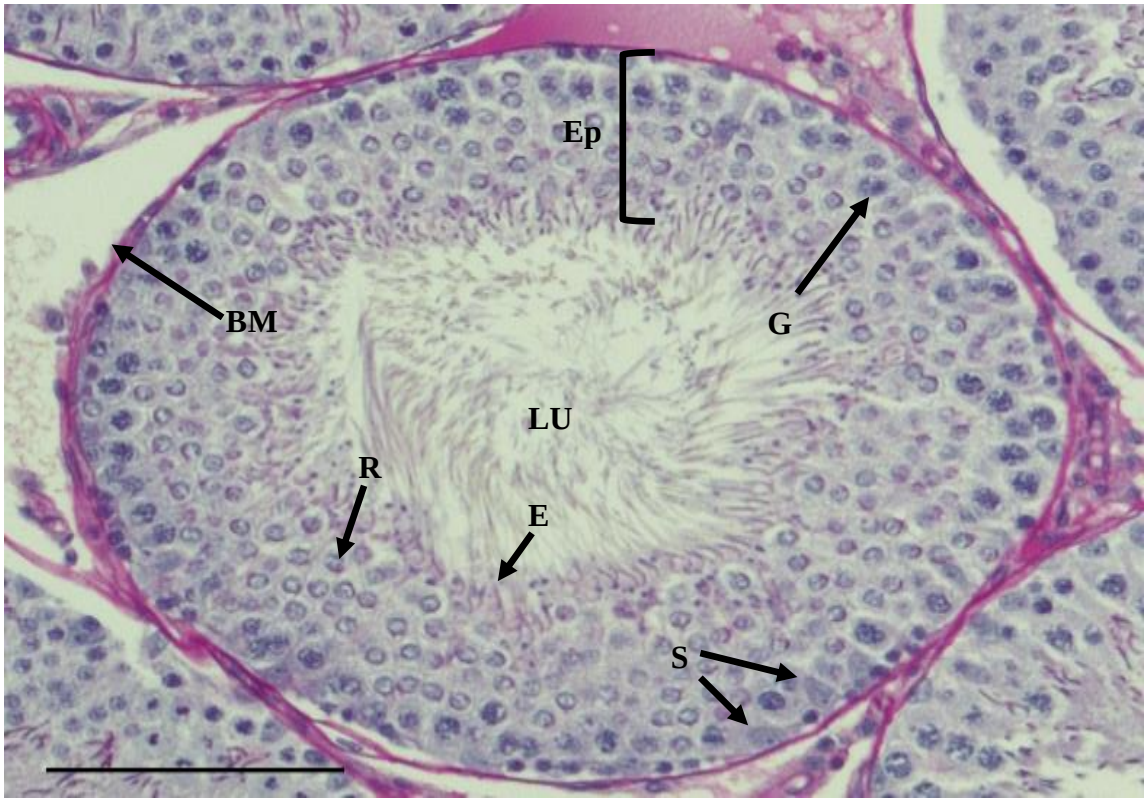

Figure 1. Cross Section of Rat Testis in Stage VI.
LU: Lumen of seminiferous tubule
BM: Basement Membrane
Ep: Epithelial layer
S: Sertoli cell nuclei
G: Germ cell nuclei
R: Round spermatid nuclei
E: Elongated spermatid nuclei
Scale Bar is 100 μm

Hormones and Spermatogenesis

Spermatogenesis is dependent upon the hormones of the hypothalamic-pituitary-gonadal axis (HPG axis). The combined contributions of all three glands maintain proper reproductive physiology in adulthood. Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus stimulates the release of LH and FSH from the anterior pituitary gland. FSH directly activates Sertoli cells to release factors necessary for spermatogenesis (Smith and Walker, 2015). Examples include DMRT responsible for sex determination, the iron transporter transferrin, and factors that assist with proliferation of germinal stem cells such as VEGF. LH stimulates Leydig cells to produce and release testosterone which also stimulates Sertoli cells. The HPG axis is highly conserved between mammal species, so effects observed within animal models can be reasonably attributed across mammalian species, including humans. The importance of FSH and testosterone for spermatogenesis is well established (Walker and Cheng, 2005). The FSH receptor is a G protein-coupled receptor present primarily within Sertoli cells. Binding of FSH to their receptors causes increased cAMP production that varies depending on the stage of the seminiferous tubule. Higher cAMP raises the levels of PKA which increases phosphorylation of multiple proteins in the Sertoli cell responsible for regulating the expression of transcription factors. Testosterone binds to intracellular receptors to recruit coactivator proteins and stimulate gene transcription. Both FSH and testosterone can activate MAP kinase pathways in Sertoli cells to stimulate proliferation. Similar to FSH, testosterone can also elevate Ca^{2+} levels in Sertoli cells, but does not act to elevate cAMP production (Loss et al., 2007). Together, FSH and testosterone are essential for spermatogenesis and their shared mechanisms allow a level of redundancy in supporting spermatogenesis.

Other hormones such as estradiol also contribute to male fertility/infertility. Similar to testosterone, the production of estrogen within the testis requires support from the Sertoli cells and Leydig cells. The enzymatic complex aromatase is responsible for the conversion of testosterone to estradiol. Expression of aromatase has been observed within Sertoli cells, Leydig cells, spermatocytes and round spermatids, and evidence suggests that germ cells could be the main source within the testis (Carreau et al., 2003; Hess 2003). Estrogen receptors (ESR1/ESR2) are expressed within the testis and epididymis of multiple species, including humans and rats, indicating the male reproductive tract as a source and target for estrogen regulation (Carreau and Hess, 2010; Hess et al. 2011). The two receptors are located in Leydig cells and the efferent ductule epithelium. ESR1 is also expressed in Sertoli cells and germ cells of the rat testis, where the hormone appears to impact modulation of libido, erectile function, and spermatogenesis (Schulster et al., 2016). However, specifics regarding the potential effects of estrogen on spermatogenesis are still being defined.

Estradiol binds to the estrogen receptors widely distributed throughout the testis in both testicular cells and germ cells (Carreau et al., 2011). ESRl appears to regulate spermiogenesis while ESR2 influences spermatocyte apoptosis and spermiation, the release of elongated spermatids from the seminiferous tubule (Dumasia et al, 2016). The receptors have varying affinities to environmental estrogens, and multiple studies have attempted to pinpoint the exact role of the hormone in reproductive physiology with conflicting results. Estradiol treatment of hypogonadal mice induced increased testicular development (Ebling et al., 2000). Estradiol may also increase the stimulatory effects of FSH on testicular maturation in rats (Kula et al., 2001). Other evidence points to possible negative effects of estrogen within the testis. Elevated estradiol in the male testis of mice and rats may cause increased germ cell apoptosis and disrupt the

functions of Sertoli cells and Leydig cells (Walczak-Jedrzejowska et al., 2013; Yu et al., 2014; Leavy et al., 2017). There is a clear need to better understand how estrogen and estrogen-like compounds interact with and impact the male testis.

Given the potential effects of estrogen on spermatogenesis, dietary phytoestrogens have become a point of study in recent years. These phytoestrogens are often found in soy based products common to eastern cultures and are becoming increasingly prevalent in western culture. Among the most common phytoestrogens found in human diets and rat feed are the isoflavones genistein and daidzein (Degen et al., 2002). The structural similarities to estradiol (Fig. 2) allow phytoestrogens to bind estrogen receptors, particularly ESR2, and often with higher affinity (Lund et al., 2004; Sullivan et al., 2011). Multiple mammalian studies have demonstrated a disruption to reproductive function due to phytoestrogen exposure. Rodent diets containing high amounts of phytoestrogens appear to increase germ cell apoptosis (Assinder et al., 2007; Jefferson et al., 2012). These changes are often attributed to disruption of the HPG axis, such as decreasing basal FSH secretion and total FSH production (Arispe et al., 2013). This endocrine disruption can also affect testosterone levels. Exposure to phytoestrogens can cause an overall decrease in testosterone within the rat testis (Weber et al., 2001; Hancock et al., 2009; Napier et al., 2014). The sensitivity of specific stages in the spermatogenic cycle to endocrine disruption is also known to occur. The conversion of round spermatids between stages VII and VIII within Sprague-Dawley rats is highly testosterone-dependent, and manipulation of diet has been shown to impact spermatogenesis in a stage specific fashion (Kainz et al., 1988; O'Donnell et al., 1994; Gonzales et al., 2013).

While high isoflavone intake may reduce overall fertility and sperm production in humans, there appears to be no significant effects on sperm motility, sperm morphology or

ejaculate volume (Chavarro et al., 2008). Long-term exposure of dietary phytoestrogens did not appear to affect LH and androgen levels in mice, but did cause reduced proportions of haploid germ cells and affected androgen-response gene expression in Sertoli cells (Cederroth et al., 2010). The majority of studies using rat models focus on the pre-pubertal developmental effects of phytoestrogens (Casanova et al., 1999; Odum et al., 2001; Degen et al., 2002; Sherrill et al., 2010; Napier et al., 2014). However, the effects of manipulating phytoestrogen levels in adult mammalian males remains sparse and with variable results (Assinder et al. 2007; Trifunović et al. 2014).

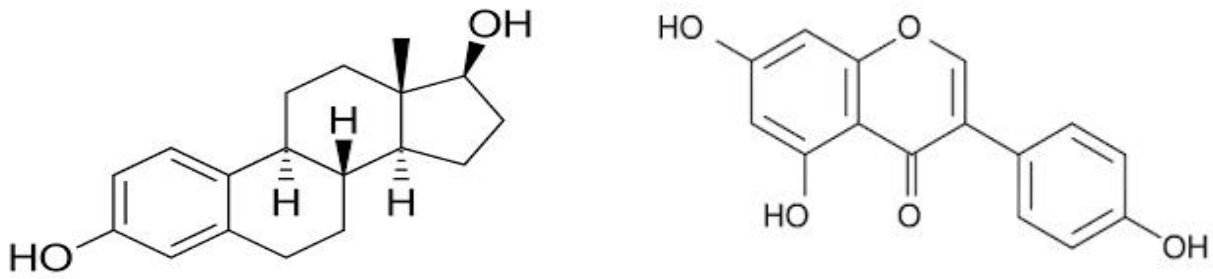

Figure 2: Comparison of Chemical Structure: 17β-estradiol (left) and genistein (right) commonly found in soy based diets.

Aging and Spermatogenesis

The effect of aging on spermatogenesis is also an area of recent interest given that a higher percentage of the population is delaying the time they attempt to conceive and have children. Human males experience a depletion of germ cells, reduced testosterone production, and lower motility of sperm with age (Sibert et al., 2014). Degeneration of the seminiferous tubule during stages VII-VIII in aged hamsters was accompanied with decreased spermatogonia proliferation (Bernal-Mañas et al., 2014). Other evidence supports a decrease in basement membrane thickness coinciding with a drop in germs cells leading to hypospermatogenesis in humans (Paniagua et al., 1987; Pop et al., 2011).

Recently, the role of Sertoli cells in maintaining spermatogonial stem cells has also received attention. Sertoli cells form a spermatogonial stem cell niche on the basement membrane to support the essential maintenance of stem cell populations in the testis. Furthermore, this stem cell niche has variable activity, with reduced replication of stem spermatogonia during stage VII-VIII of the seminiferous cycle (Johnston et al., 2011; Grasso et al., 2012). A measurable decrease in the Sertoli cell populations of the testes with age has been reported in humans, and this decrease correlates with diminished sperm production (Petersen et al., 2015).

Among the general population, approximately 15% of couples are unable to conceive and are thus considered infertile. Of these couples, the male is responsible or is a contributing factor in 40-50% of the cases. Male infertility results from improper testicular function, however the precise cause for testicular dysfunction is unclear in about 50% of cases. Previous work in our lab investigated hormone concentrations and sperm production in aging rat models (Clark and Pearl, 2014). The results showed that testosterone levels did not significantly decline, but

estradiol concentrations began dropping at 15 months of age which corresponds to an adult human roughly 47 years old. There was also a significant drop in sperm production after 15 months of age. This decline in sperm production was reduced in animals given supplemental estrogen during aging. These results suggest a role for estrogen in maintaining spermatogenesis and male fertility. Data also suggests that this time period (between 15 and 18 months of age) may be a target for disruption by phytoestrogens. The purpose of this study was to determine the effects of aging and dietary phytoestrogens on testicular function. Dietary phytoestrogens may negatively affect testicular function during aging, and removal of these endocrine disruptors would improve the age-related decline of spermatogenesis.

Chapter 3: Methodology

Animals and Experimental Design

Sprague Dawley rats are a well-established model for the study of male reproductive tract function and testicular aging. Rats have strong similarities to humans when compared to other animal models (Iannaccone and Jacob, 2009) and changes observed in human males during aging also occur in rats. Fifteen rats were acquired from Harlan Laboratories at nine months of age after breeding retirement and housed in the animal facility at Western Michigan University with approval of the WMU Institutional Animal Care and Use Committee (IACUC). Animals were assigned to one of three groups: 1) 15-month phytoestrogen diet; 2) 18-month phytoestrogen diet; or 3) 18-month phytoestrogen-free diet containing 0-20 mg/kg of phytoestrogens. Prior to 15 months of age, all animals were fed a standard rodent diet (Tekland 2014; Harlan) which contains 350-650 mg/kg of phytoestrogens. The most common isoflavones found in such diets are genistein and daidzein. At 15 months of age, one group of animals (n=5) was shifted to a phytoestrogen-free diet, one group of animals (n=5) was maintained on the diet containing phytoestrogens until 18 months of age, and one group of animals (n=3) were euthanized (Fig. 3). At 18 months of age, animals were euthanized for testis and blood sample collection. One testis from each animal was weighed, divided into two halves along the longitudinal axis and snap frozen. One half was used for determination of sperm production and the other half for hormone concentration analysis. The other testis was placed in bouins fixative for morphometric analyses. Samples were also collected from animals (n=3) sacrificed at 15 months of age. The 15-month group of animals originally contained 5 animals, but two animals died prior to 15 months.

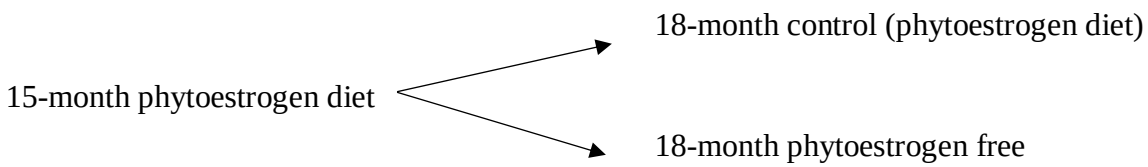

Figure 3. Experimental Outline

Daily Sperm Production

Daily sperm production (DSP) was determined using procedures similar to those previously published (Clarke and Pearl, 2014). Frozen testis samples were thawed and homogenized in 0.9% NaCl/0.05% Triton X-100 at room temperature using an OmniTip tissue homogenizer. The homogenate was brought to a total volume of 30 ml, stored at 4°C for twenty-four hours, and the number of homogenization-detergent resistant spermatids was counted using a hemocytometer. The hemocytometer is comprised of two sides containing 25 squares each. Five squares on each side were counted together and multiplied by five to account for the entire grid. This provided the number of elongated spermatids in the homogenate. The number of elongated spermatids counted, divided by the weight of testis homogenized provided sperm/gram of testis. Sperm/gram of testis provided a measure of efficiency for spermatogenesis. Sperm/gram multiplied by total testis weight equals the number of elongated spermatids. Daily sperm production was calculated as the total number of elongated spermatids per testis divided by 6.1 days, which is the amount of time for elongated spermatids to be found in the rat seminiferous tubule.

Morphological Analysis

Fixed testis samples were embedded in paraffin, cut into sections of approximately 5 μm thickness utilizing a Leica RM 2125 rotary microtome, and placed on superfrost plus slides. The sections were deparaffinized in citrisolv and hydrated in an alcohol series (100%, 95%, 70%, water). The resultant sections were then stained with periodic acid Schiff (PASH) reagent, and counter stained with hematoxylin. Sections were visualized and image captured at a 20x magnification using an Eclipse Ni-U microscope capable of bright field microscopy with digital camera and NiS imaging software. The imaging allowed analysis of seminiferous tubule diameter and epithelial height. Seminiferous tubule diameters, a marker of spermatogenic capabilities, were measured from round tubules and averaged based on four measurements in each tubule. As the actual site of germ cell development, the height of the epithelium could provide more specific insight into the spermatogenic capacity within the tubules. Five measurements from the basement membrane to the apical surface were averaged for each imaged tubule. All tubules were also examined to determine the stage within the spermatogenic cycle. Approximately 75 tubules were examined in each of the three groups.

The number of Sertoli cells was determined by labeling a Sertoli cell specific transcription factor, GATA-4. Tissue sections were deparaffinized and hydrated as described above. Antigen retrieval was performed by placing slides in Coplin jars in a steamer and heating to 93°C for five minutes before the slides were cooled to room temperature. Tissues were blocked with goat serum for 20 minutes and incubated overnight at 4°C with rabbit anti-human GATA-4 (1:100; sc-1237; Santa Cruz Biotechnology). Following primary antibody incubation, sections were incubated with goat anti-rabbit biotinylated secondary antibody followed by an avidin-biotin HRP complex (ABC reagent, Vector). Immunostaining was visualized by

incubating tissue slides in NovaRed Chromogen for 7 minutes followed by counterstaining with ImmunoMaster hematoxylin for 30 seconds. The sections were then dehydrated in an alcohol series and coverslipped.

The number of Sertoli cells was estimated using the physical dissector method similar to that previously described by Sterio (1984) and Gundersen (1986). Images of the same tubule from two serial sections were taken using an Eclipse Ni-U microscope and NiS imaging software. A measurement frame was placed on each picture in the same location. The area of the measurement frame was the same on both pictures placed side to side to allow Sertoli cells present in one image but not both to be counted. The number of cells per paired sections and the area of the measurement frame used were recorded. Approximately 200 Sertoli cells per animal were counted. The number of Sertoli cells per unit volume was determined by first adding the total right and left side counts together, dividing by the total area and multiplying by 10. This number was then multiplied by the testis volume to get the Sertoli cell number. Testis volume is the weight of the testis multiplied by 10^{12}.

Hormone Analysis

Blood and testicular concentrations of testosterone and estradiol were determined by competitive ELISAs (Enzo Life Sciences). For testicular concentrations, samples were thawed at room temperature and minced into small pieces. Pieces were homogenized in buffer, and subsequently centrifuged to remove any large portions of unsolubilized tissue. For testosterone, serum and tissue homogenates were mixed with a steroid displacement reagent for 15 minutes and diluted in assay buffer to fall within the range of the standard curve. Testosterone samples were assayed in triplicate. The plates were analyzed using an Epoch Microplate Spectrophotometer and Gen5 statistical software. For estradiol, serum and testis samples were

extracted in diethyl ether. After freezing the aqueous layer in liquid nitrogen, the ether was allowed to evaporate at room temperature overnight before the estradiol was reconstituted in assay buffer. Reconstituted samples were run in triplicate in the ELISA. Total protein in testis homogenates was measured using a BCA protein assay. Total protein was used to normalize testicular steroid values.

Statistical Analysis

While five animals were collected in each of the 18 month groups, data from only four animals in each group was used for analysis. One animal in the phytoestrogen group was excluded because it was a unilateral cryptorchid. One animal in the phytoestrogen-free group was excluded from analysis because it displayed non-age related tubular atrophy/degeneration (Creasy et al., 2012). Data was analyzed by two-way ANOVA using GraphPad Prism statistical software and values reported as means ± SEM. The data was tested for normality to ensure the assumptions of the ANOVA were satisfied. If the overall ANOVA was significant, differences between ages and treatments were determined using Tukey's multiple comparison test. Differences were considered significant if $p \leq 0.05$.

Chapter 4: Results

Sperm Production

The average estimated body weight of the animals was changed as result of the shifted diets. Rats raised for 15 months on a standard diet had an average weight of 560.7 ± 14.34 grams. The weights of animals maintained on the standard diet until 18 months averaged 550.2 ± 6.48 grams. Rats shifted to a phytoestrogen-free diet at 15 months had an average weight of 493 ±11.82 grams at 18 months (Fig 4). Animals switched to phytoestrogen-free rat feed had significantly lower body weights than animals only exposed to standard rat feed. The average testis weight was similar between the three age groups (Fig. 5). The 15-month old animals had an average testis weight of 4.6 ± 0.26 grams. The 18-month rodents raised on standard and phytoestrogen-free diets had average testis weights of 4.29 ± 0.08 grams and 4.23 ± 0.08 grams, respectively. The number of detergent-resistant spermatids within homogenized testis tissue were counted. The number of spermatids counted divided by the weight of tissue homogenized yields sperm per gram of tissue. At 15 months, the average sperm/gram was calculated to be 101.16 ± 5.52 x 10^6. The average sperm/gram of testis of both the 18-month standard rodent diet and 18-month phytoestrogen-free diet groups were numerically similar (80.47 ± 4.64 x 10^6 and 87.32 ± 1.7 x 10^6 respectively) and lower than the 15-month group. The average sperm/gram of testis of the 18-month group raised on standard rodent feed was significantly reduced when compared to the 15-month group (Fig. 6). There was, however, no significant difference in sperm/gram between the 15-month group and the group raised for three months on a phytoestrogen-free diet.

The estimated total number of elongated spermatids was calculated by multiplying sperm/gram of testis by the total weight of both testes from the animals. Elongated spermatids are the mature sperm that will be released from the testis and have the potential to fertilize an

egg. At 15 months of age, the number of elongated spermatids was $462.89 \pm 10.51 \times 10^6$. Rats raised to 18 months on a standard rodent diet had $345.1 \pm 20.45 \times 10^6$ elongated spermatids, while the 18-month phytoestrogen-free animals had $368.74 \pm 6.13 \times 10^6$ elongated spermatids. Both 18-month groups showed a significant reduction in the number of elongated spermatids when compared to the 15-month old animals. There was no significant difference based on diet (Fig. 7).

Daily sperm production (DSP) values continued the trend of decreased production with age and no difference with diet (Fig 8). At 15 months, the rat average daily sperm production was $75.88 \pm 1.72 \times 10^6$. The 18-month old rats raised on a standard diet had an average DSP of $56.57 \pm 3.35 \times 10^6$. Animals raised to 18 months on a phytoestrogen-free diet had DSP values at $60.45 \pm 1.01 \times 10^6$.

Testicular Morphology

Cross sections of the testis were stained with PASH and analyzed using bright field microscopy to identify any structural abnormalities that could contribute to altered spermatogenic efficiency in the animals. Visual observations of the seminiferous tubules did not appear to show any gross differences between the three age groups.

Seminiferous tubule diameters serve as an indicator for spermatogenic capacity, and reduced tubule diameters might explain reductions observed in spermatogenesis. The average tubule diameter at 15 months was 288.5 ± 1.36 μm. At 18 months, animals maintained on a standard rodent diet had tubule diameters of 294.3 ± 7.37 μm whereas rats shifted to a phytoestrogen-free diet had tubule diameters at 292.9 ± 3.81 μm. Average seminiferous tubule diameters were not significantly different based on age or diet (Fig. 9). The seminiferous

epithelium is the specific region within the tubule where germ cell development occurs. Thus, the epithelial height was also separately measured as a parameter for effective sperm production. Within each age group, the tubule diameters and epithelial heights were examined at specific spermatogenic stages. Stages were consolidated into three groupings (stages I-VI, stages VII-VIII, stages IX-XIV) based on relative similarities of the tubules. Measurements of epithelial height at 15 months averaged to 82.78 ± 5.6 μm, while the 18-month groups exposed to standard or phytoestrogen-free diets had average values of 84.13 ± 1.74 μm and 89.38 ± 1.94 μm, respectively. The average epithelial heights were not significantly different by age or diet (Fig. 10). Average seminiferous tubule diameters did not show any significant changes when analyzed by spermatogenic stage (Fig. 9). However, there was a significant reduction in epithelial height for both 18 month groups between tubules in stages 1-6 to tubules in stages 7-8 (Fig. 10). This marks the transition when the tubule is preparing to release spermatozoa into the lumen.

Sertoli cells are essential to proper testicular function, and evidence suggests reduced Sertoli cell numbers can negatively impact spermatogenesis. The 15-month animals had an estimated 78.29 ± 6.74 x 10^6 Sertoli cells. Rats raised to 18 months on standard rodent feed had Sertoli cell counts of 64.48 ± 0.75 x 10^6, while animals shifted to a phytoestrogen-free diet had counts of 60.96 ± 0.88 x 10^6. Sertoli cells were significantly reduced with age, but no difference was detected between the standard rodent diet and the phytoestrogen-free rat feed (Fig. 11). Thus, reduced numbers of Sertoli cells may account for the reduced sperm production.

Hormone Levels

Both testis and serum hormone levels were measured utilizing competitive ELISAs. At 15 months, testosterone in the testis was 1.0 ± 0.3 ng/mg protein. The 18-month group fed

maintained on a standard rodent diet had 0.52 ± 0.08 ng/mg protein of testosterone. Animals fed phytoestrogen-free food from 15 to 18 months showed testosterone levels of 0.62 ± 0.15 ng/mg protein (Fig. 12). The serum levels of testosterone at 15 months were 7.63 ± 1.8 ng/ml. At 18 months, the serum testosterone was 5.75 ± 0.35 ng/ml for standard feed and 9.2 ± 1.45 ng/ml for phytoestrogen-free feed (Fig 13). Testis and serum testosterone concentrations appeared lower with age. However, these values were not significantly different with age or by dietary phytoestrogens.

Estradiol is also present within the testis and is known to be critical for maintaining spermatogenesis. Testis estradiol at 15 months was found to be 18.3 ± 115.4 pg/mg protein, 10.4 ± 2.3 pg/mg protein at 18 months on a normal diet, and 10.8 ± 1 pg/mg protein at 18 months with a phytoestrogen-free diet (Fig 14). Serum estradiol was 58.9 ± 8.69 pg/ml at 15 months, 38.5 ± 1.6 pg/ml at 18 months and a standard diet, and 35.98 ± 2.7 pg/ml at 18 months without dietary phytoestrogens (Fig. 15). There was a significant decrease in testicular estradiol with age, but no difference due to dietary phytoestrogens (Fig. 14). Serum estradiol was significantly reduced between 15 months and both the 18-month groups. No significant difference in serum estradiol levels was present between the two diets (Fig. 15).

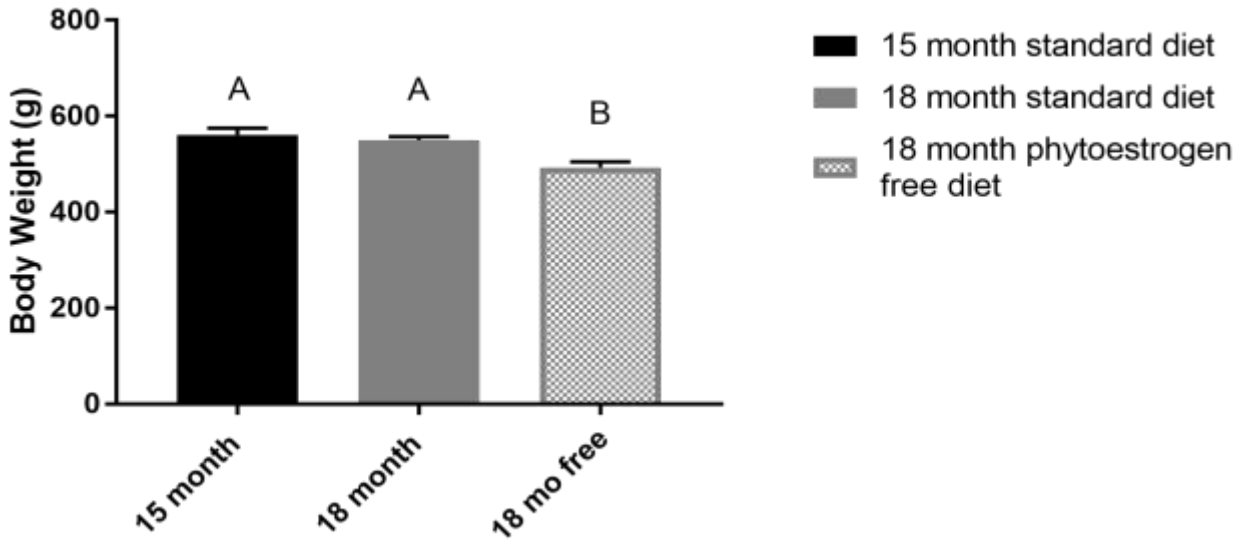

Figure 4: Body Weight
The average body weight of animals switched to a phytoestrogen-free diet was significantly lower than the weights of animals raised to 15 or 18 months on a standard rodent diet. Animals groups raised on the same diet had similar average body weights.
Columns labeled with different letters (A vs. B) indicate a significant difference based on $p \leq 0.05$.

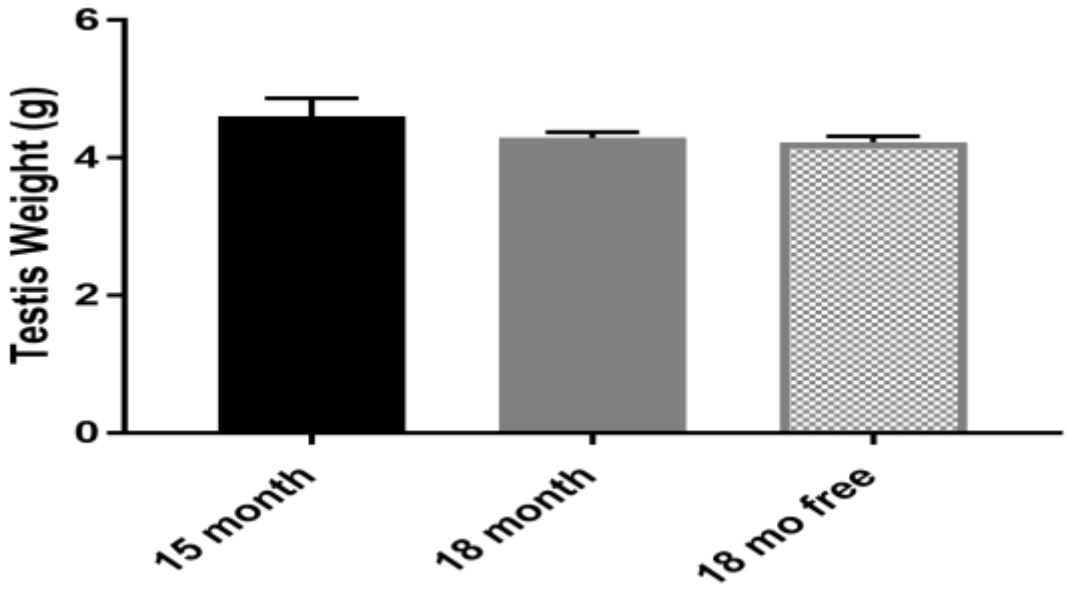

Figure 5: Paired Testis Weight
The average paired testis weights of the three groups were not significantly different.

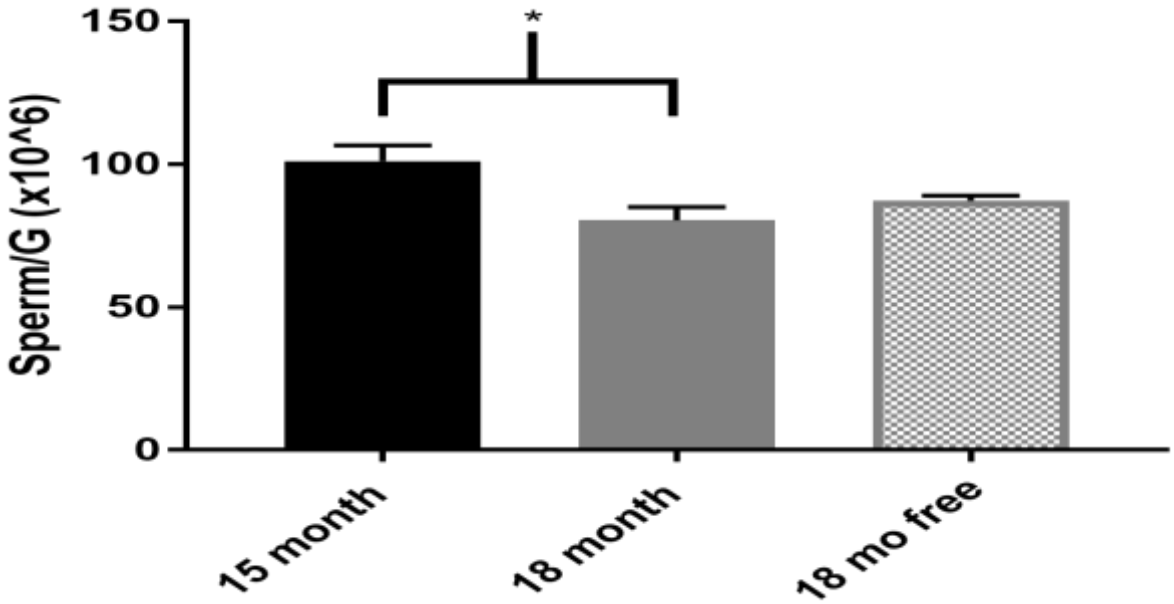

Figure 6: Sperm per gram of Testis
At 15 months, the sperm per gram of testis was significantly higher when compared to animals raised to 18 months on a standard diet, but not those raised on a phytoestrogen-free diet. While values were reduced with age, the two groups raised to 18 months were not significantly different suggesting that diet did not impact spermatogenesis.
Columns labeled with an * indicate a significant difference based on
$p \leq 0.05$.

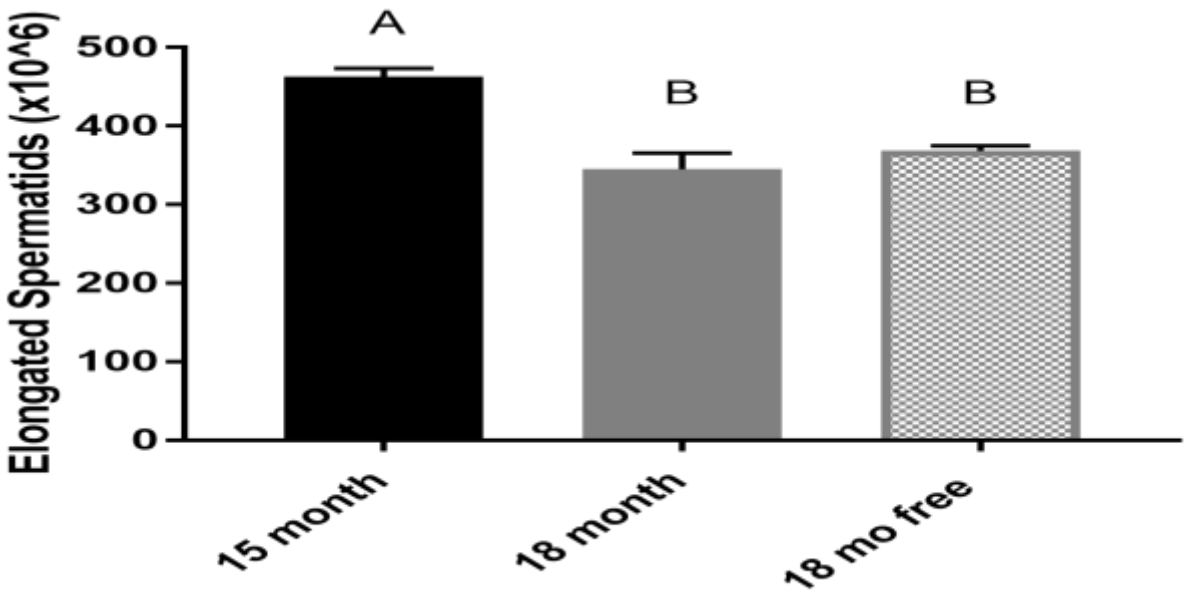

Figure 7: Elongated Spermatids (paired testis)
The number of elongated spermatids in both testes at 18 months was significantly lower than animals at 15 months. No difference was observed between the two 18-month groups. Columns labeled with different letters (A vs. B) indicate a significant difference based on $p \leq 0.05$.

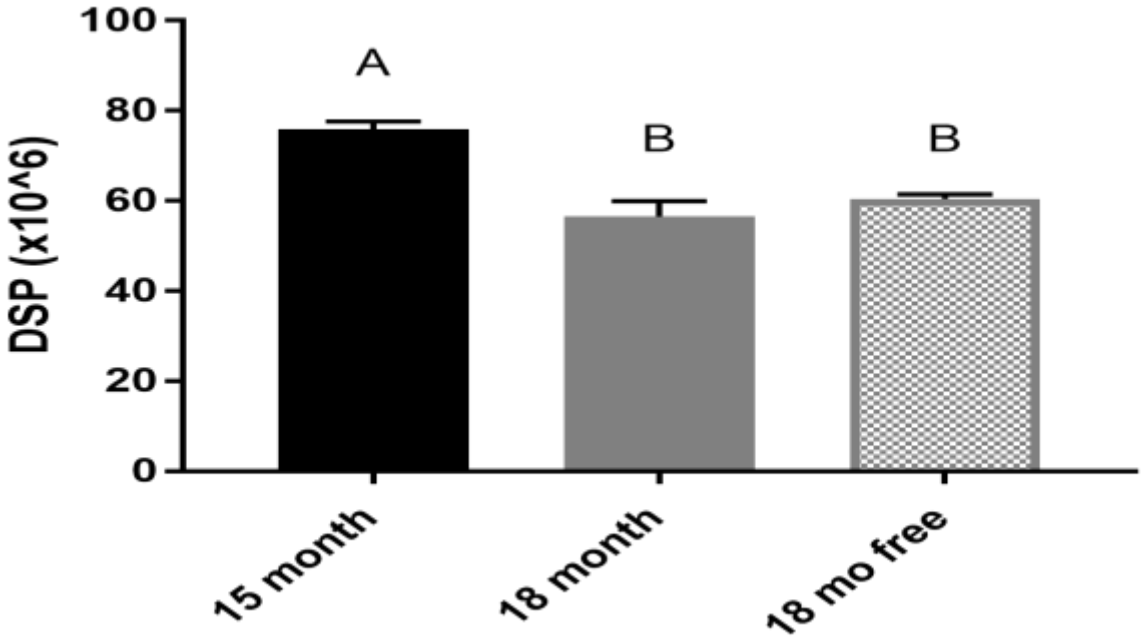

Figure 8: Daily Sperm Production (paired testis)
There was a significant reduction in sperm production between the 15-month animals and those raised to 18 months on either the standard or phytoestrogen-free diets. Daily sperm production was not altered at the 18 months based on the animal's diets.
Columns labeled with different letters (A vs. B) indicate a significant difference based on $p \leq 0.05$.

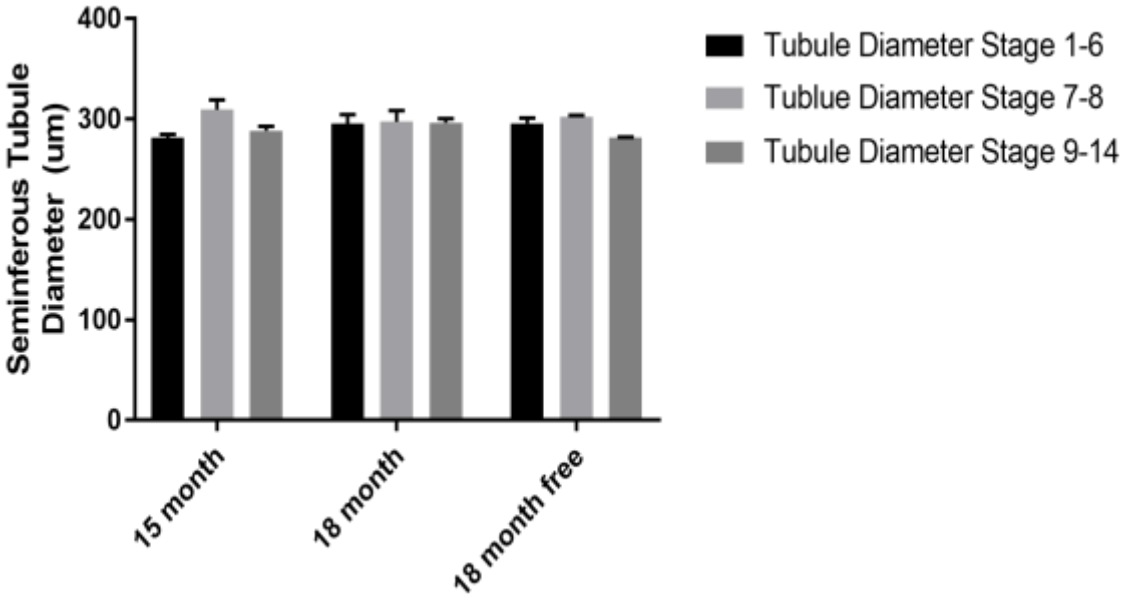

Figure 9: Seminiferous Tubule Diameter
Tubule diameters were not significantly different based on age or diet. Additionally, analyzing diameters based on spermatogenic stage within age groups did not yield any significant differences.

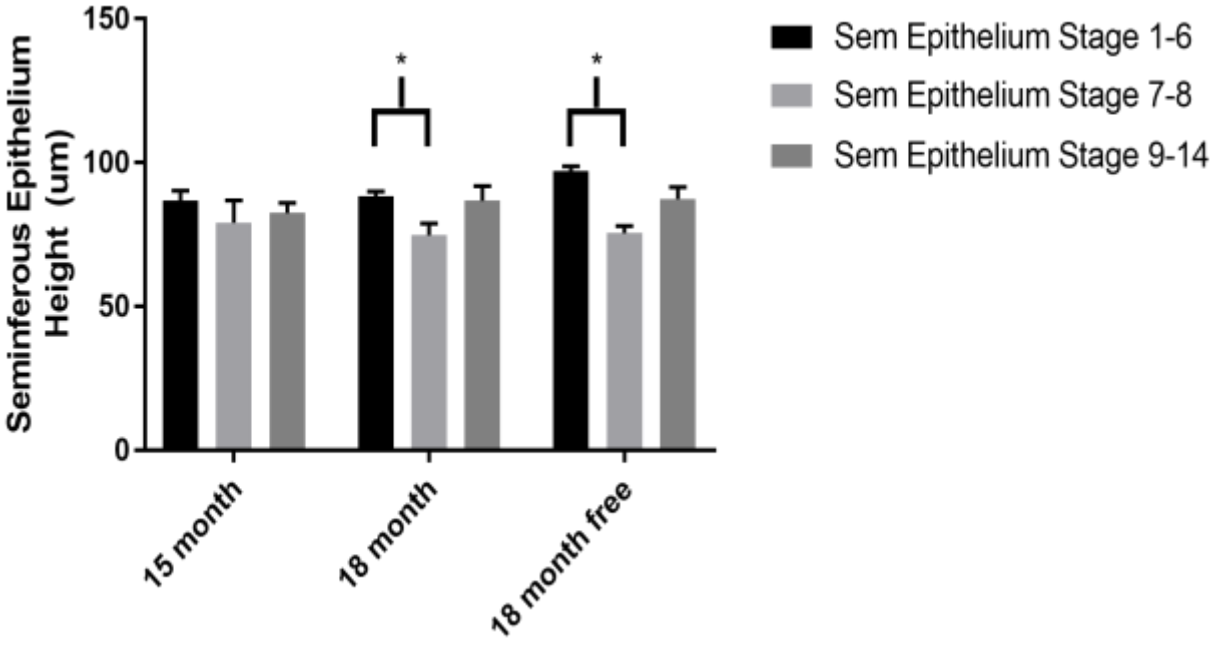

Figure 10: Seminiferous Epithelial Height
The epithelial height was not significantly different based on age or dietary phytoestrogens. When factoring in spermatogenic stage, epithelial height was reduced in the 18-month groups between stages 1-6 and stages 7-8.
An * signifies a significant difference between stages within a specific age group based on $p \leq 0.05$

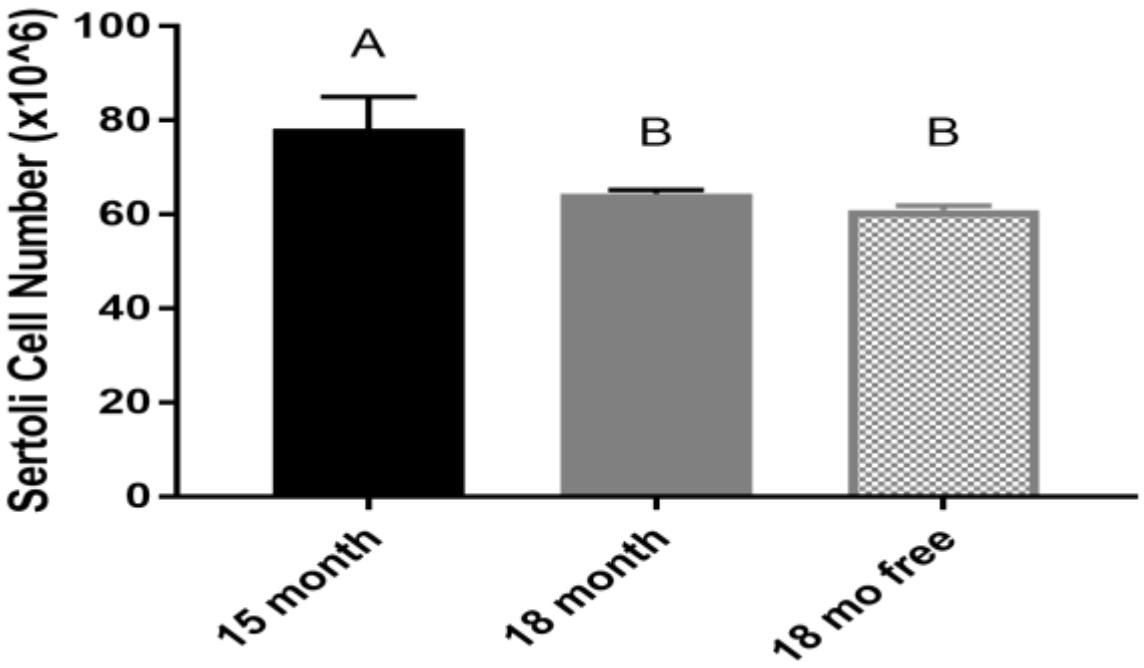

Figure 11: Sertoli Cell Counts
The number of Sertoli cells was significantly diminished when comparing the 15-month animals to the 18-month groups. There were no differences due to dietary phytoestrogens. Columns labeled with different letters (A vs. B) indicate a significant difference based on $p \leq 0.05$.

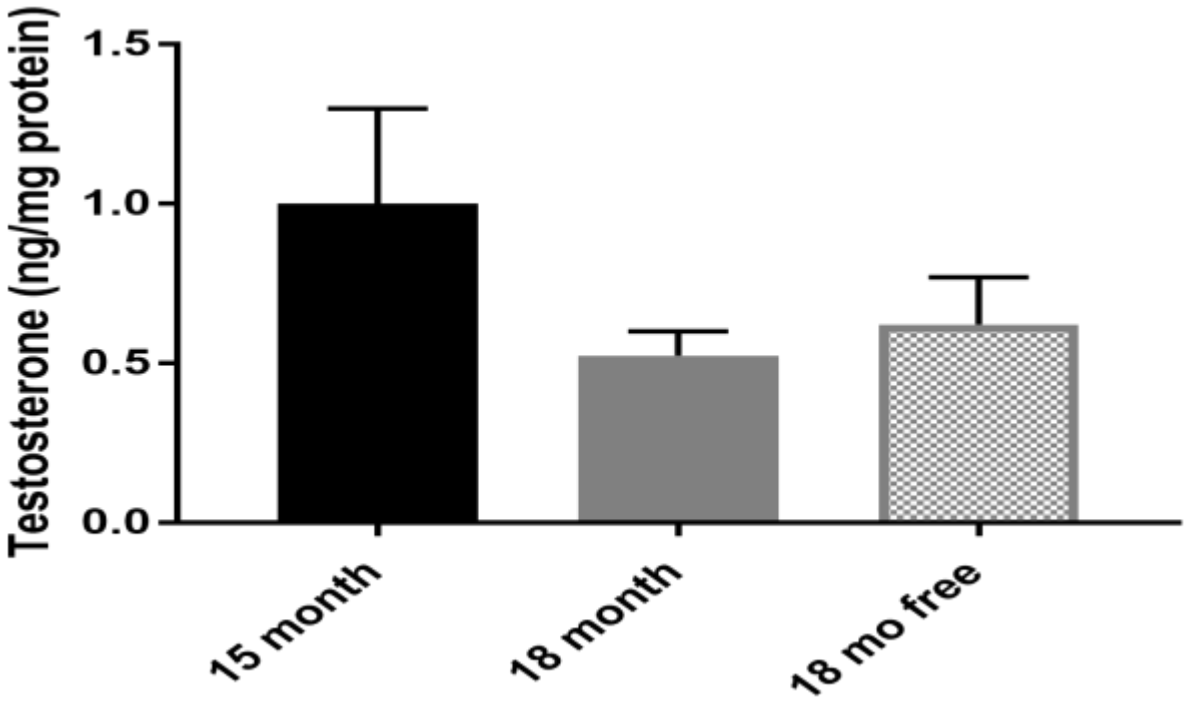

Figure 12: Testis Testosterone
Testosterone levels were not significantly different within the testis due to age or dietary phytoestrogens.

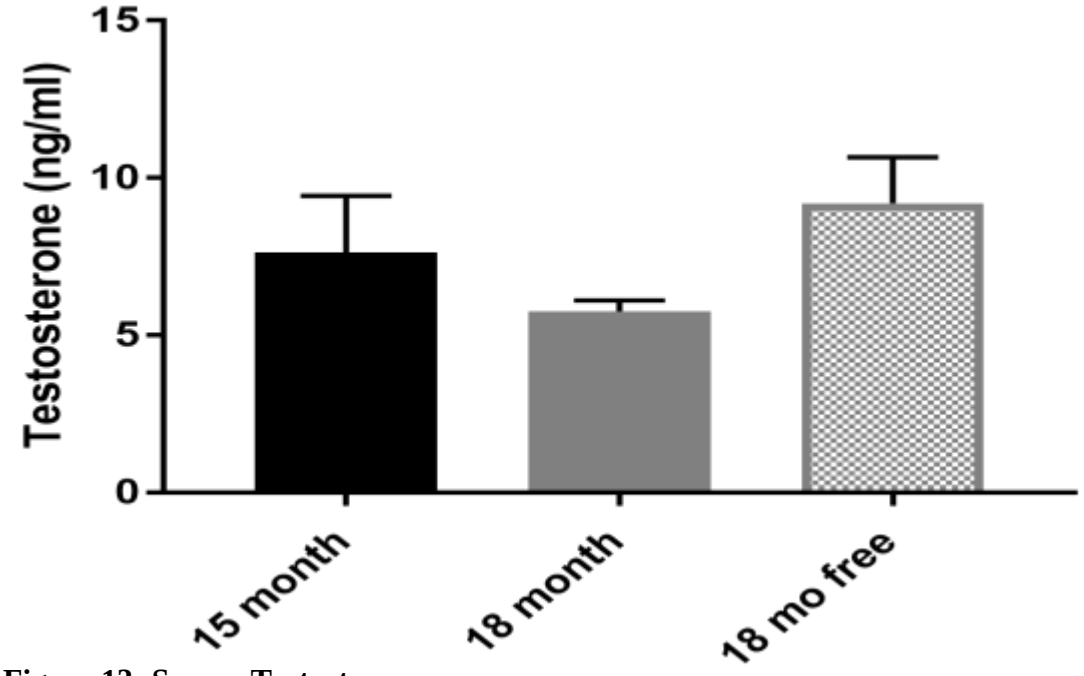

Figure 13: Serum Testosterone
Testosterone levels within the serum were not significantly different when comparing age or diet.

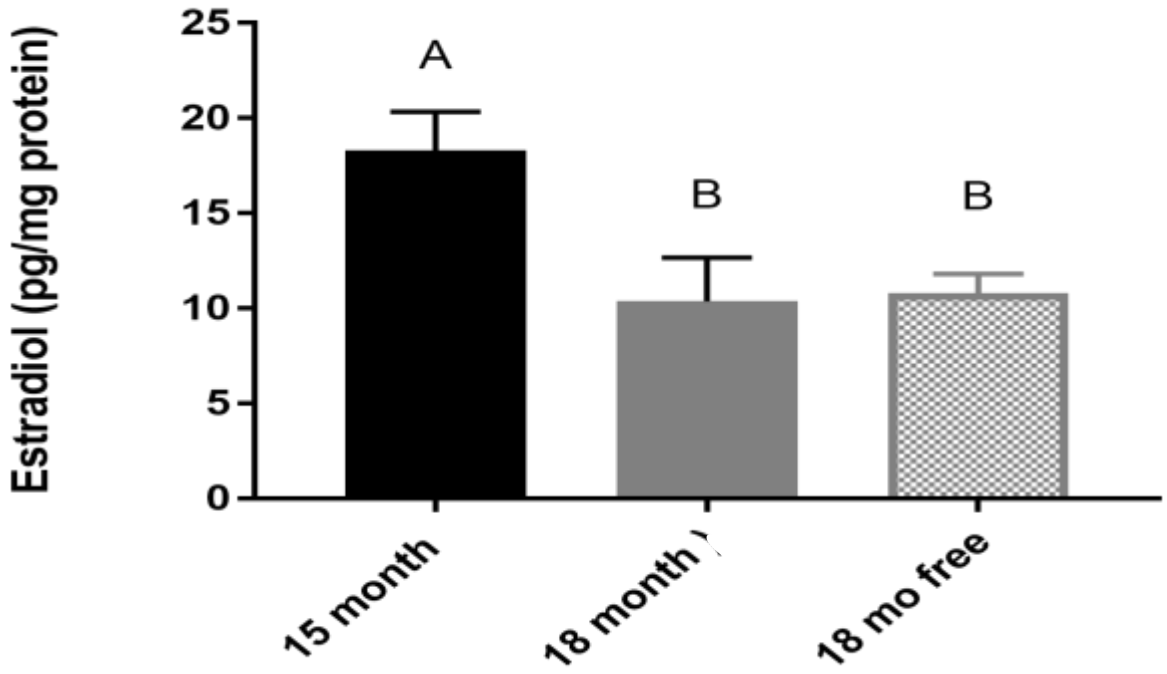

Figure 14: Testis Estradiol
Testicular estradiol values were reduced when comparing the 15-month rats to those raised to 18 months on a standard diet (p=0.044). Rats shifted to a phytoestrogen-free diet also showed diminished estradiol in the testis (p=0.054). There was no significant difference between the 18-month age groups.
Columns labeled with different letters (A vs. B) indicate a significant difference.

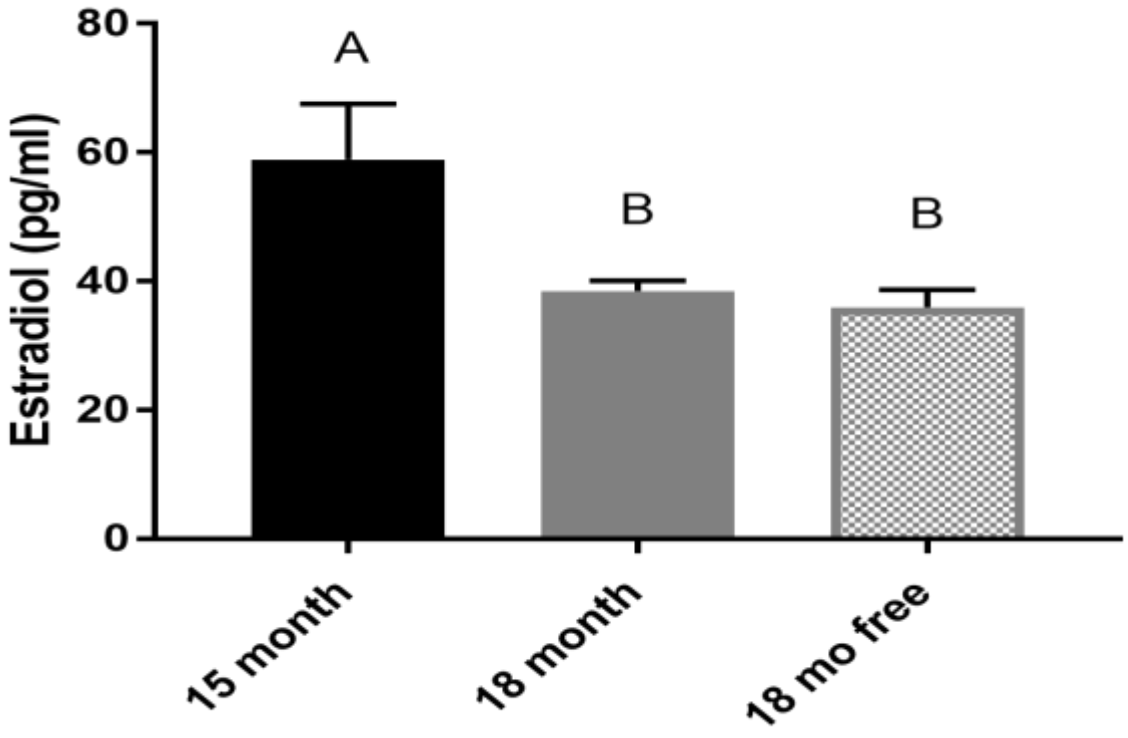

Figure 15: Serum Estradiol
Estradiol was significantly reduced between 15 and 18 months regardless of diet. The 18-month groups were not significantly different from each other based on diet.
Columns labeled with different letters (A vs. B) indicate a significant difference based on $p \leq 0.05$.

Chapter 5: Discussion and Conclusions

The purpose of this study was to investigate the effects of dietary phytoestrogens on testicular function in aging rats. It was hypothesized that the high phytoestrogen content of standard rodent diets contributed to reduced testicular function, and removal of these isoflavones would improve the age-related decline of spermatogenesis. This study demonstrated that sperm production declined with age. Both estradiol concentrations and Sertoli cell numbers also declined with age. This suggests estradiol and/or Sertoli cells having a role in maintaining sperm production with age. Altered diets in the 18-month animals did not appear to affect the age-related changes observed. This is the first study to characterize the effects of endocrine disruption during aging in adult male rats.

At 18 months of age, rats raised on either a phytoestrogen-free or phytoestrogen-containing diet showed significant loss of sperm production compared to 15 months of age. Although average sperm/gram was not significantly reduced for rats raised on a phytoestrogen-free diet, the overall average daily sperm production decreased. When accounting for the total testis weight of the animals, there was a loss of elongated spermatids/paired testis weight similar to that experienced by the 18 month animals raised on standard rodent feed. Given that both 18-month groups were not significantly different from each other suggests that the loss of sperm production was independent of diet, and therefore a result of advanced aging. This change in daily sperm production is consistent with losses previously observed in lab rats (Clarke and Pearl, 2014). Possible causes for reduced sperm numbers are lower hormone levels and Sertoli cell numbers, altered HPG-axis, and morphology (Gunes et al., 2016; Sibert et al., 2014).

Reduction in fertility may be caused by changes in testis morphology, specifically the seminiferous epithelium. Rats aged to 24 months can exhibit epithelium degeneration in up to

85% of total tubules (Wright et al., 1993). This study only examined rats between the ages of 15 and 18 months. When only considering age, changes in either seminiferous tubule diameter or height/thickness of the seminiferous epithelium were not observed. However, when analyzing the seminiferous cycle of the tubules, 18-month old animals had reduced epithelial heights in stages VII-VIII when compared to stages I-VI. Stages I-VI contain germ cells proceeding towards maturation and eventual release into the lumen. Germ cells in stages VII-VIII are localized near the lumen in preparation for release into the reproductive tract. Reduced epithelial heights are often associated with reduced sperm production because of increased germ cell apoptosis (Wang et al., 1999). Germ cells, like any cell in the body, follow a highly regulated apoptotic pathway that can become less controlled with aging. Inappropriate apoptosis can be a result of poor hormonal control or lack of communication between germ cells and Sertoli cells (Shukla et al., 2012). There may exist a genetic disposition towards developing infertility, but environmental effects are equally likely to impact spermatogenesis. Determining the unique combination of these factors is the challenge of diagnosing infertility (Kolesnikova et al., 2015). Previous studies have indicated decreased spermatogonial proliferation in animals during stages VII-VIII that may be related to Sertoli cell activity (Johnston et al., 2011; Bernal-Mañas et al., 2014). It is also worth noting that studies of rats with more advanced aging show further loss of Sertoli cells leading to regressed tubules devoid of germ cells. The loss is largely due to a breakdown of the blood-testis barrier (Levy et al., 1999).

Sertoli cells are among the most essential components of reproductive physiology in males. The cells support germ cell development throughout the entire seminiferous epithelium and help to maintain the structural integrity of the blood-testis barrier (Franca et al., 2016). This blood-testis barrier prevents leukocytes or antibodies from encountering developing germ cells

and triggering an autoimmune response (Johnson and Setchell, 1968). Sertoli cell numbers directly correlate with the efficiency of sperm production (Orth et al., 1988). The loss of Sertoli cells in conjunction with reduced sperm numbers seems to confirm this relationship. Fewer Sertoli cells may interfere with the stem cell niche leading to reduced spermatogonial renewal at the basement membrane of the seminiferous tubule. A breakdown of the blood-testis barrier due to reduced Sertoli cell activity could also explain the observed loss of seminiferous epithelium seen in the 18-month old rats. This will need to be further explored in detail in future studies.

The most likely issue related to Sertoli cell loss is diminished communication with germ cells (Syed and Hecht, 2001; Rosenstrauch et al., 1994). Control of this communication is a result of proper hormone circulation within the HPG-axis. Disruption of testosterone and FSH would prevent proper Sertoli cell function. This would likely increase germ cell apoptosis and cause the environment within the seminiferous epithelium to become less hospitable to germ cell development. There was a downward trend in testosterone concentrations with age, but the change was not significant within the 15 to 18 month time period. The lack of significance is likely a consequence of the small sample size used in this study. There was a high level of variability within the age groups when measuring testosterone. Reduced testosterone in males is also very gradual, so observations of rats over a longer time scale could demonstrate a more significant decline (Schill, 2003). The fact that testosterone levels remained unchanged at 18 months despite dietary changes suggests that endocrine disruption via testosterone did not impact spermatogenesis. Were that the case, higher sperm counts in the phytoestrogen free animals would have been observed.

The diminished sperm counts were accompanied with lower Sertoli cell numbers and estradiol values in both groups of 18 month animals. Estrogen receptors are present within

Sertoli cells (Lin et al., 2014) and estradiol is known to enhance Sertoli cell proliferation (Yang et al., 2015). Studies examining ESR1 and ESR2 determined they have an important role in early development to establish Sertoli cell populations. There has also been an association with 17beta-estradiol and regulation of apoptotic pathways in rat Sertoli cells, specifically when binding ESR2 (Dumasia et al., 2016). Estradiol can modulate nuclear transcription to trigger either proapoptotic or antiapoptotic genes (Royer et al., 2012). Estradiol can also modulate the expression of specific ion transporters, and higher estradiol levels can disrupt Sertoli cell metabolic function. (Martins et al., 2013; Bernardino et al., 2016). A majority of studies regarding estradiol's role in Sertoli cells concern early development. This study examined changes in fertility in aging adult rats. It is possible that depleted estradiol levels with age cause a drop in Sertoli cells, possibly due to increased apoptosis caused by reduced regulation via estrogen receptors.

Previous studies showed phytoestrogens have caused endocrine disruption of male reproduction. High isoflavone intake may cause lower sperm concentrations, including increased germ cell apoptosis, abnormal semen quality and hormone levels in adult male rodents and humans (Assinder 2007; Chavarro et al., 2008; Mínguez-Alarcón 2015). Similarly, infant mammals exposed to soy rich diets experience reduced fertility in adulthood (Cederroth 2010; Liu et al., 2012; Napier et al., 2014). However, isoflavone intake at levels normally seen in adults (2.14 mg/day) has not shown serious adverse effects (National Toxicology Program). The purpose of this study was to test if reproductive physiology could improve in aging animals after removing phytoestrogens from the diet. Animals switched to a phytoestrogen-free diet did not experience significant changes in fertility. This suggests that the phytoestrogen content of

standard adult rat feed does not cause significant endocrine disruption to diminish sperm production.

Phytoestrogens are known to impact is estradiol activity in the male reproduction system. Isoflavones can bind estrogen receptors with increased affinity when compared to 17beta-estradiol found in the body (Lund et al., 2004; Sullivan et al., 2011). Normally, estradiol acts to maintain fertility, and it was previously demonstrated that injection of estradiol between 15 and 18 months of age could diminish declining sperm counts in rats(Clark and Pearl, 2014). Estradiol has also exhibited inhibitory effects emphasizing the level of estradiol sensitivity of reproductive mechanisms within the testes (Schulster et al., 2016). Based on previous observations of the sensitivity to estrogen treatment in rats aged 15 to 18 months, removal of phytoestrogens could have a positive effect on hormone levels during this period. Phytoestrogens are capable of binding aromatase enzyme or even blocking expression of the gene (Lephart, 2015). Reduced aromatase would then prevent conversion of testosterone to estradiol. This study found no difference in estradiol levels at 18 months between the two diets. Results did reveal a decrease in estradiol with age that coincided with reduced sperm counts. Taken together, these results show that removing phytoestrogens from rodent diets does not improve subfertility with age. The results do however further emphasize the relationship between declining estradiol levels in the testis and reduced fertility.

Analysis of testosterone showed that hormone levels in the testis and in serum did not decline with age, thus excluding testosterone as a cause for reduced fertility in this study. Testosterone has the most direct role of maintaining fertility within mammalian males. Deficiency in either production or action of the hormone will certainly cause infertility. This is true for excessively high or low levels of testosterone in males (Kliesch, 2010). Complete

infertility may often be a result of disrupted testosterone levels, but subfertility due to aging does not appear to be a consequence of reduced testosterone. With regards to phytoestrogens, the results are consistent with previous findings. Given that testosterone levels were not different at 18 months suggests that standard diet levels do not negatively affect testosterone production.

These results confirm that removing phytoestrogens from rat diets will not significantly improve results in reproductive studies. There is also strong evidence supporting the causative agents of reduced fertility with aging. Alongside decreasing sperm counts was a loss of Sertoli cells and estradiol. Despite a marked drop in spermatogenic efficiency, a decline in testosterone was not observed. More likely, the loss in fertility was result of decreased support from Sertoli cells, and not a disruption of the HPG-axis. Results from this study also reinforce the idea that estradiol is essential for maintaining fertility in adulthood.

Subfertility with aging may be due to diminished Sertoli cell counts, caused by decreasing estradiol levels. This study has demonstrated reduced fertility with age in rats raised for three months on either a phytoestrogen-free or standard diet. The time period between 15 and 18 months was previously observed to be highly sensitive towards treatment with estradiol (Clarke and Pearl, 2014). Mammals are highly sensitive to estradiol in infancy while establishing Sertoli cell populations (Kula et al., 2001). The loss of estradiol in adulthood could serve as the impetus to increased Sertoli cell apoptosis, and eventual loss of germ cell populations. Issues of infertility in adulthood could thus be the consequence of excessively low estradiol levels. It is worth noting however that other evidence has shown that too much estradiol may have a negative impact on fertility as well (Walczak-Jedrzejowska et al., 2013; Yu et al., 2014; Leavy et al., 2017).

Future studies should aim to identify how estradiol and Sertoli cells affect spermatogenesis with age. Specifically, what mechanisms essential for spermatogenesis are impacted in adulthood. An area of interest could be analysis of how estradiol interacts with Sertoli cells in childhood, and how changes to this interaction may lead to reduced Sertoli cells in adulthood. Other studies could use this model to elucidate how the seminiferous tubule changes with age, and whether germ cell apoptosis is increased in aged mammalian seminiferous tubules.

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