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Effects of Dietary Phytoestrogens on Spermatogenesis and Male Reproductive Tract in Mice

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The signatories of the committee below indicate that they have read and approved the thesis of Trenton Allen Dalm in partial fulfillment of the requirements for the degree of Master of Health Sciences in Biomedical Science.

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Abstract

Increases in infertility over the past 50 years are linked to environmental exposures to endocrine disrupting chemicals (EDCs). One type of EDC is phytoestrogens, commonly found in soy-based diets and foods such as cauliflower, tofu, and broccoli. Phytoestrogens may bind estrogen receptors and have positive or negative effects on receptor signaling. The developing male reproductive tract is potentially susceptible to endocrine disruptors leading to changes in adult reproductive function and possibly infertility. We hypothesized that dietary phytoestrogen exposure would have a dose dependent effect with the high diet yielding the lowest sperm count and worst performance compared to the medium and low phytoestrogen diet. Male C57 mice were fed a diet containing a low, medium or high concentration of phytoestrogens during pubertal development. Testicular and epididymal sperm were counted to determine sperm production and transit times. Differences between the groups were determined by ANOVA using GraphPad Prism statistical analysis software. Daily sperm production (DSP) was not significantly different between groups, however DSP in the high diet group appeared to trend lower. Serum follicle stimulating hormone (FSH) was lowest in the high group indicating changes in pituitary hormone function. Intratesticular testosterone concentration was significantly increased in the high group, indicating a direct effect on the testis. Epididymis weight was increased in the high group suggesting changes in tissue development. Caudal transit time was the longest in the high group, a factor that could negatively affect sperm development. These results suggest that high levels of dietary phytoestrogens may alter pubertal development and sexual maturation in mice.

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Chapter 1: Introduction

A global increase in prevalence of infertility over the past 50 years has been linked to factors such as poor diet, alcohol and tobacco consumption as well as environmental exposures to endocrine disrupting chemicals such a pesticides, bisphenol-A and phthalates (Agarwal et al., 2021). One type of these chemicals, called phytoestrogens, are found commonly in soy-based diets and foods such as cauliflower, tofu, broccoli and legumes, and are similar to estrogen in structure. Phytoestrogens may bind to estrogen receptors and have either positive or negative effects, making them potential endocrine disrupting compounds.

Excess estrogen has also been shown to decrease sperm production, maturation and motility, all vital factors of male fertility (Li et al., 2001, O'Donnell et al., 2001, Sharpe, 1998). Dietary phytoestrogen exposure yielded similar results to excess estrogen in many studies. Oral gavage of a common phytoestrogen called genistein in Wistar Han rats from gestation until adulthood (10 mg/kg/day) yielded smaller litter sizes and significantly decreased sperm production (Eustache et al., 2009). CD-1 mice supplemented with dietary soy from gestation until adulthood yielded a 21% decrease in litter size and 25% decrease in sperm production (Cederroth et al., 2009). Adult male Wistar rats placed on a high phytoestrogen diet (465 mg/g) of primarily genistein and daidzein also exhibited significantly decreased epididymal sperm counts as well as Sertoli cell counts (Assinder et al., 2007).

However, other studies found no association between decreased fertility and phytoestrogen exposure. Dietary phytoestrogen exposure from gestation until adulthood in C57 (Takashima-Sasaki et al., 2006) as well as CD-1 mice (Ruhlen et al., 2008) yielded no significant decrease in factors such as sperm production, litter size, or testis and seminal vesicle weight. This discrepancy may be due to the differing windows of sexual development evaluated. For

example, mice may be more prone to phytoestrogens' negative effects on fertility if exposed prepubertally as opposed to adult mice. Adult mice have fully formed reproductive tracts that phytoestrogen exposure may not affect or more quickly recover when exposure was removed. Another possible explanation is that the mode of exposure and the chemicals used are not standardized across studies.

Purpose

The purpose of this study was to determine the effects of postnatal dietary phytoestrogen exposure during puberty on development of the male reproductive tract in mice.

Scope

Male fertility is determined by the success of spermatogenesis in the testis and maturation in the epididymis. These processes are maintained by hormones such as estrogen and testosterone. A loss of either hormone could alter sperm count and quality. The extent of the effects of dietary phytoestrogen exposure on hormone levels and spermatogenesis as it relates to male fertility was investigated. These effects were studied during the post-natal/ prepubertal developmental window to fill a potential knowledge gap as the majority of similar studies focus on in utero or adult exposures.

Assumptions

1. Estrogen is significantly involved in the maintenance and regulation of spermatogenesis.
2. Phytoestrogens are endocrine disruptors.
3. Dietary phytoestrogens are a common and relevant route of endocrine disruptor exposure.

Hypothesis

We hypothesized that dietary phytoestrogen exposure would have a dose dependent effect on spermatogenesis and testicular function with the high diet yielding the lowest sperm count and worst performance compared to the medium and low phytoestrogen diet.

Significance

Males are responsible for one third to one half of infertility cases, and 40-50% of male infertility cases are idiopathic (Agarwal et al., 2021). Phytoestrogens are a likely endocrine disruptor that may have detrimental effects on fertility with respect to dose. Diets high in phytoestrogens such as diets high in soy are becoming increasingly popular due to their claims of health benefits such as anti-inflammatory effects as well possibly reducing the risk of cardiovascular disease and some cancers such as breast cancer. Better understanding of phytoestrogens and endocrine disruptor's effects on fertility may also lead to improvements in infertility treatments.

Chapter 2: Review of Literature

Testis and Spermatogenesis

The testis is a paired organ of the male reproductive tract whose role is to facilitate spermatogenesis and synthesize steroid hormones (Fig. 1). The testis contains two compartments, the seminiferous tubules where sperm production occurs, and the interstitial space containing blood vessels, Leydig cells and peritubular myoid cells (Smith & Walker, 2015). Peritubular myoid cells function to contract the tubule and transport sperm (Mayerhofer, 2013), while Leydig cells produce androgens by steroidogenesis (Zirkin & Papadopoulos, 2018). Seminiferous tubules contain Sertoli cells that are responsible for facilitating the proper microenvironment or "niche" for spermatogonial stem cells (SSC) to divide and mature (Scadden, 2006). These SSCs line the basement membrane and develop in the seminiferous tubules, aided by direct contact with Sertoli cells that extend from the basement membrane into the lumen of the seminiferous tubule. Sertoli cells form adhesion junctions with neighboring Sertoli cells, dividing the tubule into a basal and adluminal compartment (França et al., 2016). This division, known as the blood-testis barrier, isolates the developing sperm from the blood, making them dependent on Sertoli cells to secrete growth factors and hormones necessary for spermatogenic development (Cheng & Mruk, 2012; see review Skinner, 2005).

Facilitated by direct contact with Sertoli cells (Griswold, 1998), spermatogonia undergo mitotic divisions that yields spermatogonia. Also known as germ cells, SSCs increase in number through these mitotic divisions. Diploid germ cells near the basement membrane divide into diploid primary spermatogonia who divide further into spermatocytes. Spermatocytes undergo meiotic division (meiosis I) into secondary spermatocytes and a second meiotic division (meiosis II) to produce spermatids. Spermatids undergo the process of spermiogenesis where they

differentiate into fully mature germ cells called spermatozoa. Through spermiogenesis, the nucleus of the spermatid condenses, the acrosome needed for fertilization of ova is formed, and the sperm gain motility as flagella fully develop. During spermatogenesis and spermiogenesis, as well as after the release of mature sperm into the lumen, hormones directly and indirectly influence the maturation and maintenance of sperm.

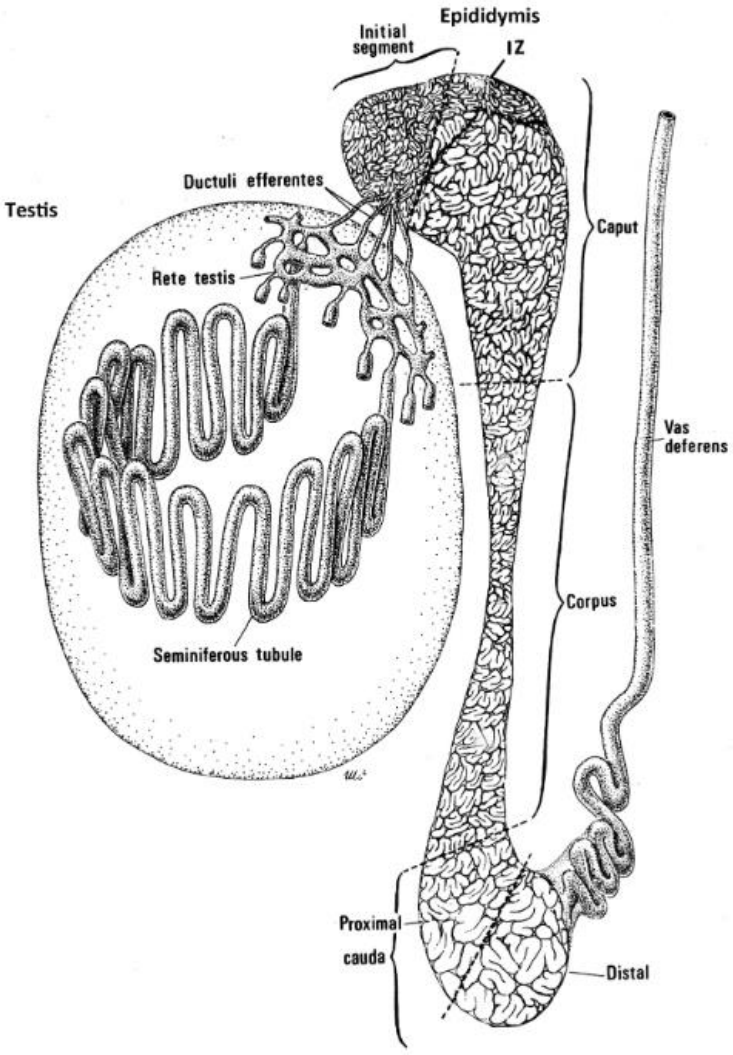

Figure 1: Testis and epididymis anatomy. (Robaire & Hinton, 2015).

Hypothalamic-Pituitary-Gonadal axis

The hypothalamic-pituitary-gonadal (HPG) axis describes the hormone system involved in regulating the initiation of spermatogenesis as well as the survival, development, and differentiation of SSCs and sperm. Gonadotropin-releasing hormone is secreted from the hypothalamus, stimulating the anterior pituitary to secrete two hormones: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These two hormones act on the testis' cells in order to regulate spermatogenesis. LH stimulates Leydig cells, as the majority of LH receptors are concentrated on Leydig cells, to secrete testosterone through the stimulation of steroidogenic enzymes (Akingbemi, 2005) and increasing intracellular cAMP and translocation of cholesterol into mitochondria (Zirkin & Papadopoulos, 2018). Testosterone inhibits LH secretion through a negative feedback loop.

Testosterone is the paramount androgen in the testis, which regulates spermatogenesis by binding to androgen receptors (AR) present on Leydig, Sertoli, and peritubular myoid cells (Berensztein et al., 2006; Rey et al., 2009; Walker, 2011). By binding to AR, a ligand-activated hormone receptor, testosterone induces cell-specific signal cascades through transcription factors leading to the cell carrying out its functions in supporting spermatogenesis (Smith & Walker, 2015). Without proper concentrations of testosterone in adult rats, spermatogenesis becomes unregulated, resulting in a change in cell signaling and proteins involved in meiosis (Stanton et al., 2012). Therefore, a loss of testosterone may cause the developing spermatids to not properly form as Sertoli cell function is inhibited. As Sertoli cells function to form the blood-testis-barrier (BTB), the barrier separating sperm and its nutrient-specific niche from circulation may become compromised. In addition, mature sperm that would normally be released into the lumen are not released and are instead phagocytized by the Sertoli cell without proper testosterone signaling

(Holdcraft & Braun, 2004). Immature sperm may also be released too early as the gap junction protein connexin between Sertoli cells and developing sperm is downregulated in absence of testosterone (Kidder & Cyr, 2016; Kopera et al., 2010).

FSH's contribution to supporting spermatogenesis operates entirely through Sertoli cells as FSH receptors are only expressed on Sertoli cells (Smith & Walker, 2015). FSH binds to FSH receptor (FSHR) proteins on Sertoli cells, activating intracellular signaling pathways such as cAMP-PKA (Walker et al., 1995) and MAPK (Crépieux et al., 2001). These pathways ultimately function to increase germ cell numbers (O'Shaughnessy et al., 2010) as well as induce the maturation and proliferation of Sertoli cells themselves (Walker & Cheng, 2005). While serum FSH remains steady, the expression of FSHR varies as FSH stimulation downregulates FSHR presentation, resulting in a pattern of stimulating SSCs to undergo division in cycles of about 12 days (Walker & Cheng, 2005). In other words, FSHR expression is highest in the earlier stages of spermatogenesis in the seminiferous epithelium, and lower in the later stages (Heckert & Griswold, 1991).

Estrogen is synthesized in the testis via the local conversion of testosterone by aromatase. Sertoli cells primarily express aromatase during sexual development, while Leydig cells account for the majority of aromatase expression during adulthood (Smith & Walker, 2015). Estrogen binds to two receptors, ESR1 and ESR2. ESR1 is expressed on Leydig and Sertoli cells as well as the epithelium of the epididymis in mice, but not that of humans (Hess & Cooke, 2018). ESR2 is more ubiquitous and is present on both Leydig and Sertoli cells as well as peritubular myoid cells (Hess & Cooke, 2018). ESR1 has been shown to play a role in differentiation of spermatids in spermiogenesis, while ESR2 is involved in spermiation or the release of mature sperm into the lumen, (Dumasia et al., 2016). Germ cells also have been shown to synthesize a considerable

percentage of the estrogen present in the testis (Carreau et al., 2006). Estrogen in the testis functions to initiate and maintain spermatogenesis, most likely through a pathway independent of the FSH pathway (Pak et al., 2002). A mutation in the genes that express ESR1 and ESR2 (Smith et al., 1994) as well as a mutation causing aromatase deficiency (Morishima et al., 1995; Carani et al., 1997; Herrmann et al., 2002) in men and rodent models (Hewitt et al., 2005) results in the loss of testicular estrogen, and abnormal and dysfunctional sperm. While many studies suggest ESR2 has a lesser effect on male fertility (Chen et al., 2010), some even claiming it to be a vestigial receptor (Gustafsson et al., 2019), ESR1 has been shown to play a larger role. ESR1-KO adult mice became infertile due to loss of fluid reabsorption in the efferent duct, ultimately causing sperm infertility and epididymal abnormalities (Hess et al., 2000). On the other hand, elevated concentrations of intratesticular estrogen have been shown to have detrimental effects on Sertoli and Leydig cell morphology and function, impaired spermatogenesis and ultimately infertility (Leavy et al., 2017). Altogether, estrogen plays a significant role in the testis and spermatogenesis in contrast to the traditional dogma that testosterone is solely responsible for regulating male fertility. Estrogen also plays a similarly significant role as we continue down the male reproductive tract into the epididymis.

Epididymis

Seminiferous tubules from the testis form the rete testis and flow into the efferent ducts. These efferent ducts join to the epididymis. This tightly coiled duct has four major anatomical regions including the initial segment, caput, corpus, and cauda (Robaire & Hinton, 2015). The epididymis is primarily responsible for sperm maturation as well as sperm transport and storage (Davis & Pearl, 2019). More specifically, the epididymis primarily functions as a space where

immature sperm from the testis are concentrated and gain motility and the ability to fertilize ova (Robaire & Hinton, 2015). The epididymis fulfills this role by maintaining distinctly unique nutrient rich environments along the epithelium of each of its four segments (Turner et al., 2003). The epididymis' segments of epithelium are themselves further partitioned by tissue septa, allowing for varying expression of genes and proteins that dictate and regulate the function of specific epididymal segments (Turner et al., 2003, Cornwall, 2009). Several cell types line the epididymal lumen and are present in varying concentrations, explaining the variance in gene and protein expression (Fig. 2).

Of those major cell types, principal cells are the most prevalent. Principal cells exist along the epididymis, making up about 65-80% of all epididymal epithelial cells (Trasler et al., 1988). Principal cells secrete proteins into the lumen that are necessary for sperm to gain motility and ability to fertilize (Frenette et al., 2006). Additionally, principal cells express transport proteins and channels to regulate the luminal environment and provide sperm and epididymal cells with proper basic nutrients such as ATP, water and bicarbonate (Belleannée et al., 2012). Principal cells also contribute to the regulation of pH in the luminal fluid. Clear cells appear to regulate luminal fluid pH via V-ATPase function (Shum et al., 2009). Clear cells also work in conjunction with principal cells as they perform endocytic function and remove proteins from the luminal fluid (Vierula et al. 1995). Basal cells, extending from the basement membrane to the lumen, regulate clear cells and principal cells and therefore regulate the luminal environment for proper sperm maturation and storage (Shum et al., 2009). These cell types along the epididymis create and maintain a nutrient-rich luminal environment vital for proper sperm maturation and storage, but they do not explain the movement of sperm through the epididymis to the rest of the male reproductive tract.

Immature sperm are propelled into the epididymis from the testis in testicular fluid that is partially driven forward by the cilia of the efferent ducts. Once in the epididymis, the propulsion of fluid and cilia is slowed as the epididymis is not lined by mobile cilia and much of the luminal fluid is absorbed in order to concentrate sperm. Smooth muscle lines the entirety of the epididymis and contracts to propel sperm through each of the four segments (Robaire & Hinton, 2015). These contractions are controlled by androgens such as testosterone that slows contraction and sperm transport, as well as estrogen that accelerates sperm transport (Fernandez et al., 2008). Alterations in sperm transit time may cause sperm to improperly mature and even decrease sperm count in ejaculate (Klinefelter, 2002). In humans and most mammals, sperm spend approximately 10 days in the epididymis in order to properly gain their mature functions (França et al., 2005). While hormones such as estrogen and androgens influence the storage and propulsion of sperm through the epididymis, their influence is broader.

While most estrogen synthesis is done in the testis, the epididymis expresses aromatase (Shayu & Rao, 2006) as well as estrogen receptors (Cooke et al., 1991). Estrogen not only causes the contraction of the epididymis but has also been shown to maintain epididymal epithelial cell function. ESR1 has been shown to regulate ion transport and fluid reabsorption in the epididymis and efferent ducts (Joseph et al., 2011). Knockout of ESR1 resulted in loss of epididymal fluid reabsorption, swelling in the rete testis and initial segment, spermiostasis, as well as dysfunctional and morphologically abnormal initial segment epithelial cells (Hess et al., 2000). Estrogen treatment improved epididymal sperm transport during aging (Davis & Pearl, 2019). Therefore, without estrogen stimulation, epididymal sperm concentrations decrease, and sperm may fail to properly mature and gain the ability to fertilize ova. Somewhat paradoxically,

exposure to high amounts of estrogen-like compounds may also disrupt sperm maturation in the male reproductive tract (Nakazumi et al., 1996).

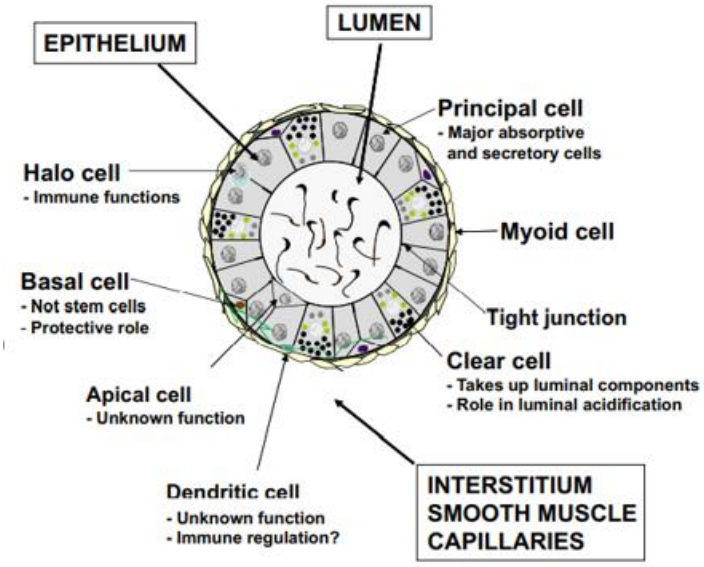

Figure 2: Epididymis epithelial cell type organization. (Robaire & Hinton, 2015)

Puberty

Puberty is commonly considered the physiological process of becoming sexually mature and able to reproduce. With respect to males, completion of the process of puberty should result in the presence of functional sperm in the epididymis allowing for reproduction. The onset of puberty is initiated by a reactivation of GnRH secreting neurons in the hypothalamus, causing a pulsatile release of GnRH into the hypothalamic-hypophyseal portal circulation (Plant et al., 2015). Increased GnRH stimulates gonadotropin secretions, namely LH and FSH, that bind to receptors on the male reproductive tract to initiate development. In other words, the HPG axis is

reawakened to begin maturation of the gonads through puberty. Prepubertal humans have low levels of circulating gonadotropins in order to undergo development and growth before puberty and sexual development begin (Watanabe & Teresawa, 1989). During this juvenile developmental state, the gonads remain undeveloped because GnRH is not secreted due to a nongonadal "brake" present in the hypothalamus. This brake is not released until after the age of 12 in humans (Wood et al., 2019) when puberty usually begins, and around 6 weeks after birth in mice (Falconer, 1984). Humans have a long hiatus before the onset of puberty, whereas in mice this time window is brief, allowing them to become sexually competent at 12 weeks.

As puberty begins, increased LH and FSH secretions influence Leydig and Sertoli cell differentiation. As these cells mature, structures such as the seminiferous tubules grow larger, lengthen, and become more contorted. Testosterone is responsible for stimulating the morphological changes in Sertoli cells at the onset of puberty to be able to secrete factors necessary for fostering sperm production and maturation (Rey et al., 2009). Both FSH and testosterone function to increase Sertoli cell proliferation in prepuberty and adults (Ramaswamy et al., 2000). This ultimately results in a significant increase in testicular size and volume from about 2mL to 20-25 mL in a human adult (Plant et al., 2015). Coupled with the initiation of spermatogenesis, the maturation of Sertoli and Leydig cells relies on an increase of circulating LH and FSH from the pituitary. GnRH pulsatile release has been noted to occur nocturnally, along with subsequent pulsatile LH release, with both frequencies decreasing as puberty progresses. This decrease is due to the increase in testosterone that functions to decrease the frequency of hypothalamic GnRH secretions through a negative feedback loop. However, testosterone not only exerts these effects at the level of the hypothalamus, but also the pituitary. Similarly, estrogen may function in this negative feedback. There is little consensus regarding

the contributions of estrogen and testosterone to this negative feedback (Lindzey et al., 1998), with some sources asserting that the AR plays a more dominant role with respect to negative feedback (Wersinger et al., 1999). These same studies also suggest that ESR1 is the primary receptor regulating estrogens effect on GnRH, LH and FSH secretions either directly or indirectly through modification of AR function and aromatization of testosterone to estrogen (Wersinger et al., 1999).

Puberty, as it pertains to male fertility, culminates in the individual becoming sexually mature or able to reproduce, marked by the first motile and differentiated sperm leaving the epididymis. This marked production of sperm occurs around day 40-55 of age in mice and rats (Glass et al., 1986; McKinney & Desjardins, 1973). In humans, the exact timing of spermatogenesis is less certain due to a lack of data requiring serial ejaculates and testicular biopsies (Plant et al., 2015). Instead, human gonadarche is marked by the large increase in testicular volume occurring around the age of 9 to 13 years (Witchel & Topaloglu, 2019). Once established, the process of spermatogenesis in both mice and rats is completed in 40-54 days (Hess & Renato de Franca, 2008), while in humans it takes 64 days for sperm to mature into differentiated spermatozoa (Heller & Clermont, 1964).

Phytoestrogens and Endocrine Disruption

Phytoestrogens are plant-derived non-steroidal compounds structurally similar to estrogen, allowing them to bind to both ESRs, with a higher affinity for ESR2 (Fig. 3) (Kuiper et al., 1998; Kuiper et al., 1997; Lund et al., 2004). Phytoestrogens are heavily concentrated in some vegetables and most notably in soy-containing products (Cederroth et al., 2010). In males, ingestion of these foods can quickly elevate circulating levels of isoflavones such as genistein

and daidzein (Watanabe et al., 1998), two of the most common phytoestrogens in humans and mammals (Degen et al., 2002), as well as equol, the most common phytoestrogen in rodents (Cederroth et al., 2010). As these phytoestrogens may bind to ERs, they can potentially influence the functions of the testis and epididymis where ERs are expressed, affecting spermatogenesis and fertility.

Phytoestrogens such as daidzein and genistein have relatively weak affinities for ERs and can have either positive or negative effects on the G protein-coupled protein depending on whether estrogen is present (Shanle & Xu, 2011). These compounds may bind to ERs and downregulate or upregulate both genomic and non-genomic pathways. Genomic pathways refer to those that are mediated by transcriptional activation of genes, while non-genomic pathways are not involved in transcription of genes and are instead mediated by the phosphorylation of proteins or an increase in intracellular calcium (Warner & Gustafsson, 2006). Both of these types of pathways are critical to organ development, as well as proteins and signaling molecules responsible for the maturation of the reproductive tract. One such example is the downregulation of transcription factors and metabolic enzymes required for testosterone synthesis by exposure to phytoestrogen compounds (Jefferson et al., 2012). Through various mechanisms, exogenous phytoestrogens or dietary phytoestrogens may alter the morphology and function of the testis and epididymis and have detrimental effects on spermatogenesis and fertility. For example, adult male rats given a diet high in phytoestrogens exhibited an increase in apoptosis of germ cells as well as decreasing elongated spermatid and epididymal sperm counts (Assinder et al., 2007). High exposure to phytoestrogens has also been shown to decrease testosterone in the rat testis, further inhibiting the maturation of sperm (Napier et al., 2014; Weber et al., 2001). The developing male reproductive tract is more susceptible to potential endocrine disruptors as they

are sensitive to and can adapt to estrogen-like compounds, causing permanent changes in the reproductive tract at the organ and cellular level possibly leading to disease or infertility (Sleiman et al., 2021). This is concerning as 30% of infants in the US are exposed to soy-based formulas that are high in these phytoestrogen compounds (Napier et al., 2014). The majority of studies concerning the effects of phytoestrogen in mice focus on the pre-pubertal time window (Casanova et al., 1999; Odum et al., 2001; Degen et al., 2002). These studies suggest that dietary phytoestrogens may be partially responsible for disturbing normal development in utero and sexual development such as delaying the onset of puberty. Studies in adult-only mice are less in number and even less in consensus. Assinder et al. (2007) suggests that a diet of high phytoestrogen content not only disrupts spermatogenesis but increases germ cell apoptosis in the testis and epididymis. On the other hand, Faqi et al. (2004) suggests that the adult male reproductive tract is mostly unaffected by long-term exposure to dietary phytoestrogens. Therefore, further study of the effects of phytoestrogens is warranted, specifically during and after the pubertal developmental window.

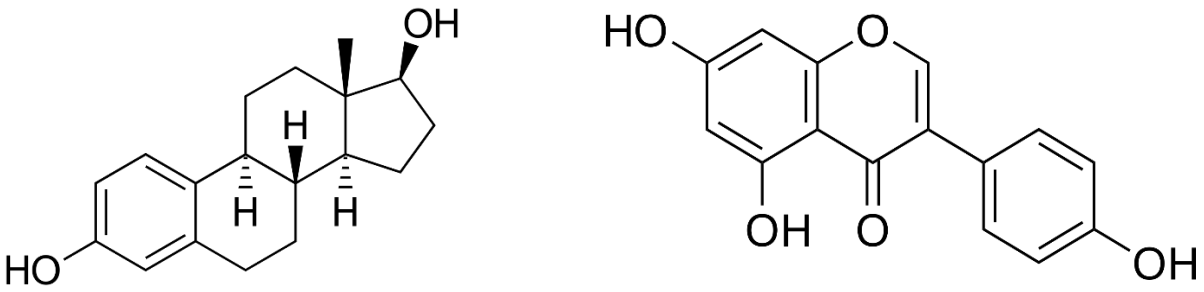

Figure 3: Chemical structure of estradiol (left) vs. Genistein (right)

Chapter 3: Methodology

Animals and Experimental Design

Male C57Bl/6 mice (n=18) were obtained from Charles River Laboratories and housed in the GVSU animal facility with ad libitum access to food and water. Mice were obtained at 4 weeks of age and randomly assigned to three different groups of phytoestrogen diets (n=6 each). Three mice were housed together in each cage. Mouse diets were obtained from Envigo (Indianapolis, IN) and were considered low (2014; Teklad global 14% protein rodent maintenance diet), medium (2018; Teklad global 18% protein rodent maintenance diet) or high (8640; Teklad 22/5 rodent diet) based on the phytoestrogen content. The low diet contained 0-20 mg/kg isoflavones consisting mainly of daidzein and genistein, the medium contained 150-250 mg/kg isoflavones, and the high diet contained 350-650 mg/kg isoflavones. Mice were euthanized using CO_2 at 12 weeks of age; animal weight, trunk blood, and tissues were collected. For each group of six animals, there was a total of twelve testes and epididymides. For each group of 12, 4 testes were frozen for hormonal analysis, 4 testes frozen for analysis of sperm production, and 4 testes fixed for morphology in Bouin solution. Similarly, 4 epididymides were frozen for sperm counts, 4 fixed for morphology and 4 used for analysis of sperm motility. Frozen tissues were stored at -80°C until assayed.

Sperm Counts

Homogenization-detergent resistant spermatids were counted to calculate sperm per gram testis as previously described (Amann and Lambiase, 1969; Robb et al., 1978; Wicks et al, 2013; Clark and Pearl, 2014). Testis tissue samples were thawed and then homogenized in a glass test

tube with 2 mL of a 0.9% NaCl and 0.05% Triton-X solution using an OmniTip tissue homogenizer. Samples were transferred to a 50 mL conical tube and diluted with Triton-X and NaCl solution to a final volume of 15 mL and stored at 4°C. Twenty four hours later sperm were counted using a hemacytometer and phase contrast microscopy. Sperm in 5 out of the 25 fields of the hemacytometer were counted and multiplied by 5 to obtain the number of sperm in the entire counting grid. Each side of the hemacytometer was counted. Counts were performed by two individuals. Sperm number in the counting area was multiplied by 10,000 to obtain sperm in 1 mL and then multiplied by total volume to obtain the number of sperm per testis. Sperm/mg testis was obtained by dividing sperm/testis by the testis weight. Daily sperm production was calculated by multiplying sperm/g testis by single testis weight and divided by 4.84 days. Dividing by 4.84 days was used because that time constitutes one cycle of the seminiferous epithelium, where spermatids may be measured as they are in a detergent and homogenization resistant stage of development (stage 14-16) (Oakberg, 1956; Kyjovska et al., 2013).

Sperm in the proximal (IS, caput, corpus) and distal (cauda) epididymis was also determined similar to testicular sperm count. Epididymis tissue was thawed, cut into proximal and distal portions using microscissors, and homogenized in a test tube similar to testis samples. Proximal epididymis samples were diluted to a final volume of 10 mL and distal epididymis samples diluted to 15 mL and stored at 4°C. Twenty four hours later sperm were counted using a hemacytometer and phase contrast microscopy. Each side of the hemacytometer was counted by two individuals. Sperm number in the counting area was multiplied by 10,000 to obtain sperm in 1 mL and then multiplied by total volume to obtain the number of sperm per region. Sperm/mg was obtained by dividing sperm/region by the tissue weight. Epididymal transit time was

calculated by dividing the number of sperm in the epididymal region by daily sperm production of the corresponding testis.

Hormonal Analysis

Frozen testis samples were thawed and homogenized in 1 mL of PBS containing protease inhibitors. Each testis sample was aliquoted into 3 tubes and frozen at -80°C until further analysis. Testosterone and estradiol concentrations of each testis were determined with ELISA kits from ENZO Life Sciences (Farmingdale, NY; ADI-900-065, ADI-900-174) and normalized to weight of homogenized tissue.

For testosterone, samples were assayed in the ELISA following kits instructions. Samples were mixed with a steroid displacement reagent and diluted in assay buffer at ratios of 1:2, 1:10 and 1:20. One hundred microliters of sample were added to duplicate wells. Fifty microliters of primary antibody were added to the well and the mixture was incubated for 1 hour at room temperature on a plate shaker at ~500rpm. We next added antibody conjugate and incubated for an additional 1 hour on a plate shaker. Antibody conjugate is enzyme-linked and produces color if bound to plate after wash and substrate are added. Wells were emptied and washed three times with wash buffer. Substrate solution was added to each well and incubated for 1 hour without shaking. Stop solution was added to each well and the plate was read at 405 nm using an EPOCH plate reader and BioTek Gen5 software.

For estradiol, samples were first extracted using diethyl ether. For extraction, 1 mL of ether was added to 250 µL of thawed homogenate samples in 13x100 borosilicate glass tubes. The sample and ether were mixed for two minutes and the layers were allowed to separate for 10

minutes. The aqueous layer was frozen by submerging the bottom of tubes in methanol with dry ice. The ether layer was decanted into 12x75 borosilicate glass tubes and allowed to evaporate overnight in a fume hood. The next morning samples were reconstituted in 250 µL assay buffer by letting sample sit in solution for 5 minutes, vortexing the tubes on rack shaker for 2 minutes and letting them sit again for 5 minutes. Samples were then diluted in assay buffer at ratios of 1:2, 1:5 and 1:10. The ELISA plate was loaded with samples and the assay run following kit instructions. The plate was read at 405 nm using an EPOCH plate reader and BioTek Gen5 software.

Trunk blood was collected by cutting the thoracic aorta and transferring blood via syringe to a serum separator tube. Blood was allowed to clot for approximately 30 minutes and then centrifuged. Serum was aliquoted and stored at -80°C. Approximately 150-200 µL were collected from each animal. One aliquot of serum from each animal was sent to the University of Virginia Ligand Core for determination of LH and FSH concentration. Samples were assayed using a LH/FSH multiplex assay in duplicate. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the Eunice Kennedy Shriver NICHD/NIH Grant R24HD102061.

Testis and Epididymis Morphology

Testis and epididymis tissues were placed into Bouin solution overnight and then transferred to 70% ethanol. Samples were placed into cassettes and embedded, sectioned and stained by Michigan State University's Investigate Histopathology Laboratory. Period acid Schiff – hematoxylin (PAS-H) stained slides were returned to the Pearl lab at GVSU. From these

sections multiple fields were visualized using a Nikon Eclipse NiU microscope equipped for bright field microscopy with digital camera and imaging software. Morphological analysis was done using the Nikon NIS-Elements Basic Research imaging software. Four seminiferous tubule diameter measurements were taken and averaged to generate a single diameter for that tubule. Seminiferous tubule diameter was measured in 20-30 tubules per animal and varied in position across the testis. Morphology was also assessed by determining the percentage of tubules with normal vs abnormal features and the percentage of normal tubules with open lumens. Normal seminiferous tubules demonstrated visually open lumens with mature sperm inside. Additionally, normal tubules demonstrated developing sperm at each stage of spermatogenesis in contact with a layer of tightly bound Sertoli cells and a clear basement membrane. Abnormal tubules may or may not demonstrate an open lumen but lacks presentation of the various stages of spermatogenesis or differentiation of sperm cells. Similarly, Sertoli cells may not be present or mature and lack a formed blood-testis barrier and basement membrane.

Sperm Motility

Sperm from the proximal and cauda regions were collected and underwent a motility assessment using the YO Male Fertility Sperm Test. Sperm were visualized using the YO Male Fertility Sperm Test camera and software and allowed for video recording of the samples. This test kit is designed as a human sperm motility test that can be used at home, but we utilized the kit to try and determine mouse sperm motility between groups. Animals in the high diet group were collected first. One epididymis from the high group was cut into proximal and distal segments, minced, vortexed and homogenized in 1 mL PBS. Three other epididymides from the high group were similarly tested but in 500 µL PBS. On day two, epididymides came from the

medium group. Each sample was minced with microscissors, compressed with blue pestle in blue microcentrifuge tube, and incubated for 10 mins in liquefaction powder. Sample was mixed by inversion and flicking, not by vortex. Samples were diluted in PBS, HBSS, or HGDMEM/10% FBS. After dilution, all samples on each day were loaded onto slides and placed into YO motility video recording device. Videos were observed to assess sperm motility.

Statistical Analysis

Data was analyzed by ANOVA using GraphPad Prism statistical analysis software. If the ANOVA was significant ($p \leq 0.05$), differences between groups were determined by using a Tukey's multiple comparison test.

Chapter 4: Results

We first measured the body weight of each animal to assess general health and development. Body weights of the three phytoestrogen diet groups were not significantly different, with mice weighing 26.8 ± 0.87g on the low diet, 28.5 ± 1.02g on the medium diet, and 26.9 ± 1.00g on the high diet (Fig. 4).

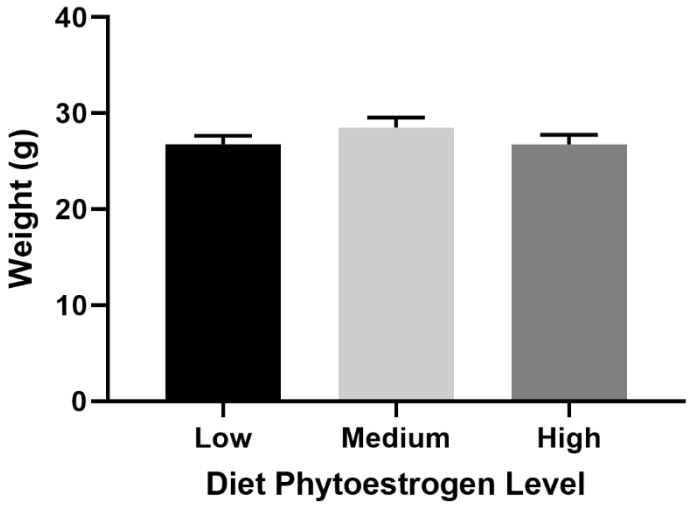

Figure 4: Animal weight. Body weights were similar between the three diet phytoestrogen groups.

One measure to determine if the treatment affected the testis is testis weight. Paired testis weight was not significantly different between treatment groups (Fig. 5). Mice in the low group had a mean testis weight of 96.9 ± 5.86 mg, 104 ± 6.07 mg for the medium group, and 93.7 ± 1.16 mg for the high group. We also measured seminiferous tubule diameter, and there were no significant differences between the three groups. Mice in the low group had a mean lumen diameter of 187.78 ± 21.39 μm, 191.54 ± 59.84 μm for the medium group, and 185.47 ± 75.10 μm for the high group. We also measured the percent abnormal seminiferous tubule lumen (Fig. 6). Mice in the low group had 8.84 ± 1.73% abnormal lumens, the medium group had 5.47 ± 2.02%, and the high group had 4.31 ± 1.26%. The groups did not significantly differ from each other with respect to the percentage of abnormal lumens.

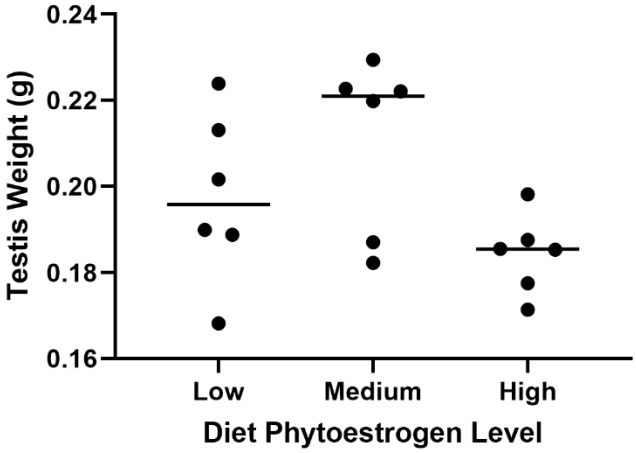

Figure 5: Paired testis weight. Testis weight showed some variability in each group but average weight was not different.

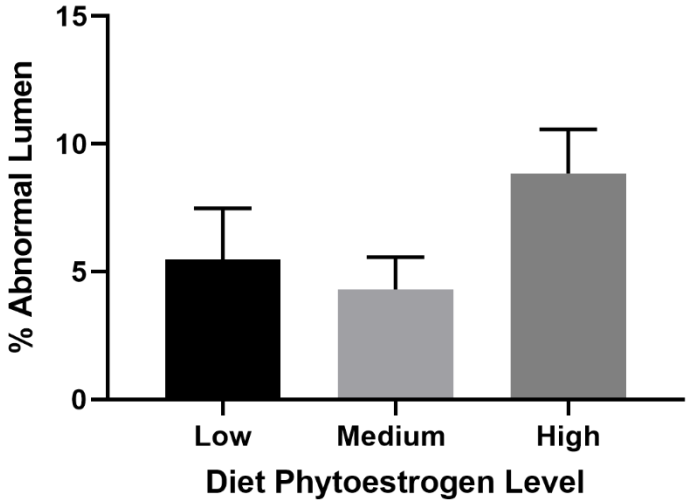

Figure 6: Percent Abnormal Lumen. Abnormal lumen count was divided by total lumen count. No significant differences were observed between groups.

Weight of both epididymides from each animal were also measured and normalized to their corresponding testis. Mice in the low diet had a paired epididymis weight of 0.0913 ± 0.0039 g, 0.0921 ± 0.0018 g for the medium group, and 0.0951 ± 0.0056 g for the high group. Mice in the low diet had an epididymis weight as % of testis of 46.2 ± 0.007%, 44.0 ± 0.016% for the medium group, and 51.6 ± .029% for the high group (Fig. 7). Epididymis weight as % testis was significantly greater in the high group compared to the medium group.

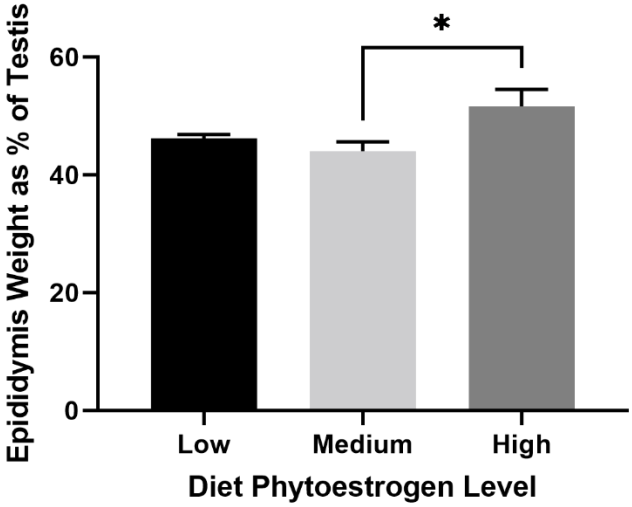

Figure 7: Epididymis Weight as % of Testis. Epididymis weight was divided by and normalized to testis weight. The high diet was significantly higher compared to the medium group ($p < 0.05$).

Serum FSH and LH were measured from trunk blood samples in order to assess possibly effects on anterior pituitary secretions. Serum FSH concentrations were significantly lower in the high group compared to the low group (Fig. 8). Serum LH was not significantly different across groups (Fig. 9).

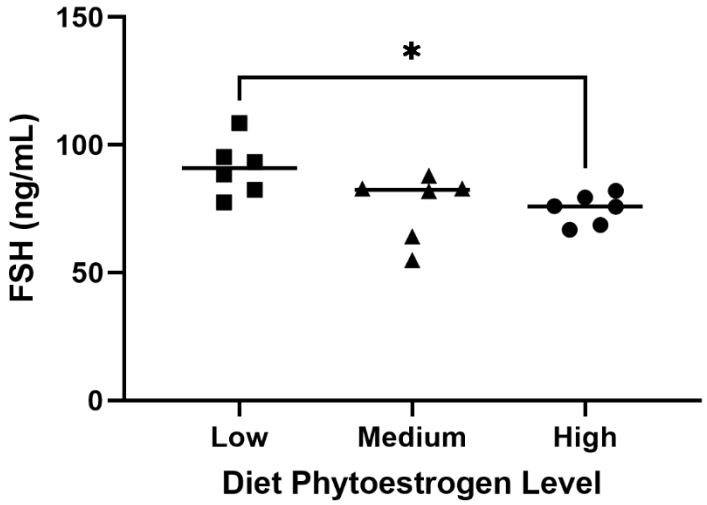

Figure 8: Serum FSH Concentrations. The high diet group had significantly lower serum FSH concentrations compared to the low diet group. Asterisk indicates a significant difference based on $p \leq 0.05$.

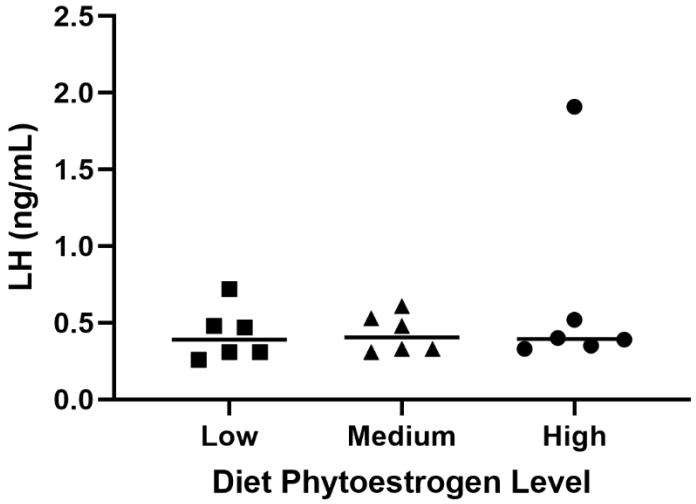

Figure 9: Serum LH concentrations. Serum LH concentrations were quantified (ng/mL) and no significant differences were observed between the groups.

As testicular hormones have a critical role in male fertility and the success of spermatogenesis, we measured testicular concentrations of testosterone (Fig. 10) and estradiol (Fig. 11). Mice in the low group had a testosterone concentration of 179 ± 11.2 ng/g, the medium group had 132 ± 27.1 ng/g, and the high group had 287 ± 42.3 ng/g. The high group had a significantly higher testosterone concentration when compared to the medium group, but not the low group.

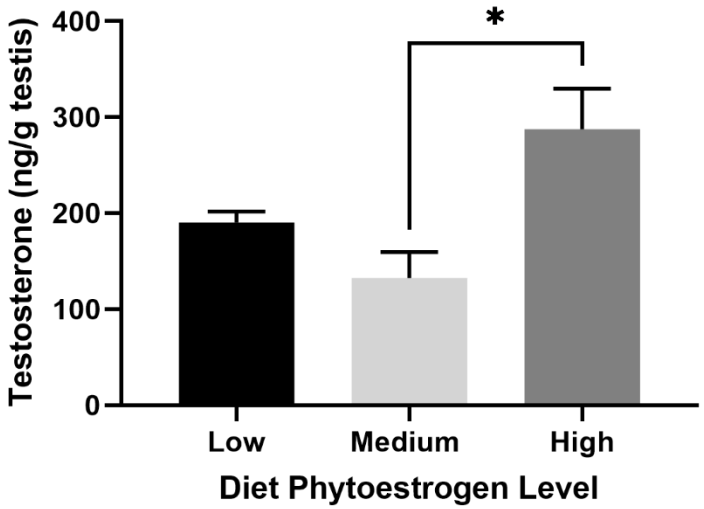

Figure 10: Testicular Testosterone Concentration (ng/g). Mice in the high group had a significantly higher concentration of testosterone compared to the medium group, but interestingly not the low group. Asterisk indicates a significant difference based on $p \leq 0.05$.

Mice in the low group had an estradiol concentration of 1789 ± 242 pg/g, the medium group had a concentration of 2648 ± 1537 pg/g, and the high group had a concentration of 1860 ± 270 pg/g. None of the groups significantly differed from each other.

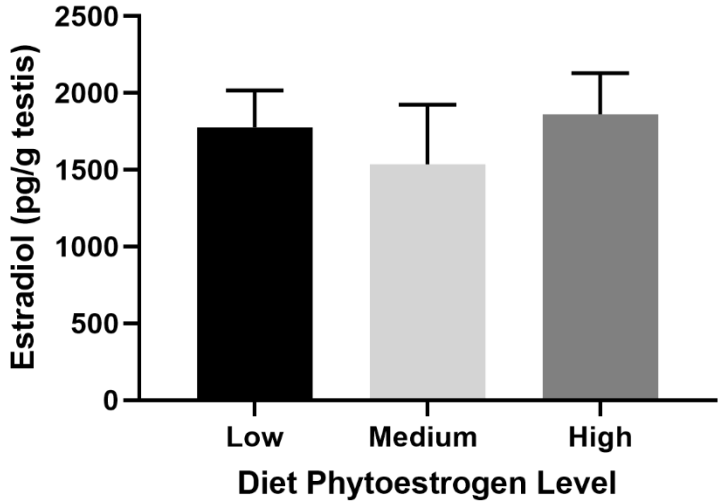

Figure 11: Testicular Estradiol Concentration (pg/g). There was no significant difference in sperm/testis between the three dietary phytoestrogen groups.

We next calculated the ratio of testosterone to estradiol by dividing the testosterone concentration by the estradiol concentration (T/E_2) (Fig. 12). Mice in the low group had a T/E_2 of 123.6 ± 1.82, 112.4 ± 9.83 for the medium group, and 178.0 ± 20.8 for the high group. Similar to testicular testosterone concentrations, the T/E_2 ratio was statistically different between the high and medium groups ($p < 0.05$).

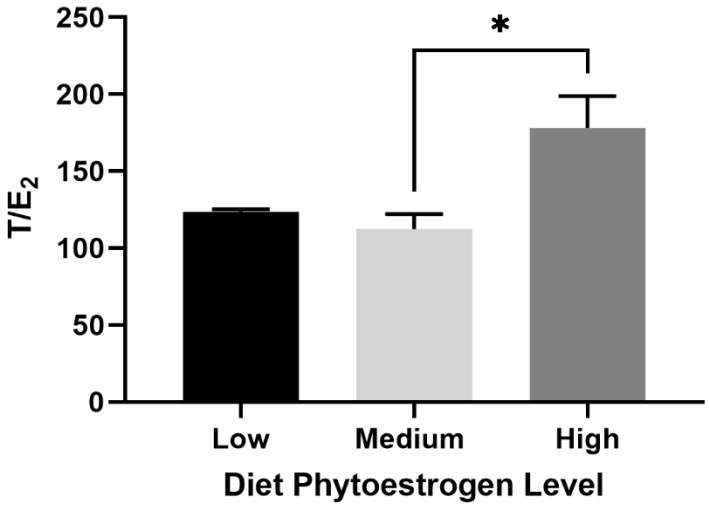

Figure 12: Testosterone/Estradiol Ratio. Mice in the high group had a greater T/E_2 ratio compared to the medium diet group ($p < 0.05$).

We next measured the total amount of sperm per testis (Fig. 13). Mice in the low group had an average of 17.0 ± 1.33 million sperm/testis, the medium group had 17.4 ± 1.31 million sperm/testis, and the high group had 13.9 ± 0.865 million sperm/testis. While the high group was numerically lower, each of the dietary phytoestrogen groups did not statistically differ from each other.

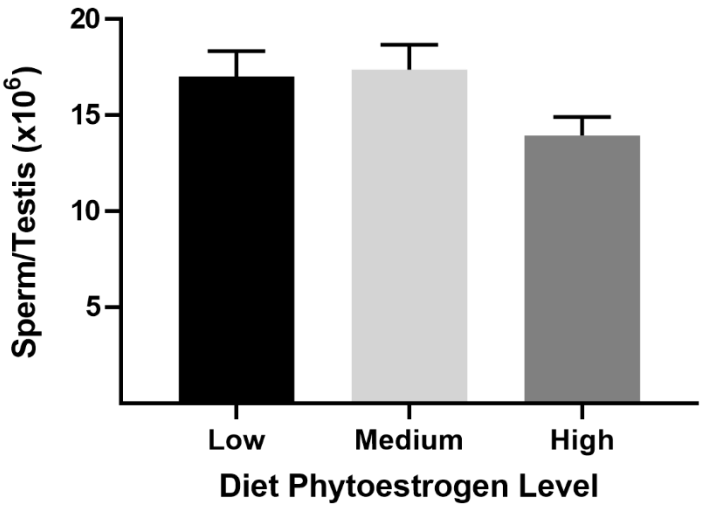

Figure 13: Sperm/Testis. There was no statistical difference in sperm/testis between the three dietary phytoestrogen groups.

We next determined sperm per milligram of testis which is a measure of spermatogenesis efficiency. While the diet phytoestrogen groups do not significantly differ, there appears to be a negative trend as dietary phytoestrogens increase (Fig. 14). Mice in the low diet had 0.176 ± 0.009 million sperm, the medium group had 0.167 ± 0.008 million sperm, and the high group had 0.149 ± 0.007 million sperm.

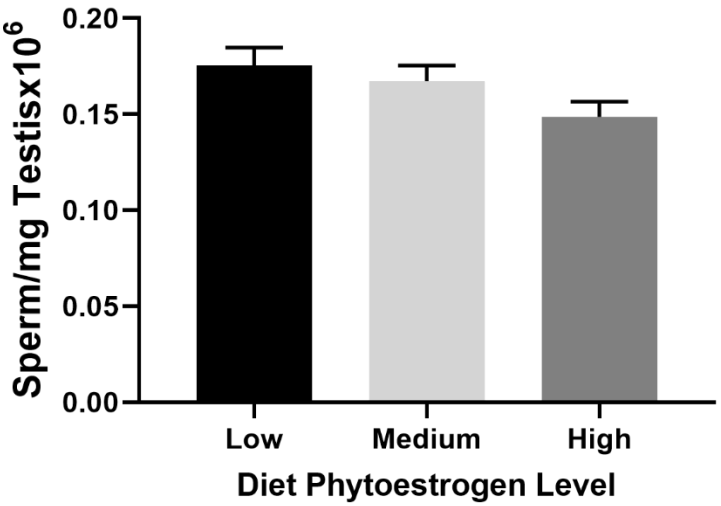

Figure 14: Sperm/mg Testis. Mice exposed to higher levels of phytoestrogens appear to trend lower in sperm present per milligram of testis.

Daily sperm production (DSP) is a measure of fertility that takes into account sperm/g testis as well as paired testis weight. There were no significant differences between treatment groups; however, the high group appears to be trending lower than the low and medium groups (Fig. 15). Mice in the low group had a DSP of 3.52 ± 0.274 million sperm, 3.59 ± 0.271 million sperm for the medium group, and 2.88 ± 0.179 million sperm for the high group.

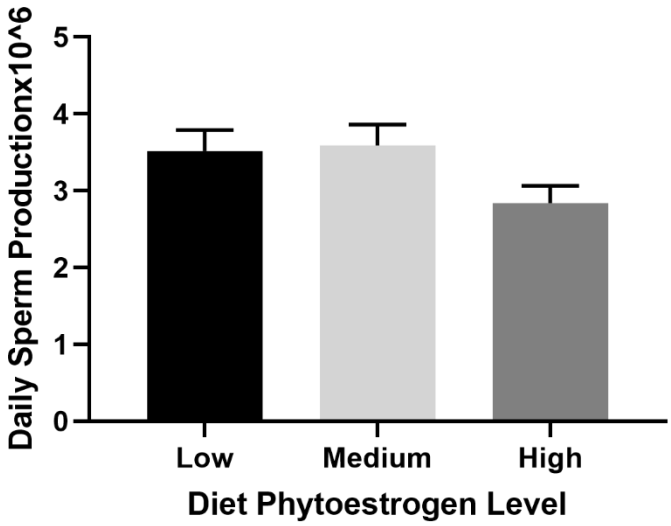

Figure 15: Daily sperm production. There was no significant difference in DSP between the three dietary phytoestrogen groups.

We next measured the number of sperm in the proximal (initial segment, caput, corpus) and distal sections (cauda) of the epididymis. For the proximal epididymis, mice in the low group had 34.3 ± 1.39 million sperm, 43.2 ± 0.980 million sperm for the medium group, and 42.7 ± 0.409 million sperm for the high group. For the distal epididymis, mice in the low group had 93.2 ± 1.69 million sperm, the medium group had 92.6 ± 0.905 million sperm, and the high group had 96.0 ± 0.979 million sperm. Total sperm differed with respect to epididymis region with the cauda being higher than more proximal regions within each diet but did not significantly differ with respect to phytoestrogen diet group (Fig. 16).

We next looked at sperm/mg epididymis to normalize total sperm in the epididymis to weight of epididymis, which also did not significantly differ with respect to phytoestrogen diet treatment. Mice in the low group had a sperm/mg epididymis of 0.927 ± 0.222 million, the medium group had 0.992 ± 0.223 million, and the high group had 1.07 ± 0.266 million. Similar to total sperm numbers, sperm per milligram was higher in the distal epididymis than the proximal epididymis but did not differ by treatment.

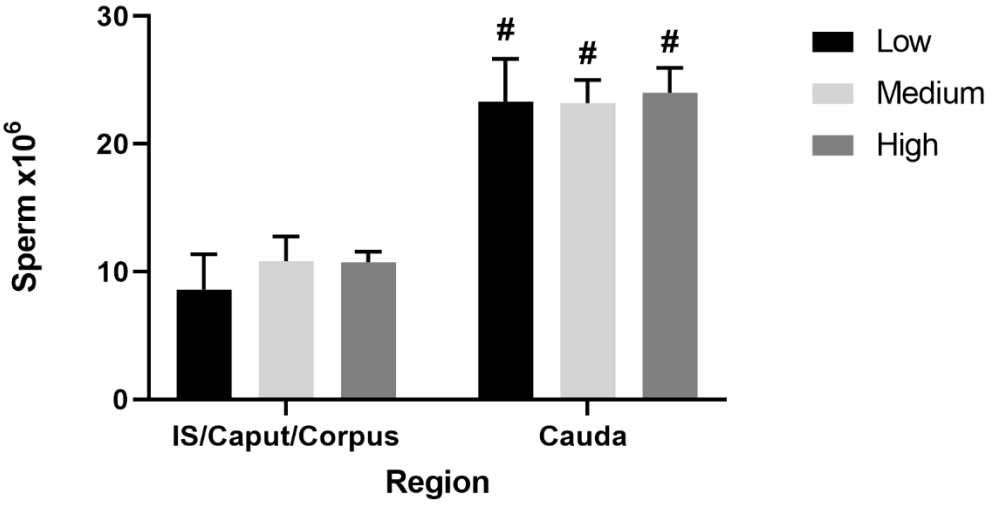

Figure 16: Sperm x10^6 by Epididymis Region. Total sperm in proximal and distal epididymis. There was no significant difference in sperm count with respect to treatment group. # denotes significant difference between regions (proximal vs distal) within a diet group.

Sperm develop as they traverse the epididymis, so we next determined total transit time (Fig. 17). Mice in the low group had a total transit time of 9.25 ± 0.556 days, the medium group had 9.58 ± 0.657 days, and the high group had 12.0 ± 0.796 days. The high group had a significantly longer transit time when compared to the low group ($p = 0.0337$).

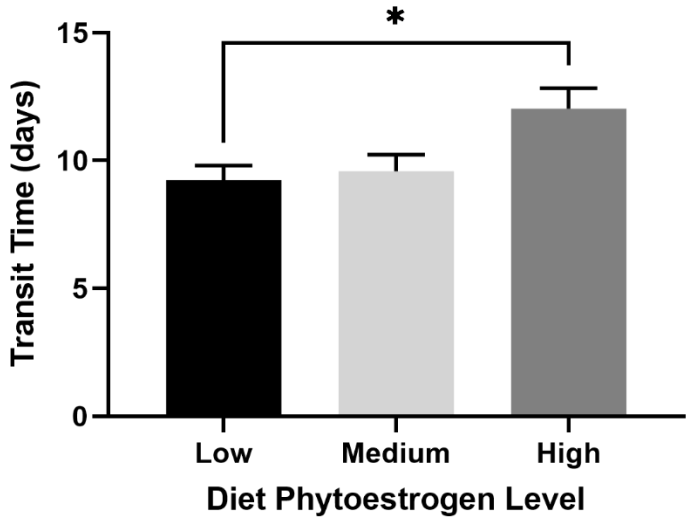

Figure 17: Total Transit Time (days). Mice in the high group had a longer transit time compared to the low group. Asterisk indicates a significant difference based on $p \leq 0.05$.

We next determined transit time in more detail by determining transit time with respect to proximal and distal epididymis (Fig. 18). In the proximal epididymis (IS/caput/corpus), the low group had a transit time of 2.49 ± 0.351 days, the medium group had 3.07 ± 0.408 days, and the high group had 3.77 ± 0.317 days. In the distal epididymis (cauda), mice in the low group had a transit time of 6.76 ± 0.236 days, the medium group had 6.51 ± 0.286 days, and the high group had 8.39 ± 0.378 days. Since the cauda stores sperm, it is not surprising that the epididymal transit time was significantly longer in the distal region compared to the proximal region.

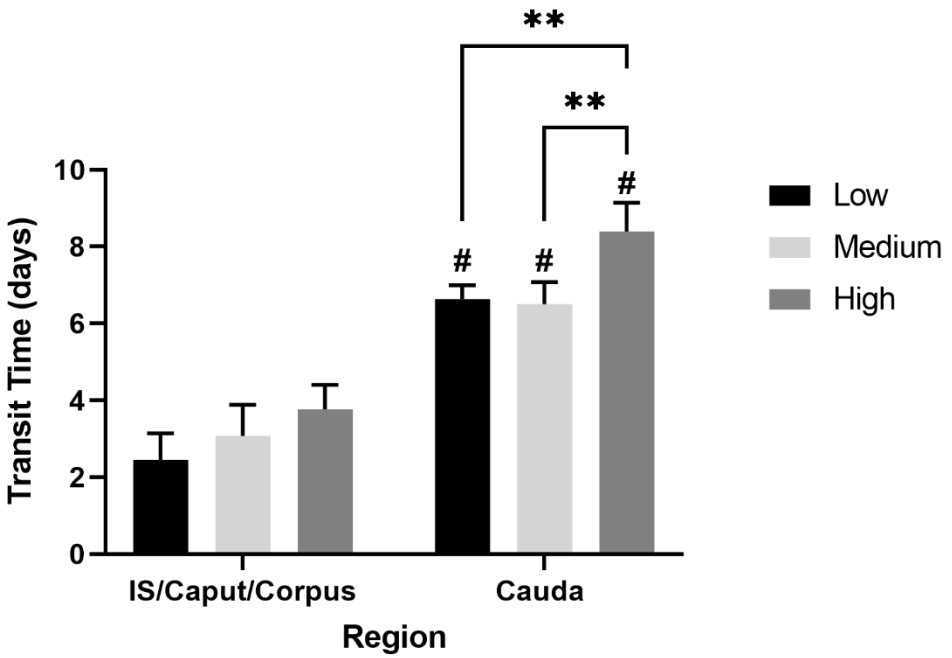

Figure 18: Transit Time per Region Epididymis. Transit time in days in proximal and distal epididymis. Mice in the high group had a longer transit time in the cauda when compared to the medium and low group. Asterisks indicate a significant difference between groups within region based on $p \leq 0.05$. # denotes significant difference between regions within a diet group.

We next examined sperm from the proximal epididymis and cauda regions using the YO Male Fertility Sperm Test. The sperm that came from the high group that were diluted in PBS showed little to no motility. The lack of motility was potentially due to a failure to activate sperm after being released from the epididymis. On the second collection day, sperm were suspended in PBS, HBSS, or DMEM with high glucose and 10% FBS. Sperm from the DMEM group showed much improved motility. Given that collection media conditions greatly impacted sperm motility, and different conditions were used for two of the three diet groups, an assessment of motility between groups was not possible. However, the results do suggest that use of the YO sperm test may be feasible in future studies if using appropriate collection media to activate sperm motility.

Chapter 5: Discussion

This study aimed to test if peri-pubertal exposure to dietary phytoestrogens would show dose-dependent adverse effects on spermatogenesis and male fertility in mice. This study demonstrated that high dietary phytoestrogens during pubertal development decreased pituitary hormone secretion, increased testicular steroid secretion and altered epididymal transit time. Sperm production parameters appeared to be lower in the high group but did not reach statistical significance. Dietary phytoestrogens did not significantly affect body weight. This suggests that the three diets provided similar nutritional value for growth and development and that overall animal health was not impaired. Together, these results suggest that high dietary phytoestrogen during puberty may affect male fertility.

Serum hormones, specifically FSH, have a direct effect on the success of spermatogenesis and therefore sperm count in the testis. More specifically, FSH is paramount in stimulating the development and survival of spermatogonia (Meachem et al., 1999). Mice in the high group had a significantly decreased serum concentration of FSH compared to the low group. Therefore, increased phytoestrogen exposure may have adverse effects on the HPG axis at the anterior pituitary level. Phytoestrogens such as genistein have been shown to decrease basal production and secretion of FSH through a mechanism dependent on ER (Arispe et al., 2013). While phytoestrogens have a lower binding affinity for ER compared to estrogen, it may compete for binding (Kuiper et al., 1998). The majority of estrogen's functions at the pituitary are through ER1 (Curtis Hewitt et al., 2000; Haydar Ali Tajuddin et al., 2020) and may be the predominant receptor acted upon by phytoestrogens to exert their effects. At the level of the testis, FSH stimulates Sertoli cells that ultimately produce the majority of intratesticular estrogen during puberty (Carreau et al., 2006). Aromatase, the enzyme that converts local testosterone to

estrogen, is expressed primarily by Sertoli cells during puberty (Carreau, 2002). A lack or excess of estrogen in the testis has been shown to cause decreased sperm counts and infertility (Robertson et al., 1999; Li et al., 2001). Intratesticular estrogen concentrations did not differ between the diet groups, suggesting that phytoestrogen exposure does not have a direct effect on intratesticular estrogen concentrations. FSH is also responsible for increasing Sertoli cell count during development as well as supporting their function. Testosterone is also involved in increasing Sertoli cell count, however FSH has been shown to have a larger role (Griswold et al., 1977). Additionally, increased testosterone concentrations could increase the negative feedback on pituitary secretions such as with the decrease in FSH, but we would also expect LH to be decreased which was not true in our case.

Serum LH is responsible for stimulating Leydig cells that produce testosterone, the paramount androgen in stimulating and regulating spermatogenesis. Similar to estrogen, a lack of testosterone may adversely affect spermatogenesis (O'Donnell et al., 1994, McLachlan et al., 2002) as well as epididymis function (Assinder et al., 2007). Although testosterone and FSH have many overlapping and synergistic effects on spermatogenesis, testosterone has positive effects on spermatogenesis and is uniquely responsible for the terminal differentiation of round spermatids, adhesion to Sertoli cells, and completion of spermiogenesis (Holdcraft & Braun, 2004). Testicular testosterone concentrations as well as the T/E_2 ratio were significantly higher in the high group compared to the medium group, but not the low group. Since there was no change in serum LH concentrations that would suggest alterations in pituitary hormone production and/or secretion, this suggests that phytoestrogen exposure acts on the testis to increase intratesticular testosterone concentrations through altering Leydig cell function. This is consistent with previous findings that genistein, one of the most common sources of

phytoestrogens and one of the chemicals used in this study, stimulates Leydig cell function and intratesticular testosterone concentrations (Kuiper et al., 1998). Developmental exposure to phytoestrogens has also been shown to stimulate proliferation in Leydig cells (Sherrill et al., 2010) in addition to increased secretion of testosterone (Akingbemi et al., 2007), however other studies found that exposure decreased testosterone concentrations without affecting LH (Weber et al., 2001). Therefore, it is possible that the significant increase in intratesticular testosterone concentrations is caused by increased Leydig cell proliferation and/or function due to phytoestrogen exposure. Phytoestrogens may be exerting these effects through ER1 and/or ER2 as these receptors are both expressed in Leydig cells. However, ER1KO mice have been shown to produce twice as much testosterone compared to wild type mice, while ER2KO mice yielded no change, suggesting ER1 is the dominant receptor in mediating testosterone production in the Leydig cell (Akingbemi et al., 2003). These findings suggest that, although phytoestrogens such as genistein and daidzein have higher binding affinities for ER2, phytoestrogens could be acting through ER1, or possibly through ER2 and have inhibitory effects on ER1 signaling.

Many of the testicular sperm count and production parameters appeared to decrease in the high phytoestrogen diet but were not statistically different from other groups. Consistent with our results, prepubertal genistein exposure resulted in a slight decrease in sperm counts (Lee et al., 2004). Another study found prepubertal genistein exposure showed no change in sperm counts but did have adverse effects on sperm and testis morphology (Tang et al., 2020). Sperm morphology was not assessed in our study but testis morphology appeared largely similar between diet groups. The effects of high phytoestrogen on FSH and testosterone could contribute to the minimal changes in sperm production. An increase in intratesticular testosterone in the

high group would positively affect spermatogenesis and could offset the negative effects from the decrease in FSH concentrations.

Total sperm counts in the proximal and distal epididymis did not differ with respect to diet. This is inconsistent with one study that found significant decreases in caudal sperm counts with exposure to genistein and daidzein (Caceres et al., 2015). Similarly, another study found that high dietary phytoestrogen exposure similar to our high diet group yielded a 25% decrease in epididymal sperm counts, although in adult mice (Cederroth et al., 2010). We expected and found that the distal epididymis contained more sperm as it functions to store sperm. Transit time is vital to the proper maturation and maintenance of sperm and is controlled by testosterone and estrogen. If transit time is too short, sperm may mature or form incorrectly (Fernandez et al., 2008), however sperm function is preserved even if transit time in the cauda is significantly increased, even to 30 days (Mc et al., 1975). In the high group, total transit time was significantly increased compared to the medium group, but not the low group. When examined in more detail, epididymal transit time in the proximal epididymis did not statistically differ between groups but trended longer and did differ in the distal epididymis. Transit time in the distal region was significantly longer in the high group compared to both the medium and low groups. These results agree with previous findings that show increased concentrations of testosterone increase transit time, opposed to estrogen that decreases transit time (Fernandez et al., 2008). This suggests that only high doses of phytoestrogen exposure have adverse effects on transit time, specifically in the cauda region. This trend of increased time spent in the maturation region as well as increased time in the cauda region of the epididymis would suggest that sperm have more time to mature. Our measurements only show an increase of about 2 days in the high group vs

the low group, which has been shown to not be detrimental to sperm counts but, in fact, cause more sperm to collect before ejaculation (Billups et al., 1990).

Pubertal development is marked morphologically in the testis by increased length and tortuosity of seminiferous tubules, as well as proper development and opening of its lumen (Koskenniemi et al., 2017). A delay in the onset of puberty, therefore, may have adverse effects on testis development. Seminiferous tubule diameter and percent open lumen were not significantly affected across diets suggesting that phytoestrogen exposure does not delay puberty. Percent abnormal lumen trended upward especially in the high diet, consistent with the downward trend in DSP. These results are inconsistent with a study that found that Wistar rats that were exposed to pure genistein and daidzin had a delayed onset of puberty (Caceres et al., 2014). A similar study done in Sprague-Dawley rats, EDC exposure to genistein in the gestational and perinatal period significantly increased body weight but had no significant effects on the endocrine system (Masutomi et al., 2003). This discrepancy may be due to differences between strains such as differences in extent and location of ER and AR expression across strains and species (Zhou et al., 2002; Plata & Pearl, 2022). Similarly, there is a lack of uniformity of methods concerning exposure route and chemical in other studies.

While phytoestrogen exposure has a clear effect on the male reproductive tract, exposure may also influence and even positively affect other systems in the body. Several studies have demonstrated that phytoestrogens have protective effects on the cardiovascular system against cardiovascular disease and hypertension. Dietary phytoestrogen exposure has been shown to reduce total cholesterol, reduce plasma triglyceride levels and lower LDL levels while increasing HDL levels (Nagamma et al., 2017). One study demonstrated that isoflavone exposure in postmenopausal women over the course of six months reduced hypertension by increasing

endothelial vasodilation through reducing cell adhesion molecule counts involved in hypertension development such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) and E-selectin (Desmawati & Sulastri, 2019). Phytoestrogens may be able to reduce overall cholesterol levels, preventing the buildup of fatty deposits in blood vessels and therefore blood clots, heart attack or stroke (Terzic et al., 2012). In Sprague-Dawley rats, 200 mg/kg genistein dietary supplementation lowered total serum cholesterol concentrations by 17% (Legette et al., 2011). Phytoestrogens have also been shown to benefit the cardiovascular system by suppressing inflammation, specifically by inhibiting (COX-2), an enzyme important in prostaglandin production for the inflammatory response. Sprague-Dawley rats treated with genistein showed inhibited COX-2 expression (Krenn & Paper, 2009). Inflammation is known to promote carcinogenesis as well as metastasis (Coussens & Werb, 2002). Female rats injected with the MCF-7 human breast cancer cell line demonstrated that genistein and capsaicin treatment inhibits MAP kinase (mitogen-activated protein kinase) function which is involved in development of cancers through increased COX-2 expression (Hwang et al., 2009). The positive benefits of phytoestrogens go beyond the cardiovascular system due to their antioxidant function. For example, dietary phytoestrogens have been shown to be neuroprotective and reduce Alzheimer's Disease related pathology and progression, although only having short-term improvements on cognitive function (Soni et al., 2014). The various health benefits that come with dietary intake of phytoestrogens such as genistein should be evaluated alongside the possible negative health effects, specifically those contributing to infertility.

Our study provides evidence to support that dietary phytoestrogen exposure during the prepubertal developmental window may have adverse effects on and male reproductive tract function but does not fully support a dose-dependent relationship between exposure and

infertility. While these effects may be multifactorial, our results support that phytoestrogen exposure primarily influences the anterior pituitary and decreases secretion of FSH, as well as the testis and increases testosterone secretion. These negative and positive effects on spermatogenesis may be counteracting or masking each other, resulting in a downward trend for spermatogenesis metrics. Therefore, if only the testis and/or sperm counts were studied with phytoestrogen exposure, the exposure's effects on disrupting the endocrine system would be missed. Further studies would benefit from studying the effects of phytoestrogen exposure on sperm motility to further assess effects on overall fertility. Additionally, Sertoli cell and Leydig cell counts as well as aromatase expression could further elucidate the mechanism for phytoestrogen's effects on the testis.

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