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Mechanisms of Effects of Phytoestrogens on Reproduction, Steroidogenesis and Steroid Action in Male Rats.

By

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Abstract

The consequences of soy isoflavone consumption on steroidogenesis were examined in F1 male rats from a multi-generation reproduction study investigating the effects of diets varying in isoflavone content. F1 male rats were obtained from a multi-generation study where the parental generation was fed diets containing alcohol-washed soy protein supplemented with increasing amounts of Novasoy, a commercially available isoflavone supplement. A control group was maintained on a soy-free casein-based diet (AIN93G). The diets were designed to approximate human consumption levels and ranged from 0 to 1046.6 mg isoflavones/kg pelleted feed, encompassing exposures representative of North American and Asian diets as well as infants fed soy-based formula. Testicular and serum androgen levels were assayed with commercial kits and were approximately doubled at postnatal day (PND) 120 for rats fed a diet of elevated levels of isoflavones. Microsomal steroidogenic enzyme activities were examined radiometrically. Testicular mRNA and protein levels were assayed by RT-PCR, western blotting and immunohistochemistry respectively. Steroidogenic enzyme activities were significantly increased at PND 28 and immunohistochemistry revealed approximately 25 % greater numbers of Leydig cells stained for steroidogenic factor 1 at both PND 28 and 120 amongst rats fed elevated levels of isoflavones, resembling high human consumption rates. These findings show that F1 male rats continuously exposed to a mixture of dietary soy isoflavones from conception onwards exhibit altered gene expression at PND 28, which may lead to increased microsomal steroidogenic enzyme activity at this age and serum and testicular androgen profiles in adulthood.

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List of Abbreviations

ABP	Androgen binding protein
ACAT	Acyl cholesterol acyl transferase
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1
AR	Androgen receptor
BNP	Brain natriuretic peptide
cAMP	Cyclic Adenosine phosphate
cDNA	Complimentary DNA
C/EBPß	member of the CCAAT/enhancer-binding protein (C/EBP) family of basic region/leucine zipper transcription factors
CEH	Cholesterol ester hydrolase
cFOS	human oncogene of FOS family
CNP	C-type natriuretic peptide
CPM	Counts per minute
CRL	Crown rump length
CYP17	Cytochrome P450 type 17
DAB	3,3'-Diaminobenzidine
DAX-1	Dosage sensitive sex reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1
DEPC	Diethyl pyrocarbonate
DES	Diethystilbestrol

DHEA	Dihydroepiandrosterone
DHT	Dihydrotestosterone
DMGA	Dimethyl glutaric acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immuno selective assay
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated protein kinase
αERKO	ERα knockout
ßERKO	ERß knockout
EtBr	Ethidium bromide
F0	Parental generation
F1	First generation
F2	Second generation
FSH	Follicle stimulating hormone
GATA	GATA consensus transcription factor
GnRH	Gonadotropin releasing hormone
G3PDH	3-glyceraldehyde phosphate dehydrogenase
GRTH	Gonadotropin regulated transcriptional helicase
hCG	Human chorionic gonadotrophin
HDL	High density lipoprotein
HPG	Hypothalamic-pituitary-gonadal axis
HPT	Hypothalamic-pituitary-testis axis

HPLC	High performance liquid chromatography
3ß-HSD	3ß-hydroxysteroid dehydrogenase
17ß-HSD	17ß-hydroxysteroid dehydrogenase
17-OHase	17-hydroxylase
IHC	Immunohistochemistry
IL	Interleukin
IP	Intraperitoneal injection
LC	Liquid chromatography
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LHRH	Luteinizing hormone releasing hormone
LuRKo	Luteinizing hormone receptor knockout
MAPK	Mitogen-activated protein kinase
MIS	Mullerian inhibiting substance
mRNA	Messenger RNA
MS	Mass spectroscopy
NO	Nitric oxide
NSS	Normal swine serum
OP	Octylphenol
P450c21	cytochrome P450 21-hydroxylase
P450-SCC	P450 cholesterol side chain cleavage enzyme
PACAP27	Pituitary adenylate cyclase-activating polypeptide

PBR	Peripheral type benzodiazepine receptor
PGF2α	Prostaglandin F2 alpha
PKA	Protein kinase A
PND	Post natal day
PVDF	Diethyl pyrocarbonate
rFtz-F1	Fushi tarazu factor 1 subfamily member rat gene encoding Ad4BP
RNA	Ribonucleic acid
RT-PCR	Reverse trancriptase polymerase chain reaction
SC	Sub-cutaneous injection
SD	S*prague Dawley*
SE	Standard error
SF-1	Steroidogenic factor-1
SHBG	Sex hormone binding globulin
STAR	Steroidogenic acute regulatory protein
SOX-9	Sox family transcription factor 9
SR-B1	Scavenger receptor class B type 1 (HDL receptor)
SRY	Testis determining factor Y
T	Testosterone
TBST	TRIS buffered saline containing 0.1% tween-20
TLC	Thin layer chromatography
U	Units
WT-1	Wilm's tumor protein 1

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Dedication

This work is in memory of Dr. Peter R. Garner MA, MSc, MB, B.Chir, FRCP(C), FACP.

Chapter 1

1.0 General Introduction

1.1 Hypothesis

Previous findings by other researchers showing that concentrated aglycone isoflavones can alter circulating androgen levels (Strauss et al., 1998; Roberts et al., 2000; Weber et al., 2001; Zhang et al., 2002a) and enzyme activities (Evans et al., 1995; Weber et al., 1999; Wong and Keung, 1999; Krazeisen et al., 2001; Whitehead et al., 2002) have led our laboratory to hypothesize that:

Testicular production of androgens in Sprague-Dawley rats exposed to a mixture of soy isoflavones in conjugated and unconjugated states during their entire life spans is increased or decreased due to changes in the steroidogenic cascade.

Steroidogenesis in the male rat is stimulated by hypothalamic gonadotropin releasing hormone (GnRH) acting to stimulate pituitary luteinizing hormone (LH) production and release, which travels via the blood to the testis to act at Leydig cell LH receptors (LHR) to upregulate androgen production.

The Leydig cells within the interstitium of the testis are the primary androgen producing cells. Under basal conditions Leydig cells will produce limited androgen, but androgen production is stimulated by LH through an immediate LH receptor mediated second messenger pathway involving increased cAMP dependent phosphorylation. Leydig cells mobilize internal cholesterol reserves by regulating acyl cholesterol acyl transferase (which produces cholesterol esters) and cholesterol ester hydrolase (which liberates free cholesterol from esters), or produce cholesterol *de novo* from acetate or use extracellular cholesterol from dietary sources with the assistance of the high density

lipoprotein receptor: scavanger receptor class I protein (SRB1). Cholesterol then moves through a cascade of enzymatic conversions to form androgens by first being shuttled to mitochondria with the help of shuttling proteins such as steroidogenic acute regulatory protein (STAR) and peripheral-type benzodiazepine receptor (PBR) to reach the cholesterol side chain cleavage enzyme (P450-SCC), which irreversibly cleaves cholesterol to pregnenolone (Payne and Youngblood, 1995; Hauet et al., 2002). Pregnenolone is then transported to the smooth endoplasmic reticulum (SER) to be further modified to androgens such as testosterone. The gene expression of many steroidogenic enzymes, shuttle proteins and receptors are, in part, regulated by a family of transcription factors including steroidogenic factor 1 (SF-1). The activity and expression of factors regulating androgen production are in part controlled by LH. LH in turn, is regulated by feedback inhibition by testosterone and dihydrotestosterone (DHT) and estradiol, as the long term effects of LH stimulation act to upregulate gene expression of key steroidogenic genes.

1.2Testis Development and Function

1.2.1 Reproduction

Reproduction is a fundamental function of all living things. Sexual reproduction in mammals requires specialized tissues and chemicals for the production of progeny. The main purpose of the male reproductive tract can be summarized as the successful production of sex steroids and spermatozoa, involving the testicular production of steroids and spermatozoa and the coordinated release and movement of the latter to the

epididymis for further maturation and storage. These tasks involve a variety of specialized cells and endocrine, paracrine and autocrine mechanisms of regulation.

1.2.2 Cell Types Involved with Testicular Function

Male reproduction relies on a number of specialized reproductive tissues. There are two testes, two epididymides and accessory glands. Testes are the product of primordial germ cells which migrate from the yolk sac to the genital ridge of the mesonephros, where they form the primary epithelium and medullary cords which combine with somatic cells from the genital ridge to form the gonad. In mammals the testes are paired, encapsulated, ovoid organs comprised of numerous seminiferous tubules separated by interstitial tissue.

The cell types found in the testis each have important specialized roles and communicate through autocrine, paracrine and endocrine mechanisms. The bulk of the testis is comprised of the seminiferous tubules where the spermatozoa are formed. The tubules in most species range in size from 200 to 250 μm in diameter, and in the rat there are approximately 12 metres of tubules (normally approximately 30 tubules per testis) per gram of testis (Setchell and Brooks, 1988). Sertoli cells, which represent the main somatic component of the tubular compartment, provide structural support and specific microenvironments needed for the developing germ cells (Konrad et al., 1998). The mechanisms which regulate Sertoli cell development and function are poorly understood. Sertoli cells are the target cells for follicle stimulating horomone (FSH) and testosterone (T), which are two major regulators of spermatogenesis (Hoeben et al., 1999). Peritubular myoid cells surround the tubule separating the basal surface of Sertoli cells

from the interstitium and this compartmentalization of the tubule allows regulation of intratubular pressure and the formation of a blood–testis barrier (Konrad et al. 1998), which protects the developing haploid germ cells from the immune system. The interstitium, located in the spaces between the seminiferous tubules, is comprised of steroidogenic Leydig cells, lymphatic endothelium, nerves, testicular macrophages, mast cells, lymphocytes, blood vessels and stromal cells. These cells partake in paracrine and autocrine regulation (Skinner et al. 1991), for example, Leydig cells that are adjacent to seminiferous tubules at spermatogenic stages VII-VIII are larger, and have increased steroidogenic capacity (Sprando et al. 1997). The interstitium of different species varies considerably, for example approximately 15% of rat testes compared with 30% of human testes are comprised of interstitium (Setchell and Brooks, 1988). Testes also contain a section referred to as the rete testis, which is a complicated set of intercommunicating channels involved in endocytotic transport of developing spermatozoa (Setchell and Brooks, 1988). In the rat, the rete testis is a sac located close to the epididymis. Through this network, spermatozoa are carried to the epididymides, via the efferent ducts. The epididymides are responsible for the further maturation (motility and fertility) and storage of spermatozoa. Downstream of the epididymides is the ductus (vas) deferens: a complex epithelium with both absorptive and secretory functions, which acts as a conduit leading spermatozoa to the urethra.

1.2.3 Testicular Steroidogenic Development

In life, androgens are critical in establishing the male phenotype very early in development (Huhtaniemi, 1995). *In utero,* Leydig cell testosterone, the HPT axis and

Sertoli-cell derived MIS, are important for the formation and development of the male Wolffian reproductive tract and the arrest of the default female Mullerian tract (Huhtaniemi, 1995; Parker et al., 1999). Early in fetal life, the reproductive tract is bipotential, yet males are masculinized by androgens and expression of SRY to form testes, epididymides, vas deferens, penis, scrotum, prostate and ejaculatory duct.

Testicular androgen production in early fetal life is regulated by a LH-independent mechanism, as proven by androgen production in LuRKO mice (Zhang et al. 2004). Factors such as vasoactive intestinal peptide, pituitary-adenylate-cyclase-stimulating polypeptide and, recently, atrial, brain and C-type natriuretic peptides have been implicated as endocrine and paracrine regulators of LH-independent embryonic testicular steroidogenesis (reviewed in El-Gehani et al., 2001).

Later in fetal life, endocrine hormones regulating testicular steroidogenesis are produced by the pituitary and androgen target tissues develop responsiveness by the expression of receptors (Huhtaniemi, 1995). GnRH has been found in male rat brains as early as gestational day 15, and acts on the neonatal pituitary to stimulate LH production for signalling to testicular LHRs present as early as gestational day 15.5 (Ojeda and Urbanski, 1998). LH and chorionic gonadotropin regulate androgen levels by stimulating Leydig cells and also lead to the secretion of other paracrine factors from the Leydig cell such as a paracrine factor that modulates Sertoli cell function (PmodS) by peritubular myoid cells (Skinner, 1991). In both males and females sex steroids have important feedback roles on the HP-axis, as negative feedback reduces the production of GnRH in the hypothalamus, leading to reduced LH and FSH release from the pituitary.

LH levels in prepubertal mammals are low but rise sharply during puberty when spermatogenesis is beginning; terminally differentiating immature Leydig cells. In rats, the second to third weeks post-natally lead to important developments such as the opening of the tubular lumen on post-natal-day (PND) 10, the blood-testis barrier begins to form on PND 15, while Sertoli cell proliferation ceases on PND 15 (Weber et al. 2001). Leydig cell differentiation and increased testosterone production begin at approximately PND 20, which coincides with increased serum LH levels, testis weights and the production of enzymes for steroidogenesis (Miyachi et al, 1973). The molecular mechanisms involved in the differentiation and initiation of steroidogenic function in immature Leydig cells are poorly understood. At puberty testosterone levels rise in the serum and become a significant source of negative feedback on the hypothalamic-pituitary-gonadal axis. Intratesticular testosterone levels in adults are roughly 100 times greater than in serum. Reduced androgen levels have been proposed as a means of sensitizing the reproductive tract to exogenous estrogens, since the balance of estrogens and androgens may regulate normal development in some regions of the male reproductive tract (Rivas, et al. 2002).

1.2.4 Spermatogenesis

In the early postnatal rat testis, fetal gonocytes differentiate into spermatogonia at approximately PND 5, then spermatogonia divide and develop into spermatocytes in the seminiferous tubules from PND 10 to 19, and the first spermatozoa appear at approximately PND 45 (Clermont et al. 1957, Kula 2001). It has been proposed (Kula et al. 2001) that the mechanism triggering seminiferous tubule maturation is under

hormonal regulation, potentially involving androgens, estrogens, FSH and prolactin and the production of spermatozoa is dependent on the secretion of Leydig cell derived testosterone and the pituitary gonadotropins FSH and LH (McLaughlan et al. 2002). Spermatogenesis can be broken down into 4 steps: 1) spermatogonial development which involves stem cells and mitotic divisions; 2) meiosis involving DNA synthesis to produce haploid spermatids; 3) spermiogenesis which involves development and formation of structures such as head and tail; and 4) spermiation where spermatozoa are moved to the tubule lumen for export (McLaughlan et al. 2002).

1.2.5 Steroidogenesis

Steroids are signalling molecules common to a broad variety of aquatic and terrestrial plants and animals. Steroids are planar lipid structures containing a cyclopentanoperhydrophenanthrene heterocyclic ring and are largely insoluble in water. Testosterone is a potent androgenic steroid produced by the testis. It can be further metabolized locally in androgen target tissues to the more potent androgen DHT by the enzyme 5α-reductase. The function of androgen target tissues such as the seminiferous tubules, prostate, brain, skin and epididymis depend on the presence of testosterone and DHT.

Sex steroids originate from the non-steroid precursor cholesterol, which is formed *de novo* in the liver or is ingested in the diet. Cholesterol is absorbed from the blood by steroidogenic cells, via the high-density lipoprotein receptor (HDL-receptor) (SR-B1) to be stored in lipid droplets (cholesterol esters) in steroidogenic cells. In the Leydig cell, free cholesterol is transported to the mitochondrial membrane to be converted to

androgens. The first enzyme-mediated modification of cholesterol, is carried out by P450- SCC located in the inner-mitochondrial membrane, which cleaves cholesterol to pregnenolone (Payne and Youngblood, 1995). Pregnenolone is then transported to the smooth endoplasmic reticulum where it is converted by the enzymes 3ß-hydroxysteroid dehydrogenase (3β-HSD), 17-hydroxylase C-17,20 lyase (CYP17), 17β-hydroxysteroid dehydrogenase (17ß-HSD), 5α-reductase and aromatase into the hormonally active steroids progesterone, testosterone, 5α-DHT and estradiol (Payne and Youngblood, 1995).

In 1948, Li and Evans showed that hypophysectomized animals exhibited reduced androgen synthesis. Since then, research had shown that LH pulses from the pituitary, acting via Leydig LHR, increase testosterone production via cAMP/PKA and Ca^{2+} dependent mechanisms (Li and Evans, 1948; Hall, 1994). This endocrine regulation by LH ranges from short-term stimulation of the mobilization of cholesterol from lipid stores and STAR mediated cholesterol transport to the mitochondria (minutes), to the longer-term up-regulation of the synthesis of steroidogenic enzymes (hours) (Hall, 1994). The regulation of elements of the testicular steroidogenic cascade such as CYP17, P450-SCC, 3ß-HSD, STAR, SR-B1, MIS and LHß is complex involving transcription factors such as SF-1 and LH endocrine regulation (Morohashi and Omura, 1996; Kawabe et al., 1999; Lopez et al., 1999; Morohashi, 1999; Parker et al., 1999; Parker and Schimmer, 2002; Gyles et al., 2001; Lopez et al., 2001).

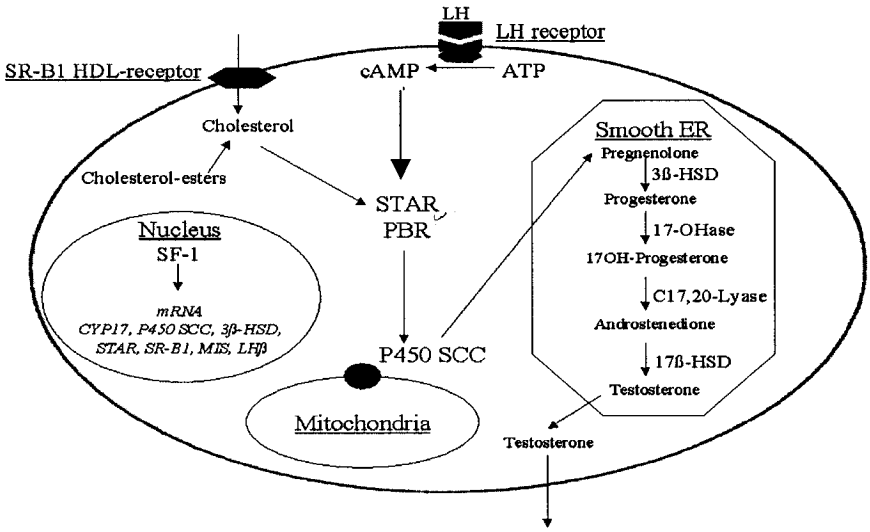

Figure 1 Leydig cell steroidogenesis.

Depiction of Leydig cell LH stimulated testosterone production involving the stepwise conversion of cholesterol with steroidogenic enzymes and cholesterol shuttling proteins.

1.2.6 Androgen Function

The production of testosterone (Figure 1) and estradiol in testicular Leydig cells is necessary for spermatogenic mitotic and meiotic divisions (McLauchlan et al., 2002), androgen target tissue function and libido. At puberty spermatogenesis requires a high androgen production particularly at stages 7 to 8 of spermatogenesis, which are coincident with meiotic division (McLauchlan et al., 2002). Androgens regulate the transcription of androgen dependent genes (Leung et al., 2002). In early perinatal stages, the male brain receives androgens in order to develop, and in rat brains, there exists a critical period in which testosterone actively promotes the development of structural and functional sexually dimorphic characteristics that, in the absence of androgens, would be female (Huhtaniemi, 1995; Suzuki et al., 2002).

1.3 Isoflavones

1.3.1 Soy Isoflavones

Phytoestrogens are plant-derived molecules that are found in fruits, vegetables, legumes, whole-grains and soy products, which act as pigments, waste products, or as fungistatic defense mechanisms (Clevenger, 1964; Naim et al., 1974). Phytoestrogens are classified into three categories: lignans, coumestans and isoflavones (Humfrey, 1998). The structure of isoflavone compounds (Figure 2) includes the flavone nucleus of two benzene rings linked via a heterocyclic pyrane ring (Messina et al., 2001).

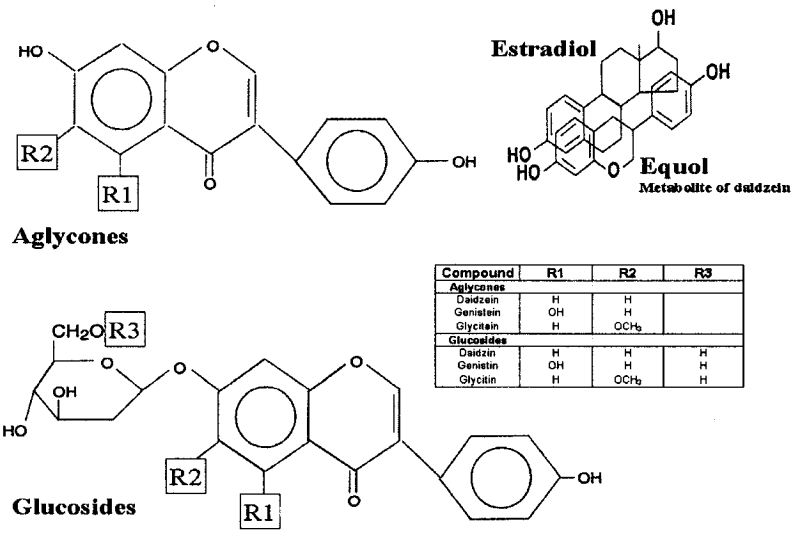

Figure 2 Dietary soy isoflavone structures

Structures of soy isoflavones (aglycones) or conjugated forms (glucosides) [left]. Estradiol superimposed with equol[right].

Isoflavones are found in at least 300 plant sources and are referred to as phytoestrogens, due to their weak estrogenic activity, which is 10^5 fold less than estradiol in mammals (Kurzer and Xu, 1997; Messina et al., 2001). Humans consume isoflavones in a variety of fruits and vegetables such as corn, wheat and soy. Soy foods and soybean constituents

are being researched to determine their physiological effects, pharmacokinetics and metabolic effects since they are growing in popularity amongst vegetarians, vegans, and those with allergies to milk products. Soybeans are a major source of phytochemicals such as saponins, sphingolipids, phenolic acids, Bowman-Birk trypsin inhibitor and isoflavones (Hendrich and Murphy, 2001). Soy has the highest concentration of isoflavones of any food consumed by humans (Walz, 1931), and isoflavanoid composition and quantities in foods can be affected by a variety of conditions such as environmental factors, pathogens, soil nutrients, fertilizer, day length, cultivation under glass, temperature and humidity (Whitten et al., 1995).

1.3.2 Isoflavone Metabolism

Research of soy isoflavones was accelerated with the discovery of equol (metabolite of daidzein) in human urine and the correlation that isoflavones in urine and blood were found at levels far greater than endogenous estrogen levels after soy consumption (Setchell et al, 1984; Axelson et al., 1994). It has been known since 1931 that soybeans contain high concentrations of isoflavones (Walz, 1941) and that soy foods contain high levels of isoflavones, primarily as β-glycoside conjugates (Murphy, 1982; Coward et al., 1993; Setchell et al., 2001). When phytoestrogens from soy are ingested, the conjugated isoflavones undergo hydrolysis by intestinal bacterial β-glucosidases in the jejunum, releasing the principal bioactive aglycone forms (Kurzer and Xu, 1997, Setchell et al., 2001). The conjugated forms of isoflavones are very poorly absorbed and have not yet been detected in human blood or urine (Setchell et al., 1984; Yasuda et al., 1994). The aglycones are absorbed by the intestines and then conjugated mainly with

glucuronic acid by an as yet, uncharacterized UDP-glucuronyl transferase, for enterohepatic recycling (Sfakianos et al., 2000; Hendrich and Murphy, 2001; Setchell et al., 2001).

Daidzein metabolism differs from that of genistein in that it is converted to sulfate and sulfate/glucuronide conjugates in rats, likely allowing for faster elimination in the urine. Genistein is more hydrophobic, leading to a lengthening of its retention time in the body (Sfakianos et al., 1997; Hendrich and Murphy, 2001). Humans exhibit considerable differences in blood plasma and urine levels of isoflavones and this variability has been correlated to individuals with specific intestinal bacterial strains capable of metabolizing daidzein to equol.

1.3.3 Potential Benefits of Isoflavone Consumption

It has been proposed that biologically active concentrations of isoflavones confer health benefits that may explain the lower incidence of some hormone-dependent diseases in Eastern countries such as Japan, where high consumption rates of dietary soy are evident (Setchell et al., 1984, Wang et al., 2004). Isoflavone usage has been stimulated by correlations between soy isoflavone intake levels in Asian diets and the reduced incidences of coronary heart disease, prostate, colon and breast cancers, suggestive of protective effects of isoflavones (Clarkson et al., 1995; Adlercreutz and Mazur, 1997; Humfrey, 1998; Tikkanen and Adlercreutz, 2000). Phytoestrogen intake has been implicated in reducing platelet aggregation and thrombus formation, relaxation of arteries and reducing oxidized lipid levels in serum, all of which may potentially help to prevent heart disease (Goldwyn et al., 2000). Monkeys fed soy protein and

isoflavones exhibited reductions in serum triacylglycerol and cholesterol and increased high-density lipoprotein (HDL) (Goldwyn et al., 2000). Other potential beneficial aspects of soy consumption include: the prevention of osteoporosis (Humfrey, 1998); as hormone replacement therapy in menopause (Brige, 2000); as well as having antioxidant effects; and antipromotional effects, since genistein can prolong certain tumor latencies or decrease tumor multiplicity (Wei et al., 1995; Kurzer and Xu, 1997). In guinea pigs, genistein lowered nitric oxide (NO) in animals with inflammatory bowel disease, which is characterized by high expression of NO synthase (Goldwyn et al., 2000).

1.3.4 Potential Risks of Isoflavone Consumption

In the 1940's Australian researchers linked infertility in sheep to the hormonal activity of ingested red clover isoflavones, namely formonentin and biochanin A (Bennetts et al., 1946; Kurzer and Xu, 1997; Mesiano et al., 1999). Additionally, evidence of phytoestrogens affecting female human health was accumulated from Dutch women who exhibited uterine bleeding and menstrual effects after ingesting high levels of phytoestrogens from eating tulips during the Second World War (Humfrey, 1998). Some animal studies investigating the safety of soy isoflavone consumption have demonstrated some deleterious effects on reproductive success and health (Bennetts et al., 1946, Shutt, 1976; Humfrey, 1998, Cassanova et al., 1999; Nagao et alk., 2001; Lewis et al., 2003). Genistein and daidzein lengthen the female rat estrus cycle and may even cause chromosomal aberrations (Whitten et al., 1995; Kurzer and Xu, 1997; Goldwyn et al., 2000) and male animals may also be sensitive to isoflavones since genistein can inhibit the growth and proliferation of testicular cell lines (Kumi-Daika et al., 1998). In

rats, Delclos and colleagues (2001) have shown that genistein can disrupt spermiation and decrease the number of spermatozoa present in the epididymis. This may be the result of isoflavones perturbing the levels of androgens and estrogens, thereby influencing the growth and development of the male reproductive tract (Rivas et al. 2002). Early exogenous estrogen exposure may increase the risk of testicular cancer in male offspring as mothers exhibiting excessive nausea in early pregnancy have shown a correlation to having male offspring with testicular cancer possibly due to long term changes in SHBG/ABP levels (Hawkins, and Miaskowski. 1996).

Isoflavone studies using different experimental animal models have found significant reductions in serum testosterone in rats (Roberts et al., 2000; Weber et al., 2001), mice (Strauss et al., 1998) and fish (Zhang et al., 2002a). Phytoestrogens have been shown to both increase and decrease steroidogenic enzyme activities that are important for androgen production such as 3ß-HSD (Wong and Keung, 1999; Whitehead et al., 2002), 17ß-HSD (Kraeisen et al., 2001; Whitehead et al., 2002) and 5α-reductase (Evans et al., 1995; Weber et al., 1999), which could impact the production of androgens within the testis. Therefore, isoflavones through effects on androgen biosynthesis, may cause improper brain and reproductive tract differentiation (Humfrey 1998). Humfrey has postulated that early age exposure to phytoestrogens could reduce semen quality and increase male congenital malformations (cryptorchidism, hypospadias) (Humfrey, 1998), indicative of impaired androgen production or action. Isoflavones have been also shown to effect androgen biosynthesis by increasing or decreasing the levels of serum LH (Strauss et al., 1998; Roberts et al., 2000; Lund et al., 2003). Changes in enzymes and endocrine factors of the steroidogenic cascade by isoflavones could be due to changes in

gene expression. The regulation of enzymes, cholesterol transport proteins, developmental factors and receptors involved with steroidogenesis such as CYP17, P450-SCC, 3ß-HSD, STAR, SR-B1, MIS and LHß are, at least in part, controlled by steroidogenic factor 1 (SF-1) (Morohashi and Omura, 1996; Lopez et al., 1999; Parker et al., 1999; Kawabe et al., 1999; Morohashi, 1999; Gyles et al., 2001; Lopez et al., 2001; Parker and Schimmer, 2002). It is of interest, therefore, that the chemicals diethylstilbestrol (DES) and 4-octylphenol (OP) reduced fetal rat SF-1 mRNA levels (Jury et al., 2000), and it is possible that isoflavones, due to their estrogenicity, could have a similar effect on SF-1 thus affecting overall androgen production.

1.3.5 **Mechanisms of Isoflavone Action**

Soy isoflavone research has uncovered a myriad of cellular effects. Many of these effects are due to the agonistic or antagonistic action of the isoflavones on estrogen receptor activation, potentially influencing genes regulated by estrogen. Additionally phytoestrogens can bind ABP/SHBG, (albeit with affinities several orders of magnitude lower than estradiol) and can stimulate production of SHBG (Goldwyn et al., 2000; Pino et al., 2000; Hodgert-Jury et al., 2000).

Since genistein has been shown to affect both estrogen receptor positive and negative cells there must be other mechanisms of action that are independent of estrogen receptor-mediated action. Genistein has a broad range of effects in the body. For example, genistein causes cell cycle arrest in G2/M phase; acts as a scavenger for hydrogen peroxide; and plays a stimulatory role for natural killer cells (Humfrey, 1998; Hendrich and Murphy, 2001). Genistein has also been shown to be an inhibitor of

tyrosine kinase and topoisomerase II (Humfrey, 1998; Goldwyn et al., 2000). Isoflavones bind and can modify membrane environments (Lehtonen et al., 1996; Arora et al., 2000), which could in turn cause changes to proteins involved in the androgen producing steroidogenic cascade.

1.3.6 Human Consumption of Isoflavones

The fact that Asian populations have been consuming large quantities of soy in their diets and lead normal healthy lives, with low incidences of certain cancers indicates that the consumption of phytoestrogens for humans is safe and potentially beneficial. Until the mid 1970's soy containing foods were unfamiliar to North Americans, or considered unappealing products that were only consumed by vegetarians. It is critical that research in this area continues as North Americans have, over the last few decades, accepted soy into their diets, probably as a result of food and pharmaceutical companies promoting beneficial effects on physical health. Today, soy foods such as soymilk, tofu, miso, tempeh, natto, soy sauce and kinako are popular. Soy protein has been a component of infant formulae in the West for nearly 100 years. Modern soy based infant formulas are fed to approximately 36% of infants in North America with (~1.4 million infants per year in the United States alone) and are carefully regulated by the American Academy of Pediatrics recommendations and Infant Formula Act (1980 and subsequently amended in 1986) (reviewed in Merritt and Jenks, 2004). However, even with the potential benefits of soy consumption, research into the possible adverse effects of soy phytoestrogens must continue. Some researchers have argued against the risks of feeding infants soy formula, claiming that isoflavones are extensively metabolized by

infants into glucuronide and sulfate conjugates which have low or negligible biological activity (Humfrey, 1998). Animal studies have demonstrated some deleterious effects on reproductive success and animal health, thus further studies are needed to confirm the safety of high levels of dietary soy isoflavones in humans. The present multigeneration study examined the effects of exposure to a mixture of dietary soy isoflavones (Novasoy concentrate which contains a mixture of soy isoflavones: genistein, daidzein and glycitein in conjugated and unconjugated states (glycone and aglycone)) in F1 male rats for their entire lives. The diets used are relevant to human consumption of isoflavones ranging from low to no consumption (0 to 1 mg isoflavones/ kg bodyweight/ day (mg/kg/d)) typical of North American diets, to modest vegetarian and Asian consumption levels (1 to 3 mg/kg/d), to high infant consumption levels (10 mg/kg/d) (Cassidy et al., 1994; Irvine et al., 1998; Strom et al., 1999; Kirk et al., 1999; Wakai et al., 1999; Zung et al., 2001) and a diet level which represented approximately 5 times the infant and 10 times the adult consumption level, representative of a consumer of soy isoflavone supplements (30 mg/kg/d).

1.4 Importance of Isoflavone Research

The research proposed will add to the current knowledge of the effects of isoflavones on male reproduction. The study involved dosing *Sprague Dawley* (SD) rats with a soy protein diet containing increasing amounts of mixed phytoestrogens in different (glycone/aglycone) conjugational states. Few studies to date have examined the effects of various concentrations of phytoestrogens over a long period of time. Many studies have been done on small populations of experimental animals and do not include

multiple generations of animals. This study will examine the effects in rats of dietary isoflavone levels simulating normal human consumption levels on male reproductive tract development and function in terms of androgen production. Dietary soy isoflavone exposure was investigated using a multigeneration study with 2 generations of SD rats fed diets containing 0 to 1046.6 mg isoflavones / kg feed (Appendix 2). To examine the effects of dietary soy isoflavones on F1 male rat steroidogenesis the serum and testicular androgen levels (T and DHT) as well as steroidogenic enzyme activities were profiled across various ages (PND 28, 70, 120, 240 and 360). Levels of mRNA and protein were assessed for steroidogenic enzymes and other factors involved with the regulation of testicular androgen production. Expression levels of mRNA of genes involved with steroid regulation was examined in an attempt to find biomarkers for effects of phytoestrogens on male rats for potential use in predicting health problems. The development and validation of these biomarkers such as protein expression, enzyme activity and specific genes or clusters could prove to be valuable in biochemical/molecular and epidemiological studies as early tools in diagnosing adverse physiological outcomes and assessing genetic susceptibilities to these conditions.
The multigeneration study would allow monitoring of any changes in physiology of first generation offspring whose parents are naïve consumers of soy isoflavones. This study could prove useful for determining safe ranges of dietary soy consumption and potential effects of soy consumption.

Chapter 2

2.0 Increased serum and testicular androgen levels in F1 rats with lifetime exposure to soy isoflavones.

McVey M.J., Cooke G.M., Curran I.H.A., "Increased serum and testicular androgen levels in F1 rats with lifetime exposure to soy isoflavones." Reproductive Toxicology (2004); 18:677-685.

The initial focus of my investigations was to examine the androgen levels in F1 generation male rats at different stages of postnatal life (PND 28, 70, 120, 240 and 360) to determine if dietary soy isoflavones, at levels obtainable by human consumption, could influence circulating and intra-testicular testosterone and dihydrotestosterone levels.

Increased serum and testicular androgen levels in F1 rats with lifetime exposure to soy isoflavones.

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2.1 Abstract

The consequences of dietary soy isoflavones on serum and testicular androgen levels were examined in F1 male rats from a multi-generation study investigating the effects of diets varying in isoflavone content. Rats were fed either a soy-free casein based diet (AIN93G) or a diet in which alcohol-washed soy protein replaced casein as the protein source and to which increasing amounts of Novasoy, a commercially available isoflavone supplement were added. Analysis of these diets showed that the isoflavone content in each diet was 0 (diet 1; casein based control), 31.7 (diet 2; alcohol washed soy-based diet control), 36.1 (diet 3), 74.5 (diet 4), 235.6 (diet 5) and 1046.6 (diet 6) mg total isoflavones/kg pelleted diet. The levels of isoflavones in diet 1 would represent a daily intake level of 0 mg isoflavones, diets 2 and 3 estimate a low soy-containing human diet (e.g. North American), diet 4 would correspond to Asian diets (e.g. Japanese) or adult humans taking isoflavone supplements, diet 5 approximates the isoflavone intake by babies fed soy based infant formula and diet 6 approximates 5-fold the intake levels by babies or 10-fold the intake levels of adults consuming high isoflavone containing diets. Serum testosterone (T) from F1 male rats of each diet sacrificed on postnatal days (PND) 28, 70, 120, 240 and 360 were low at PND 28 (0.4 ng/ml), increased approximately 5 to 6-fold at PND 70 (2.5 – 3.0 ng/ml) and thereafter declined to a steady state level of ~1 ng/ml by PND 120. However, rats on diets 5 and 6 demonstrated altered serum testosterone profiles such that at day 120, testosterone levels remained significantly elevated at ~3 ng/ml ($P<0.05$). Serum dihydrotestosterone levels exhibited similar profiles and the levels in PND 120 rats on diets 5 or 6 were also significantly elevated (2 to 3-fold, $P<0.05$). The intra-testicular testosterone concentration in rats on diet 5 was also elevated at PND 120 compared with diet 1 ($P<0.05$). These findings show that F1 male rats continuously exposed to a mixture of dietary soy isoflavones from conception onwards exhibit altered serum and testicular androgen profiles.

2.2 Introduction

A wide range of plants naturally possess compounds that have estrogenic properties and are referred to as phytoestrogens. They serve a variety of roles for the plant such as anti-fungal defence [1,2]. Certain phytoestrogens, the isoflavones are found in foods such as legumes, lentils, chickpeas. For humans, the main sources of dietary isoflavones are soy foods [3,4]. Human consumption of soy is increasing as more soy products become available and vegetarianism and veganism grow in popularity. Soy foods, such as soybeans, soy milk products, soy flour and soy-based infant formula, contain large quantities of isoflavones (18 –1980 mg/kg) [4,5]. Adult humans on a western diet can attain serum isoflavone levels in the high nanomolar to low micromolar range by ingesting phytoestrogen food supplements [6]. These serum levels resemble those found in Japanese men consuming a traditional Japanese diet [7]. Isoflavone usage has been stimulated by correlations between soy isoflavone levels in Asian diets and the lower incidence of coronary heart disease, prostate, colon and breast cancers which suggest a possible protective effect of isoflavones [1, 8-10]. Other potential beneficial aspects of soy consumption include the prevention of osteoporosis [10], hormone replacement therapy in menopause [11], as well as having antioxidant and antipromotional effects [12].

Some animal studies investigating the safety of soy isoflavone consumption have demonstrated some, albeit minimal, deleterious effects on reproductive success and health [13-16]. However phytoestrogen research since the 1940's has considered possible deleterious effects, following the discovery that animals grazing on plants rich in phytoestrogens aborted their fetuses [17]. Male animals may also be sensitive to the

effects of isoflavones since genistein can inhibit the growth and proliferation of testicular cell lines [18] and, in rats, Delclos and colleagues have shown that genistein can disrupt spermiation and decrease the number of spermatozoa present in the epididymis [19]. Successful spermatogenesis requires androgen production from Leydig cells (see review by McLachlan and colleagues [20]. Androgens regulate the function of androgen target tissues, such as the seminiferous tubules, prostate, seminal vesicles, epididiymis and skin by controlling gene expression by binding to the androgen receptor, a ligand-activated transcription factor [21].

Several isoflavone studies using different experimental models have found significant reductions of serum testosterone production in rats [22, 23], mice [24] and fish [25]. A recent study by Yi *et al.* 2002, showed a significant increase in total serum androgen levels and a decrease in serum DHT in isoflavone supplemented rats compared with isoflavone free controls [26]. Isoflavone consumption has been shown to have long-term effects on serum androgen levels. For example, multigeneration feeding studies have examined the effects of isoflavones on early development and reproductive success [19,27-29] and have shown that isoflavones can modulate the level of sex steroids in rats [28,29]. Fritz *et al.* [30], found that rats (70 days old) exposed to genistein (25 and 250 mg/kg diet) for their entire lives had significantly elevated serum T levels compared with genistein-free controls [30] and Dalu *et al.* (2002) showed that 140-day-old F1 male rats fed isoflavones (500 mg/kg) also exhibited significantly elevated serum androgens [28]. Furthermore, Laurenzana *et al.* (2002) found that F1 rats exposed to genistein (1250 mg/kg) in the feed showed significant changes in liver testosterone metabolism [29]. Thus, the ability of soy isoflavones to influence multiple pathways

leading to the modulation of sex steroid levels in males could have repercussions on secondary sex-tissue function, libido and spermatogenesis.

The present multigeneration study examined the effects of exposure to a mixture of dietary soy isoflavones (Novasoy concentrate which contains a mixture of soy isoflavones: genistein, daidzein and glycitein in conjugated and unconjugated states (glycone and aglycone) in F1 male rats for their entire lives. The diets used are relevant to human consumption of isoflavones [31-36], ranging from low to no consumption (North American diet), to modest vegetarian and Asian consumption levels, to high infant consumption levels.

2.3 Methods

2.3.1 Chemicals

Novasoy soy isoflavone concentrate #152-400 and alcohol washed soy protein (Pro Fam 930) were obtained from Archer Daniels Midland Company (Decatur, IL). Casein protein (90 % purity as determined by manufacturer) was purchased from ICN (Cleveland, OH). Dimethyl sulfoxide (DMSO), TRIS, ß-mercaptoethanol, genistein and daidzein (both >98% purity) were purchased from Sigma Chemical Co., St Louis, MO. Sucrose was purchased from BDH Inc., Toronto, Canada. [1,2,6,7-^{3}H]-Testosterone (95.0 Ci/mmol) and aquasol were obtained from Dupont/NEN, Boston, MA. Methanol, hexanes, acetone and chloroform were purchased from EM Science Merck KgaA, Darmstadt, Germany.

2.3.2 Experimental Diets

Six semi-purified diets were formulated according to American Institute of Nutrition (AIN) specifications [37]. The diets for this study were formulated by adding increasing amounts of a commercial soya extract containing high concentrations of isoflavones (Novasoy), to a base diet similar to AIN93G, but with casein replaced by alcohol-washed soy protein concentrate (Pro Fam 930) to yield a highly reduced isoflavone level soya-based diet with levels of crude protein similar to AIN93G (diet 2). This diet was supplemented with increasing concentrations of isoflavones from Novasoy to create subsequent diets (diets3-6). In addition, a soya-free, casein protein (AIN93G), diet was used to control for effects of non-isoflavone soya components (diet 1). The total content of three specific isoflavone species in the diets (genistein, daidzein and glycitein), was kindly determined by Dr. Sarwar Gilani and Mr. Patrick Robertson of Health Canada using HPLC (linear gradient with UV detection at 254 nm) [38] of ß-glucuronidase digested extracts of the diets. The results are presented in Table 1. All diets were provided to the animals ad libitum in pelleted form.

2.3.3 Animals

This study utilized rats from a multi-generation study examining the effects of dietary isoflavones on growth, development, reproduction and general physiology of rats. The multi-generation study was conducted according to the Organization for Economic Cooperation and Development (OECD) Guideline 416 (OECD TG416, 2001). The description and results from this larger study will be provided elsewhere (Curran, I.H.A,

Cooke G.M., and Gilani, G.S. in preparation). Animal handling and care followed the guidelines of the Canadian Council for Animal Care and all procedures were reviewed and approved by the Health Canada Animal Care Committee.

Pubertal Sprague-Dawley rats (Charles River, St-Constant, PQ) were pair housed with a 12 hours light/dark cycle in hanging polycarbonate cages (Health Guard System, Research Equipment Company, Inc. Byran, TX) containing corncob bedding and free access to food and fresh water. Parental generation rats were acclimatized until 50 days of age at which point they were assigned to experimental diets. After 70 days exposure to the experimental diets, rats were mated on postnatal day (PND) 120 and the resulting (F1) progeny were weaned at 21 days of age and provided with the same diet as their parents. Weekly food consumption was recorded at regular intervals throughout the study. The rat chow was analyzed every 100 kg consumed for isoflavone content (data not shown). Estimates of daily isoflavone intake for F1 generation male rats at PND 28 were 0, 0.9, 1.2, 2.0, 6.3, and 29.5 (mg isoflavones/ kg body weight (BW)/ day) for diets 1-6 respectively, and 0, 0.9, 1.0, 2.1, 7.3, and 28.7 (mg/ Kg BW/ day) for PND 120 rats for diets 1-6.

Male F1 rats were sacrificed on PND 28, 70, 120, 240 and 360 by exsanguination by cardiac puncture under isoflurane anaesthesia. Serum was collected using serum separator tubes (Becton Dickinson and Company, Franklin Lakes, NJ), centrifuged (1000g for 15 minutes), and stored at –20 °C until assayed for serum T and DHT. To reduce the possible variability of serum androgen quantitation due to circadian rhythms, collection of blood at necropsy was always conducted between 9-11 AM. Testes were

weighed and immediately frozen in liquid nitrogen and stored at –80 °C until assayed for T (PND 28, 70, 120, 240, 360) and DHT (PND 28 only).

2.3.4 Serum Androgen Determination

Serum T and DHT assays were conducted using commercial ELISA kits (IBL, Hamburg Cat# RE521-51 for T and Cat# DB520-21 for DHT). The quoted cross reactivity of DHT in the T assay was 0.05%. The quoted cross reactivity of the antibody for T in the DHT assay of 8.7 % does not influence the test results of this ELISA due to a specific complexing buffer system in the kit which blocks the binding of testosterone to the antibody (www.IBL-Hamburg.com). The cross-reactivities of phytoestrogens in the T and DHT assays were investigated by titrating a mixture of purified (>98%) genistein and daidzein (1:1 relative concentrations), dissolved in DMSO and diluted with 50 mM TRIS buffer (pH 7.4), across a range of 40 pg/ml to 0.6 mg/ml but neither assay was influenced by 50 mM TRIS buffer, DMSO or isoflavones at any of the concentrations tested. Inter-assay variation (CV %) for serum T was 5.7 (n = 8) and 23.6 (n = 8) for serum DHT. The intra-assay variation for serum T was 4.5 (n = 3) and for serum DHT was 6.3 (n = 3).

2.3.5 Intra-testicular Androgen Quantitation

Testes were thawed, decapsulated and approximately 0.90 ± 0.15 grams testis tissue (adults) or 0.29 ± 0.07 grams testis tissue (28 day old rats) was homogenized with a Pro250 Polytron homogenizer (Pro Scientific, Monroe, CT) in 50 mM TRIS buffer containing 0.25 M sucrose, 25 mM KCl, 5mM $MgCl_2$ and 7 mM mercaptoethanol (pH 7.4). Aliquots of the testis homogenate (50 µl for PND 28 or 100 µl for adults (PND>28))

were spiked with tritiated testosterone (approximately 85,000 CPM) and extracted twice with hexanes (1.5 ml). Samples were vortexed vigorously for 1 minute, centrifuged at 800 x g for 10 minutes and the organic phase was collected. Combined extracts were evaporated to dryness using ultra pure nitrogen gas (N-EVAP Analytical Evaporator Organomotion, South Berlin, MA) and residues were dissolved in 50 µL of chloroform : acetone (1:1) and evaporated to dryness. Residues were resuspended in 10 µL of DMSO, vortexed and diluted with 240 µl (PND 28) or 990 µL (PND>28) of 50 mM TRIS buffer (pH 7.4) and androgen levels quantified by ELISA. Intra-testicular androgen levels were extrapolated using 2^{nd} order decay non-linear analysis (Graphpad Prism V3.02 1994-2000. Graphpad Software Inc.) of the androgen calibration curve. When necessary, samples were further diluted with TRIS buffer such that values were within the calibration curve of the ELISA kits. Recoveries of ^{3}H-T from both samples and calibration standards were quantified by aliquoting 10 % of the resuspension into 5 ml of aquasol for analysis (Packard Liquid Scintillation Analyzer Tri-Carb 2100TR). The mean extraction efficiency was 87.1 % with a standard error of 1.4% (n = 206). No androgen was detected in blank samples processed through the extraction protocol, indicating that neither solvent residues nor buffers interfered with the ELISA. The intra-testicular T and DHT had intra assay variation of 3.5% (n = 4) and 3.0 % (n = 3), while the inter assay variation was 15.3 (n = 8) and 20.2 (n = 3) repectively.

2.3.6 Statistical analysis

One-way analysis of variance (ANOVA) was conducted for PND 28 intra-testicular DHT values and for the intra-testicular T:DHT ratio. Two way ANOVA was

used for all other comparisons and to test for significant time and dose interactions. All pairwise Tukey's test was used to compare androgen levels between dose groups (Sigma Stat V2.03 1992-1997 for Windows (SPSS Inc.)). Intra- and inter-assay CV % for ELISA assays were experimentally derived from the data (standard deviation / mean x 100%). Grubb's test was used for the detection of outliers of serum and testicular androgen levels [39].

2.4 Results

2.4.1 Body and Testis Weights

The body weights (mean ± SE) for diet 1 rats were 99.1 ± 3.8g, 444.7 ± 16.7g, 623.9 ± 20.3g, 845.6 ± 33.9g and 981.2 ± 48.0 grams for PND 28, 70, 120, 240 and 360 respectively. There were no significant differences in body weight or in food consumption (g/kg body weight/day) between diet groups for any time examined ($P>0.05$).

Testis weights from PND 28 rats were increased by about 30% for isoflavone diets 3-6 compared with diet 1 (Figure 1) ($P<0.05$). Mean testis weights for diet 1 at PND 70, 120, 240 and 360 were 1.65 ± 0.03 g, 1.66 ± 0.6 g, 1.80 ± 0.05 g and 1.76 ± 0.08 grams. There were no significant effects of diet on testis weights at any other age examined ($P>0.05$).

2.4.2 Serum Androgens

There was a rise in the mean serum T concentration between PND 28 and PND 70, which represents development through puberty to adulthood (Figure 2). At PND 70,

serum T levels in all dose groups had risen to levels ranging from (mean ± SE) 2.18 ± 0.4 ng/ml (diet 4) to 3.43 ± 0.8 ng/ml (diet 6). The divergence in serum T levels between the diets became statistically significant at PND 120 ($P<0.05$) where diet 5 (3.03 ± 0.5 ng/ml) and diet 6 (2.87 ± 0.5 ng/ml) were significantly higher than diet 1 (1.19 ± 0.2 ng/ml) ($P<0.05$; Figure 2). T levels began to decrease with age after PND 70, such that at PND 240 and 360, the levels were below 1.5 ng/ml. There was a significant interaction between time and dose for serum T levels ($P=0.037$).

Serum DHT at PND 28 ranged from (mean ± SE) 84.7 ± 5.8 pg/ml (diet 4) to 122.5 ± 14.6 pg/ml (diet 2), and rose by PND 70 to levels ranging from 335.9 ± 41.9 pg/ml (diet 2) to 489.1 ± 79.7 pg/ml (diet 6). Serum DHT levels showed statistically significant differences ($P<0.05$) at PND 120 where diet 5 (456 ± 54.4 pg/ml) and diet 6 (460.1 ± 65.5 pg/ml), were higher compared with diet 1 (233.0 ± 42.4 pg/ml) ($P<0.05$). By PND 360, mean serum DHT levels in diets 1 and 2 groups were approximately 250 pg/ml (Figure 3). The high serum DHT levels at PND 360 within diets 2 and 4 were not statistically significant from values for diets 1 ($p>0.05$; Figure 3). There was a significant interaction between time and dose for serum DHT levels ($P=0.005$).

Serum T and DHT data were sampled from the same animals allowing examination of the effects of dietary isoflavone on the ratios of the serum T/DHT levels. The mean serum T/DHT ratios (mean ± SE) ranged from: 3.7 ± 0.4, 7.3 ± 0.7, 5.2 ± 0.3, 5.8 ± 0.6 and 4.0 ± 0.1 for diet 1 at PND 28, 70, 120, 240 and 360 respectively (data not shown). Two-way-ANOVA of all diet groups examined at PND 28, 70, 120, 240 and 360 revealed no significant differences from the diet 1 ratios ($P>0.05$).

2.4.3 Intra-testicular Androgens

At PND 28 the intra-testicular T levels were all less than 50 ng T/g testis with the exception of diet 1, which exhibited a higher but statistically non-significant level ($P>0.05$) (Figure 4). There was a marked increase in mean T concentrations (ng/g) at PND 70 ranging from 231.9 ± 36.6 (diet 1) to 368.9 ± 32.4 (diet 6) and at PND 120, ranging from 259 ± 67.4 (diet 1) to 551.9 ± 101.4 (diet 5). At the later time points examined no diets were significantly different ($P>0.05$) from diet 1 where mean concentrations of 146.4 ± 30.1 ng/g and 260.4 ± 73.6 ng/g at PND 240 and 360 respectively were determined. The time point with the highest testicular T concentration was at PND 120 where the concentration was 551.9 ± 101.4 ng/g for rats fed diet 5 and which was significantly higher ($P<0.05$) than the mean diet 1 level 259.6 ± 67.4 ng/g (Figure 4). All other comparisons were not significant ($P>0.05$). The intra-testicular T data were also analyzed in terms of ng T/ testis, which showed the same significant differences at PND 120 with diet 5 compared to diet 1 (data not shown).

Mean intra-testicular DHT levels at PND 28, varied from 132.5 ± 72.7 ng/g for diet 1 rats to 22.4 ± 4.7 ng/g for diet 4 rats (Table 2), however compared with the diet 1 group no significant differences were observed either on a ng DHT/g testis or ng DHT/testis basis ($P>0.05$).

The intra-testicular T/DHT ratio for PND 28 exhibited little variation between diets and no statistically significant differences were observed ($P>0.05$).

2.5 Discussion

The current study has shown that rats exposed to soy isoflavones at levels comparable to human infants fed soy based infant formula exhibit significant changes in developmental profiles in terms of increased testis weights at PND 28 followed by an increase in testicular T and serum T and DHT levels at PND 120. These findings support those of previous studies and suggest that moderate levels of dietary isoflavones can influence the endocrine regulation of male reproductive function in rats.

At PND 28 the testes of rats of diet group 3,4,5 and 6 showed significantly higher masses compared with rats fed diet 1 with no soy isoflavones ($P<0.05$). The increase was transient; as by PND 70 there were no longer any differences in testis mass between any of the diet groups. The increase in testis weights at PND 28, have not been observed in other studies using pure genistein, where there were either no changes in testis weights [19] or decreased testis weights evident at adulthood [22]. It is possible that in the present study the glycoside genistin, or other isoflavones: daidzein or its metabolite equol, could be influencing testicular weights at PND 28. Atanassova *et al.* (2000), have shown the weak environmental estrogen octylphenol caused increases in testis weights in rats at PND 18; but in adulthood the increased testicular weights were not longer evident [40]. Neonatal treatment of rats with polychlorinated biphenyls caused decreases in serum thyroxine (T_4) levels, which the authors proposed as the cause of increased testis weights [41]. However, in the current study no significant changes in thyroid hormone levels were evident at PND 28 (Curran, I.H.A, Cooke G.M., and Gilani, G.S. in preparation). Alternatively, mice in which the gene encoding steroidogenic factor 1 (SF-1) was selectively inactivated in the pituitary, have gonads weighing approximately 5%

of the weight of wild-type gonads [42]. Isoflavones may upregulate pituitary SF-1, leading to an over development of gonads seen at PND 28 in the present study. This intriguing possibility is currently under investigation. F1 male body weights were not significantly affected by varying isoflavone concentrations at the time points examined. Some studies have shown no changes in body weights [22,30] or reproductive organ weights [14,28,30] with isoflavone exposure, whereas, other studies have found changes in body weights [14,28] and reproductive organ weights such as the prostate [23,27] and epididymis [22] being affected by isoflavones.

Androgen levels in post partum rats are low prior to puberty. Throughout puberty, serum androgen levels rise towards their maximal adult levels but then slowly decline as testosterone production by Leydig cells is reduced in aging adult animals [43-45]. Serum T and DHT levels in the current study were similar to historical control levels [46,47]. The serum T and DHT profiles for the different isoflavone diet groups began to diverge at PND 70 where diets 5 and 6 caused higher serum T levels although these were not significant when compared with diet 1. However by PND 120, the increases in serum T for rats fed diets 5 and 6 were statistically significant. By PND 240, androgen levels had reconverged in all dose groups, possibly the result of a lowering of the steroidogenic potential of the testis that occurs in normal ageing rats [45]. Isoflavones have been shown to alter 5α-reductase activity [48,49], yet in the current study this was not apparent, as the T/DHT ratio was not significantly altered between diet groups at any time examined.

Dietary isoflavones have been shown to alter the levels of androgens in the serum of experimental animals [22-26,28,30]. A study of fish (Japanese medaka) dosed with genistein for 10 days showed significantly lowered plasma T levels [25]. Similarly 10

month old male mice dosed subcutaneously (SC) with 2.5 mg/kg (body weight) genistein for 9 days, showed significantly reduced pituitary LH and decreased serum and testicular T levels [24]. In studies using adult male Sprague-Dawley rats fed test diets for 35 days, a 50% reduction in serum T was seen in phytoestrogen (600 mg/kg) fed rats [23]. This decrease in T was not accompanied by any significant changes in plasma LH levels or testicular weights [23]. Male offspring fed genistein in a developmental study exhibited decreased serum T at PND 21 and decreased serum LH levels at PND 28 and 130 [22]. Not all animal studies have shown reductions in serum androgens due to isoflavone consumption. In a recent study, male rats fed soy extract or a soy isoflavone supplemented diet, an increase in serum androgen (T + DHT) with soy extract, and a significantly higher total serum androgen level for the isoflavone supplemented group was observed [26]. Similarly, Fritz *et al.* (2001), using pure genistein in feed, found that exposure to 25 mg/kg and 250 mg/kg genistein from conception onwards, caused an increase in serum T levels in 70 day old rats accompanied by an increase, though not statistically significant, in serum DHT [30]. A multigeneration rat study by Dalu *et al.* 2002 using pure genistein in rat chow, found that for F1 males at PND 140 serum T and DHT positively correlated with increasing genistein dose and was significantly increased in rats exposed to 500 ppm genistein [28]. Consequently, it appears that short-term studies dosing via feed, SC, or intraperitoneally (IP) in various animal models have shown that isoflavones can lower serum androgen levels. However, long term feeding studies suggest that isoflavones cause increased serum androgen levels when rats are exposed to isoflavones throughout development, resulting in elevated serum androgen levels ranging from 70 to 140 days of age [28,30]. To date most studies have typically

involved dosing with pure isoflavone-aglycones [22,24,25,28,30] as opposed to a mixture of soy isoflavones as used in the present study.

Other researchers have found testicular and serum T and DHT profiles in rats show T levels at birth [50] or early in life (PND 14-20) [51-53] decreased to a minimal value around PND 25-40 at which time T rises to maximal adult levels (PND 60-90), while DHT levels tend to rise slowly from PND 14 onwards to reach maximal levels in adulthood [51,52]. Intra-testicular T and DHT levels in our study were consistently higher than those typically seen in historical controls [46]. This may have been due to sampling an entire testis homogenate; as opposed to the interstitial fluid, testicular vein blood or seminiferous tubules [46,47].

At PND 28 there were no significant differences seen between T/DHT ratios of each diet group, suggesting that testicular 5α-reductase activity [54] was not affected by dietary soy isoflavone exposure. In addition, at PND 28, we observed that the intratesticular T levels for diet 1 had a greater variation in the data compared with diets 3-6. Although not significantly different, this may suggest an effect of isoflavones, altering the timing of testicular development and maturation. Isoflavones have been shown to alter early androgen dependent events such as delaying preputial separation [55].

At later time points examined, testicular T levels for diets 2-6 were higher than diet 1. Diet 1 exhibited modest increases in testicular T levels from PND 28 to 120 while rats fed diets 2-6 showed larger increases, which became significantly higher at PND 120 for diet 5 compared with diet 1. By PND 120, testicular T profiles resembled serum profiles in terms of increased T levels compared with diet 1, for rats fed isoflavones.

There have been few studies of the effects of isoflavone exposure on testicular androgen levels. Fish of a study of Japanese medaka (*Oryzias latipes*) dosed with genistein exhibited decreased *ex vivo* T release from whole testis [25]. However, genistein treated testes showed a statistically insignificant increase in *ex vivo* estradiol production, which may partially explain the lower amounts of androgenic steroids since they may have been converted to estrogens [25].

The results of the present study showing significantly elevated testicular (diet 5) and serum (diets 5 and 6) androgens could be attributable to altered enzyme activities caused by isoflavone exposure as has been seen in other studies examining steroidogenic enzymes *in vivo* and *in vitro* [48,49,56-59]. In the current study the observations of increased androgens in both serum and testes of test animals, fed high levels of isoflavones are indicative of soy isoflavones being able to alter testicular steroid production at PND 120. Alternative causes of changes in serum T and DHT profiles due to isoflavones include differences in steroid metabolism and clearance via the liver [29], altered sex hormone binding globulin (SHBG) levels or altered free versus bound androgen due to SHBG-isoflavone interactions [60]. There have been changes reported in LH levels of animals exposed to isoflavones, which could affect feedback regulation of testicular androgen production [22,24]. Lund *et al.* (2004) found increased circulating LH levels after dosing with equol (major metabolite of daidzein), which could lead to increased testicular androgen production [61]. This group also found increased plasma DHT levels (though not statistically significant) with equol treatment [61]. Changes in circulating LH levels in animals exposed to isoflavones, may reflect changes in the developmental programming of the hypothalamic-pituitary-testicular axis. LH levels

could not be assessed in the current study, as other required endpoints reduced the amount of serum available. The increased masses at PND 28 or increased androgen levels at PND 120 may be a consequence of altered cellular profiles, as was observed in marmoset monkeys exposed to isoflavones via infant formula, where increased numbers of Leydig cells were evident, although, paradoxically, this was matched with lower serum T levels [62].

There have been numerous human isoflavone consumption studies. Short term studies with humans show no negative effects in terms of sperm quality [6,63] or reproductive hormone levels [63,64]. Yet other human studies have found significant modulation of steroids where isoflavones increased plasma DHT levels [65] and soy products caused decreases in serum T [66].

The results of this study are of significance since rats fed dietary soy isoflavones exhibited increasing testis mass and altered testicular T and DHT levels at an early age (PND 28), as well as changing the developmental timing and magnitude of changes in serum and testicular androgen levels at adulthood (PND 120). Thus early androgen dependent events such as preputial separation and, later in life, spermatogenesis, libido and androgen target tissue function, could be impacted. The findings of this study that both serum and testis show increases in androgen levels indicate that dietary soy isoflavones alter Leydig cell steroidogenesis, although at this time, the mechanism of this action is not known. Future research will address the mechanisms responsible for the increases in androgen levels caused by isoflavones. Such research is necessary since not only does it impact on the health and safety advisories to human consumers of soy products, but it also demonstrates that isoflavones typically found in rodent chow [67]

can significantly affect androgen levels within the testis and serum of male rats, thereby potentially confounding endocrinological interpretation of some rodent studies.

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2.6 References

[1] Adlercreutz H, Mazur W, Phytoestrogens and Western diseases. Ann Med. 1997; 29:95-120.

[2] Naim M, Gestetner B, Zikah S, Birk Y, Bondi A, Soybean isoflavones. Characterization; determination, and antifungal activity. J Agr Food Chem. 1974; 22(5):806-810.

[3] Mazur W, Adlercreutz H, Naturally occurring oestrogens in food. Pure Appl. Chem. 1998; 70:1759-1776.

[4] United States Department of Agriculture (USDA). USDA-Iowa State University Database on Isoflavone Content of Foods. http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html. (1999)

[5] Coward L, Barnes NC, Setchell KDR, Barnes S, Genistein, daidzein and their ß-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. J. Agric. Food Chem. 1993; 41:1961-1967.

[6] Mitchell JH, Cawood E, Kinniburgh D, Provan A, Collins AR, Irvine DS, Effect of a phytoestrogen food supplement on reproductive health in normal males. Clinical Science 2001; 100:613-618.

[7] Morton MS, Arisaka O, Miyake N, Morgan LD, Evans BAJ, Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. J. Nutr. 2002; 132:3168-3171.

[8] Clarkson TB, Anthony MS, Hugher CLJr., Estrogenic soybean isoflavones and chronic disease. Risks and benefits. Trends Endocrinol. Metab. 1995; 6:11-16.

[9] Tikkanen MJ, Adlercreutz H, Dietary soy-derived isoflavone phytoestrogens: could they have a role in coronary heart disease prevention? Biochemcial Pharmacology 2000; 60:1-5.

[10] Humfrey CDN, Phytoestrogens and human health effects: weighing up the current evidence. Natural Toxins 1998;6:51-59.

[11] Birge SJ, Soy phytoestrogens: an adjunct to hormone replacement therapy? Menopause: the Journal of The North American Menopause society 2000; 4:209-212.

[12] Wei H, Bowen R, Cai Q, Barnes S, Wang Y, Antioxidant and antipromotional effects of the soybean isoflavone genistein. P.S.E.B.M. 1995;208:124-130.

[13] Cassanova M, You L, Gaido KW, ArchibequeEngle S, Janszen DB, Heck HD, Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. Toxicol. Sci. 1999; 51:236-244.

[14] Lewis RW, Brooks N, Milburn GM, Soames A, Stone S, Hall M, Ashby J, The effects of the phytoestrogen genistein on the postnatal development of the rat. Toxicol. Sci. 2003; 71:74-83.

[15] Nagao T, Yoshimura S, Saito Y, Nakagomi M, Usumi K, Ono H, Reproductive effects in male and female rats of neonatal exposure to genistein. Reprod. Toxicol. 2001; 15:399-411.

[16] Shutt DA, The effects of plant oestrogens of dietary origin. Endeavour 1976; 35:110-113.

[17] Bennetts HW, Underwood EJ, Shier FL, A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aust. J. Agric. Res. 1946; 22:131-138.

[18] Kumi-Daika J, Rodriguez R, Goudaze G, Influence of genistein (4',5,7-trihydroxy-isoflavone) on the growth and proliferation of testicular cell lines. Biol Cell 1998; 90:349-354.

[19] Delclos KB, Bucci TJ, Lomax LG, Latendresse JR, Warbritton A, Weis CC, Newbold RR, Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. Reprod. Toxicol. 2001; 15:647-663.

[20] McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, Krester DM, Pratis K, Robertson DM, Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys and man. Recent Prog Horm Res. 2002; 57:149-179.

[21] Leung PS, Wong TP, Chung YW, Chan HC, Androgen dependent expression of AT1 receptor and its regulation of anion secretion in rat epididymis. Cell Biol Int. 2002; 26:117-22.

[22] Roberts D, Veeramachaneni DNR, Schlaff WD, Awoniyi CA, Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats. Endocrine 2000; 13:281-286.

[23] Weber KS, Setchell KDR, Stocco DM, Lephart ED, Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5 alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J. Endocrinol. 2001, 170:591-599.

[24] Strauss L, Makela S, Joshi S, Huhtaniemi I, Santti R, Genistein exerts estrogen-like effects in male mouse reproductive tract. Mol.Cell. Endocrinol. 1998; 144:83-93.

[25] Zhang L, Khan IA, Foran CM, Characterization of the estrogenic response to genistein in Japanese medaka (*Oryzias latipes*). Comparative Biochemistry and Physiology 2002; Part C 132:203-211.

[26] Yi MA, Son HM, Lee J-S, Kwon C-S, Lim JK, Yeo YK, Park YS, Kim J-S, Regulation of male sex hormone levels by soy isoflavones in rats. Nutrition and Cancer 2002; 42:206-210.

[27] Kang KS, Che JH, Lee YS, Lack of adverse effects in the F1 offspring maternally exposed to genistein at human intake dose level. Food and Chemical Toxicology 2002; 40:43-51.

[28] Dalu A, Blaydes BS, Bryant CW, Latendress JR, Weis CC, Delclos KB, Estrogen receptor expression in the prostate of rats treated with dietary genistein. Journal of Chromatography B 2002; 777:249-260.

[29] Laurenzana EM, Weis CC, Bryant CW, Newbold R, Delclos KB, Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P450 enzymes, and estrogen receptor alpha expression. Food Chem. Toxicol. 2002; 40:53-63.

[30] Fritz WA, Wang J, Eltoum IE, Lamartiniere CA, Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate. Mol. Cell. Endocrinol. 2002; 186:89-99.

[31] Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Babaian RJ, Pillow PC, Hursting SD, Phytoestrogen intake and prostate cancer: a case-control study using a new database. Nutr. Cancer 1999; 33:20-25.

[32] Kirk P, Patterson RE, Lampe J, Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults. J. Am. Diet. Assoc. 1999; 99:558-563.

[33] Cassidy A, Bingham S, Setchell KDR, Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am. J. Clin. Nutr. 1994; 60:330-340.

[34] Wakai K, Egami I, Kato K, Kawamua T, Tamakoshi A, Lin Y, Nakayama T, Wada M, Ohno T, Dietary intake and sources of isoflavones among Japanese. Nutr Cancer 1999; 33:139-145.

[35] Irvine CHG, Fitzpatrick MG, Alexander SL, Phytoestrogens in soy-based infant foods: concentrations, daily intake, and possible biological effects. Proc. Soc. Exp. Biol. Med 1998; 217:247-253.

[36] Zung A, Reifen R, Kerem Z, Zadik Z, Phytoestrogens: the pediatric perspective. Journal of. Pediatric Castroenterology and Nutrition 2001; 33:112-118.

[37] Reeves PG, Nielsen FH, Fahey GCJr, AIN-93 purified diets for laboratory rodents: final report of the American Instititute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 1993; 123:1939-1951.

[38] Wang HJ, Murphy PA Isoflavone content in commercial soybean foods. J Agric. Food Chem. 1994; 42:1666-1673.

[39] Grubbs FE, Procedures for detecting outlying observations in samples. Technometrics.1969; 11:1-21.

[40] Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM, Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: evidence for stimulatory effects of low estrogen levels. Endocrinology 2000; 141:3898-3907.

[41] Cooke PS, Zhao Y-D, Hansen LG, Neonatal polychlorinated biphenyl treatment increases adult testis size and sperm production in the rat. Toxicology and Applied Pharmacology (1996); 136:112-117.

[42] Zhao L, Bakke M, Parker KL, Pituitary-specific knockout of steroidogenic factor 1. Mol. Cell. Endocrinol. (2001); 185:27-32.

[43] Gruenewald DA, Naai MA, Marck BT, Matsumoto AM, Age-related decrease in hypothalamic gonadotropin-releasing hormone (GnRH) gene expression, but not pituitary responsiveness to GnRH, in the male Brown Norway rat. J. Androl 2000; 21: 72-84.

[44] Tenover JS, Matsumoto AM, Clifton DK, Bremner WJ, Age-related alterations in the circadian rhythms of pulsatile luteinizing hormone and testosterone secretions in healthy males. J. Gerontology 1988; 43(6):M163-169.

[45] Zirkin BR, Chen H, Luo L, Leydig cell steroidogenesis in aging rats. Experimental Gerontology 1997; 32(4/5):529-537.

[46] Turner TT, Ewing LL, Jones CE, Howards SS, Zegeye B, Androgens in various fluid compartments of the rat testis and epididymis after hypophysectomy and gonadotropin supplementation. J. Androl. 1985; 6:353-358.

[47] Turner TT, Jones CE, Howards SS, Ewing LL, Zegeye B, Gunsalus GL, On the androgen microenvironment of maturing spermatozoa. Endocrinology 1984; 115: 1925-1932.

[48] Evans BA, Griffiths K, Morton MS, Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavanoids. J. Endocrinol. 1995; 147:295-302.

[49] Weber KS, Jacobson NA, Setchell KD, Lephart ED, Brain aromatase and 5alpha-reductase, regulatory behaviors and testosterone levels in adult rats on phytoestrogen diets. Proc. Soc. Exp. Biol. Med. 1999; 221:131-5.

[50] Resko JA, Feder HH, Goy RW, Androgen concentrations in plasma and testis of developing rats. J. Endocr. (1968); 40:485-491.

[51] Podesta EJ, Rivarola MA, Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development. Endocrinology 1974; 95:455-461.

[52] Knorr DW, Vanha-Perttula T, Lipsett MB, Structure and function of rat testis through pubescence. Endocrinology (1970); 86:1298-1304.

[53] Miyachi YE, Nieschlag E, Lipsett M, The secretion of gonadotropins and testosterone by the neonatal male rat. Endocrinology (1973); 92:1-5.

[54] Killian J, Pratis K, Clifton RJ, Stanton PG, Robertson DM, O'Donnell L, 5(alpha)-reductase isoenzymes 1 and 2 in the rat testis during postnatal development. Biol Reprod. 2003; 68:1711-1718.

[55] Wisniewski AB, Klein SL, Lakshmanan Y, Gearhart JP, Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. J. Urol. 2003; 169(4):1582-1586.

[56] Ibrahim AR, Abdul-Haj YJ, Aromatase inhibition by flavanoids. J. Steroid Biochem. Mol. Biol. 1990; 37:257-260.

[57] Whitehead SA, Cross JE, Burden C, Lacey M, Acute and chronic effects of genistein, tyrphostin and lavendustin A on steroid synthesis in luteinizing human granulosa cells. Human Reprod. 2002; 17:589-594.

[58] Wong CK, Keung WM, Bovine adrenal 3ß-hydroxysteroid dehydrogenase (E.C. 1.1.1.145)/5-ene-4-ene isomerase (E.C. 5.3.3.1): characterization and its inhibition by isoflavones. J. Steroid Biochem. Mol. Biol. 1999; 71:191-202.

[59] Krazeisen A, Breitling G, Moller G, Adamski J, Phytoestrogens inhibit human 17ß-hydroxysteroid dehydrogenase type 5. Mol. Cell. Endocrinol. 2001; 171:151-162.

[60] Cassidy A, Faughnan M, Phytoestrogens through the life cycle. Proc. Nutr. Soc. 2000; 59:489-496.

[61] Lund TD, Munson DJ, Haldy ME, Setchell KDR, Lephart ED, Handa RJ, Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback. Biol. Reprod. 2004; (in press).

[62] Sharpe RM, Martin B, Morris K, Greig I, McKinnell C, McNeilly AS, Walker M, Infant feeding with soy formula milk: effect on the testis and on blood testosterone levels in marmoset monkeys during the period of neonatal testicular activity. Human Reprod. 2002; 17:1692-1703.

[63] Kurzer MS, Hormonal effects of soy in premenopausal women and men. J. Nutr 2002; 132:570S-573S.

[64] Allen NE, Appleby PN, Davey GK, Key TJ, Soy milk intake in relation to serum sex hormone levels in British men. Nutr. Cancer 2001; 41:41-46.

[65] Lewis JG, Morris JC, Clark BM, Elder PA, The effects of isoflavones extract ingestion, as Trinovin, on plasma steroids in normal men. Steroids 2002; 67:25-29.

[66] Nagata C, Inaba S, Kawakami N, Kakizoe T, Shimizu H, Inverse association of soy product intake with serum androgen and estrogen concentrations in Japanese men. Nutr. Cancer 2000; 36:14-18.

[67] Degen GH, Janning P, Diel P, Bolt HM, Estrogenic isoflavones in rodent diets. Toxicol. Lett. 2002; 128:145-157.

Table 1 Isoflavones levels in diets

Isoflavones were determined by HPLC in ß-glucuronidase digested extracts of experimental diets. Diets 2 to 6 contained an alcohol-washed soy protein concentrate and in diets 3 to 6, Novasoy was added to supplement the diets with isoflavones. (N.D. not detectable),

Diets	Isoflavones (mg/kg pelleted dry diet)			
	Total Isoflavones	Daidzein	Glycitein	Genistein
Diet 1 (Casein)	ND	ND	ND	ND
Diet 2 (Soy protein)	31.7	10.5	2.6	18.6
Diet 3	36.1	12.3	2.8	21.0
Diet 4	74.5	27.6	7.6	39.3
Diet 5	235.8	90.9	20.5	124.4
Diet 6	1046.6	412.3	89.5	544.8

Table 2 Intra-testicular dihydrotestosterone levels (ng/g) in PND 28 F1 rats.

No statistically significant differences were observed. Grubbs' tests were used to eliminate outliers in diets 1,2 and 3.

Dose Group	[DHT] (ng/g)	SE	N
Diet 1	132.5	72.7	5
Diet 2	71.5	45.7	4
Diet 3	39.1	9.9	5
Diet 4	22.4	4.7	6
Diet 5	29.9	3.6	6
Diet 6	24.3	4.2	6

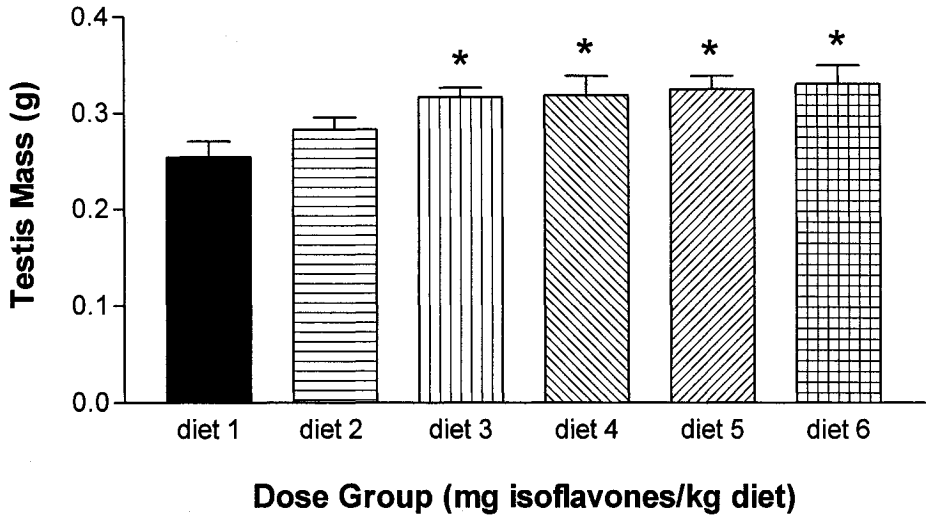

Figure 1 Day 28 Testicular Mass of F1 Rats (g)

Testicular masses of 28 day old male rats Mean ± SE. Diets were as described in Methods. * Statistically significant compared with diet 1 ($P<0.05$). Older animals' testes were larger, ranging from 1.5 to 2.0 grams with no significant variation between time points (day 70, day 120,day 240, day 360) or dose. The number of animals sampled at 28 days of age were n = 5 for diets 1 to 3 and n = 6 for diets 4 to 6.

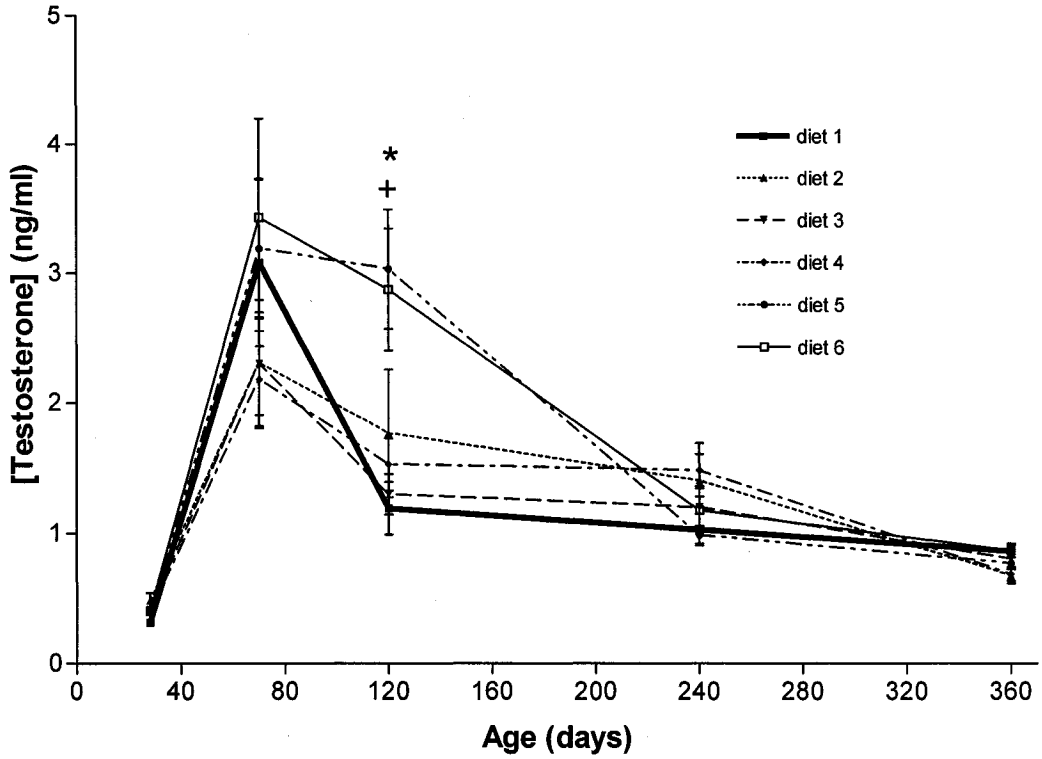

Figure 2 Serum Testosterone Levels in F1 Male Rats (ng/ml)

Serum Testosterone levels (ng/ml) Mean ± SE in male rats. Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed (+) diet 5 and (*) diet 6 were significantly higher than diet 1 at 120 days of age ($P<0.05$). The number of animals sampled was n = 12 for all time points except for rats of 360 days of age where n = 6.

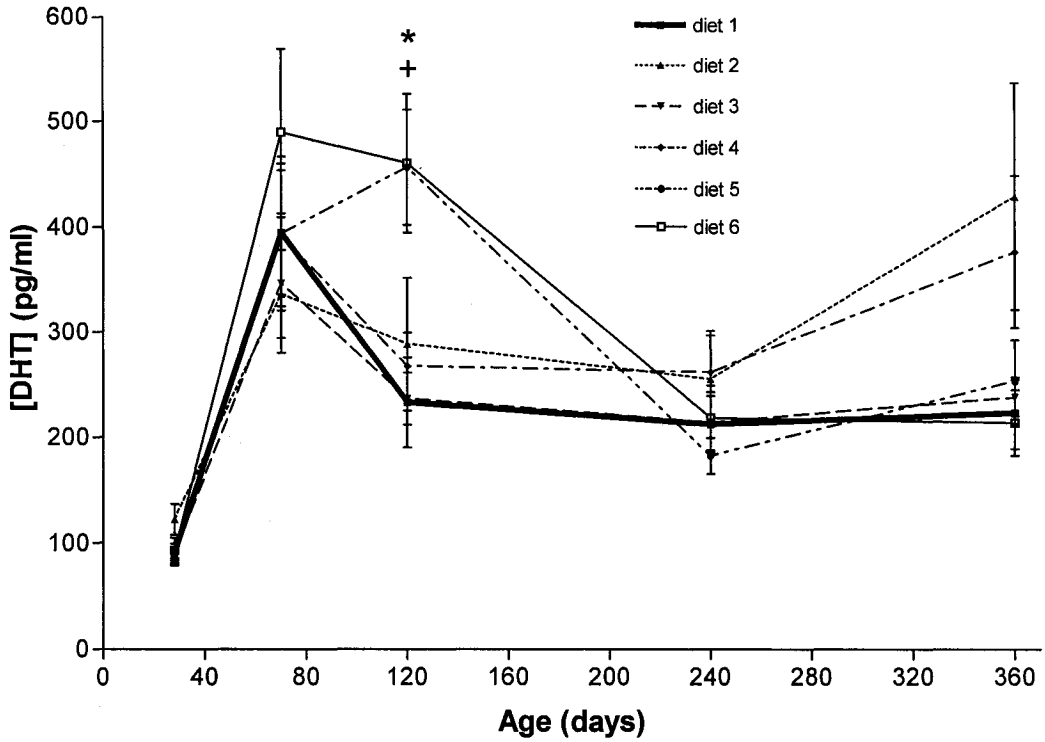

Figure 3 Serum Dihydrotestosterone Levels in F1 Rats (pg/ml)

Serum dihydrotestosterone levels (pg/ml) Mean ± SE in male rats. (+) Diet 5 and (*) diet 6 were significantly higher than diet 1 at 120 days of age ($P<0.05$). The number of animals sampled was n = 12 for all time points except for rats of 70 days of age for diet 3 n = 10 and diet 4 n = 10 and for rats 360 days of age which had an n = 6.

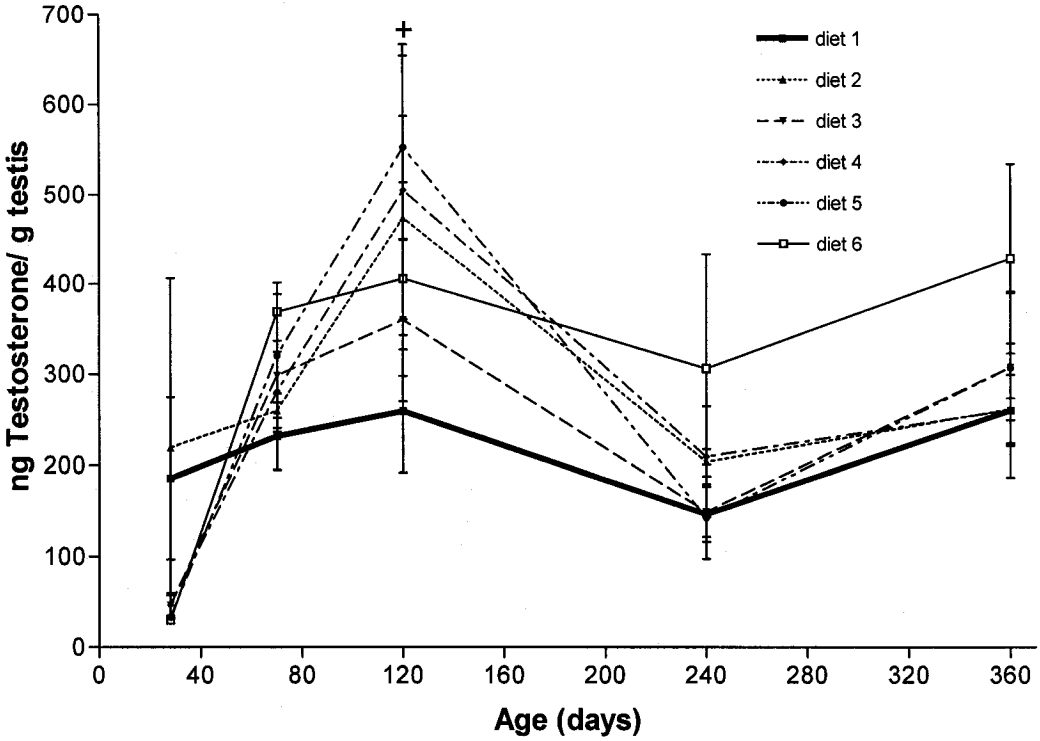

Figure 4 Intra-testicular Testosterone Levels in F1 Rats (ng/g)

Testicular testosterone levels (ng/g testis) Mean ± SE in male rats. (+) Diet 5 was significantly higher than diet 1 at 120 days of age ($P<0.05$). The number of animals sampled was n =6 for all time points and doses with the exceptions of at 28 days of age diet 1 and 3 n = 5, diet 2 n = 4, at 70 days of age diet 2 n = 4 and diet 6 n = 5, at 120 days of age diets 3 and 6 n = 5, at 240 days of age diets 2,4,5 n = 5 and at 360 days of age diets 1,4,5 n = 5.

Chapter 3

3.0 Altered testicular microsomal steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones.

McVey M.J., Curran I.H.A., Cooke G.M., "Altered testicular microsomal steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones." Journal of Steroid Biochemistry and Molecular Biology (2004); (accepted).

Following the findings of increased serum and testicular androgen levels at PND 120, the focus of this investigation was to determine the mechanism(s) by which isoflavones had increased androgen levels at PND 120 amongst rats fed high levels of isoflavones (Diets 5 and 6). Isoflavones have been shown to cause increases (and in some cases decreases) in the activity of steroidogenic enzymes. Therefore testicular steroidogenic enzyme activities determined in radiometric assays, were determined in an attempt to explain the increase in serum and intra-testicular androgen levels.

Altered testicular microsomal steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones.

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Abbreviated form of the title:

Isoflavones affect rat testicular steroidogenic enzyme activities.

Keywords:

3ß-HSD, CYP17, testicular enzyme activity, soy isoflavones

This work was presented in part at The Canadian Fertility and Andrology Society Meeting November 2003 in Victoria, BC.

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3.1 Abstract

Androgen production in the testis is carried out by the Leydig cells, which convert cholesterol into androgens. Previously, isoflavones have been shown to affect serum androgen levels and steroidogenic enzyme activities. In this study, the effects of lifelong exposure to dietary soy isoflavones on testicular microsomal steroidogenic enzyme activities were examined in the rat. F1 male rats were obtained from a multi-generational study where the parental generation was fed diets containing alcohol-washed soy protein supplemented with increasing amounts of Novasoy, a commercially available isoflavone supplement. A control group was maintained on a soy-free casein protein-based diet (AIN93G). The diets were designed to approximate human consumption levels and ranged from 0 to 1046.6 mg isoflavones/kg pelleted feed, encompassing exposures representative of North American and Asian diets as well as infant fed soy-based formula. Activities of testicular 3ß-hydroxysteroid dehydrogenase (HSD) (3ß-HSD), P450c17 (CYP17), 17ß-HSD were assayed on post natal day (PND) 28, 70, 120, 240 and 360 while 5α-reducatase was assayed on PND 28. At PND 28, 3ß-HSD activity was elevated by approximately 50% in rats receiving 1046.6 mg total isoflavones/kg feed compared to those on the casein only diet. A similar increase in activity was observed for CYP17 in rats receiving 235.6 mg total isoflavones/kg feed, a level representative of infant exposure through formula, compared to those receiving 0 mg isoflavones from the casein diet. There were no other significant changes for other diet levels at other time points. These results demonstrate that rats fed a mixture of dietary soy isoflavones showed significantly altered enzyme activity profiles during development at PND 28 as a result of early exposure to isoflavones at levels obtainable by humans.

3.2 Introduction

Many plants contain phytoestrogens, a class of compounds that have estrogenic properties and appear to function in anti-fungal defence [1,2]. Isoflavones are a type of phytoestrogen found in foods such as legumes, lentils and chickpeas. Soy foods such as soybeans, soy milk products, soy flour and soy-based infant formulas [3,4] have the highest recorded levels of isoflavones in food, and as such represent a large source of dietary isoflavones [3,5]. Human consumption of soy is on the rise as more soy based foods and supplements are becoming available and vegetarianism grows in popularity. Published benefits of soy isoflavones include therapeutic value for hormone replacement therapy in menopause as well as antioxidant and antipromotional effects against coronary heart disease, various cancers and osteoporosis [1,6-10].

Research examining possible deleterious effects of phytoestrogens began during the 1940's, following the discovery that consumption of plants rich in phytoestrogens caused animals to abort their fetuses [11]. Some animal studies examining the safety of isoflavone consumption have not shown impairment of male sperm production or reproductive tract development [12,13], while other studies have shown deleterious effects in terms of reproductive success and health [14-17]. The isoflavone genistein has been shown to inhibit the growth and proliferation of testicular cell lines [18], as well as disrupt spermiation and decrease spermatozoa number present in epididymis of rats [19]. In addition a number of isoflavone studies have found significant alterations of serum testosterone production in rats [20-25], mice [26] and fish [26]. Previous work from this laboratory has shown that levels of serum and testicular testosterone (T) and dihydrotestosterone (DHT) levels, were significantly elevated in rats at PND 120

following consumption of isoflavones at levels comparable to those found in some human diets [28].

Testicular biosynthesis and metabolism of male steroid hormones involves a cascade of cholesterol transport proteins and steroidogenic enzymes regulated in part by the hypothalamic pituitary axis [29-31]. The mechanisms by which isoflavones may affect hormone production and circulating levels have not been investigated in detail. However, some phytoestrogens have been shown to alter the enzyme activities of 3ß-hydroxysteroid dehydrogenase (3ß-HSD) [32,33], 17ß-hydroxysteroid dehydrogenase (17ß-HSD) [32,34] and 5α-reductase [35,36], which could impact the production of androgens within the testis.

The following studies were designed to explore the mechanisms responsible for androgen level changes previously detected in F1 rats fed a mixture of dietary isoflavones containing genistein, daidzein, and glycitein in conjugated (glycone) and unconjugated (aglycone) states for their entire lives [28]. The rats were fed diets containing various levels of isoflavones ranging from 0 mg isoflavones (diet 1) to low amounts representative of a typical North American diet (diets 2-3) [37,38], to modest vegetarian or Asian consumption levels (diet 4) [39,40], to high dietary levels experienced by infants fed on soy-based formulae (diet 5) [41,42]. Diet 6 represents approximately 5 times the consumption of infants and 10 times the consumption of adults. The present investigation examined the effects of dietary isoflavones on the activities and expression of mRNA and protein levels of the steroidogenic enzymes 3ß-HSD, 17-hydroxylase/C-17,20 lyase (CYP17), 17ß-HSD and 5α-reductase.

3.3 Experimental

3.3.1 Materials

Unlabelled steroids were purchased from Steraloids Inc. (Newport, RI). Aquasol and labelled steroids : [1,2,6,7-^{3}H] DHEA (60.0 Ci/mmol), [1,2,6,7-^{3}H] progesterone (114.4 Ci/mmol), [1,2,6,7-^{3}H], [1,2,6,7-^{3}H] testosterone (95.0 Ci/mmol) and [1,2,6,7-^{3}H] androstenedione (74.0 Ci/mmol) were bought from Dupont/NEN (Boston, MA). Organic solvents were purchased from EM Science Merck KgaA (Darmstadt, Germany) with the exception of formaldehyde, which was purchased from Fisher Scientific (Nepean, ON). Plastic coated Whatman^{TM} PE SIL G silica gel chromatography plates were bought from Chromatographic Specialties (Montreal, QC). Novasoy soy isoflavone concentrate (152-400) and alcohol washed soy protein (Pro Fam 930) were obtained from Archer Daniels Midland Company (Decatur, IL). Casein protein was purchased from ICN (Cleveland, OH). Dimethyl sulfoxide (DMSO), TRIS, diethyl pyrocarbonate (DEPC), nicotinamide cofactors (NADPH and NAD^+), and ß-mercaptoethanol were purchased from Sigma Chemical Co. (St Louis, MO). Sucrose was purchased from BDH Inc. (Toronto, ON). Ethidium bromide was purchased from Gibco BRL (Gaithersburg, MD). Agarose, RNAse H ribonuclease and dNTPs were purchased from Roche Diagnostics Corporation (Indianapolis, IN). Superscript II RNAse H-Reverse transcriptase, oligo d(T_{15}) primers, custom PCR primers (Table 1) and TRIzol were purchased from Life Technologies Inc. (Burlington, ON). Chill wax for PCR reactions was obtained from MJ Research Inc (Boston, MA). Taq DNA polymerase and PCR buffers were purchased from Boehringer Mannheim (Laval, QC). NE-PER nuclear and cytoplasmic extraction reagents,

Coomassie Plus Protein Assay reagents, bovine serum albumin standards and Supersignal ULTRA chemiluminescent substrate were purchased from Pierce (Rockford, IL). SDS-polyacrylamide and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Richmond, CA). Immobilon-P, polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). TRIS buffered saline containing 0.1 % tween-20 (TBS) was purchased from ICN Biochemicals (Cleveland, OH); and 4% skim milk powder, was purchased from Nestle (Don Mills, ON). Streptavadin was obtained from DAKO Diagnostics Canada Inc. (Mississauga, ON).

3.3.2 Animals

The male *Sprague-Dawley* (SD) rats used in this study were the F1 generation produced during a multi generation study examining the effects of dietary isoflavones on growth, development and reproductive physiology. The study adhered to the protocol for the Organization for Economic Cooperation and Development (OECD) Guideline 416 (OECD TG416, 2001). Results from this larger study will be provided elsewhere (Curran, I.H.A, Cooke G.M., and Gilani, G.S. in preparation). Animal care was provided according to the guidelines of the Canadian Council for Animal Care and all procedures were reviewed and approved by the Health Canada Animal Care Committee. The parental generation (F0) were purchased from Charles River (St-Constant, PQ) at the pubescent stage of development and were pair housed with a 12 hours light/dark cycle in hanging polycarbonate cages (Health Guard System, Research Equipment Company, Inc. Byran, TX) containing corncob bedding and free access to food and fresh water. Weekly food consumption was recorded at regular intervals throughout the investigation. Parental generation rats (F0) were acclimatized until postnatal day (PND) 50 at which

point they were assigned randomly to experimental diets. After 70 days exposure to the experimental diets, rats were mated on PND 120 and the resulting (F1) progeny were weaned at PND 21 and provided with the same diet as their parents.

Male F1 progeny were sacrificed on PND 28, 70, 120, 240 and 360 by exsanguination, through cardiac puncture under isoflurane anaesthesia. Testes were weighed, immediately frozen in liquid nitrogen and stored at –80 °C until assay for steroidogenic enzyme activities.

3.3.3 Experimental diets

Six semi-purified diets were formulated according to American Institute of Nutrition (AIN) specifications [43]. One diet was casein-based (AIND93G) while the other diets were formulated by adding increasing amounts of a commercial mixture of isoflavones (Novasoy), to a base diet resembling AIN93G, but containing alcohol-washed soy protein concentrate (Pro Fam 930) in place of casein.

Once the diets were prepared, soy isoflavone content (genistein, daidzein and glycitein) was determined by HPLC [44] anlaysis of ß-glucuronidase digested extracts of the diets as shown in Table 1 (Dr. Sarwar Gilani and Mr. Patrick Robertson of Health Canada, personal communication). All diets were provided to the animals *ad libitum* in pelleted form.

3.3.4 Specific enzyme activities

Decapsulated testes were homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 7 mM mercaptoethanol and 100 µM

NADPH. For PND 28 testes, the homogenates were centrifuged (1000 x g_{max}, 10 minutes, 4°C) to pellet the nuclear fraction, which was then resuspended in dimethyl glutaric acid (DMGA) buffer (50 mM DMGA, 50 mM NaOH, 250 μM NADPH, 5 % glycerol, pH 6.5) for immediate use in 5α-reductase enzyme assays. The 1000 g_{max} supernatant was centrifuged at 10000 x g_{max} for 10 minutes at 4°C and the supernatants recovered and recentrifuged under the same conditions to obtain a post-mitochondrial supernatant. The microsomal fractions for all assays at all time points, obtained by centrifugation of the post-mitochondrial supernatantant (176000 x g_{max}), were resuspended in the 50 mM Tris-HCl buffer pH 7.4 containing 250 μM NADPH for immediate use in microsomal 3ß-HSD, CYP17 (17-OHase, C17,20-Lyase), 17ß-HSD and 5α-reductase assays. Microsomal and nuclear protein were estimated using the method of Lowry *et al.* [45].

Testicular microsomal (3ß-HSD, CYP17, 17ß-HSD, 5α-reductase) and nuclear (5α-reductase) steroidogenic enzyme activities were assayed according to the modified methods of Cooke *et al.* [46,47]. At PND 28, testes from the six diet groups of rats were tested for all enzyme activities, while only 3ß-HSD, CYP17 and 17ß-HSD were tested at PND 70, 120, 240 and 360 as young rats aged 20 to 40 days of age are able to convert T to the more potent androgen DHT by the testicular enzyme 5α-reductase, but as the rat approaches adulthood, this testicular activity diminishes to unmeasurable levels [31].

Aliquots of appropriate microsomal fractions were added to (1) 3 ml Tris-HCl buffer (pH 8.4) containing DHEA (10^{-6} M, 40000 cpm ^{3}H/ml) for the 3ß-HSD assay, (2) 3 ml Tris-HCl buffer (pH 7.4) containing progesterone (10^{-6} M, 40000 cpm ^{3}H/ml) for the CYP17 assay, (3) 3 ml Tris-HCl buffer (pH 7.4) containing 4-androstenedione (10^{-6} M,

40000 cpm ^{3}H/ml) for the 17ß-HSD assay and (4) 3 ml of DMGA buffer (pH 4.5 or 6.5) containing testosterone (10^{-6} M, 40000 cpm ^{3}H/ml) for the 5α-reductase assay. Enzyme reactions with labeled substrates were stopped after 30, 60 and 90 minute incubations with hexanes and appropriate carrier steroids were added to facilitate localization of substrates and products by UV-light and exposure to iodine vapor after separation by thin layer chromatography plates using 9:1 = chloroform:methanol as a solvent. Regions corresponding to the substrate and product carrier steroids were excised and quantified by scintillation counting (Packard Liquid Scintillation Analyzer (Tri-Carb 2100TR)). Enzyme specific activities were determined by linear regression analysis ($r > 0.95$) and expressed as pmol product formed/minute/mg protein, µmol product formed/minute/g testis tissue and µmol product formed/minute/testis. Optimal conditions were determined for all enzymes with respect to substrate and protein concentrations and linearity of product formation with time.

3.3.5 RNA Isolation and RT-PCR

Total RNA isolation of 20 mg of thawed testes tissue were carried out with TRIzol according to manufacturer's specifications. The concentrations and purity of total RNA isolated was determined spectrophotometrically, based on optical density at 260 nm and $A_{260/280}$ ratio respectively. RNA quality was verified by denaturing ethidium bromide stained formaldehyde RNA electrophoresis through agarose gel [48]. All RNA samples were stored at –80°C until reverse transcription reactions were carried out.

cDNA was synthesized using 5 µg of total RNA and 200 U Superscript II RNAse H-Reverse transcriptase in a total reaction volume of 20 µl. The RT-reaction and

subsequent steps were performed as per manufacturer's recommendations (Life Technologies Inc., Burlington, ON). The cDNA was then used immediately or stored at –20°C until needed for subsequent amplifications. Control RT-PCR reactions were conducted using RNA to test for possible false priming from genomic DNA (Appendix 1).

3.3.6 Gene Amplification

Target cDNA was amplified using 50 ng of initial total RNA from the cDNA reaction in a 50 µl volume containing (1x PCR buffer + Mg as per manufacturer's kit) 0.25 mM each of forward and reverse primers (Table 2). Primers were designed using SciEdCentral Clone Manager Professional Suite (Scientific and Educational Software, Durham, NC). Premature initiation of the reaction was prevented by the addition of chill wax (25 µl) before addition of 2.5 U Taq DNA polymerase. PCR reactions were optimized for each primer pair by temperature and cycle gradients (example shown in Appendix 4).

The following were used as PCR cycles after initial denaturing 95°C (3 min.): 95°C (60 sec.), 58°C (70 sec.), 72°C (70 sec.). G3PDH, SCC and CYP17 were amplified with 25 cycles, while 23 cycles were used for 3ß-HSD; PCR products were subjected to an extended final incubation (72°C, 5 min.) to fully extend all partial DNA fragments. Thermocycled reaction (optimized with cycle and temperature gradients) products were separated by 2% agarose gel electrophoresis followed by ethidium bromide staining and images captured (Kodak Digital Science Image Station 440 CF) for comparative

densitometric analysis (Kodak ID Image Analysis Software, Eastman Kodak Company, Rochester, NY).

3.3.7 Western Blotting Analysis

Cytoplasmic and nuclear fractions from 50 mg of testis tissue were isolated with NE-PER nuclear and cytoplasmic extraction reagents according to manufacturer's instructions. Protein concentrations were determined using Pierce Coomassie Plus Protein Assay reagents using bovine serum albumin as a standard. Equal amounts of protein (35 μg) were resolved with 10% SDS-polyacrylamide gel electrophoresis as described by Laemmli [49] and transferred to Immobilon-P PVDF membranes as described by Matsudaira [50]. Proteins were visualized on 10% SDS-polyacrylamide gels and PVDF membranes by staining with Coomassie Brilliant Blue R-250 to test for equal loading of wells and equal transfer from gel to membrane (data not shown). The western blots were blocked with TRIS buffered saline containing 0.1% tween-20 (TBST) and 4% skim milk powder (1 h, room temperature.). A number of antibodies were used to probe the western blots: (1) a polyclonal rabbit antibody against rat SCC (1/10000 dilution) (Chemicon International, Temecula, CA), (2) a polyclonal rabbit antibody against purified human placental 3ß-HSD1 (1/500 dilution) (generous gift from Dr. James L. Thomas, Mercer University, School of Medicine at Macon, GA), (3) a polyclonal rabbit-anti porcine CYP17 antisera (1/10000 dilution) (kindly supplied by Dr. Buck Hales, University of Illinois at Chicago, IL [51]). Each antibody was added with streptavadin- horseradish peroxidase conjugate (1/3000 dilution) in TBST with 4% skim-milk powder. Blots were washed 3 x 5 minutes with TBST before the addition of a

donkey secondary anti-rabbit antibody coupled to horseradish peroxidase (1/5000 dilution) (Jackson Immuno Research Laboratories Inc., West Grove, PA) in TBST with 4% skim-milk powder. Blots were washed 6 x 5 minutes before the chemiluminescent detection of antigens by addition of SuperSignal ULTRA Chemiluminescent Substrate as per manufacturer's instructions in conjunction with a Kodak Digital Science Image Station 440 CF and Kodak ID Image Analysis Software.

3.3.8 Statistical analysis

Linear and non-linear regressions were carried out using Prism V3.02 1994-2000 (Graphpad Software Inc., San Diego, CA) of radiometric steroid assay data. One-way ANOVA was used to analyze 5α-reductase enzyme activities and comparative densitometry analysis of cDNA. Two-way ANOVA was used for analysis of 3ß-HSD, CYP17, 17ß-HSD, 17-hydroxyprogesterone : androstenedione ratio and microsomal protein concentrations, followed up by all pairwise Tukey's tests. (Sigma Stat V2.03 1992-1997 for windows (SPSS Inc., Chicago, IL)).

3.4 Results

3.4.1 Microsomal Protein Determination

The mean testis microsomal protein concentrations (mean ± SE) for diet 1 rats were 20.3 ± 1.7, 17.0 ± 0.5, 18.5 ± 1.5, 20.9 ± 0.7 and 17.1 ± 1.5 milligrams per ml for PND 28, 70, 120, 240 and 360 respectively. There were no significant differences in protein concentrations between diet groups for any time examined ($P>0.05$).

3.4.2 3ß-Hydroxysteriod Dehydrogenase Activity

3ß-HSD activities tended to decrease with age, for example at PND 28 the lowest values were 938.8 ± 91.4 nmol androstenedione produced/min/mg protein (diet 1), which was decreased by 50% at PND 70, and then at later time points, all values tended to range from 219.4 ± 37.3 (PND 120 diet 3) to 412.9 ± 56.2 (PND 360 diet 4) nmol androstenedione produced/min/mg protein (Figure 1). At PND 28, rats fed 1046.6 mg isoflavones/kg feed (diet 6) showed a significant increase in 3ß-HSD activity compared with those consuming 0 or 31.7 mg/kg (approximately 42% and 47%; respectively) ($P<0.05$). Similarly, animals which had consumed 235.6 mg/kg (diet 5) exhibited a significant increase in 3ß-HSD activity when compared with diets containing 0 or 31.7 mg /kg (approximately 28% and 24% respectively; $P<0.05$). When the data were expressed as androstenedione formed per minute per gram testis tissue, both diets 5 and 6 were approximately 50% and 46% higher than diet 1 with 0 mg/kg ($P<0.05$) (Figure 1 inset). There was a statistically significant interaction between time and isoflavone levels as determined by 2-way ANOVA ($P = 0.017$) indicating a relationship between increased enzyme activities and duration of dosing. Results obtained when expressed as androstenedione formed/min/testis showed no significant differences between different diet groups at any age examined (data not shown). Time points examined after PND 28 showed less variation between enzyme activities for the different diet groups.

3.4.3 CYP17 Activity

Progesterone is converted in a two step process to 17-hydroxyprogesterone and then to androstenedione by one enzyme: 17-hydroxylase/C17,20-lyase (CYP17). The CYP17 enzyme activity (nmol androstenedione/min/mg protein) detected in rats fed diet 5 at PND 28 were significantly higher (approximately 38% and 52%) compared with those found in rats fed diets 1 and 2 respectively ($P<0.05$). When the data were analyzed as µmol androstenedione produced/min/g testis, diet 5 activity was again found to be significantly higher than the activity of diet 1 (approximately 60%) at PND 28 (Figure 2 inset), although at diet 6 this difference was no longer observed. Furthermore, when the data were expressed as µmol androstenedione produced/min/testis, similar trends were evident at PND 28 but no statistically significant changes in activities were detected ($P>0.05$). Statistically significant interactions occurred between time and dose of isoflavones as determined by 2 way ANOVA for all three analyses of CYP17 activity ($P<0.001$) indicating a relationship between increased enzyme activities and duration of dosing. Activities from ages later than PND 28 exhibited no significant differences between diet groups ($P>0.05$). In these assays, the ratio of 17-hydroxyprogesterone : androstenedione was not significantly different indicating that there were no selective effects on C17,20-lyase activity ($P>0.05$) (data not shown).

3.4.4 17ß-Hydroxysteroid Dehydrogenase Activity

The profile of 17ß-HSD activity with age resembled the profiles for 3ß-HSD and CYP17. At PND 28, the 17ß-HSD activities ranged from 17.3 ± 2.1 (diet 2) to 21.7 ± 3.4 (diet 6) nmol testosterone/min/mg protein, then decreased at PND 70 and for all later time

points examined into the range of 5.2 ± 0.6 (PND 360 diet 5) to 10.9 ± 1.5 (PND 120 diet 6) nmol testosterone/min/mg protein (Figure 3). There were no significant differences in 17ß-HSD activity between dose groups at any ages when the data were examined in terms of nmol testosterone produced/min/mg protein, µmol testosterone produced/min/testis and µmol testosterone produced/min/g testis (P>0.05).

3.4.5 5α-Reductase Activity

The testis of PND 28 animals contained measurable 5α-reductase activity within microsomal and nuclear testicular extracts at a reaction pH of 6.5 but no activity was detectable at pH 4.5. Furthermore, at pH 6.5, there were no significant differences observed for 5α-reductase activities (nmol dihydrotestosterone/min/mg protein) between the 6 diets examined for either the microsomal or nuclear 5α-reductase activities (P>0.05) (Figure 4). Similarly, when the data were examined in terms of µmol dihydrotestosterone/min/g testis and µmol dihydrotestosterone/min/testis activity, neither analysis showed any significant differences between diet groups (P>0.05) (data not shown).

3.4.6 Steroidogenic Enzyme mRNA Expression Levels

The initial step in androgen production is the conversion of cholesterol to pregnenolone through the action of the mitochondrial cholesterol side chain cleavage enzyme (P450 SCC). Enzyme activities were not determined for SCC, as appropriate radiolabelled substrates are not available commercially. However, isoflavone effects on SCC gene expression were examined by semi-quantitative RT-PCR in testis samples

from each diet group at PND 28 (age when changes in other steroidogenic enzyme activities were observed) and at PND 120 (age when changes in serum and testicular androgen levels were evident [28]). Amplifications of SCC cDNA relative to a constitutive control (G3PDH) showed no significant changes in mean mRNA ratios of arbitrary densitometry units between the 6 diets examined at PND 28 ($P>0.05$) (Figure 5 A) or at PND 120 (Figure 5 B). The steroidogenic enzymes that exhibited changes in activity at PND 28 (3ß-HSD and CYP17) were also examined for differences in mRNA expression. RT-PCR analysis of both enzymes revealed no statistically significant differences between mean ratios of different diet groups ($P>0.05$) (data not shown).

3.4.7 Western Blot Protein Levels of Steroidogenic Enzymes

Western blot analyses of SCC, 3ß-HSD and CYP17 were performed to test for possible differences in enzyme protein levels between rats fed different diets. Western blots showed no visible differences in intensity between immunoreactive SCC protein levels between diets 3-6 compared with intensities seen in diets 1 and 2 at both PND 28 and 120 (Figure 6A,B). Similarly 3ß-HSD and CYP17 protein levels were not different between diets at PND 28 (appendix 5).

3.5 Discussion

The current study was undertaken to investigate whether lifetime exposure of male rats to isoflavones that resulted in elevated serum and testicular androgen levels [28] also altered testicular enzyme activities. Earlier work from our laboratory demonstrated that exposure of rats to soy isoflavones at levels attainable by adults and infants, result in

increased serum and testicular androgen levels in adulthood (PND 120) [28]. The present studies show that at PND 28 there are significantly higher enzymatic activities of 3ß-HSD and CYP17 in the pathway converting cholesterol to androgens due to consumption of dietary isoflavones. CYP17 showed the greatest dose-dependent increases in enzyme activity in rats fed isoflavones, followed closely by 3ß-HSD, while 5α-reductase activity and 17ß-HSD activities were not affected by isoflavones. In addition, at PND 28, subtle differences were observed according to whether the 3ß-HSD and CYP17 enzyme activities were expressed in terms of mg microsomal protein, g testis tissue or whole testis, possibly due to differences in testis weights between the diet groups that were seen at this age [28]. At PND 28 a dose dependent increase in testis mass was observed as the amount of soy isoflavones was increased, which did not persist at older ages [28]. At this time point the increased steroidogenic potential may have increased with the larger testis of rats fed high levels of isoflavones. These findings agree with the work of others who have found decreased steroidogenic enzyme activities as a result of isoflavones [32-35], and more specifically, increased steroidogenic enzyme activity in tissues following isoflavone administration [36,52]. The results of the present studies, while not able to explain the mechanism of increased serum and testicular androgen levels seen in adult rats described previously [28], add a new layer of complexity by revealing alterations in enzyme activities during early development.

Estrogenic chemicals have been shown to alter steroid hormone enzyme activities. Majdic *et al.*, (1996), found that fetal male rats exposed *in utero* to the exogenous estrogenic chemicals diethylstilbestrol (DES) or 4-octylophenol (OP) exhibited altered expression of mRNA coding for CYP17, protein levels of CYP17 and 17α-

hydroxylase/C17-20-lyase activity [53]. The effects of isoflavones on enzyme activities have shown mixed results in terms of inhibition and activation. While some influence of genistein on steroid hormone signalling may be due to its potent inhibitory action on enzymes such as tyrosine kinase [54], genistein and daidzein are known inhibitors of steroidogenic enzymes in a variety of bacterial [55] and mammalian cell lines [32,33,56]. Both genistein and daidzein inhibit human placental 17ß-HSD activity [56], purified human 17ß-HSD type 5 [57], while genistein can inhibit human granulosa cell 17ß-HSD [32]. Structural homology between genistein and daidzein compared with endogenous steroidogenic substrates is postulated as a mechanism leading to competitive inhibition at the active site of steroidogenic enzymes [58]. Recently Ohno *et al.* (2002) have shown that daidzein is a competitive inhibitor of 3ß-HSD type II enzyme activity (K_m = 6.6 μM) within human H295R adrenocortical cells with a K_i of 2.9 μM [57]. Krazeisen *et al.* (2001) postulate that inhibitory potency increases with hydroxylation of the flavonoids, and inhibition occurs through interference with hydrophilic cofactor binding sites, as opposed to interference with substrate binding [34].

In contrast, other studies such as those by Weber *et al.* [36], have shown increased steroidogenic enzyme activities due to exposure to isoflavones. A short term animal study where PND 45 male SD rats were fed diets containing 200 mg isoflavones/kg (50% genistin, 40% daidzin and 10 % glycitin) for 29 days, revealed increased 5α-reductase activity within the amygdala region, while medial basal hypothalamic-preoptic area 5α-reductase activity was significantly decreased [36]. Similarly, Laurenzana *et al.* [24] found significantly increased 5α-reductase activity in male and female rat liver microsomes, accompanied by non-significant increases in hepatic cytochrome P450

enzyme protein levels, after exposure from gestational day 7 until sacrifice at PND 50, with 250 mg genistein/ kg feed. However, at 1250 mg/kg genistein, decreased 5α-reductase activity was found indicating a non-linear dose response in males [24]. In human fetal, postnatal and adult adrenal cell lines, genistein and daidzein lowered ACTH-stimulated cortisol production to basal levels, while postnatal and adult adrenal cells exhibited increased DHEA and DHEA-S levels [52]. The increased androgen due to genistein and daidzein may have been caused by decreasing cortisol production leading to either increased availability of substrate for androgen production or increased steroidogenic enzyme activity of P450scc or CYP17, though no changes in mRNA expression of these enzymes were found [52].

Timing and duration of exposure and the route of dosing of pure or mixed isoflavones may be important variables determining isoflavone action on steroidogenic enzymes. *In vitro* research involving pure isoflavones directly added to cell cultures has typically shown inhibitory actions of isoflavones on steroidogenic enzymes [32-34,55-57], while *in vivo*, animal studies have shown soy isoflavones increase certain steroidogenic activities [24,36], as seen in the current study.

The enzyme activities examined in the present study are down-stream of the rate-determining step of steroidogenesis, the movement of cholesterol to the side chain cleavage enzyme. Enzyme activities for P450 SCC were not examined due to difficulties in acquiring labelled cholesterol substrate to monitor conversion to pregnenolone, though activity does not correlate to expression exactly deficitsor increases in enzyme protein were investigated. However at PND 28 and 120, no differences were found in expression levels of mRNA or protein for P450 SCC in any of the diet groups examined. Similarly

mRNA and protein levels of 3ß-HSD and CYP17 revealed no changes due to isoflavones at PND 28. It is possible that altered enzyme activity, independent of changes in mRNA and protein levels result from different substrate availability or alterations in the microenvironment. For example, altered P450c21 enzymatic activities due to isoflavones, could not be explained by corresponding changes in expression, and it was proposed that this was due to the upstream inhibition of 3ß-HSD which lowered the amount of substrate available for P450c21 [52]. Alternatively, it has been shown that isoflavones can bind to and modify membrane environments [59,60]. Changes in microsomal membrane structure in the immediate area surrounding steroidogenic enzymes such as 3ß-HSD [61] could impact enzyme activity. The membrane environment has been shown to at least partially regulate enzyme activity *in vitro* [62-64].

It is possible that the differences in enzyme activities seen at PND 28 are related to changes in the rate of development between rats of different diet groups. In previous work, our lab has shown increased intratesticular androgen levels in diets 1 and 2 compared with diets 3-6 at PND 28 [28]. Although these changes were not statistically significant, they could indicate a delay in androgen dependent development due to isoflavone consumption. It is possible that the increased steroidogenic enzyme activities seen with high dietary levels of isoflavones are a homeostatic overcompensation to redress delays in androgen dependent development. Isoflavones have been shown to modify endocrine regulation of androgen production by altering levels of serum LH [65-67]. Lund et al. (2003) have found increased circulating LH and statistically insignificant increased plasma DHT levels due to dosing with equol (a major metabolite of daidzein)

[67]. Interestingly this group proposed that equol binds directly to DHT preventing it from providing negative feedback within the hypothalamic-pituitary-testicular (HPT) axis. Therefore, isoflavones or their metabolites (e.g. equol) could have led to changes in the normal development of the HPT axis, which in turn may have altered steroid production and circulating levels that were observed in our study. An examination of serum and tissue genistein, daidzein, glycitein and equol is underway and will be important for deciphering mechanisms of isoflavone action and extrapolating the current findings to the human situation, where isoflavone metabolism varies between individuals who have different gut bacteria and diets leading to subpopulations who tend to be high equol excreters, similar to rodents, or who are low equol excreters [68,69].

At PND 28 testicular weights of rats fed diets 3-6 were significantly higher compared with diet 1 [28]. These isoflavone dose dependent differences could reflect altered cellular profiles. Marmoset monkeys exposed to isoflavones via infant formula exhibited increased numbers of Leydig cells, though this was paradoxically matched with lower serum T levels [70]. In our previous findings, androgen levels at PND 28, tended to be lower for diets 3-6 compared with diets 1-2 ($P>0.05$) [28], yet the corresponding enzyme activities are higher, possibly due to an increased number of Leydig cells in rats fed diets 5 and 6. We are presently pursuing the intriguing possibility that changes seen at PND 28 could be due to altered number of Leydig cells or altered signalling in the steroidogenic cascade.

It was of interest that steroidogenic enzyme activities were not altered at PND 120, an age where diets 5 and 6 caused elevated serum T and DHT compared with diets 1 and 2 [28]. However, other possible explanations for the increased androgens at this age

may include: altered metabolism of androgens by modification of testicular and/or peripheral aromatase activity [58], changes in steroid metabolism and clearance via the liver [24,71], altered androgen binding protein/sex hormone binding globulin (ABP/SHBG) production or altered free versus bound androgen profile due to ABP/SHBG-isoflavone interactions influencing the ability of steroids to be transported [71].

In our earlier report, isoflavones significantly altered testicular T, serum T and serum DHT at PND 120 [28] and our current work reveals altered steroidogenic enzyme activities at PND 28 in rats fed isoflavones at levels obtainable by human infants [41,42,72]. Studies involving humans have found dose dependent modulation of plasma steroid levels by isoflavones, such as increased plasma DHT levels [73] or decreased serum T [74]. The impact of isoflavones on human *in vivo* steroidogenesis is not fully understood as present research largely focuses on cell culture [24,32,57,58]. The altered activities seen at PND 28 in the present study are at the same age as when isoflavones were seen to significantly increase testicular weights, and though not statistically significant, decreased testicular T and DHT levels [28]. These findings show it is possible that isoflavones affect early development in male rats possibly delaying the production and level of testicular androgens. We have limited our investigation of steroids to Leydig cell derived T and DHT at PND 28, as their levels were not significantly affected by isoflavones, although examination of 3α-androstanediol, a major androgen at this age, may form part of future investigations [75,76]. Additionally, the enzyme activity profiles examined in the current study do not account for the increased

androgen production seen at PND 120 [28], and therefore future work will address regulatory systems upstream of the conversion of cholesterol to androgen.

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3.6 References

[1] H. Adlercreutz , W. Mazur, Phytoestrogens and Western diseases, Ann Med. 29 (1997) 95-120.

[2] M. Naim, B. Gestetner, S. Zikah, Y. Birk, A. Bondi, Soybean isoflavones. Characterization; determination, and antifungal activity, J Agr Food Chem. 22 (1974) 806-810.

[3] United States Department of Agriculture (USDA). USDA-Iowa State University Database on Isoflavone Content of Foods. http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html. (1999).

[4] L. Coward, N.C. Barnes, K.D.R. Setchell, S. Barnes, Genistein, daidzein and their ß-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets, J. Agric. Food Chem. 41 (1993) 1961-1967.

[5] W. Mazur, H. Adlercreutz, Naturally occurring oestrogens in food, Pure Appl. Chem. 70 (1998) 1759-1776.

[6] T.B. Clarkson, M.S. Anthony, C.L.Jr Hugher, Estrogenic soybean isoflavones and chronic disease. Risks and benefits, Trends Endocrinol. Metab. 6 (1995) 11-16.

[7] M.J. Tikkanen, H. Adlercreutz, Dietary soy-derived isoflavone phytoestrogens: could they have a role in coronary heart disease prevention?, Biochemcial Pharmacology 60 (2000) 1-5.

[8] C.D.N. Humfrey, Phytoestrogens and human health effects: weighing up the current evidence, Natural Toxins 16 (1998) 51-59.

[9] S.J. Birge, Soy phytoestrogens: an adjunct to hormone replacement therapy?, Menopause: the Journal of The North American Menopause Society 4 (2000) 209-212.

[10] H. Wei, R. Bowen, Q. Cai, S. Barnes, Y. Wang, Antioxidant and antipromotional effects of the soybean isoflavone genistein, P.S.E.B.M. 208 (1995) 124-130.

[11] H.W. Bennetts, E.J. Underwood, F.L. Shier, A specific breeding problem of sheep on subterranean clover pastures in Western Australia, Aust. J. Agric. Res. 22 (1946) 131-138.

[12] T. Nagao, S. Yoshimura, Y. Saito, M. Nakagomi, K. Usumi, H. Ono, Reproductive effects in male and female rats of neonatal exposure to genistein, Reprod. Toxicol. 15(2001) 399- 411.

[13] A.S. Faqi, W.D. Johnson, R.L. Morrissey, D.L. McCormick, Reproductive toxicity assessment of chronic dietary exposure to soy isoflavones in male rats, Reprod Toxicol. 18 (2004) 605-11.

[14] M. Cassanova, L. You, K.W. Gaido, S. ArchibequeEngle, D.B. Janszen, H.D. Heck, Developmental effects of dietary phytoestrogens in *Sprague-Dawley* rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta *in vitro*, Toxicol. Sci. 51 (1999) 236-244.

[15] R.W. Lewis, N. Brooks, G.M. Milburn, A. Soames, S. Stone, M. Hall, J. Ashby, The effects of the phytoestrogen genistein on the postnatal development of the rat, Toxicol. Sci. 71 (2003) 74-83.

[16] T. Nagao, S. Yoshimura, Y. Saito, M. Nakagomi, K. Usumi, H. Ono, Reproductive effects in male and female rats of neonatal exposure to genistein, Reprod. Toxicol. 15 (2001) 399-411.

[17] D.A. Shutt, The effects of plant oestrogens of dietary origin, Endeavour 35 (1976) 110-113.

[18] J. Kumi-Daika, R. Rodriguez, G. Goudaze, Influence of genistein (4',5,7-trihydroxy-isoflavone) on the growth and proliferation of testicular cell lines, Biol. Cell. 90 (1998) 349-354.

[19] K.B. Delclos, T.J. Bucci, L.G. Lomax, J.R. Latendresse, A. Warbritton, C.C. Weis, R.R. Newbold, Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats, Reprod. Toxicol. 15 (2001) 647-663.

[20] D. Roberts, D.N.R. Veeramachaneni, W.D. Schlaff, C.A. Awoniyi, Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats, Endocrine 13 (2000) 281-286.

[21] K.S. Weber, K.D.R. Setchell, D.M. Stocco, E.D. Lephart, Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5 alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male *Sprague-Dawley* rats, J. Endocrinol. 170 (2001) 591-599.

[22] M.A. Yi , H.M. Son, J-S. Lee, C-S. Kwon, J.K. Lim, Y.K. Yeo, Y.S. Park, J-S. Kim, Regulation of male sex hormone levels by soy isoflavones in rats, Nutrition and Cancer 42 (2002) 206-210.

[23] A. Dalu, B.S. Blaydes, C.W. Bryant, J.R. Latendress, C.C. Weis, K.D. Delclos, Estrogen receptor expression in the prostate of rats treated with dietary genistein, Journal of Chromatography B 777 (2002) 249-260.

[24] E.M. Laurenzana, C.C. Weis, C.W. Bryant, R. Newbold, K.D. Delclos, Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P450 enzymes, and estrogen receptor alpha expression, Food Chem. Toxicol. 40 (2002) 53-63.

[25] W.A. Fritz, J. Wang, I.E. Eltoum, C.A. Lamartiniere, Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate, Mol. Cell. Endocrinol. 186 (2002) 89-99.

[26] L. Strauss, S. Makela, S. Joshi, I. Huhtaniemi, R. Santti, Genistein exerts estrogen-like effects in male mouse reproductive tract, Mol.Cell. Endocrinol. 144 (1998) 83-93.

[27] L. Zhang, I.A. Khan, C.M. Foran, Characterization of the estrogenic response to genistein in Japanese medaka (*Oryzias latipes*), Comparative Biochemistry and Physiology PART C 132 (2002) 203-211.

[28] M.J. McVey, G.M. Cooke, I.H.A. Curran, Increased serum and testicular androgen levels in F1 rats with lifetime exposure to soy isoflavones, Reprod. Toxicol. 18 (2004) 677-685.

[29] A. Payne, G. Youngblood, Regulation of expression of steroidogenic enzymes in Leydig cells, Biol. Reprod. 52 (1995) 217-225.

[30] W.L. Miller, J.F. Strauss, Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, STAR, J. Steroid Biochemistry and Molecular Biology 69 (1999) 131-141.

[31] J. Killian, K. Pratis, R.J. Clifton, P.G. Stanton, D.M. Robertson, L. O'Donnell, 5(alpha)-Reductase Isoenzymes 1 and 2 in the Rat Testis During Postnatal Development, Biol. Reprod. 68 (2003) 1711-1718.

[32] S.A. Whitehead, J.E. Cross, C. Burden, M. Lacey, Acute and chronic effects of genistein, tyrphostin and lavendustin A on steroid synthesis in luteinized human granulosa cells, Human Reproduction 17 (2002) 589-594.

[33] C.K. Wong, W.M. Keung, Bovine adrenal 3ß-hydroxysteroid dehydrogenase (E.C. 1.1.1.145)/5-ene-4-ene isomerase (E.C. 5.3.3.1): characterization and its inhibition by isoflavones, J. Steroid Biochem. Mol. Biol. 71 (1999) 191-202.

[34] A. Krazeisen, R. Breitling, G. Moller, J. Adamski, Phytoestrogens inhibit human 17ß-hydroxysteroid dehydrogenase type 5, Molecular and Cellular Endocrinology 171 (2001) 151-162.

[35] B.A. Evans, K. Griffiths, M.S. Morton, Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavanoids, J. Endocrinol. 147 (1995) 295-302.

[36] K.S. Weber, N.A. Jacobson, K.D.R. Setchell, E.D. Lephart, Brain aromatase and 5alpha-reductase, regulatory behaviors and testosterone levels in adult rats on phytoestrogen diets, Proc Soc Exp Biol *Med.* 221 (1999) 131-5.

[37] S.S. Strom, Y. Yamamura, C.M. Duphorne, M.R. Spitz, R.J. Babaian, P.C. Pillow, S.D. Hursting, Phytoestrogen intake and prostate cancer: a case-control study using a new database, Nutr. Cancer 33 (1999) 20-25.

[38] P. Kirk, R.E. Patterson, J. Lampe, Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults, J. Am. Diet. Assoc. 99 (1999) 558-563.

[39] A. Cassidy, S. Bingham, K.D.R. Setchell, Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women, Am. J. Clin. Nutr. 60 (1994) 330-340.

[40] K. Wakai, I. Egami, K. Kato, T. Kawamua, A. Tamakoshi, Y. Lin, T. Nakayama , M. Wada, T. Ohno, Dietary intake and sources of isoflavones among Japanese, Nutr Cancer 33 (1999) 139-145.

[41] C.H.G. Irvine, M.G. Fitzpatrick, S.L. Alexander, Phytoestrogens in soy-based infant foods: concentrations, daily intake, and possible biological effects, Proc. Soc. Exp. Biol. Med. 217 (1998) 247-253.

[42] A. Zung, R. Reifen, Z. Kerem, Z. Zadik, Phytoestrogens: the pediatric perspective, Journal of Paediatric Gastroenterology and Nutrition 33 (2001) 112-118.

[43] P.G. Reeves, F.H. Nielsen, G.C.Jr Fahey, AIN-93 purified diets for laboratory rodents: final report of the American Instititute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet, J. Nutr. 123 (1993) 1939-1951.

[44] H.J. Wang, P.A. Murphy, Isoflavone content in commercial soybean foods, J Agric. Food Chem. 42 (1994) 1666-1673.

[45] O.H. Lowry, N.J. Rosebrough, A.L. Far, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265-271.

[46] G.M. Cooke, C.A. Price, R.J. Oko, Effects of *In Utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on serum androgens and steroidogenic enzyme activities in the male rat reproductive tract, J. Steroid Biochem. Molec. Biol. 67 (1998) 347-354.

[47] G.M. Cooke, F. Pothier, B.D. Murphy, The effects of progesterone, 4,16-androstadien-3-one and MK-434™ on the kinetics of pig testis microsomal testosterone-4-ene-5α-reductase activity, J. Steroid Biochem. Molec. Biol. 60 (1997) 353-359.

[48] J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular cloning: A laboratory manual 2nd Ed. USA:Cold Spring Harbor Laboratory Press. (1989).

[49] U.K. Laemmli, Cleavage of structural proteins during assembly of the head of bacteriophage T4, Nature 227 (1970) 680-685.

[50] P. Matsudaira, Sequence from picomole quantitites of proteins electroblotted onto polyvinylidene difluoride membranes, J. Biol. Chem. 262 (1987) 10035-10039.

[51] D.B. Hales, L. Sha, A.H. Payne, Testosterone inhibits cAMP-induced de novo synthesis of Leydig cell cytochrome P45017α by an androgen receptor-mediated mechanism, J. Biol. Chem. 262 (1987) 11200-11206.

[52] S. Mesiano, S.L. Katz, J.Y. Lee, R.B. Jaffe, Phytoestrogens Alter Adrenocortical Function: Genistein and Daidzein Suppress Glucocorticoid and Stimulate Androgen Production by Cultured Adrenal Cortical Cells, J. Clin. Endocrinol. Metab. 84 (1999) 2443-2448.

[53] G. Majdic, R.M. Sharpe, P.J. O'Shaugnessy, P.T.K Saunders, Expression of cytochrome P450 17α-hydroxylase /C17-20 Lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens, Endocrinology 137 (1996) 1063-1070.

[54] M. Tarsounas, R.E. Pearlman, P.B. Moens, Meiotic activation of rat pachytene spermatocytes with okadaic acid: the behaviour of synaptonemal complex components SYN1/SCP1 and COR1/SCP3, J. Cell Sci. 112 (1999) 423-434.

[55] W.M. Keung, Dietary estrogenic isoflavones are potent inhibitors of ß-hydroxysteroid dehydrogenase of *P. testosteronii*, Biochem. Biophys. Res. Commun. 215 (1995) 1137-1144

[56] J-C. Le Bail, Y. Champavier, A-J. Chulia, G. Habrioux, Effects of phytoestrogens on aromatase, 3ß and 17ß-hydroxysteroid dehydrogenase activities and human breast cancer cells, Life Sci. 66 (2000) 1281-1291.

[57] S. Ohno, S. Shinoda, S. Toyoshima., H. Nakazawa, T. Makino, S. Nakajin, Effect of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells, J. Steroid Biochem. Mol. Biol. 80 (2002) 355-363.

[58] Y.C. Kao, C. Zhou, M. Sherman, C.A. Laughton, S. Chen, Molecular basis of the inhibition of human aromatase (estrogen synthase) by flavone and isoflavone phytoestrogens: a site-directed mutagenesis study, Environ. Health Prospect. 106 (1998) 85-92.

[59] J.Y.A. Lehtonen, H. Adlercreutz, P.K.J. Kinnunen, Binding of daidzein to liposomes, Biochim. Biophys. Acta 1285 (1996) 91-100.

[60] A. Arora, T.M. Byrem, M.G. Nair, G.M. Strasburg, Modulation of liposomal membrane fluidity by flavanoids and isoflavanoids, Arch. Biochem. Biophys. 373 (2000) 102-109.

[61] G.M. Cooke, Identification and mechanism of action of phospholipids capable of modulating rat testicular microsomal 3ß-hydroxysteroid dehydrogenase-isomerase activity in vitro, Biol. Reprod. 41 (1989) 438-445.

[62] G.M. Cooke, B. Robaire, Phospholipases modulate the rat testicular androgen biosynthetic pathway *in vitro*, Biol. Reprod. 39 (1988) 329-339.

[63] A. Machino, H. Nakano, B.I. Tamaoki, Influence of physical and chemical treatments upon the microsomal enzymes of the testes related to androgen biosynthesis, Endocrinol. Jpn. 16 (1969) 37-46.

[64] K. Bogovich, A.H. Payne, Purification of rat testicular microsomal 17-ketosteroid reductase: evidence that 17-ketosteroid reductase and 17ß-hydroxysteroid dehydrogenase are distinct enzymes, J. Biol. Chem. 260 (1980) 5552-5559.

[65] D. Roberts, D.N.R. Veeramachaneni, W.D. Schlaff, C.A. Awoniyi, Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats, Endocrine 13 (2000) 281-286.

[66] L. Strauss, S. Makela, S. Joshi, I. Huhtaniemi, R. Santti, Genistein exerts estrogen-like effects in male mouse reproductive tract, Mol.Cell. Endocrinol. 144 (1998) 83-93.

[67] T.D. Lund, D.J. Munson, M.E. Haldy, K.D.R. Setchell, E.D. Lephart, R.J. Handa , Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback, Biol. Reprod. 70 (2004) 1185-1195.

[68] E. Bowey, H. Adlercreutz, I. Rowland, Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats, Food Chem Toxicol. 41 (2003) 631-6.

[69] N.G. Coldham, C. Darby, M. Hows, J. King, A.Q. Zhang, J. Saur, Comparative metabolism of genistein by human and rat gut microflora: detection and identification of the end-products of metabolism, Xenobiotica. 32 (2002) 45-62.

[70] R.M. Sharpe, B. Martin, K. Morris, I. Greig, C. McKinnell, A.S. McNeilly, M. Walker, Infant feeding with soy formula milk: effect on the testis and on blood testosterone levels in marmoset monkeys during the period of neonatal testicular activity, Human Reprod. 17 (2002) 1692-1703.

[71] A. Cassidy, M. Faughnan, Phytoestrogens through the life cycle, Proceedings of the Nutrition Society 59 (2000) 489-496.

[72] K.D.R. Setchell, L. Zimmer-Nechemias, J. Cai, J.F. Heubi, Exposure of infants to phytoestrogens from soy-based infant formula, Lancet 350 (1997) 23-27.

[73] J.G. Lewis, J.C. Morris, B.M. Clark, P.A. Elder, The effects of isoflavones extract ingestion, as Trinovin, on plasma steroids in normal men, Steroids 67 (2002) 25-29.

[74] C. Nagata, S. Inaba, N. Kawakami, T. Kakizoe, H. Shimizu, Inverse association of soy product intake with serum androgen and estrogen concentrations in Japanese men, Nutr. Cancer 36 (2000) 14-18.

[75] E.J. Podesta, M.A. Rivarola, Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development, Endocrinology, 95 (1974) 455-461.

[76] R.G. Foldesy, J.H. Leathem, Simultaneous measurements of testosterone and three 5α-reduced androgens in the venous effluent of immature rat testes in situ, Steroids, 35 (1980) 621-631.

Table 1 Isoflavone levels in diets

Isoflavones were determined by HPLC in ß-glucuronidase digested extracts of experimental diets. Diet 1 contained casein protein, while diets 2 to 6 contained an alcohol-washed soy protein concentrate and in diets 3 to 6, Novasoy was added to supplement the diets with isoflavones. (N.D. not detectable) Data depicted as mg isoflavones/kg pelleted feed.

Diet	Protein	Genistein	Daidzein	Glycitein	Total isoflavones
diet 1	casein	ND	ND	ND	ND
diet 2	soy	18.6	10.5	2.6	31.7
diet 3	soy	21	12.3	2.8	36.1
diet 4	soy	39.3	27.6	7.6	74.5
diet 5	soy	124.4	90.9	20.5	235.6
diet 6	soy	544.8	412.3	89.5	1046.6

Table 2 PCR primer pairs

Target Gene	Forward Primer	Reverse Primer	Fragment Size (Base Pairs)	GeneBank Accesion #
G3PDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452	NM_017008
SCC	ATC ACA GAG ATG CTG GCA GGA	GCA CGT TGA TGA GGA AGA TGG	481	J05156
3ß-HSD	ACT GGC AAA TTC TCC ATA GCC	TTC CTC CCA GGT GAC AAG TGG	402	L17138
CYP17	GTG CTG GCA CAC GAC AAG GAG	GCC AGG ATC CAC TTG AGC ACA	478	NM_012753

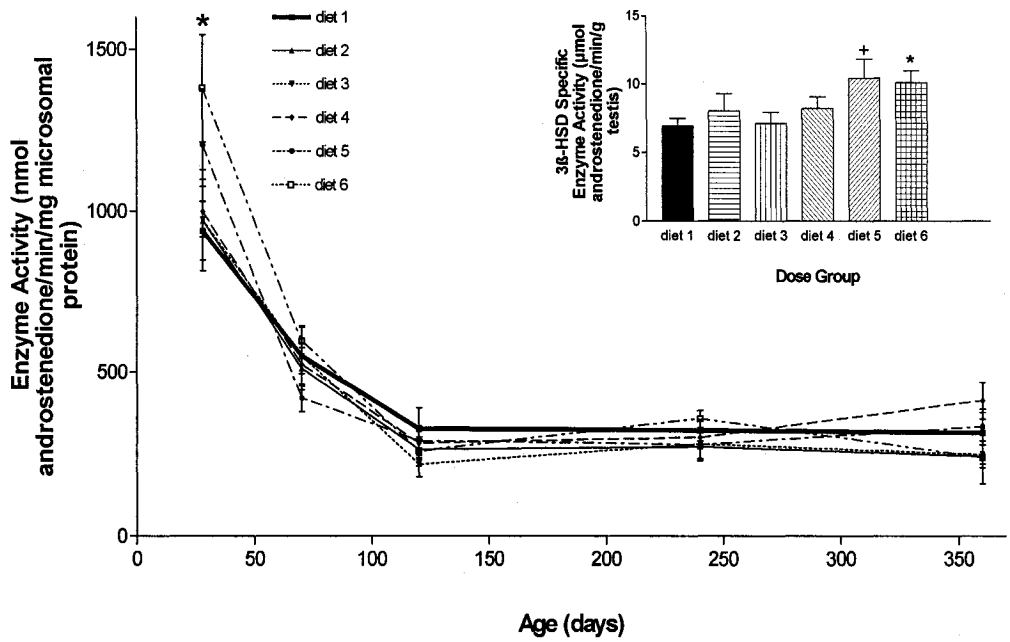


Figure 1 3ß-HSD enzyme activity/mg microsomal protein F1 rats.

3ß-hydroxysteroid dehydrogenase (3ß-HSD) enzyme activity (nmol androstenedione (A4) / min / mg protein mean ± SE of male rats). Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed a significant increase in enzyme activity for diet 6 (1379.3 ± 167) compared to diet 1 (938.8 ± 91) (* $P<0.05$) at PND 28. The inset represents 3ß-HSD activity (µmol A4 / min / g testis mean ± SE) at PND 28 in male rats. Statistical analysis showed a significant increase in enzyme activity for diet 5 (10.4 ± 1.4) (+) and diet 6 (10.1 ± 0.9) (*) compared with diet 1 (6.9 ± 0.6) ($P<0.05$). The number of animals sampled at PND 28 n = 5 for diets 1-3, n = 6 for diets 4-6. For PND 70 rats n = 9 for diets 1 and 6, n = 8 for 2,4 and 6, and n = 6 for diet 3. There were 8 animals sampled per dose for PND 120 except for diets 1 and 6 which had n = 9. For PND 240 there were 8 rats sampled for all groups except for diets 3 and 5 in which 7 rats were sampled. PND 360 rats had 6 rats examined per dose with the exception of diet 4, which had 5 rats examined.

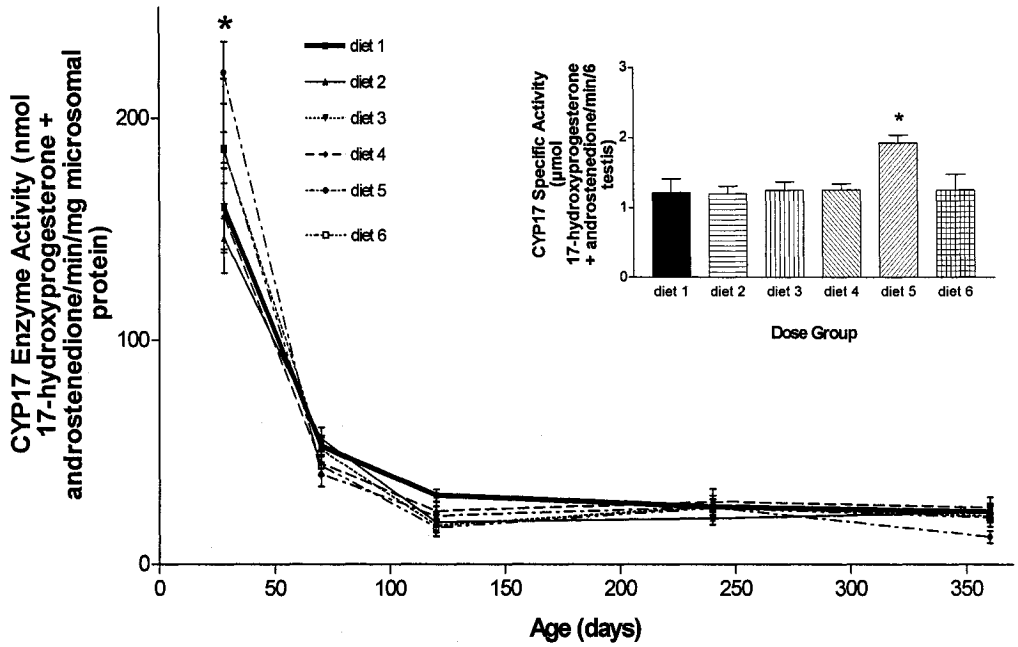


Figure 2 CYP 17 enzyme activity/mg microsomal protein in F1 rats

CYP17 (17-OHase and C17,20-Lyase) enzyme activity (nmol 17-hydroxyprogesterone (17OH-P) + androstenedione (A4) / min / mg protein mean ± SE) of male rats. Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed a statistically significant increase in activity seen for diet 5 (220.5 ± 14) compared with diet 1(159.7 ± 20) (* $P<0.05$) at PND 28. The inset depicts CYP17 activity (µmol 17OH-P + A4 / min / g testis mean ± SE) of PND 28 male rats. Statistical analysis showed a significant increase in activity for diet 5 (1.92 ± 0.1) compared with diet 1 (1.21 ± 0.2) (* $P<0.05$). The number of animals sampled at PND 28 were n = 5 for diets 1-3 and 6, n = 6 for diets 4-5. For PND 70 rats n = 8 for diets 1,2,4 and 5 n = 6 for diet 3 and n = 9 for diet 6. There were 6 animals sampled from diets 5-6, n = 7 for diet 1, n = 3 for diet 3 and n = 8 for diet 6 for PND 120 rats. For PND 240 rats there were 8 rats sampled for all groups except for diets 3 and 6 which had 8, and diet 1 which had 7 rats. There were 6 rats examined per dose with the exception of diet 4, which had 5 rats examined at PND 360.

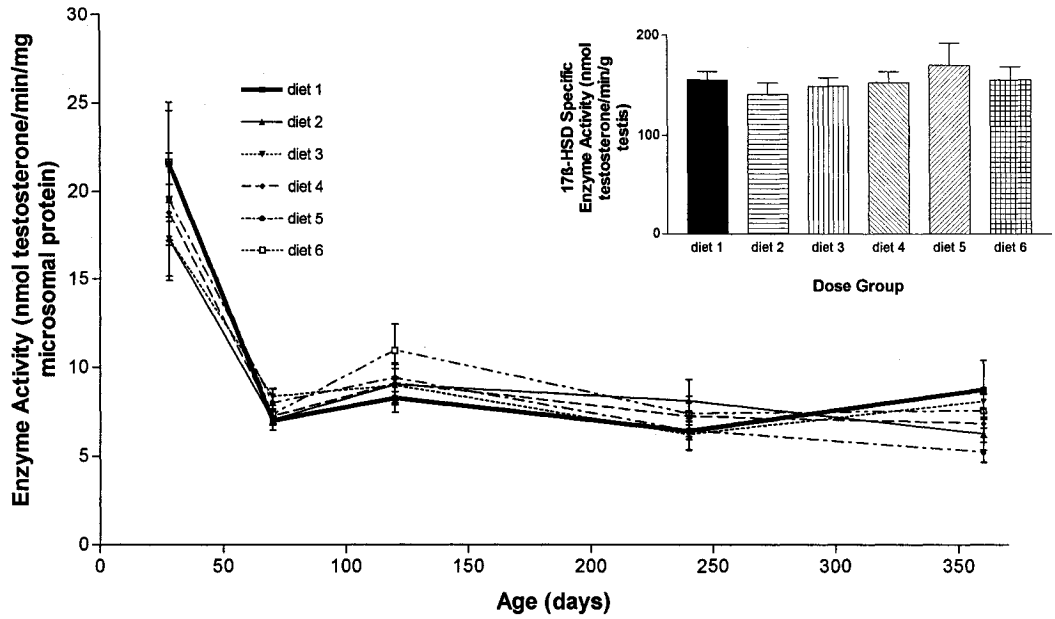


Figure 3 17ß-HSD enzyme activity/mg microsomal protein in F1 rats

17ß-Hydroxysteroid dehydrogenase (17ß-HSD) enzyme activity (nmol testosterone/min/ mg protein mean ± SE) of male rats. Statistical analysis (2 way-ANOVA followed by all pairwise Tukey's tests) showed no statistically significant differences in enzyme activity seen for 17ß-HSD activities (P>0.05). The inset depicts 17ß-HSD activity (µmol testosterone/min/testis mean ± SE) of PND 28 male rats. Statistical analysis of the inset data showed no significant changes in enzyme activity (P>0.05) for PND 28 rats. The number of animals sampled at PND 28 were n = 5 for diets 1-3, n = 6 for diets 4-6. For PND 70 animals n = 9 for diets 1,6, n = 8 for diets 2,4,5, and n = 6 for diet 3. There were n = 8 animals sampled per dose for PND 120 rats except for diet 1 (n = 9) and diet 6 (n = 7). For PND 240 rats there were 8 rats sampled for all groups except for diet 4 (n= 7). There were 6 rats examined per dose with the exception of diet 4 (n=5) for PND 360.

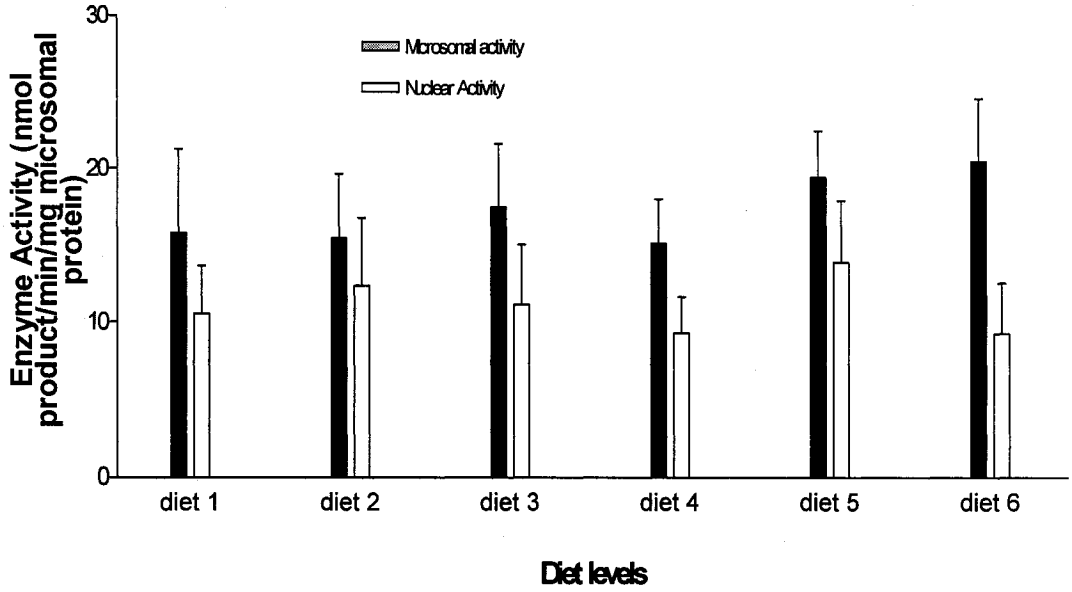
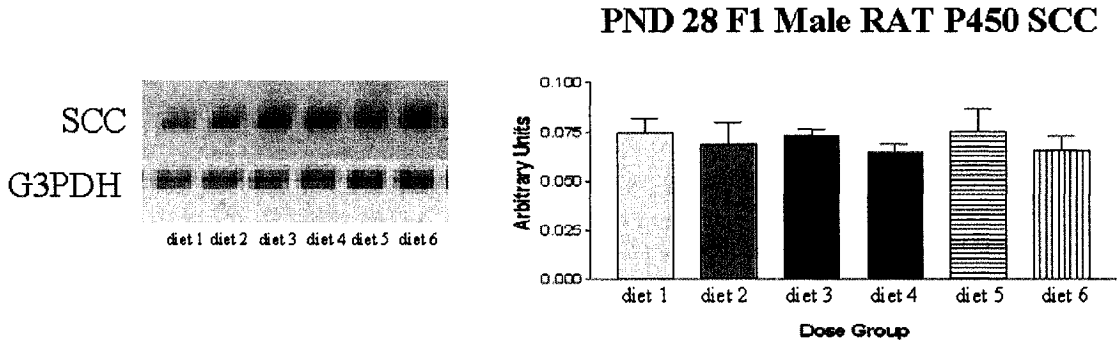


Figure 4 5α-Reductase activity/mg microsomal or nuclear protein in F1 rats

5α-reductase enzyme activities (nmol dihydrotestosterone/min/mg protein mean ± SE) of PND 28 male rats. Enzyme assays were carried out both at pH 4.5 (not shown) and pH 6.5. The enzyme assays were conducted on both microsomal and nuclear testis extracts. No statistically significant differences (1 way-ANOVA) were seen between the enzymatic rates between different diet groups ($P>0.05$). The number of animals sampled at 28 days of age were n = 5 for diets 1-3, n = 6 for diets 4-6.

A



B

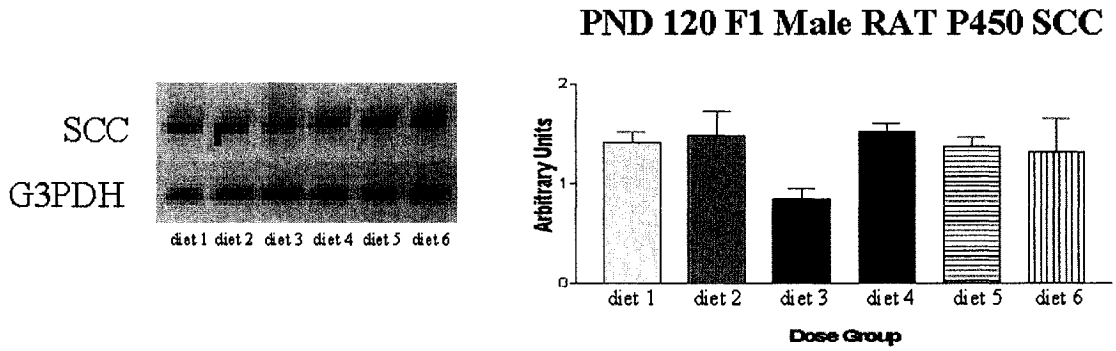


Figure 5 mRNA levels of P450 SCC in PND 28 and 120 F1 male rats

Mean levels of mRNA of (A) PND 28 SCC, (B) PND 120 SCC, examined by RT-PCR. Data are depicted as a single representative (on the left) of each diet level from the populations examined, which were examined statistically (on the right) (Mean arbitrary units ± SE). The Y-axis represent arbitrary units developed from the ratio of densitometry of the mRNA of SCC divided by the constitutive control G3PDH. No statistically significant differences were seen between mean diet dose group expression of mRNA examined by 1-way analysis of variance ($P>0.05$). The number of animals sampled at PND 28 was 6 for all doses examined with the exception of diet 3 ($n=5$). The number of animals sampled at PND 120 was 6 except for diets 2,3 ($n = 5$).

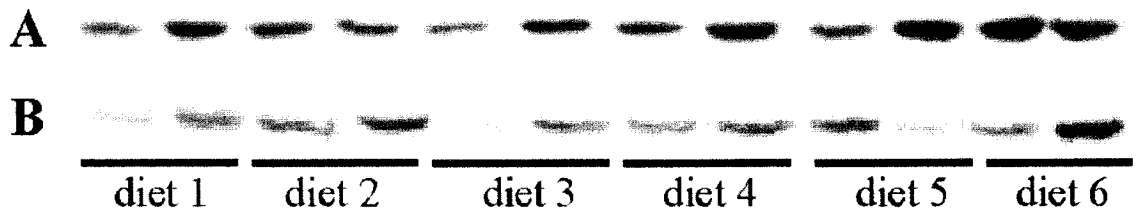


Figure 6 Protein levels of P450 SCC in PND 28 and 120 F1 male rats

Immunoreactive protein levels of A) PND 28 SCC, B) PND 120 SCC examined by Western blot. (For all data shown there were 2 rats sampled for each diet group at PND 28 and 120 – A stained gel was used to tests loading consistency).

Chapter 4

4.0 Altered developmental gene expression in testes of rats with lifetime exposure to soy isoflavones.

McVey M.J., Curran I.H.A, Cooke G.M., "Altered developmental gene expression in testes of rats with lifetime exposure to soy isoflavones." Submitted to Biology of Reproduction. (2004).

The finding that 3ß-HSD and CYP17 enzyme activities were changed at PND 28, while indicative of isoflavones affecting early development, did not explain the increased androgen levels seen at PND 120. Therefore, investigations of other components of the steroidogenic cascade that lead to testosterone production were examined. An examination of mRNA levels of several genes involved in the regulation of steroidogenesis (transcription factors, cholesterol shuttling proteins, receptors, enzymes, and other factors) was done. Western blotting and IHC were used to follow changes in protein expression in genes that had altered levels of mRNA due to dietary isoflavones.

Altered developmental gene expression in testes of rats with lifetime exposure to soy isoflavones.

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Abbreviated form of the title:

Isoflavones affect rat developmental genes.

Keywords:

SF-1, WT1, SOX9, DAX-1, soy isoflavones, testis, rat, steroidogenesis

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4.1 Abstract

The effects of lifelong exposure to dietary soy isoflavones on the levels of mRNA and protein for genes involved in development and steroidogenesis were examined in F1 male rat testes. F1 male rats were obtained from a multi-generational study where the parental and F1 rats were fed either a soy-free casein-based control diet (AIN93G) or diets containing alcohol-washed soy protein supplemented with increasing amounts of Novasoy, a commercial isoflavone supplement. Diets were designed to approximate ranges of human consumption levels and included 0 to 1046.6 mg isoflavones/kg pelleted feed. It was previously determined that F1 male rats exhibited increased serum and testicular androgen levels at postnatal day (PND) 120 and increased steroidogenic enzyme activities at PND 28. Our current research has revealed that at PND 28 there were significant increases in testicular mRNA levels for the transcription factors: *wt1, sox9* and *dax-1*. These changes in mRNA expression did not coincide with changes in protein levels as determined by western blot analysis, but immunohistochemical examination showed that the number of Leydig cells positive for SF-1 were increased, whereas those for DAX-1 were decreased in rats fed isoflavones. Altered levels of SF-1 and DAX-1 in Leydig cells could be in part responsible for changes in steroidogenic enzyme activities and androgen production previously detected in these rats.

4.2 Introduction

During puberty serum androgen levels rise to significant levels, and provide negative feedback regulation on the hypothalamic-pituitary-testicular (HPT) axis, regulating stimulatory endocrine signalling. Luteinizing hormone (LH) released from the anterior pituitary, stimulates testicular Leydig cell androgen production through increased cholesterol mobilization from lipid stores by a cAMP dependent mechanism, and secondly by increasing the transcription of enzyme-encoding genes [1].

The movement of cholesterol from lipid stores in Leydig cells to the cholesterol side chain cleavage enzyme (P450 SCC) in the mitochondria for conversion to pregnenolone is the rate-determining step of steroidogenesis. Steroidogenic acute regulatory protein (STAR) is a short half-life protein, which is rapidly synthesized in response to LH to increase the transfer of cholesterol to P450 SCC [2]. The regulation of STAR mRNA and protein levels is complex, involving many factors such as steroidogenic factor 1 (SF-1), the zinc finger transcription factor GATA-4, dosage sensitive sex reversal-adrenal hyperplasia congenital critical region on the X-chromosome (DAX-1), activator protein 1 (AP-1), cFOS, PGF2α, ERK2/1, arachidonic acid, and phosphatases and kinases [3]. Other proteins have been implicated in the movement of cholesterol to P450 SCC, such as the peripheral type benzodiazepine receptor (PBR), which facilitates cholesterol transfer to the inner mitochondrial membrane [4,5] possibly via a LH dependent mechanism [6]. Other factors that are able to influence steroidogenesis include proinflammatory cytokines [7], gonadotropin regulated testicular helicase (GRTH: an RNA helicase which acts as a translational activator upregulated by hCG) [8] and scavenger receptor class B type I (SR-B1) the

HDL receptor responsible for selective cholesteryl ester uptake from HDL particles [9]. The rat SR-B1 gene is largely regulated by a SF-1 mediated-cAMP dependent mechanism [10].

In male rats, SF-1 functions as a transcription factor increasing the transcription of genes important for development and steroidogenesis such as LHß, sex-determining-region –Y gene (SRY), 17-hydroxylase/C17,20-lyase (CYP17), CYP-19, 3ß-hydroxysteroid dehydrogenase (3ß-HSD), P450-SCC, STAR, SR-B1, DAX-1 and Mullerian inhibiting substance (MIS) [10-17]. SF-1 transcriptional activity can be modified by cell specific transcription factors such as Wilms tumor related 1 (WT1), SOX family protein (SOX9), DAX-1 and GATA-4 [12,18]. DAX-1 is an important transcriptional repressor involved in development and steroidogenesis [11-14], whose transcription is controlled at least in part by SF-1 [15]. Levels of Leydig cell DAX-1 can repress SF-1 transcriptional activation [11] and have been linked to the control of steroidogenesis due to its role in controlling transcription of genes such as SR-B1 [14]. In the current study we examined the proportion of SF-1/DAX-1 per testis to look for possible inhibition or activation of SF-1 activity by isoflavones.

Research has shown that the environmental estrogenic compounds diethylstilbestrol (DES) and 4-octylphenol (OP) can reduce fetal rat expression of SF-1 [19]. Phytoestrogens are estrogenic compounds with roles such as anti-fungal defence within plants [20,21]. Isoflavones are phytoestrogens that are common in soy, legumes, lentils and chickpeas. Soy foods and soy-based infant formulas [22,23] have the highest recorded levels of isoflavones in food, and as such represent a major source of dietary isoflavones [22,24]. Research involving consumption of soy isoflavones has investigated

possible therapeutic benefits for hormone replacement therapy in menopause as well as antioxidant and antipromotional effects against coronary heart disease, various cancers and osteoporosis [20,25-29]. However, not all research concludes that dietary isoflavones are beneficial, for example, in the 1940's, it was observed that consumption of plants rich in phytoestrogens caused sheep to abort their fetuses [30]. Subsequently, studies have shown that isoflavone consumption has deleterious effects on reproductive success and health, for example, genistein impairs the growth of testicular cell lines and disrupts rat spermatogenesis [31-36].

Isoflavone studies have found both significantly increased [37] and reduced production of serum testosterone (T) in rats [38-42], mice [43] and fish [44]. Our laboratory has shown recently, that male rats exhibit significantly elevated levels of serum and testicular T and dihydrotestosterone (DHT) at postnatal day (PND) 120 following consumption of soy isoflavones at levels obtainable by human consumption [45-52]. Furthermore, at PND 28, significantly increased testicular 3ß-HSD and CYP17 steroidogenic enzyme activities were evident [46]. Isoflavones have been shown to both increase and decrease serum LH levels [37,43,53], but they may also modify the expression of other factors, which regulate testicular steroidogenesis. Our current interest is to examine other possible targets of isoflavone action that could explain the changes seen in our earlier investigation of androgen levels [45] and enzyme activities [46].

The present investigation of rats fed a mixture of soy isoflavones in conjugated and unconjugated (glycone and aglycone) states, involved the examination of testicular levels of mRNA and if changes were seen then also protein levels for genes pertinent to rat steroidogenesis and steroid action: STAR, PBR, SF-1, WT1, SOX9, DAX-1, GATA-

1,4,6, GRTH, SR-B1, androgen receptor (AR), luteinizing hormone receptor (LHR), estrogen receptor (ER) ERα, Erß, androgen binding protein/sex hormone binding globulin (ABP/SHBG), cFOS, IL-1α, IL-ß and aromatase. Altered *wt1, dax-1* and *sox9* mRNA levels prompted the examination of testicular protein levels for SF-1, SOX9, DAX-1 and WT1. SF-1 and DAX-1 as well as the SF-1/DAX-1 ratio were examined with immunohistochemical (IHC) staining to test for possible cell specific effects, as these genes are reportedly expressed in both Leydig cells of the interstitium as well as Sertoli cells in the seminiferous tubules, while WT-1 and SOX-9 are strictly expressed postnatally in Sertoli cells.

4.3 Materials and Methods

4.3.1 Materials

Organic solvents were purchased from EM Science Merck KgaA (Darmstadt, Germany) with the exception of formaldehyde, which was purchased from Fisher Scientific (Nepean, ON). Novasoy soy isoflavone concentrate (152-400) and alcohol washed soy protein (Pro Fam 930) were obtained from Archer Daniels Midland Company (Decatur, IL). Casein protein was purchased from ICN (Cleveland, OH). Diethyl pyrocarbonate (DEPC) was purchased from Sigma Chemical Co. (St Louis, MO). Ethidium bromide was purchased from Gibco BRL (Gaithersburg, MD). Agarose, RNAse H ribonuclease and dNTPs were purchased from Roche Diagnostics Corporation (Indianapolis, IN). Superscript II RNAse H-Reverse transcriptase, $d(T_{15})$ primers, custom PCR primers (Table 1) and TRIzol were purchased from Life Technologies Inc. (Burlington, ON). Chill wax was obtained from MJ Research Inc, (Boston, MA). Taq

DNA polymerase and PCR buffers were purchased from Boehringer Mannheim (Laval, QC). NE-PER nuclear and cytoplasmic extraction reagents, Coomassie Plus Protein Assay reagents, bovine serum albumin standards and Supersignal ULTRA chemiluminescent substrate were purchased from Pierce (Rockford, IL). SDS-polyacrylamide and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Laboratories (Richmond, CA). Immobilon-P, polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). TRIS buffered saline containing 0.1 % Tween-20 (TBS) was purchased from ICN Biochemicals (Cleveland, OH), and 4 % skim milk powder was purchased from Nestlé (Don Mills, ON). Streptavidin and antibody diluent buffer were obtained from DAKO Diagnostics Canada Inc. (Missassauga, ON). Two polyclonal rabbit antibodies were generously donated: (1) a human placental 3ß-hydroxysteroid dehydrogenase-1 (Dr. James L. Thomas, Mercer University, School of Medicine at Macon, GA), (2) a bovine Ad4BP/SF-1 (Dr. Ken-Ichirou Morohashi, Department of Developmental Biology, National Institute of Basic biology, Okazaki, Japan). Three affinity purified polyclonal antibodies were of commercial origin (Santa Cruz Biotechnology, Inc. Santacruz, CA): (1) a rabbit antibody raised against a peptide mapping the amino (A)-terminal of human DAX-1, (2) a rabbit antibody raised against a peptide mapping the carboxy (C)-terminal of human WT1 and (3) a goat antibody raised against a peptide mapping the A-terminal of human SOX9. Normal swine serum and avidin/biotin blocking kits were purchased from Vector Laboratories Inc. (Burlingame, CA). PBS buffer was purchased from Sigma Chemical Co. (St Louis, MO). Slides were mounted with micromount medium purchased from Surgipath Medical Industries Inc. (Grayslake, IL).

4.3.2 Experimental Diets

Six semi-purified diets were formulated according to specifications of the American Institute of Nutrition (AIN) [54]. One diet was casein-based (AIND93G) (diet 1) while the other diets were formulated by adding increasing amounts of a commercial mixture of isoflavones (Novasoy) (diets 3-6), to a base diet resembling AIN93G, but containing alcohol-washed (to remove isoflavones) soy protein concentrate (Pro Fam 930) in place of casein (diet 2). Soy isoflavone content (genistein, daidzein and glycitein, Table 2) was analyzed by HPLC [55] using ß-glucuronidase digests of each diet (Dr. Sarwar Gilani and Mr. Patrick Robertson of Health Canada, personal communication). All diets were provided to rats *ad libitum* in pelleted form.

4.3.3 Animals

The male *Sprague-Dawley* rats used for this study were the F1 generation of a multi generation study examining the effects of dietary isoflavones on growth, development and reproductive physiology. The study design adhered to the protocol for the Organization for Economic Cooperation and Development (OECD) Guideline 416 (OECD TG416, 2001). Results from this larger study will be provided elsewhere (Curran, I.H.A, Cooke G.M., and Gilani, G.S. in preparation). Animal care was provided following the guidelines of the Canadian Council for Animal Care and was approved by the Health Canada Animal Care Committee. The parental generation (F0) purchased from Charles River (St-Constant, PQ) at the pubescent stage of development were pair housed with a 12 hours light/dark cycle in hanging polycarbonate cages (Health Guard System, Research Equipment Company, Inc. Byran, TX) containing corncob bedding and free access to food and fresh water. F0 rats were acclimatized until PND 50 at which

point they were assigned randomly to experimental diets. After 70 days exposure to the experimental diets (PND 120), rats were mated and the resulting (F1) progeny were weaned at PND 21 and provided with the same diet as their parents. Weekly food consumption was recorded for randomly selected rats from each diet group throughout their lives (data not shown). Estimated daily intake of soy isoflavones for F1 male rats at PND 28 was 0, 0.9, 1.2, 2.0, 6.3 and 29.5 (mg isoflavones/kg body weight (BW)/day) for diets 1-6 respectively, and 0 0.9, 1.0, 2.1, 7.3, and 28.7 mg/kg BW/day for PND 120 rats for diets 1-6 respectively. No differences were seen in food consumption amongst rats of different diet groups. Male F1 progeny were sacrificed by exsanguination, through cardiac puncture under isoflurane anaesthesia on PND 28 and 120. Testes were weighed and snap-frozen in liquid nitrogen before being stored at –80°C until assay.

4.3.4 RNA Isolation and RT-PCR

Testes from time points when changes in steroidogenic enzyme activities were observed (PND 28) [46] and when changes in serum and testicular androgen levels were evident (PND 120) [45] were subjected to total RNA extraction followed by semi-quantitative RT-PCR of genes involved with steroidogenesis.

RNA isolations from 20 mg of thawed testes tissue were carried out using TRIzol according to manufacturer's specifications. Concentrations and purities of the RNA isolated were determined spectrophotometrically (optical density at 260 nm and $A_{260/280}$ ratio) and quality was verified by denaturing ethidium bromide stained formaldehyde RNA electrophoresis through agarose gels [56]. RNA samples were stored at –80°C until required for cDNA synthesis. cDNA was obtained using 5 μg of total RNA and 200 U

Superscript II RNAse H-Reverse transcriptase in a total reaction volume of 20 µl. The reverse transcription reaction and subsequent steps were performed as per manufacturer's recommendations (Life Technologies Inc., Burlington, ON). cDNA was used immediately or stored at –20°C until needed for gene amplification. Samples were tested for genomic DNA contamination by primer amplification from RNA (Appendix 1), which revealed no products formed.

4.3.5 Gene Amplification

Target cDNA was amplified from 50 ng of RNA from the cDNA reaction in a 50 µl volume containing (1x PCR buffer + Mg as per manufacturer's kit) 0.25 mM each of forward and reverse primers (Table 1). Primers were designed using SciEdCentral Clone Manager Professional Suite (Scientific and Educational Software, Durham, NC) or purchased from Ambion Inc. (*cfos* cat # 5402, IL-1ß cat # 5420) (Austin, TX). Premature initiation of the reactions was prevented by the addition of 25 µl chill wax before addition of 2.5 U Taq DNA polymerase. PCR reactions were previously optimized for primer pairs by temperature and cycle gradients (Appendix 4). The following conditions were used as PCR cycles after initial denaturing 95°C (3 min.): 95°C for 1 minute, followed by a specific annealing temperature (Table 2) for 70 seconds each followed by 72°C for 70 seconds. After the last amplification cycle (Table 2), PCR products were subjected to an extended final incubation at 72°C for 5 minutes to fully extend all partial DNA fragments. Thermocycled reaction products were separated by 2% agarose gel electrophoresis followed by ethidium bromide staining and images were captured (Kodak Digital Science Image Station 440 CF) for comparative semi-quantitative densitometric analysis (Kodak ID Image Analysis Software, Eastman Kodak Company, Rochester, NY).

4.3.6 Western Blotting Analysis

Nuclear and cytoplasmic protein fractions from 50 mg of testis tissue were isolated with NE-PER nuclear and cytoplasmic extraction kits according to the manufacturer's instructions. Protein concentrations were estimated with Pierce Coomassie Plus Protein Assay reagents using a bovine serum albumin (BSA) standard. Equal quantities of protein (35 μg) were resolved by 10 % SDS-polyacrylamide gel electrophoresis [57] and transferred to Immobilon-P PVDF membranes [58]. Proteins were visualized on 10 % SDS-polyacrylamide gels and PVDF membranes followed by staining with Coomassie Brilliant Blue R-250 to test for equal loading of wells and equal transfer from gel to membrane (data not shown). The western blots were blocked with TRIS buffered saline containing 0.1% tween-20 (TBST) and 4 % skim milk powder for 1 hour at room temperature. The dilutions of each antibody (see materials) used to probe the western blots were determined experimentally (data not shown): (1) 3ß-HSD1 [1/500], (2) Ad4BP/SF-1 [1/2000], (3) DAX-1 [1/5000], (4) WT1 [1/5000] and (5) SOX9 [1/2000]. Antibodies were added with streptavidin horseradish peroxidase conjugate [1/3000] in TBST with 4% skim-milk powder. Blots were washed 3 x 5 minutes with TBST before the addition of a donkey secondary anti-rabbit antibody coupled to horseradish peroxidase [1/5000] or donkey secondary anti-goat antibody coupled to horseradish peroxidase [1/5000] (Jackson Immuno Research Laboratories Inc., West Grove, PA) in TBST with 4% skim-milk powder. Blots were washed 6 x 5 minutes before chemiluminescent detection of antigens with SuperSignal ULTRA Chemiluminescent Substrate as per manufacturer's instructions in conjunction with a Kodak Digital Science Image Station 440 CF with Kodak ID Image Analysis Software.

4.3.7 Immunohistochemistry

At the time of sacrifice, testes were immersed in Bouins fixative for 6 hrs, followed by 3 x 70 % ethanol rinses and finally embedded in paraffin [59]. IHC staining was carried out as previously described [59] using the avidin-biotin-peroxidase complex method using 4 μm sections of testis mounted on silinated slides. Deparaffinized slides had endogenous peroxidases blocked with a peroxide treatment, followed by avidin-biotin blocking before incubation with primary antibody. Concentrations of primary antibodies (see materials) used for incubations were (1) 3ß-HSD [1/100], (2) DAX-1 [1/600] and (3) SF-1 [1/100], diluted with diluent buffer and 15% normal swine serum. The secondary antibody incubation was carried out with biotinylated rabbit anti-mouse antibody [1/100] and streptavidin-horseradish peroxidase conjugate [1/200] diluted with diluent buffer and 15 % normal swine serum. Peroxidase binding on sections was visualized by diaminobenzidine reaction [60]. Negative controls consisted of sections treated as above with the exception that normal serum replaced the primary antibody. Sections were counter stained with hematoxylin prior to mounting with Micromount medium. Sections were examined with a Zeiss Axiophot Microscope and Axiocam (Tampa, FL) using a 40X lense for 3ß-HSD, DAX-1 and SF-1. Leydig cell numbers and the number of SF-1 and DAX-1 stained Leydig cells were determined by counting 25 fields of view at 400X magnification (approximately 1000 Leydig cells per animal).

4.3.8 Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze mRNA data and Leydig cell counts followed by all pairwise Tukey's tests. If data analysed by one-way ANOVA failed normality tests, then one-way ANOVA tests were conducted on ranks,

after which differences were identified by all pairwise Dunn's tests using Sigma Stat V2.03 1992-1997 for windows (SPSS Inc., Chicago, IL).

4.4 Results

4.4.1 mRNA Expression Levels of F1 Male Rat Testes

Levels of mRNA of genes involved in Leydig cell steroidogenesis (*aromatase*, *grth*, *cfos*, *gata-1*, *gata-4*, *gata-6*, *lhr*, *erα*, *erß* and *abp/shbg*) were examined at PND 28, the age in which the rats exhibited significantly increased testicular microsomal steroidogenic enzyme activities [46]. These same genes excluding *aromatase* were examined at PND 120 as well as other genes (*star*, *pbr*, *il-1α*, *il-1ß*, *sr-b1* and *ar*), which may have been involved in the elevated serum and testicular androgen levels seen at this age [45] (data not shown). The levels of *aromatase*, *grth*, *cfos*, *gata-1*, *gata-4*, *gata-6*, *lhr*, *erα*, *erß*, *abp*, *star*, *pbr*, *il-1α*, *il-1ß*, *sr-b1* and *ar* mRNA showed no significant changes in mean mRNA ratios between the 6 diets examined relative to a constitutive control (*g3pdh)* after analysis with 1 way ANOVA ($P>0.05$) (data not shown).

The SF-1 mRNA levels were not significantly different between diet levels at PND 28 and 120 (Figure 1) ($P>0.05$). The repressor DAX-1 exhibited significant increases in mRNA levels at PND 28 and 120 with increasing dietary isoflavones (Figure 1). At PND 28, mean *dax-1* ratios of diets 5 and 6 were increased significantly (approximately 44 % and 33 % respectively) compared with diet 1 ($P<0.05$). At PND 120, mean *dax-1* ratios of diet 6 were significantly higher than diet 1 by approximately 56 % ($P<0.05$).

The tumour suppressor WT1 and transcription factor SOX9, exhibited significant increases in mRNA levels in rats fed isoflavones (diets 5 and 6) compared with rats fed casein protein (Figure 1). Statistical analysis of *wt1* mRNA levels at PND 28 revealed diet 6 was significantly higher than the mean ratio of diet 1 by approximately 50 % ($P<0.05$). At PND 120 there were no significant differences seen amongst the diet groups ($P>0.05$). For *sox9* mRNA, at PND 28 the ratios of diets 5 and 6 were significantly higher than diet 1 by 62 % and 69 % respectively ($P<0.05$) but at PND 120, no significant differences for SOX-9 mRNA levels were evident ($P>0.05$).

4.4.2 Western Blot Analysis of SF-1, WT1, and DAX-1 Proteins in F1 Rat Testes.

Selective staining of one immunoreactive band for WT1 (data not shown), SF-1, DAX-1 and 3ß-HSD was obtained (Figure 2). 3ß-HSD was examined, as it was used to identify Leydig cells with immunohistochemical analysis. 3ß-HSD protein levels (42 KDa) from cytoplasmic extracts of testicular protein homogenates appeared consistent across the six diet levels examined at PND 28 (Figure 2). SF-1, DAX-1 and WT-1 were examined using nuclear protein extracts of testes homogenates. SF-1 (52 KDa), DAX-1 (52.7 KDa) and WT-1 (52 KDa) protein levels did not appear to be altered by increasing amounts of isoflavones in rat diets (Figure 2).

4.4.3 Histology and Immunohistochemistry of DAX-1 and SF-1 in F1 Male Rat Testes

IHC analysis was conducted to look for a possible change in SF-1 or DAX-1 protein levels specific to a single cell type, which may have been undetected by Western

analysis of testicular extracts. IHC of 3ß-HSD was conducted at PND 28 as a positive marker to identify Leydig cells within the interstitium (Figure 3 – 3ß-HSD). IHC staining of 3ß-HSD was not different between diet groups (data not shown), and showed uniform strong cytoplasmic staining of Leydig cells regardless of stage of spermatogenic development of adjacent tubules, and no staining within tubules (Figure 3 – 3ß-HSD – arrowhead). Leydig cells positive for SF-1 were evident at both PND 28 and 120 (Figure 3 – SF-1 Diet 1,5,6 PND 28 and 120 – arrowheads). Diet 5 and 6 increased the numbers of positive Leydig cells for SF-1 (approximately 20%) compared with diet 1 at both PND 28 and 120 (Figure 4, $P<0.05$). SF-1 labelled Leydig cells appeared to have consistently strong cytoplasmic staining and variable nuclear staining of SF-1 protein regardless of their position relative to seminiferous tubules at different developmental stages of spermatogenesis. The SF-1 antibody reacted with developing germ cells within tubules (Figure 3 – SF-1 – tailed arrows), but Sertoli cells were not visibly stained (Figure 3 – diamonds). No staining of fibroblasts (Figure 3 – F) or endothelial cells (Figure 3 – stars) was seen with the SF-1 antibody in any samples.

There were no significant differences between the numbers of stained cells for DAX-1 protein within Leydig cells of any diet group at PND 28 ($P>0.05$), while at PND 120 diets 5 and 6 reduced the number of DAX-1 positive cells ($p < 0.05$; Figure 4). Additionally IHC staining of DAX-1 at PND 28 and 120 revealed no differences in the intensity of Leydig or Sertoli cell staining between diet groups examined (examples of PND 120 DAX-1 IHC shown in Appendix 6).

Since the SF-1 and DAX-1 staining was examined from consecutive tissue sections from the same rat, and since SF-1 is involved in the regulation of DAX-1, the

data was reanalyzed using the ratio of the number of positive Leydig cells for SF-1 over DAX-1 at PND 28 and 120. One-way ANOVA revealed that, at PND 28, diet 6 increases this ratio compared with diet 1 (Figure 4, $P<0.05$). Additionally at PND 120 both diet 5 and 6 were found to be significantly elevated for SF-1/DAX-1 staining compared with diet 1 (Figure 4, $P<0.05$).

Analysis of testis slides did not indicate any differences in spermatogenesis at PND 28 or 120 between rats of different diet groups, in terms of tubular abnormalities (data not shown). The number of Leydig cells per field of view did not change significantly within different diet groups examined (4 rats per diet), indicating no change in Leydig cell number with increasing the amount of isoflavones in diets (data not shown, $P>0.05$).

4.5 Discussion

The present research was undertaken to investigate the mechanisms by which isoflavones have previously been shown to increase androgen levels at PND 120 [45] and steroidogenic enzyme activities and testicular weights at PND 28 [46] in the F1 male rats from the present study. Early developmental genes, which play a role in the postnatal regulation of sex steroid levels by acting as transcriptional regulators of steroidogenic genes [10-13,18], exhibited isoflavone dependent changes in mRNA levels. An examination of SF-1, WT1, and DAX-1 protein levels with a small number of rats suggested there were no concomitant increases in protein levels between rats consuming different diets. SOX9 protein levels could not be reliably assessed due to technical difficulties concerning the specificity of the primary antibody. SOX9 and WT1 are

strictly expressed within Sertoli cells of the tubule, 3ß-HSD is expressed in Leydig cells, and DAX-1 and SF-1 are expressed in both cell types [11,12]. IHC was used to investigate possible cell specific changes in SF-1 or DAX-1 protein levels, which may have been masked by Western blot analysis of whole testis proteins. IHC for SF-1 and DAX-1 revealed a cell-specific increase in the number of Leydig cells stained for SF-1 in diets 5 and 6 compared with diet 1, while the number of cells stained for DAX-1 did not change between diets at PND 28 or decreased at PND 120 for diets 5 and 6 in Leydig cells. The ratio of cells stained for SF-1 to DAX-1 revealed that the ratio increased for diets 5 and 6 compared with diet 1.

Curiously there was minimal staining of SF-1 in Sertoli cells but some stage specific staining of germ cells was evident. Studies examining SF-1 expression in specific cell types with age using IHC and Western blots have shown Sertoli cell expression is reduced after birth, while Leydig cell expression is not [16]. It is possible that the expression of Sertoli SF-1 was undetectable under the conditions used in the current study. Neither the increased number of Leydig cells stained for SF-1 nor the increased intensity of staining within Leydig cells seen with diets containing isoflavones, was associated with any particular stage of spermatogenesis in the neighbouring tubules.

Other studies using IHC and RNase protection assays have shown that fetal rat testes, exposed to the estrogenic chemicals DES or OP, had reduced Sertoli and Leydig cell levels of SF-1 and lower testicular mRNA levels for *sf-1* [19]. It was suggested that the changes in expression of *sf-1* mRNA levels were through the interaction of DES and OP with estrogen receptors [19]. Genistein has been shown to stimulate the transcriptional activity of estrogen receptors, and phytoestrogens have been shown to

interact estrogenically and anti-estrogenically with ERα and ERß depending on the tissue in question [61,62]. Other researchers have found changes in expression of receptors as a result of isoflavones in liver ERα [41], testis ERα and AR [43,63], prostate ERα, Erß and AR [40,42,64] and brain ERα [65]. In the current study testicular ERα, Erß and AR mRNA levels revealed no significant differences between diet groups at PND 28 or 120, which could have altered sex steroid regulated expression.

The consequences of increased SF-1 levels in rats fed diets 5 and 6 at PND 28 and 120, could have led to the increased steroidogenic activity (PND 28 [46]) and androgen levels (PND 120 [45]) previously observed within the same rats, as the transcription of genes involved with steroidogenesis are influenced by SF-1. SF-1, DAX-1, SOX9 and WT1 genes are involved in the regulation of expression of developmental factors such as MIS [11,12,66-68] and SRY [11,12], which regulate the regression of the Mullerian ducts during male sexual differentiation during early fetal life and postnatal androgen levels. Sertoli cell derived MIS has been shown to inhibit Leydig cell T synthesis in adult rats possibly by lowering the expression of CYP17 [67], LHR [67] and P450 SCC mRNAs [68]. At present the SF-1 targets MIS and SRY have not been examined, but CYP17, P450-SCC, 3ß-HSD, STAR, aromatase, SR-B1 and LHß did not reveal any isoflavone dependent changes in mRNA levels [10-17,69]. Exogenous estrogenic chemicals OP and DES reduced fetal rat testicular *sf-1* mRNA levels [19] and enzymatic activities of CYP17 [70]. In the current study, we observed elevated testicular levels of *sf-1* mRNA and protein with no changes in P450 SCC, CYP17 or 3ß-HSD mRNA or protein levels, suggesting that isoflavone effects are mediated by different mechanisms than those proposed for OP and DES [46]. Additionally, receptors and other genes such as

transcription factors (GATA-1,4,6) cholesterol (PBR) or sex steroid (ABP/SHBG) shuttling proteins, cytokines, cFOS, helicases or enzymes involved with steroidogenesis which are not under SF-1 transcriptional control did not exhibit altered mRNA levels after exposure to isoflavones in the present study.

Marmoset monkeys exposed to isoflavones via infant formula exhibited increased numbers of Leydig cells [71]. Enumeration of Leydig cells in rats of the current study revealed no increase in Leydig cell numbers between isoflavone diet groups, which may have explained the increased testis weights, steroidogenic enzyme activities [46] and steroid levels [45] previously detected. It would appear that isoflavones are not affecting the number of adult Leydig cells in the current study, which tend to be deermined before birth and remain unchanged throughout postnatal life [72].

Our lab has previously shown that, though not statistically significant, there are increased intratesticular androgen levels in diets 1 and 2 compared with diets 3-6 at PND 28 [45], which could be indicative of a delay in androgen dependent development. It is possible that the increased steroidogenic enzyme activities seen with high dietary levels of isoflavones at PND 28 are a homeostatic SF-1 mediated overcompensation to redress delays in androgen dependent development. Wisniewski et al. 2003, have found rats exposed to genistein during early development have smaller anogenital distances, reduced testis size, delayed preputial separation, lowered plasma androgen levels and altered reproductive behaviour in adulthood [73]. F1 rats of the present study did not exhibit any significant changes in anogenital distances (Curran I.H.A., Cooke G.M., and Gilani G.S., in preparation). Altered steroidogenesis as a result of isoflavone consumption, may alter the balance of estrogens and androgens needed for the differentiation, development and

function of the testes, epididymides, prostate and spermatozoa [61,74,75], possibly due to changes in the programming of the HPT axis.

Altered endocrine signalling due to early life exposure to dietary soy isoflavones, could have led to the changes in mRNA levels, steroidogenic enzyme activities [46] and androgen levels previously observed in the rats of the present study [45]. Isoflavones have been shown to modify endocrine regulation of androgen production, since isoflavones can alter levels of LH [37, 43, 53]. In our study, life-long isoflavone exposure could have altered the development of the HPT axis. SF-1 knockout mice have shown SF-1 to play an important role in gonadotropin action as follicle-stimulating hormone (FSH) and LH were markedly decreased in the pituitary-specific SF-1 knockout mice [76]. However, circulating LH or FSH levels could not be determined due to the limited quantities of serum available for our studies. We determined testicular mRNA levels of Leydig cell LHR, but these were not altered by isoflavones.

Expression of the rat Fushi tarazu factor 1 subfamily member (rFtz-F1) gene, which encodes SF-1, is not entirely understood and is postulated as being regulated by mechanisms independent of hypothalamic-pituitary control [66], such as auto-regulation by the gene product itself [16]. There appear to be important regulatory elements involved with testicular SF-1 expression, namely an Ebox, CCAAT box and Sp-1 binding sites, which interact with specific proteins, which have been proposed to be cell specific between Leydig and Sertoli cells [77]. The authors theorized there may be Leydig cell specific proteins mediating SF-1 regulation of steroidogenesis [78]. It is possible that in rats of the present study, isoflavones are somehow increasing the transcription and or

translation of SF-1 in a Leydig cell specific manner, through mechanisms presently unknown.

Currently the mechanism of action of increased SF-1 in rats fed isoflavones is not well understood, as genes transcriptionally controlled by SF-1, which at least in part regulate testicular steroid production, were not altered. This lack of SF-1 mediated transcriptional activation could have been due to the increase in testicular *dax-1* mRNA levels at both PND 28 and 120, yet IHC or Western blots did not reveal any changes between DAX-1 levels or rather decreased staining at PND 120 in Leydig cells. The ratio of SF-1/DAX-1 could be important in regulating the transcription of steroidogenic genes, as SF-1's activity can be lowered by DAX-1's suppression of SF-1 mediated transcription [14,15]. The ratio of SF-1/DAX-1 was seen to increase in the current study as the isoflavone content in diets increased, indicating that DAX-1 levels were not increasing similar to SF-1 levels possibly allowing for greater SF-1 activity. Although the SF-1/DAX-1 ratio increased, it is difficult to relate this directly to the level of SF-1 transcriptional activation, as there may be involvement of other testicular repressors of SF-1 activity, such as Alien, which are recruited by DAX-1 [78]. Another possibility for a lack of SF-1 mediated activation would involve post-transcriptional modification of SF-1 activity since SF-1 function is upregulated by serine phosphorylation [79] and, while genistein has been shown to directly inhibit tyrosine kinases [80], it indirectly stimulates phosphorylation of serine residues of p53 [81,82].

The results of the present study indicate that isoflavones may alter early developmental genes, which could lead to changes in steroidogenesis both in early post-natal life and later in adulthood. Our findings of altered levels of testicular mRNA for

dax-1, wt1 and *sox9* and Leydig cell SF-1 and DAX-1 proteins due to isoflavone consumption at levels obtainable by human infants [51,52], suggest a role for SF-1 in the changes seen in steroidogenesis [46] and androgen levels [45]. However, due to the lack of changes in SF-1 target genes involved with steroidogenesis, the mechanism is presently unknown. Studies of humans have found dose dependent modulation of plasma steroid levels by isoflavones, for example, increased plasma DHT levels [83] or decreased serum T [84], although at present the involvement of SF-1 has not been investigated and the mechanisms of action remain largely unknown. Further work is needed to investigate SF-1 levels within endocrine tissues pertinent to steroidogenesis, such as the ventromedial hypothalamic nucleus of animals exposed to dietary soy isoflavones, which regulates the expression of LH and FSH [76]. Additionally further work is needed to investigate the influence of isoflavones on the phosphorylation of SF-1 and other factors such as the potential for Leydig specific proteins, which could influence the expression and activity of SF-1.

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4.6 References

1 Payne A, Youngblood G. Regulation of expression of steroidogenic enzymes in Leydig cells. Biol Rep. 1995; 52:217-225.

2 Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. Endocrine Reviews. 1996; 17:221-244.

3 Christenson LK, Strauss JF. Steroidogenic acute regulatory protein: An update on its regulation and mechanisms of action. Archives of Medical Research. 2001; 32:576-586.

4 Papadopoulos V, Mukhin AG, Costa E, Krueger KE. The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. Journal of Biological Chemistry 1990; 265:3772-3779.

5 Krueger KE, Papadopoulos V. Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. Journal of Biological Chemistry 1990; 265:15015-15022.

6 Boujrad N, Gaillard JL, Garnier M, Papadopoulos V. Acute action of choriogonadotropin on Leydig tumor cells: induction of a higher affinity benzodiazepine-binding site related to steroid biosynthesis. Endocrinology 1994; 135:1576-1583.

7 Hedger MP, Meinhardt A. Cytokines and the immune-testicular axis. Journal of Reproductive Immunology. 2003; 58:1-16.

8 Tang PZ, Tsai-Morris CH, Dufau ML. A novel gonadotropin-regulated testicular RNA helicase. A new member of the DEAD box family. Journal of Biological Chemistry. 1999; 274:37932-37940.

9 Temel RE, Trigatti B, DeMattos RB, Azhar S, Krieger M, Williams DL. Scavanger receptor (SR-BI) is the major route for the delivery of HDL-choesterol to the steroidogenic pathway in cultured mouse adrenocortical cells. Proc Natl Acad Sci USA 1997; 94:13600-13605.

10 Lopez D, Sandhoff TW, McLean MP. Steroidogenic factor-1 mediates 3'5'-cyclic adenosine monophosphate regulation of the high-density lipoprotein receptor. Endocrinology 1999; 140:3034-3044.

11 Parker KL, Schedl A, Schimmer BP. Gene interactions in gonadal development. Annu Rev. Physiol. 1999; 61:417-33.

12 Parker KL, Schimmer BP. Genes essential for early events in gonadal development. Ann Med 2002; 34:171-178.

13 Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud SJ, Jones PM. ERKs regulate cyclic AMP-induced steroid synthesise through transcription of the steroidogenic acute regulatory protein (STAR) gene. The Journal of Biological Chemistry 2001; 276:34888-34895.

14 Lopez D, Shea-Eaton W, Sanchez MD, McLean MP. DAX-1 represses the high-density lipoprotein receptor through interaction with positive regulators sterol regulatory element-binding protein-1a and steroidogenic factor-1. Endocrinology 2001; 142:5097-5106.

15 Kawabe K, Shikayama T, Tsuboi H, Oka S, Oba K, Yanase T, Nawata H, Morohashi KI. DAX-1 as one of the target genes of Ad4BP/SF-1. Molecular Endocrinology. 1999; 13:1267-1284.

16 Morohashi KI, Omura T. Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J. 1996; 10:1569-1577.

17 Morohashi KI. Gondal and extragonadal functions of Ad4BP/SF-1: Developmental aspects. TEM 1999; 10:169-173.

18 Tremblay JJ, Viger RS. GATA factors differentially activate multiple gonadal promoters through convserved GATA regulatory elements. Endocrinology 2001; 142:977-986.

19 Majdic G, Sharpe RM, Saunders PTK. Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor-1 (SF-1/Ad4BP) in the fetal rat testis. Molecular and Cellular Endocrinology 1997; 127:91-98.

20 Adlercreutz H, Mazur W. Phytoestrogens and Western diseases. Ann Med. 1997; 29:95-120.

21 Naim M, Gestetner B, Zikah S, Birk Y, Bondi A. Soybean isoflavones. Characterization; determination, and antifungal activity. J Agr Food Chem. 1974; 22:806-810.

22 United States Department of Agriculture (USDA). USDA-Iowa State University Database on Isoflavone Content of Foods. http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html. (1999)

23 Coward L, Barnes NC, Setchell KDR, Barnes S. Genistein, daidzein and their ß-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. J. Agric. Food Chem. 1993; 41:1961-1967.

24 Mazur W, Adlercreutz H. Naturally occurring oestrogens in food. Pure Appl. Chem. 1998; 70:1759-1776.

25 Clarkson TB, Anthony MS, Hugher CL. Estrogenic soybean isoflavones and chronic disease. Risks and benefits. Trends Endocrinol. Metab. 1995; 6:11-16.

26 Tikkanen MJ, Adlercreutz H. Dietary soy-derived isoflavone phytoestrogens: could they have a role in coronary heart disease prevention? Biochemcial Pharmacology 2000; 60:1-5.

27 Humfrey CDN. Phytoestrogens and human health effects: weighing up the current evidence. Natural Toxins 1998; 16:51-59.

28 Birge SJ. Soy phytoestrogens: an adjunct to hormone replacement therapy? Menopause: the Journal of The North American Menopause society 2000; 4:209-212.

29 Wei H, Bowen R, Cai Q, Barnes S, Wang Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. P.S.E.B.M. 1995; 208:124-130.

30 Bennetts HW, Underwood EJ, Shier FL. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aust. J. Agric. Res. 1946; 22:131-138.

31 Cassanova M, You L, Gaido KW, ArchibequeEngle S, Janszen DB, Heck HD. Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. Toxicol. Sci. 1999; 51:236-244.

32 Lewis RW, Brooks N, Milburn GM, Soames A, Stone S, M Hall M, Ashby J. The effects of the phytoestrogen genistein on the postnatal development of the rat. Toxicol. Sci. 2003; 71:74-83.

33 Nagao T, Yoshimura S, Saito Y, Nakagomi M, Usumi K, Ono H. Reproductive effects in male and female rats of neonatal exposure to genistein. Reprod. Toxicol. 15 (2001) 399-411.

34 Shutt DA. The effects of plant oestrogens of dietary origin. Endeavour 1976; 35:110-113.

35 Kumi-Daika J, Rodriguez R, Goudaze G. Influence of genistein (4',5,7-trihydroxy-isoflavone) on the growth and proliferation of testicular cell lines. Biol Cell 1998; 349-354.

36 Delclos KB, Bucci TJ, Lomax LG, Latendresse JR, Warbritton A, Weis CC, Newbold RR. Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. Reprod. Toxicol. 2001; 15: 647-663.

37 Yi MA , Son HM, Lee J-S, Kwon C-S, Lim JK, Yeo YK, Park YS, Kim J-S. Regulation of male sex hormone levels by soy isoflavones in rats, Nutrition and Cancer 2002; 42:206-210.

38 Weber KS, Setchell KDR, Stocco DM, Lephart ED. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5 alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male *Sprague-Dawley* rats, J. Endocrinol. 2001; 170:591-599.

39 Roberts D, Veeramachaneni DNR, Schlaff WD, Awoniyi CA. Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats, Endocrine 2000; 13:281-286.

40 Dalu A, Blaydes BS, Bryant CW, Latendress JR, Weis CC, Delclos KD. Estrogen receptor expression in the prostate of rats treated with dietary genistein, Journal of Chromatography B 2002; 777:249-260.

41 Laurenzana EM, Weis CC, Bryant CW, Newbold R, Delclos KDR. Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P450 enzymes, and estrogen receptor alpha expression, Food Chem. Toxicol. 2002; 40:53-63.

42 Fritz WA, Wang J, Eltoum IE, Lamartiniere CA. Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate, Mol. Cell. Endocrinol. 2002; 186:89-99.

43 Strauss L, Makela S, Joshi S, Huhtaniemi I, Santti R. Genistein exerts estrogen-like effects in male mouse reproductive tract, Mol.Cell. Endocrinol. 1998; 144: 83-93.

44 Zhang L, Khan IA, Foran CM. Characterization of the estrogenic response to genistein in Japanese medaka (*Oryzias latipes*), Comparative Biochemistry and Physiology PART C 2002; 132:203-211.

45 McVey MJ, Cooke GM, Curran IHA. Increased serum and testicular androgen levels in F1 rats with lifetime exposure to soy isoflavones. Reproductive Toxicology 2004; 18-5:677-685.

46 McVey MJ, Cooke GM, Curran IHA. Altered testicular microsomal steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones. Journal of Steroid Biochemistry and Molecular Biology 2004; submitted.

47 Strom SS, Yamamura Y, Duphorne CM, Spitz MR, Babaian RJ, Pillow PC, Hursting SD. Phytoestrogen intake and prostate cancer: a case-control study using a new database, Nutr. Cancer 1999; 33:20-25.

48 Kirk P, Patterson RE, Lampe J. Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults, J. Am. Diet. Assoc. 1999; 99:558-563.

49 Cassidy A, Bingham S, Setchell KDR. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am. J. Clin. Nutr. 1994; 60:330-340.

50 Wakai K, Egami I, Kato K, Kawamua T, Tamakoshi A, Lin Y, Nakayama T, Wada M, Ohno T. Dietary intake and sources of isoflavones among Japanese, Nutr Cancer 1999; 33:139-145.

51 Irvine CHG, Fitzpatrick Mg, Alexander SL. Phytoestrogens in soy-based infant foods: concentrations, daily intake, and possible biological effects, Proc. Soc. Exp. Biol. Med. 1998; 217:247-253.

52 Zung A, Reifen R, Kerem Z, Zadik Z. Phytoestrogens: the pediatric perspective, Journal of Paediatric Gastroenterology and Nutrition 2001; 33:112-118.

53 Lund TD, Munson DJ, Haldy ME, Setchell KDR, Lephart ED, Handa RJ. Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback, Biol. Reprod. 2003; Papers in Press. (BOR).

54 Reeves PG, Nielsen FH, Fahey GCJr. AIN-93 purified diets for laboratory rodents: final report of the American Instititute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 1993; 123:1939-1951.

55 Wang HJ, Murphy PA. Isoflavone content in commercial soybean foods. J Agric. Food Chem. 1994; 42:1666-1673.

56 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual 2nd Ed. USA:Cold Spring Harbor Laboratory Press. 1989.

57 Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4, Nature 1970; 227:680-685.

58 Matsudaira P. Sequence from picomole quantitites of proteins electroblotted onto polyvinylidene difluoride membranes, J Biol. Chem. 1987; 262:10035-10039.

59 Lok E, Mehta R, Jee P, Laver G, Nera EA, McMullen E, Clayson DB. The effect of butylated hydroxytoluene on the growth of enzyme-altered foci in male Fischer 344 rat liver tissue. Carcinogenesis 1995; 16:1071-1078.

60 Graham RC, Karnofsky MJ. Early stage of absorption of injected horseradish peroxidase in proximal tubules of mouse kidney-ultrastructural cytochemistry by a new technique. J. Histochem. Chytochem. 1966; 14:291-303.

61 Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor ß. Endocrinology 1998; 139:4252-4263.

62 Carreau S, Genissel C, Bilinska B, Levallet J. Sources of oestrogen in the testis and reproductive tract. Int. J. Androl. 1999; 22:211-223.

63 Adachi T, Ono Y, Koh K-B, Takashima K, Tainaka H, Matsuno Y, Nakagawa S, Todaka E, Sakurai K, Fukata H, Iguchi T, Komiyama M, Mori C. Long-term alteration of gene expression without morphological change in testis after neonatal exposure to genistein in mice: toxicogenomic analysis using cDNA microarrays. Food and Chemistry Toxicoloyg 2004; 42:445-452

64 Lund TD, Munson DJ, Adlercreutz H, Handa R, Lephart ED. Androgen receptor expression in the rat prostate is down-regulated by dietary phytoestrogens. Reproductive Biology and Endocrinology 2004; 2:5.

65 Patisaul HB, Dindo M, Whitten PL, Young LJ. Soy isoflavones supplements antagonize reproductive behavior and estrogen receptor α- and ß-dependent gene expression in the brain. Endocrinology. 2001; 142:2946-2952.

66 Nomura M, Kawabe K, Matsushita S, Oka S, Hatano O, Harada N, Nawata H, Morohashi K-I. Adrenocortical and gonadal expression of the mammalian Ftz-F1 gene encoding Ad4BP/SF-1 is independent of pituitary control. J Biochem 1998; 124:217-224.

67 Sriraman V, Niu E, Matias JR, Donahoe PK, MacLaughlin DT, Hardy MP, Lee MM. Mullerian inhibiting substance inhibits testosterone synthesis in adult rats. Journal of Andrology 2001; 22(5):750-758.

68 Trbovich AM, Sluss PM, Laurich VM, O'Neill FH, MacLaughlin DT, Donahoe PK, Teixeira J. Mullerian inhibiting substance lowers testosterone in Luteinizing hormone-stimulated rodents. PNAS 2001; 98(6):3393-3397.

69 Pezzi V, Sirianni R, Chimento A, Maggiolini M, Bourguiba S, Delalande C, Carreau S, Ando S, Simpson ER, Clyne CD. Differential expression of SF-1/AD4BP and LRH-1/FTF in the rat testis: LRH-1 as a potential regulator of testicular aromatase expression. Endocrinology 2004; Jan 21 [epub ahead of print].

70 Majdic G, Sharpe RM, O'Shaugnessy PJ, Saunders PTK. Expression of cytochrome P450 17α-hydroxylase /C17-20 Lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens, Endocrinol. 1996; 137:1063-1070.

71 Sharpe RM, Martin B, Morris K, Greig I, McKinnell C, McNeilly AS, Walker M. Infant feeding with soy formula milk: effect on the testis and on blood testosterone levels in marmoset monkeys during the period of neonatal testicular activity, Human Reprod. 2002; 17:1692-1703.

72 Lejeune H, Habert R, Saez JM. Origin, proliferation and differentiation of Leydig cells. Journal of Molecular Endocrinology 1998; 20:1-25.

73 Wisniewski AB, Klein SL, Lakshmanan Y, Gearhart JP. Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. The Journal of Urology 2003; 169:1582-1586.

74 Umar A, Ooms MP, Luider TM, Grootegoed JA, Brinkmann AO. Proteomic profiling of epididymis and vas deferens: identification of proteins regulated during rat genital tract development. Endocrinology 2003; 144:4637-47.

75 McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, Kretser DM, Pratis K, Robertson DM. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog Horm Res. 2002; 57:149-79.

76 Zhao L, Bakke M, Parker KL. Pituitary-specific knockout of steroidogenic factor 1. Mol. Cell. Endocrinol. 2001; 185:27-32.

77 Scherrer SP, Rice DA, Heckert LL. Expression of steroidogenic factor 1 in the testis requires an interactive array of elements within its proximal promoter. Biology of Reproduction 2002; 67:1509-1521.

78 Altincicek B, Tenbaum SP, Dressel U, Thormeyer D, Renkawitz R, Baniahmad A. Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita, J Biol Chem. 2000; 275:7662-7667.

79 Babu PS, Bavers DL, Shah S, Hammer GD. Role of phosphorylation, gene dosage and DAX-1 in SF-1 mediated steroidogenesis. Endocrine Research 2000; 26:985-994.

80 Tarsounas M, Pearlman RE, Moens PB. Meiotic activation of rat pachytene spermatocytes with okadaic acid: the behaviour of synaptonemal complex components SYN1/SCP1 and COR1/SCP3, J. Cell Sci. 1999; 112:423-434.

81 Ye R, Bodero A, Zhou B-B, Khanna K.K., Lavin M.F., Lees-Miller S.P. The plant isoflavanoid genistein activates p53 and Chk2 in an ATM-dependent manner. The Journal of Biological Chemistry 2001; 276:4828-4833.

82 Darbon J-M, Penary M, Escales N, Casagrande F, Goubin-Gramatica F, Baudouin C, Ducommun B. Distinct Chk2 activation pathways are triggered by genistein and DNA-damaging agents in human melanoma cells. J. Biol. Chem. 2000; 275:15363-15369.

83 Lewis JG, Morris JC, Clark BM, Elder PA. The effects of isoflavones extract ingestion, as Trinovin, on plasma steroids in normal men, Steroids 2002; 67:25-29.

84 Nagata C, Inaba S, Kawakami N, Kakizoe T, Shimizu H. Inverse association of soy product intake with serum androgen and estrogen concentrations in Japanese men, Nutr. Cancer 2000; 36:14-18.

Table 1. PCR primer pairs

Target Gene	Forwards Primer	Reverse Primer	Fragment Size (base pairs)	GeneBank Accesion #
G3PDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452	NM_017008
PBR	CCT CCG CTG GTA TGC TAG CTT G	CTC GCC GAC CAG AGT TAT CAC G	398	NM_012515
STAR	ACT GAG GCA TCA AGC TGT GCT A	ATG CGG TCC ACC AGT TCT TCA T	354	NM_031558
SF-1	CCA CCG GAC TAC ATG TTA CC	TCC TCC TCT GGC TCT AGT TG	314	AF511594
WT1	TCC ATC CGC ACC CAA GGA TAC A	TTC AAG GTA GCT CCG AGG TTC A	317	NM_031534
DAX-1	TCA CCT GCA CTT CGA GAT GAT G	TCA GCC GAT CTG ATC TGG TAC T	378	X99470
SOX9	ATG TCG GAG GAC TCG GCT GGT T	TCT TGC TGA GCT CCG CGT TGT G	337	XM_213519
GATA-1	GGA CTG CAC TGC CTA CAT CAC T	CTT GCG GTT CCT CGT CTG GAT T	479	NM_012764
GATA-4	CTG TGC CAA CTG CCA GAC TAC C	CAG GAC CAG GCT GTT CCA AGA G	484	NM_144730
GATA-6	TTG GAC TGT CCT GTG CCA ACT G	CTG TCG CAC CGA GGA TCT ACC T	434	NM_019185
GRTH	CAA CAT CCG GCA GTA TTA CGT G	TAT ACG GTG GAG GTA GGT CTC A	367	AF142629
SRB1	GGT GCT CAA GAA TGT CCG CAT A	CAT TAG CAG CTT CAG GCT TGT G	361	AB002151
AR	CCC AGG ATT TCC TGT GCA TGA AAG C	CCC CAA GGC ACT GCA GAG AAG TAG T	450	M23264
LHR	TTG GCA ACC TGA CAG TCC TCT T	AGA GCC ATC CTC CGA GCA TAA T	354	NM_012978
ABP/SHBG	CCT GCA ACC TGG ACT GTT CTT C	TTG GTC CTT GGC TCA AGG CTA C	305	NM_012650
ERα	CAG CAG CAG GTC ATA GAG AG	TCC TAA CTT GCT CTT GGA CAG G	409	NM_012689
ERß	AAA GCC AAG AGA AAC GGT GGG CAT	GCC ATT CAT GTG CAC CAG TCC CT	204	NM_012754
cFOS	AMBION COMMERCIAL KIT	AMBION COMMERCIAL KIT	266	V00727
IL-1α	CTA AGA ACT ACT TCA CAT CCG CAG C	CTG GAA TAA AAC CCA CTG AGG TAG G	623	NM_017019
IL-1ß	AMBION COMMERCIAL KIT	AMBION COMMERCIAL KIT	240	X04964
Aromatase	TGC CTG GCA AGC ACT CCT TAT C	AGC CGT CAA TCA CGT CAT CCT C	491	M33986

Table 2. PCR conditions summary

PCR conditions concerning annealing temperatures and cycles amplified for the various genes examined at both PND 28 and 120 amongst male F1 rats from RNA isolations from whole-decapsulated testis.

Gene	Annealing Temperature (°C)	PND 28	PND 120
sox9	55	25	25
sf-1	55	27	28
wt1	55	25	25
grth	55	23	23
abg/shbg	55	23	23
g3pdh	58	24	24
ar	58	27	27
il-1ß	59	----	40
star	60	26	26
pbr	60	23	23
lhr	60	24	25
il-1(alpha)	60	----	28
er(alpha)	60	27	27
sr-b1	62	26	26
cfos	63	31	30
gata-1	64	25	25
gata-4	64	25	25
gata-6	64	25	25
dax-1	64	24	24
aromatase	64	----	30
erß	64	37	37

Table 3. Isoflavones levels in diets

Isoflavones levels in diets determined by HPLC of ß-glucuronidase digested extracts. Diet 1 contained casein protein, while subsequent diets contained an alcohol-washed soy protein concentrate. Diets 3 to 6 contained increasing amounts of Novasoy added to supplement the diets with isoflavones. (N.D. not detectable) Data depicted as mg isoflavones/kg pelleted feed.

Diet	Protein	Total isoflavones	Genistein	Daidzein	Glycitein
diet 1	casein	ND	ND	ND	ND
diet 2	soy	31.7	18.6	10.5	2.6
diet 3	soy	36.1	21	12.3	2.8
diet 4	soy	74.5	39.3	27.6	7.6
diet 5	soy	235.6	124.4	90.9	20.5
diet 6	soy	1046.6	544.8	412.3	89.5

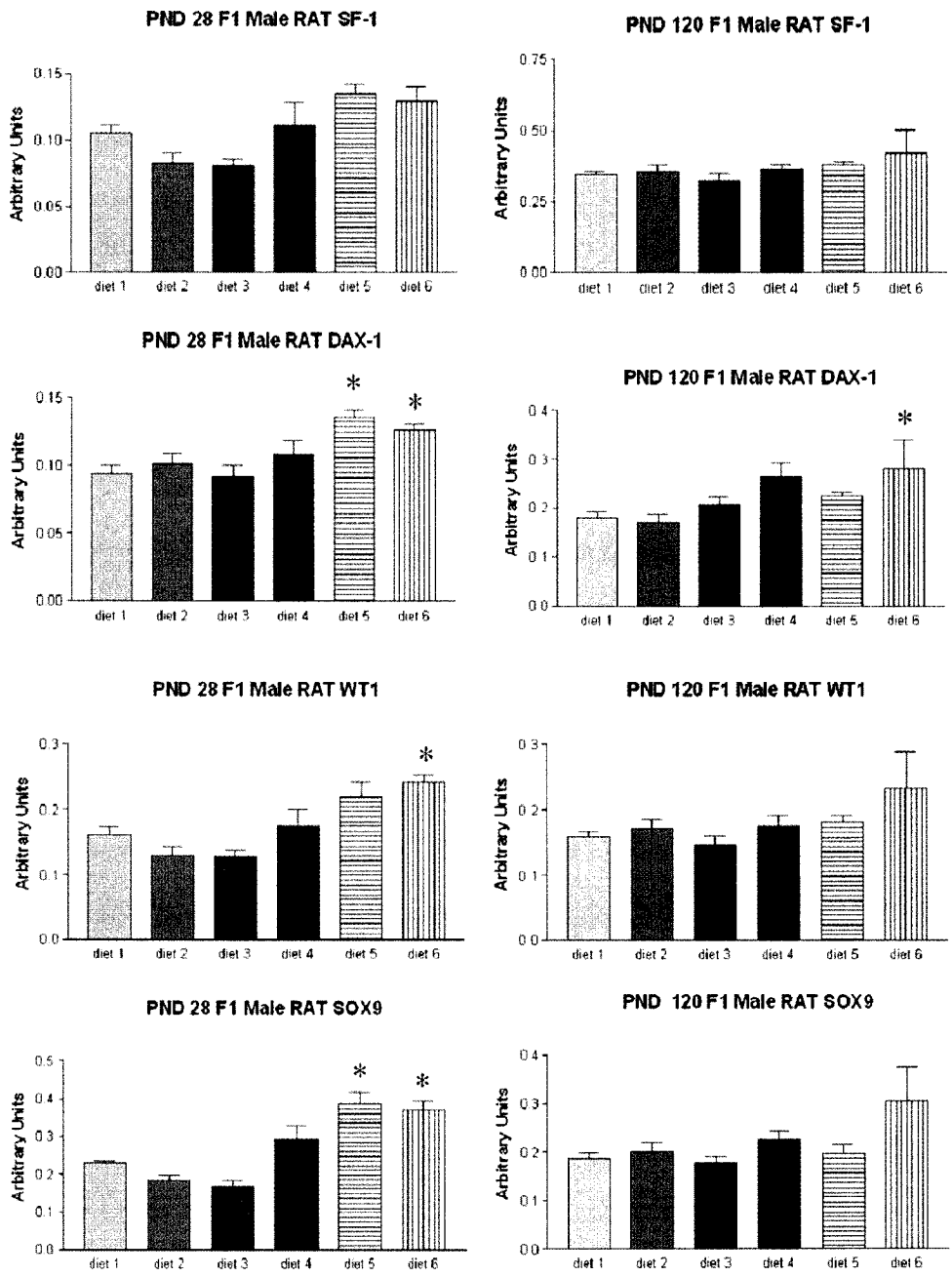
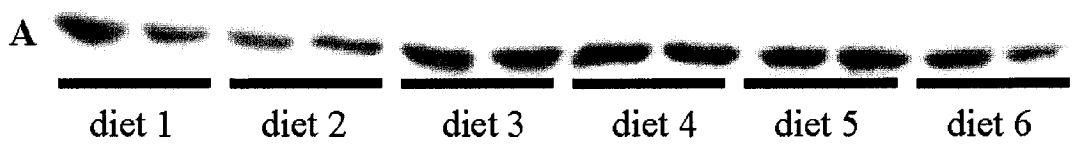


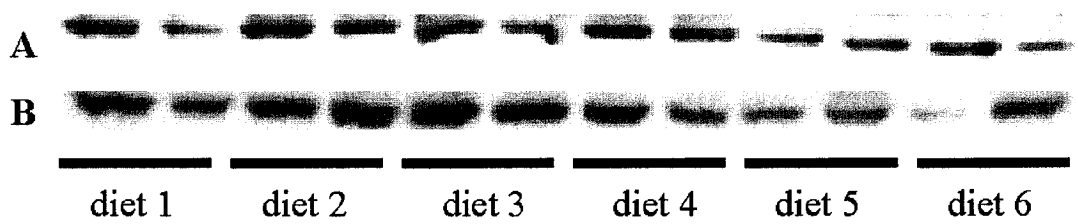
Figure 1 mRNA of developmental genes regulating testicular steroidogenesis

mRNA abundance (mean ± SE) at postnatal day (PND) 28 and 120 of SF-1, DAX-1, WT1 and SOX9 determined by RT-PCR. n = 6 for all diets examined with the exception of diet 3 at PND 28 and diets 2 and 3 at PND 120 which had n =5. The Y-axis represents arbitrary units from the ratio of densitometry of the mRNA in question divided by the densitometry of the constitutive control G3PDH. mRNA levels of diets marked (*) were statistically elevated compared with diet 1 ($P<0.05$).

I 3ß-HSD PND 28 (A)



II SF-1 PND 28 (A) and PND 120 (B)



III DAX-1 PND 28 (A) and PND 120 (B)

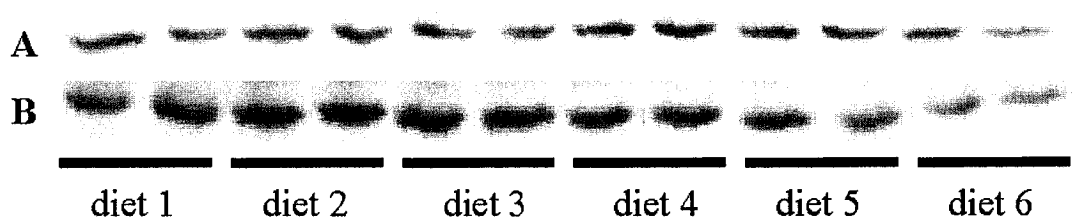


Figure 2 Western blot analysis of 3ß-HSD, SF-1 and DAX-1

Immunoreactive protein levels of I) A PND 28 3ß-HSD, II) A PND 28 SF-1, II) B PND 120 SF-1, III) A PND 28 DAX-1, III) B PND 120 DAX-1, examined by Western blot for 2 rats per diet level.

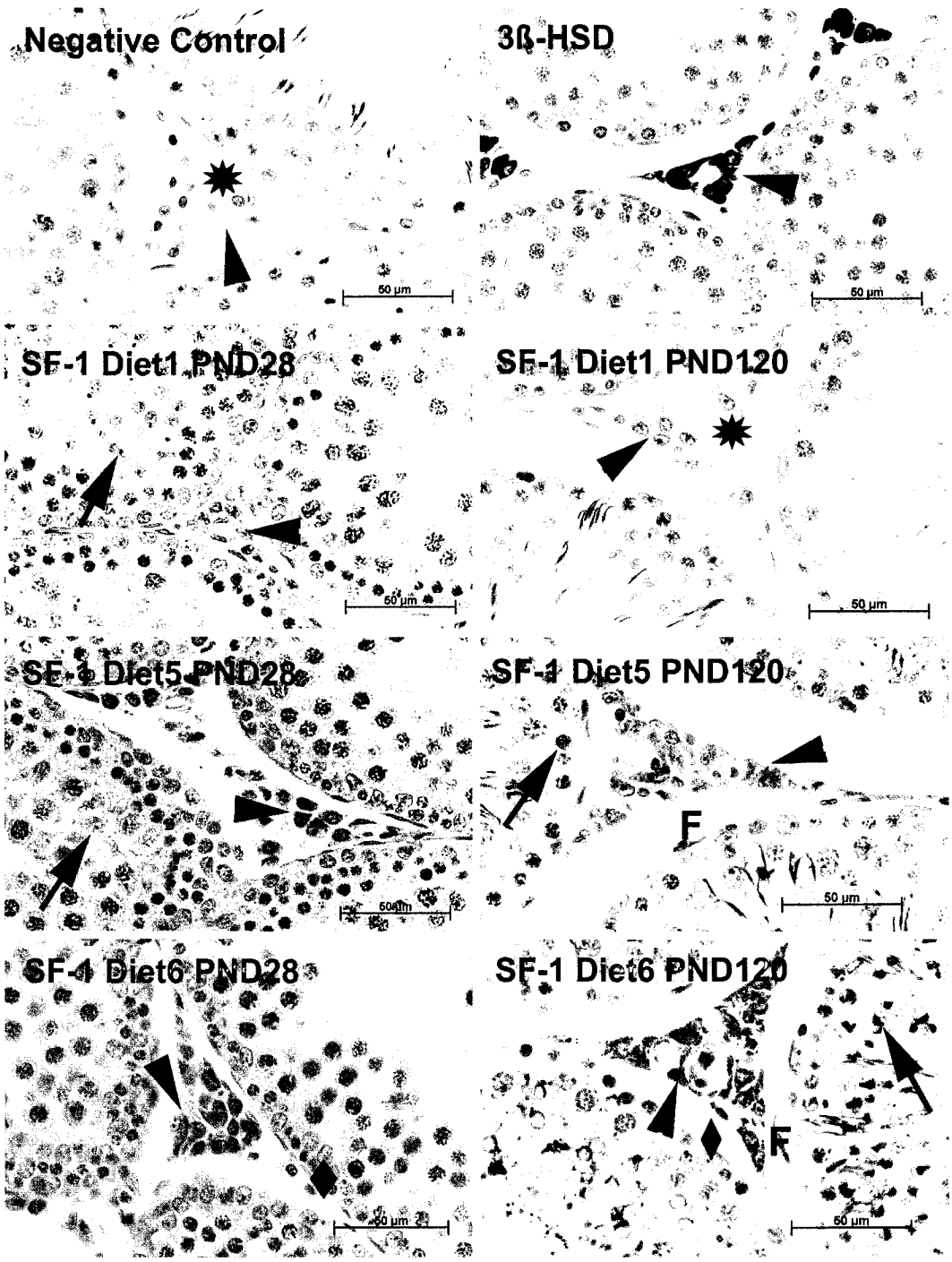


Figure 3 Immunohistochemistry of rat testis 3ß-HSD and SF-1

Immunohistochemical staining from rat testis tissue of a negative control for staining using normal swine serum in place of primary antibody at postnatal day (PND) 120, 3ß-HSD PND 28, and SF-1 Diet 1, 5 and 6; PND 28 and 120. Images were magnified 400x (n =4). Arrowheads depict Leydig cells, while tailed arrows depict developing spermatozoa, diamonds depict Sertoli cells, F depict fibroblasts and stars show endothelial cells.

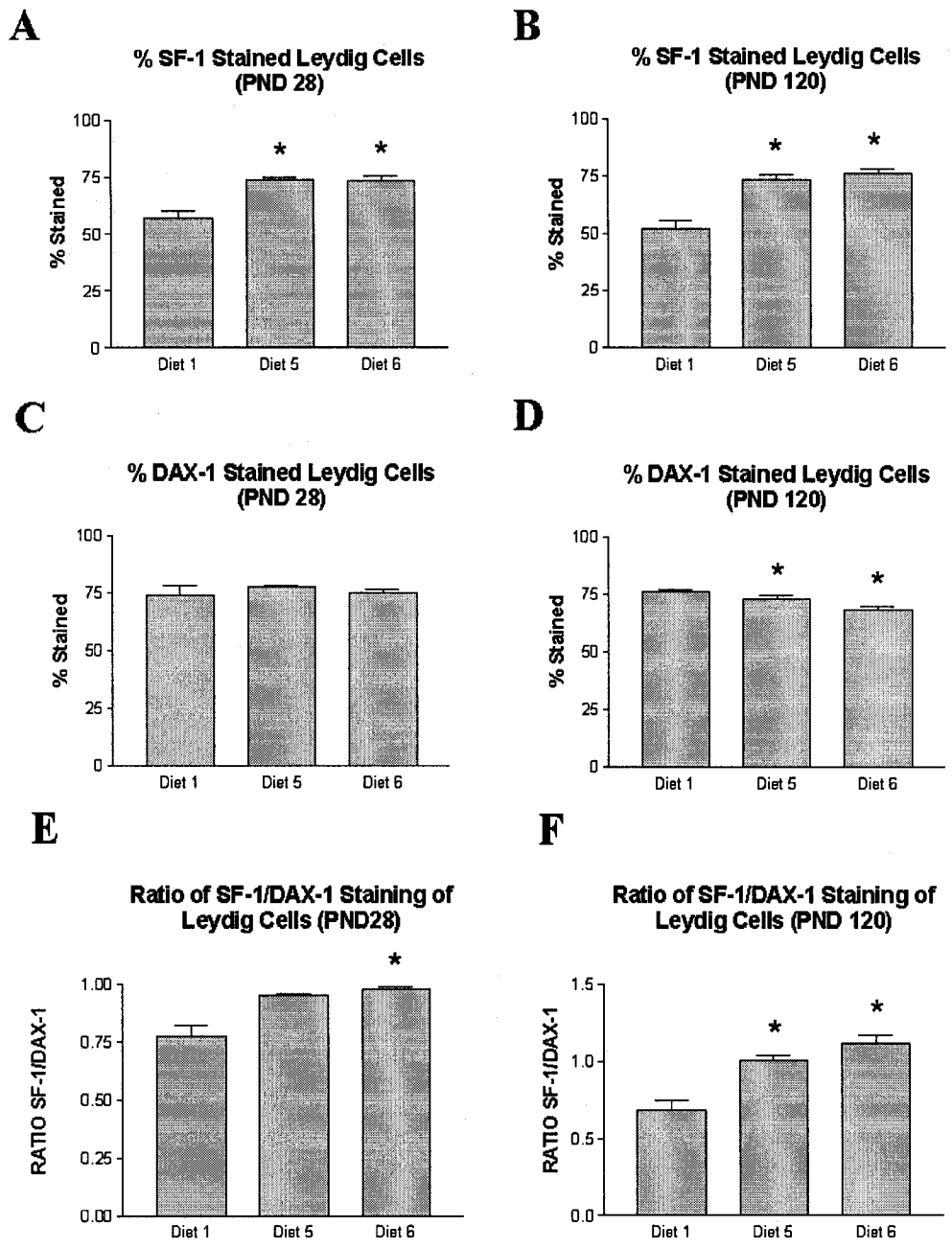


Figure 4 Percentage of Leydig cells stained for SF-1, DAX-1 and SF-1/DAX-1

Percentage of stained Leydig cells at (A) postnatal day (PND) 28 and (B) PND 120 for SF-1 protein, (C) PND 28 and (D) PND 120 for DAX-1 protein and (E) PND 28 and (F) PND 120 ratios of the number of SF-1 stained cells to DAX-1 stained cells (SF-1/DAX-1). Statistics were conducted using arcsine transformed proportionalized data. For A-C and E-F (*) indicates diet group in question was significantly higher than diet 1 or for D, that diets 5 or 6 were lower than diet 1 ($P<0.05$). Data were obtained by counting Leydig cells magnified 400X for 25 fields of view (approximately 1000 cells) for n = 4 testes per diet group.

Chapter 5

5.0 General Discussion

5.1 Summary of Results

Testicular production of androgens in Sprague-Dawley rats exposed to a mixture of soy isoflavones in conjugated and un-conjugated states during their entire lives is increased at PND 120.

The significant elevations in androgen levels seen at PND 120 in rats fed isoflavones at levels obtainable by human infants and supplement consumers (Diets 5 and 6) were further examined in terms of potential mechanisms of isoflavone action. It was shown that at PND 28 there are significantly higher enzymatic activities of 3ß-HSD and CYP17, while 5α-reductase activity and 17ß-HSD activities were not affected by isoflavones (see chapter 3 figures 1-4, pages 85-88). The changes in enzyme activity were not related to 3ß-HSD, CYP17 and P450-SCC mRNA or protein levels (see Chapter 3 Figures 5 and 6, pages 89-90). No significant changes in enzyme activity were seen at the older time points examined.

To elucidate the mechanisms by which isoflavones elevated PND 28 microsomal steroidogenic enzyme activities and PND 120 androgen levels, an examination of genes involved in the regulation of steroidogenesis was conducted. The developmental genes: SF-1, WT1, SOX9, DAX-1 as well as GATA factors (Parker et al., 1999; Parker and Schimmer, 2002; Tremblay and Viger, 2001) are important in early embryonic life for the formation of the reproductive tract and later in life play a role in the regulation of sex steroid levels by acting as transcriptional regulators of enzymes, receptors and transport proteins involved with steroidogenesis (Lopez et al., 1999; Gyles et al., 2001). Increased levels of WT1, SOX9 and DAX-1 mRNA were observed with increased isoflavone levels

in feed (see chapter 4 figure 1, page 125). These genes regulate transcription of other developmental factors such as MIS and SRY (Parker et al., 1999; Parker and Schimmer, 2002; Sriraman et al., 2001; Trovich et al., 2001), which in turn regulate male development and androgen levels by regulating SR-B1, aromatase, LHR, P450-SCC, STAR, CYP17 and 3ß-HSD (Parker et al., 1999; Parker and Schimmer, 2002). The changes in mRNA levels for DAX-1, SOX9 or WT1 observed, did not correspond to similar changes in Western blots. Although, SF-1 did not show significantly increased mRNA levels ($P>0.05$), IHC found an increased number of stained Leydig cells in rats fed isoflavones compared with control animals and the intensity of SF-1 protein staining per cell was seen to be elevated in diets 5-6 (see Chapter 4 Figures 1,3 and 4, page 125.127-128). Changes in SF-1 levels in Leydig cells, may be linked to the changes in steroidogenic enzyme activities seen at PND 28 and the increased levels of androgens found at PND 120 due to its role as a transcriptional regulator of these genes (Figure 1, page 10).

5.2 Mechanisms of Isoflavone Action

5.2.1 Direct Enzyme Effects

The effects and consequences of isoflavone exposure on various biological systems are variable. Conditions such as the route and magnitude of the dose, the composition of the dose, and the duration will affect the metabolism and bioavailability of the isoflavones. Phytoestrogens have been shown to decrease steroidogenic enzyme

activities that are important for androgen production (Evans et al., 1995; Weber et al., 1999; Wong and Keung, 1999; Krazeisen et al., 2001; Whitehead et al., 2002) and isoflavones are able to reduce serum testosterone production (Strauss et al., 1998; Roberts et al., 2000; Weber et al., 2001; Yi et al., 2002; Yhang et al., 2002). In contrast, increased steroidogenic enzyme activities due to exposure to isoflavones have also been reported in a short-term animal study in which 45-day-old male *SD* rats were fed diets containing 200 mg isoflavones/kg feed (50% genistin, 40% daidzin and 10% glycitin) for 29 days (Weber et al., 1999). Studies *in vitro*, where pure isoflavones are added directly to cell cultures, have typically shown inhibitory actions of isoflavones on steroidogenic enzymes (Keung, 1995; Wong and Keung, 1999; LeBail et al., 2000; Krazeisen et al., 2001; Ohno et al., 2002; Whitehead et al., 2002), while studies *in vivo*, have shown soy isoflavones increase certain steroidogenic activities (Weber et al., 1999; Laurenzana et al., 2002), as was seen in our study. Studies using adreno-cortical cells found decreased P450c21 enzymatic activity caused by isoflavones, which could not be explained by corresponding changes in expression, and it was proposed that inhibition of steroidogenic enzymes upstream of P450c21 reduced the amount of substrate available (Mesiano et al., 1999). Similarly in the current study, increased P450 SCC activity or substrate availability could lead to elevated levels of serum testosterone and DHT.

5.2.2 Indirect Effects on Steroidogenic Enzymes

The increased steroidogenic enzyme activities seen in PND 28 rats fed high doses of isoflavones (diets 5 and 6) may have arisen due to soy isoflavones modifying the local microenvironment surrounding the microsomal enzymes affected. Isoflavones have been shown to bind to and interact with select phospholipid structures altering membrane fluidity (Lehtonen et al., 1996; Arora et al., 2000). The phospholipid environment has been shown to at least partially regulate enzyme activity *in vitro* (Machino et al., 1969; Bogovich and Payne, 1980; Cooke and Robaire, 1988; Cooke, 1989).

5.2.3 Transcriptional Regulation of Genes Involved with Steroidogenesis

The regulation of SF-1 expression is not entirely understood. The rFtz-F1 gene, which encodes SF-1 is postulated as being regulated by mechanisms that are independent of hypothalamo-pituitary control (Nomura et al., 1998), but which involve an Ebox, CCAAT box, autoregulation and Sp-1 binding sites (Morohashi and Omura, 199). Recent work investigating SF-1 transcriptional regulation, has led to the discovery of differences in SF-1 regulation in Leydig and Sertoli cells that involve differences in Sp3 binding sites and currently unidentified cell specific proteins (Scherrer et al., 2002). In our study isoflavones increased the protein levels of SF-1 in a cell specific manner due to isoflavones interacting through SP-1 binding sites. Previous research by Majdic *et al.* (1997) led to the postulation that SF-1 expression was, indirectly, under the regulatory control of an estrogen receptor-dependent mechanism. The changes in SF-1 levels due to isoflavone consumption seeen in our study could have been mediated through changes in ER and AR expression or occupancy. Isoflavones have been shown to have estrogen-

dependent and estrogen-independent effects on biological systems. The ability of isoflavones to modulate the expression levels of androgen and estrogen receptors has been studied extensively showing mixed results in terms of up and down regulation depending on the isoflavones, method of dosing, species and tissues examined (Strauss et al., 1998; Kuiper et al., 1998; Patisaul et al., 2001; Dalu et al., 2002; Laurenza et al., 2002; Fritz et al., 2002; Lund et al., 2004; Adachi et al., 2004). Additionally isoflavones are able to bind with estrogen receptors and increase the transcriptional activity of ERα and, more preferentially, ERß (Kuiper et al., 1998). In the current study the changes seen in Leydig cell specific SF-1 protein levels appear to be not linked to the testicular mRNA expression levels of ERα, ERß or AR, as these were unaffected.

An additional factor in estrogen and androgen receptor function in target tissues such as the testis, is the level of circulating hormones. Isoflavones are able to bind ABP/SHBG, a chaperone for steroids in circulation, and also regulate its production (Martin et al., 1996; Jury et al., 2000). Interactions with ABP/SHBG could alter circulating endogenous sex steroid levels due to competition for the occupancy of these chaperones, and would thus impact the amount of free bioactive steroids. Future work is needed to clarify the involvement of ABP/SHBG in the regulation of steroidogenesis in the current study.

Increased SF-1 protein levels seen in rats fed diets 5 and 6 could be indicative of increased steroidogenic activity due to increased transcriptional activation of downstream steroidogenic target genes involved with the regulation of androgen production (Figure 1, page 10). The products of the SF-1, DAX-1, WT1 and SOX9 genes work in concert to regulate genes such as Mullerian inhibiting substance (MIS), which causes regression of

the Mullerian ducts during male sexual differentiation *in utero* and later, postnatally, regulates adult steroidogenesis through a paracrine mechanism involving Leydig and Sertoli cells (Sriraman et al., 2001).

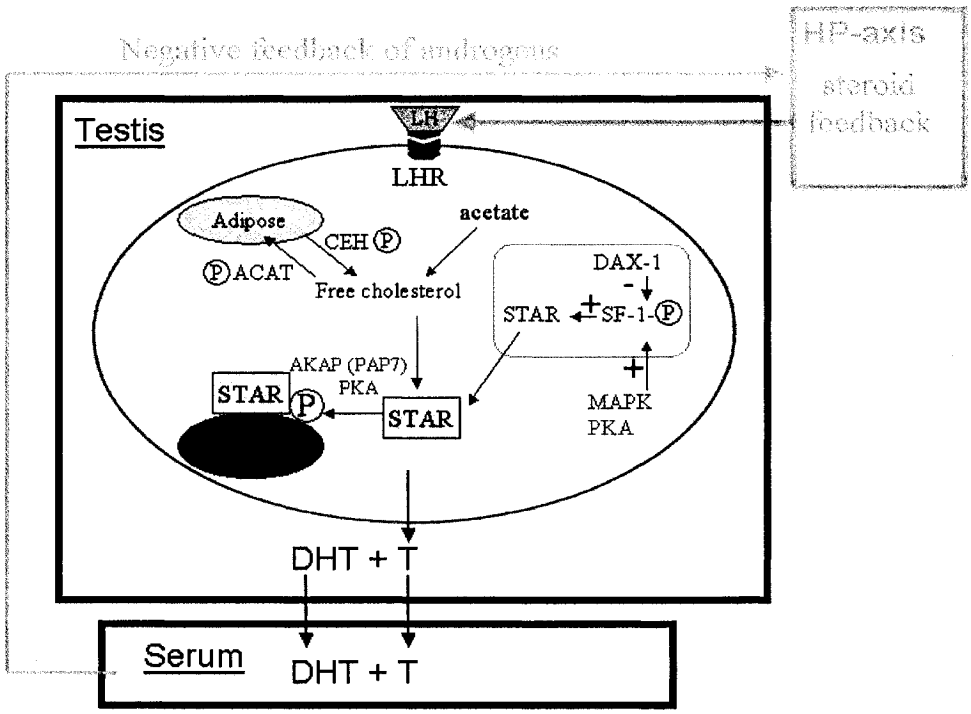


Figure 1 Possible mechanisms of isoflavone action on testicular steroidogenesis

Depiction of the steroidogenic cascade of the rat Leydig cell showing de novo synthesis of cholesterol from acetate, the movement of cholesterol from adipose reserves by acyl cholesterol acyl transferase (ACAT) and cholesterol ester hydrolase (CEH) which can both be phosphorylated. Additionally shown are Ledyig cell factors such as transcription factors SF-1 and DAX-1, which regulate expression of genes such as LHR and STAR. STAR is phosphorylated to carry out its role as a choesterol shuttling protein leading to cholesterol's cleavage to testosterone by the enzymes of the mitochondria and SER. Shown in orange is the endocrine feedback of sex steroids to the hypothalamus and pituitary, which can inhibit the release of LH and therefore LH stimulated androgen production at the testes.

The lack of upregulation of SF-1 target genes could have been due to a concomitant increase in transcriptional repression by DAX-1, which inhibits transcriptional activation by SF-1 (Figure 1). In the current study, mRNA levels of DAX-1 rose at high doses of isoflavones, though analysis of the protein levels via

Western blot did not show an increase in DAX-1 levels. However since the ratio of SF-1/DAX-1 staining of Leydig cells by immunohistochemistry rose with diets 5 and 6 compared with diet 1, it is possible that additional SF-1 transcriptional activation is suppressed by DAX-1 or other testicular repressors that DAX-1 is able to recruit (Altincicek et al., 2000).

Other genes, independent of SF-1-mediated transcription, but which are involved with steroid regulation (GRTH, PBR, aromatase, GATA-1. GATA-4, GATA-6, and the interleukins IL-1α and IL-1ß), were examined as potential mechanisms of isoflavone action. However, expression of these genes was unaffected by isoflavone consumption.

5.2.4 Phosphorylation State Modulation Due to Isoflavones.

Genistein has both direct and indirect influences on the phosphorylation state of proteins (Tarsounas et al., 1999; Darbon et al., 2000; Ye et al., 2001). In a recent study the expression of SF-1 was suppressed by LH in granulosa cells, whereas a MAPK inhibitor could block this effect and further elevate SF-1 levels (Tajima et al. 2003). Genistein's inhibitory action on tyrosine kinases may allow inhibition of MAPK signalling pathways thereby increasing LH levels (Tarsounas et al., 1999). The regulation of steroidogenic gene transcription are multifactorial, entailing developmental, tissue-specific, constitutive, and cAMP-dependent mechanisms (Morohashi and Omura, 1996; Kawabe et al., 1999; Lopez et al., 1999; Morohashi, 1999; Parker et al., 1999; Parker and Schimmer, 2002; Gyles et al., 2001; Lopez et al., 2001). Furthermore the transcription activation or enzymatic function can require the binding of coactivator proteins and post-translational modification (Sewer and Waterman, 2003). The function

of SF-1 is upregulated by the phosphorylation of serine 203 located at the hinge region of the protein, and in support of this possibility genistein has been shown to enhance the phosphorylation of the serine 15 residue on p53 protein (Figure 1, page 135; Babu et al., 2000; Darbon et al., 2000; Ye et al., 2001). There appear to be important upstream regulatory elements, such as Sp1 and Sp3 sites, involved in testicular SF-1 expression, which interact with unidentified proteins that are cell specific for Leydig and Sertoli cells (Scherrer et al., 2002). It is possible that these unidentified proteins are also in part regulated by phosphorylation and could be influenced by isoflavones.

The transcriptional regulation of STAR is at least partially controlled by SF-1, and was a strong candidate for the changes in androgen levels seen in the present study (Gyles et al., 2001), but curiously STAR mRNA levels were not altered by dietary isoflavones. Other studies have found altered androgen levels caused by isoflavones to be independent of changes in expression of of STAR or steroidogenic enzymes (Mesiano et al., 1999; Weber et al., 2001). Although no changes in STAR mRNA were seen in the present study, STAR activity could still have been modified in an isoflavone-dependent manner, by changes in phosphorylation status (Figure 1, page 135; Zwiller et al., 1991; Mesiano et al., 1999; Miller and Strauss, 1999; Shea-Eaton et al. 2002).

Isoflavones could impact steroidogenesis by modifying P450 enzyme activities by altering their phosphorylation states. It has been shown that CYP17 activity is increased by a cAMP dependent phosphorylation mechanism (Biason-Lauber et al., 2000a; Biason-Lauber et al., 2000b), while conversely CYP2B1 is inactivated by PKA mediated phosphorylation (Oesch-Bartlowmowicz and Oesch, 2003).

5.2.5 Testicular Cell Profile Changes Regulating Steroid Production

The increased testes masses and enzyme activities at PND 28 and the increased androgen levels seen at PND 120 could have been caused by altered cellular profiles. Increased numbers of Leydig cells were observed in marmoset monkeys exposed to isoflavones via infant formula, although, paradoxically, this was matched with lower serum testosterone levels (Sharpe et al., 2002). In the current study immunohistological examination of Leydig cells identified with 3ß-HSD antibody staining, revealed no increase in Leydig cell number with any dose of isoflavones (McVey et al., 2004c). The increased testis weights seen at PND 28, may have been due to an anti-estrogenic activity of soy isoflavones, as exposure to estrogen-antagonists during early life stages (PND 30-60) caused increased testis and accessory weights in male rats (Dhar et al., 1998). The importance of SF-1 in gonadal development was demonstrated in mice where the gene encoding SF-1 was selectively inactivated in the anterior pituitary causing testis weights to be approximately 5% of the weights of wild-type gonads (Zhao et al., 2001). Therefore, in our study, isoflavones may have upregulated pituitary SF-1 mRNA and protein levels leading to the increased gonadal weights observed at PND 28 (McVey et al., 2004a).

5.2.6 Endocrine Modulation

Other researchers have shown that isoflavones can modify endocrine regulation of androgen production, by altering the serum levels of LH (Strauss et al., 1998; Roberts et al., 2000; Lund et al., 2003). Altered endocrine signalling due to isoflavone consumption, could have led to changes in steroidogenic enzyme activities (McVey et al.,

2004b) and testicular and serum androgen levels (McVey et al., 2004a). Lund *et al.* (2003) found increased circulating LH but no significant changes in DHT levels in rats dosed with equol, and suggested that equol binds directly to DHT preventing it from providing negative feedback within the hypothalamo-pituitary-testicular (HPT) axis. It is possible that in the current study, soy isoflavones, including the metabolite equol, are acting anti-estrogenically in the hypothalamus and pituitary and are preventing potent endogenous steroids from exerting negative feedback through a competitive mechanism. Rats of 28 days of age, have relatively low circulating androgen (Resko et al., 1968; Knorr et al., 1970; Podesta and Rivarola, 1974) and estrogen (Saksena and Lau, 1979) levels and therefore, at this age the circulating isoflavones may act estrogenically at the hypothalamus and pituitary exerting negative feedback. It has been shown that genistein can act as an ER agonist alone and as an ER antagonist in the presence of estrogen (Ratna, 2002; Morito *et al.* 2002; Mueller *et al.* 2004). The involvement of estrogen receptors in HPT regulation has been conducted with αERKO and ßERKO (estrogen receptor knockout) female mice, which has revealed that ERα is crucial for estrogen-dependent negative feedback in the regulation of pituitary LH secretion (Couse et al. 2003). Since estradiol also provides negative feedback regulation of androgen production in males as well, a similar mechanism may explain the effects of isoflavones seen in our study (Steinberger et al. 1977; Grotjan and Steinberger 1978; Cohen et al. 1988) due to the low abundance of endogenous steroids in PND 28 male rats, isoflavones may act as estrogen agonists increasing estrogenic transcription (regulating SF-1 expression) or signalling within the HPT axis. Similarly at PND 120, there were elevated endogenous

androgen levels (McVey et al., 2004a), which may have been caused by circulating isoflavones suppressing negative feedback by endogenous steroids.

SF-1 knockout mice have shown that SF-1plays an important role in gonadotropin action as serum FSH and LH were markedly decreased in the pituitary-specific SF-1 knockout mice (Zhao et al., 2001). Limited quantities of available serum hindered our investigation of circulating LH or FSH levels and therefore we limited our investigation to testicular mRNA levels of Leydig cell LHR, but these were not altered by isoflavones. Elevated pituitary SF-1 levels may lead to elevated levels of LH, which would promote increased androgen biosynthesis in the current study.

5.3 Consequences of Exposure to Dietary Soy Isoflavones

5.3.1 Steroidogenesis

Isoflavone studies using different animal models have found significant reductions of serum testosterone production and enzyme activities (Evans et al., 1995; Strauss et al., 1998; Weber et al., 1999; Wong and Keung, 1999; Roberts et al., 2000; Weber et al., 2001; Krazeisen et al., 2001; Whitehead et al., 2002; Yi et al., 2002; Zhang et al., 2002b) and also increases in serum testosterone (Fritz et al., 2001; Dalu et al., 2002). The elevation of PND 120 testicular and circulating androgen levels seen in the current study are, in all probability, not significant enough to cause impaired reproductive function such as abnormal sperm production. Although, it is possible that there are subtle changes in androgen dependent gene transcription, spermatogenesis and libido in the rats fed high (diets 5-6) levels of soy isoflavones, which could affect individuals consuming levels of isoflavones similar to infants or adults ingesting soy isoflavone supplements.

5.3.2 Sexual Function

Exogenous estrogen exposure *in utero* has been suggested as a potential mechanism of increasing reproductive abnormalities in human males (Sharpe and Skakkebaek, 1993). Early androgen dependent events such as preputial separation and, later in life, spermatogenesis, libido and androgen target tissue function, could be impacted by isoflavone consumption. However, in our study, developmental reproductive endpoints some of which are androgen dependent (anogenital distance) and the number and development of offspring (crown rump lengths, body weights of male pups) were unaffected by soy isoflavones, indicative of normal sexual development (Appendix 2).

5.3.3 Reproductive Success

In the current study all rats were able to reproduce successfully and produce viable offspring (Appendix 2). As a result it would appear that there were no harmful, long term reproductive consequences for rats consuming soy isoflavones at the concentrations examined (McVey et al., 2004a, Appendix 2 and 3) such as altered litter sizes or developmental parameters. However, other researchers have observed lowered number of viable offspring, as a result of exposure to isoflavones ranging from average human consumption levels to 40 mg isoflavones / kg body weight / day (roughly 10-20 times high human consumption levels) in a number of species (Bennetts et al., 1946; Carter et al. 1955; Maltrone et al., 1955; Leopold et al. 1976; Shutt, 1976; reviewed in Price and Fenwich 1985; Cassanova et al., 1999; Nagao et al., 2001; Lewis et al., 2003).

5.3.4 Human Health

The data from the current investigation show that a mixture of soy isoflavones at levels that are reasonably attained by human consumers, was not harmful in terms of lowering testicular or circulating androgen levels, which could have led to impaired spermatogenesis and reproductive function (Bennetts et al., 1946; Shutt, 1976; Kumi-Daika et al., 1998; Cassanova et al., 1999; Nagao et al., 2001; Delclos et al., 2001; Lewis et al., 2003). Additionally isoflavones in this study did not elevate androgen levels to sufficiently high levels that may have led to physiological problems (Soler et al., 2000; Zhang et al., 2002; Watt et al, 2003). The approximate doubling of circulating androgen levels seen amongst rats consuming levels of isoflavones similar to human infants (diet 5) or adults mega-dosing with supplements (diet 6) could have subtle effects such as increased libido or transcriptional activation in androgen target tissues such as the skin, pituitary, epididymis etc. If the findings in the current study using rats were extrapolated to humans, the combination of soy isoflavones tested could prove to be a safe non-invasive mechanism for increasing endogenous androgen production. Increasing androgen levels could be therapeutically useful for patients with AIDS, hyogonadism, rheumatoid arthritis, cardiovascular conditions, chronic renal failure, chronic obstructive airway disease and for therapies for ageing and hormone replacement therapy (Wu and von Eckardstein, 2003). Human consumption of soy has been popular in Asian nations for countless generations, and they have not reported any significant adverse reproductive function due to isoflavone consumption (Clarkson et al., 1995; Adlercreutz and Mazur 1997; Humfrey, 1998; Setchell, 1984; Tikkanen and Aldercreutz, 2000; Lamartiniere, 2000), yet certain North American or European soy consumers may respond differently,

exhibiting different metabolism and/or bioavailability or even exhibit different genetic susceptibilities towards soy-mediated effects. The current study was well suited to studying this phenomenon, as we used rats to simulate naïve contact with soy isoflavones (P0), and their progeny which were the first generation to be soy consumers for their entire lives. Future examination of subsequent generations will show whether changes in gene expression occur as a result of consistent soy consumption.

5.4 Conclusion

Our initial finding of altered androgen levels at PND 120 led to the investigation of mechanisms involved in the regulation of steroidogenesis. Our research has shown changes in genes involved with *in utero* development and postnatal steroidogenesis (WT1, SOX9, DAX-1 and SF-1). Increased levels of Leydig cell SF-1 protein were not accompanied by corresponding increases in DAX-1 protein levels, which as a result, could allow increased SF-1 activity, as DAX-1 acts to suppress SF-1 mediated action (Figure 1). At present the exact mechanism of isoflavone action is not understood, as we have focussed our investigation to the mRNA levels of many of the steroidogenic target genes of SF-1, which were not altered, and other factors involved with SF-1 action such as phosphorylation state and the involvement of other regulatory proteins need to be examined (Figure 1).

5.5 Future Work

SF-1 is an intriguing target gene of soy isoflavone action and deserves further scrutiny, as its expression is found in a variety of endocrine and steroidogenic tissues in males such as the testis, adrenals, the pituitary and SF-1 levels in the ventromedial hypothalamic nucleus regulate the expression of LH and FSH (Zhao et al., 2001). Therefore, examination of the regulation of SF-1 Ledyig cell expression and protein function (phosphorylation) is needed to discern the means by which soy isoflavones are able to increase steroidogenesis (Figure 1, page 135).

The other generations of rats from the present multigenerational study are available to assess the effects of soy isoflavones and to compare the changes seen with the findings from the F1 generation. Genomic and proteomic analysis of the rats from the different generations could shed light on key genes such as SF-1, DAX-1, SOX9, WT1 which could be altered across the generations and may be potential biomarkers for physiological changes due to soy isoflavone exposure.

Since the expression of the genes that were changed in our study influence postnatal testicular functions such as steroidogenesis and sperm production and yet their functions prior to parturition are also critical to reproductive success; it would be interesting to examine the expression and activity of SF-1, DAX1, WT1 and SOX-9 in fetal rats exposed to the isoflavones and to examine when, where and how the changes were occurring in the various tissues expressing these factors.

The current examination of regulatory genes, which may have been influenced directly or indirectly by isoflavones, and which could have led to the changes in steroidogenesis or androgen levels, was extensive but not exhaustive. Other regulatory

systems remain which influence steroid levels such as: testicular macrophages which can regulate Leydig cell function via cytokine signalling (reviewed in Hales DB, 2002), nitric oxide regulation of steroidogenesis which can limit Leydig cell steroid production (Dobashi et al. 2001), metabolic factors such as leptin which stimulate steroidogenesis (reviewed in Tena-Sempere et al. 2002) as well as phosphorylation of proteins in the steroidogenic cascade required for androgen production.

The proposed future work should help elucidate the mechanism(s) of action of a mixture of dietary soy isoflavones at levels obtainable by human consumption on serum and testicular androgen levels amongst male rats.

References

Adachi T., Y., Ono, K-B., Koh, K., Takashima, H., Tainaka, Y., Matsuno, S., Nakagawa , E., Todaka, K., Sakurai, H., Fukata, T., Iguchi, M., Komiyama, C., Mori, 2004. Long-term alteration of gene expression without morphological change in testis after neonatal exposure to genistein in mice: toxicogenomic analysis using cDNA microarrays. Food and Chemistry Toxicoloyg 42, 445-452.

Adlercreutz, H., W., Mazur, 1997. Phytoestrogens and Western diseases. Ann Med. 29, 95-120.

Altincicek, B., S.P., Tenbaum, U., Dressel, D., Thormeyer, R., Renkawitz, A., Baniahmad, 2000. Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita, J Biol Chem. 275,7662-7667.

Arora, A., T.M. Byrem, M.G. Nair, G.M. Strasburg, 2000. Modulation of liposomal membrane fluidity by flavanoids and isoflavanoids, Archives in Biochemistry and Biophysics. 373, 102-109.

Atanassova, N., C., McKinnell, J.J., Turner, M., Walker, J.S., Fisher, M., Morley, M.R>, Millar, N.P., Groome, R.M., Sharpe, 2000. Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: evidence for stimulatory effects of low estrogen levels. Endocrinology 141, 3898-3907.

Axelson, M., J., Sjovall, B., Gustafsson, K., Setchell, 1984. Soya-A dietary source of the non-steroid oestrogen equol in humans and animals. J Endocrinol 102, 49-56.

Babu P.S., D.L., Bavers, S., Shah, G.D., Hammer, 2000. Role of phosphorylation, gene dosage and DAX-1 in SF-1 mediated steroidogenesis. Endocrine Research 26, 985-994.

Bennetts, H.W., E.J., Underwood, F.L., Shier, 1946. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Aust. J. Agric. Res. 22, 131-138.

[A]Biason-Lauber, A., M., Zachmann, E.J., Schoenle, 2000. Effect of leptin on CYP17 enzymatic activities in human adrenal cells: new insight in the onset of adrenaarche. Endocrinology. 141, 1446-1454.

[B]Biason-Lauber, A., B., Kempken, E., Werder, M.G., Forest, S., Einaudi, M.B., Ranke, N., Matsuo, V., Brunelli, E.J., Schonle, M., Zachmann, 2000. 17alpha-hydroxylase/ 17,20-lyase deficiency as a model to study enzymatic activity regulation: role of phosphorylation. Journal of Clinical Endocrinology and Metabolism. 85, 1226-1231.

Birge, S.J., 2000. Soy phytoestrogens: an adjunct to hormone replacement therapy? Menopause: the Journal of The North American Menopause society 4, 209-212.

Bogovich K., A.H., Payne, 1980. Purification of rat testicular microsomal 17-ketosteroid reductase: evidence that 17-ketosteroid reductase and 17ß-hydroxysteroid dehydrogenase are distinct enzymes, J. Biol. Chem. 260, 5552-5559.

Bogovich, K., A.H., Payne, 1980. Purification of rat testicular microsomal 17-ketosteroid reductase: evidence that 17-ketosteroid reductase and 17ß-hydroxysteroid dehydrogenase are distinct enzymes, J. Biol. Chem. 260, 5552-5559.

Byrne, G.C., Y.S., Perry, J.S., Winter, 1986. Steroid inhibitory effects upon human adrenal 3β-HSD activity. J clin Endocrinol Metab 62, 413-418.

Calogero, A.E., N., Burrello, A.M., Ossino, P., Polosa, R., D'Agata, 1998. Activin-A stimulates hypothalamic gonadotropin-releasing hormone release by the explanted male rat hypothalamus: interaction with inhibin and androgens. J Endocrinol. 156, 269-274.

Casagrande, F., J-M., Darbon, 2000. $p21^{CIP1}$ id Dispensible for the G2 Arrest Caused by Genistein in Human Melanoma Cells. Exp Cell Res. 258, 101-108.

Cassanova, M, L., You K.W., Gaido S., ArchibequeEngle D.B., Janszen H.D., Heck, 1999. Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. Toxicol. Sci. 51, 236-244.

Carter, M.W., G., Matrone, W.W.G., Smart, 1955. Effects of genistein on reproduction of the mouse. J. Nutr. 55, 639-645.

Cassidy, A., S., Bingham, K.D.R., Setchell, 1994. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am. J. Clin. Nutr. 60, 330-340.

Cassidy, A., M., Faughnan, 2000. Phytoestrogens through the life cycle. Proc. Nutr. Soc. 59, 489-496.

Clarkson, T.B., M.S., Anthony C.L.Jr, Hugher, 1995. Estrogenic soybean isoflavones and chronic disease. Risks and benefits. Trends Endocrinol. Metab.6, 11-16.

Clermont, I., B., Percy, 1957. Quantitative study of the cell population of the seminiferous tubules in immature rats. Am. J. Anat. 100, 241-267.

Clevenger, S., 1964. Flower Pigments. Sci American 210, 85-92.

Cohen, H., P., Guillaumot, I., Sabbagh, 1988. Dissociated estradiol (E2) action on the pituitary-testicular axis in a genetically hypoprolactinemic rat (IPL nude rat). J Steroid Biochem. 30, 407-410.

Cooke, P.S., Y-D., Zhao, L.G., Hansen, 1996. Neonatal polychlorinated biphenyl treatment increases adult testis size and sperm production in the rat. Toxicology and Applied Pharmacology. 136, 112-117.

Cooke, G.M., B., Robaire, 1988. Phospholipases modulate the rat testicular androgen biosynthetic pathway *in vitro*, Biol. Reprod. 39, 329-339.

Cooke, G.M., 1989. Identification and mechanism of action of phospholipids capable of modulating rat testicular microsomal 3ß-hydroxysteroid dehydrogenase-isomerase activity in vitro, Biol. Reprod. 41, 438-445.

Couse, J.F., M.M., Yates, V.R., Walker, K.S., Korach, 2003. Characterization of the hypothalamic-pituitary gonadal axis in estrogen receptor (ER) null mice reveals hypergonadism and endocrine sex reversal in females lacking ERα but not ERß. Molecular Endocrinol. 17, 1039-1053.

Coward, L., N.C., Barnes, D., Setchell, 1993. Genistein and daidzein, and their beta-glycoside conjugates: anti tumor isoflavones in soybean foods from American and Asian diets. J. Agr Food Chem 41, 1961-1967.

Dalu A., , B.S. Blaydes, C.W. Bryant, J.R. Latendress, C.C. Weis, K.D. Delclos, 2002. Estrogen receptor expression in the prostate of rats treated with dietary genistein, Journal of Chromatography B 777, 249-260.

Darbon J-M., M., Penary, N., Escales, F., Casagrande, F., Goubin-Gramatica, C., Baudouin, B., Ducommun, 2000. Distinct Chk2 activation pathways are triggered by genistein and DNA-damaging agents in human melanoma cells. J. Biol. Chem.275, 15363-15369.

Delclos, K.B., T.J., Bucci, L.G., Lomax, J.R., Latendresse, A., Warbritton, C.C., Weis, R.R., Newbold, 2001. Effects of dietary genistein exposure during development on male and female CD (Sprague-Dawley) rats. Reprod. Toxicol. 15, 647-663.

Dhar J.D., R., Mishra, B.S., Setty, 1998. Estrogen, androgen and antiestrogen responses in the accessory organs of male rats during different phases of life. Endocr. Res. 24, 159-169.

Dobashi, M., M., Fujisawa, T., Yamazaki, Y., Okuda, M., Kanzaki, N., Tatsumi, T., Tsuji H., Okada, S., Kamidono, 2001. Inhibition of steroidogenesis in Leydig cells by exogenous nitric oxide occurs independently of steroidogenic acute regulatory protein (STAR) mRNA. Archives of Andrology. 47, 203-209.

El-Gehani F, M., Tena-Sempere, H., Ruskoaho, I., Huhtaniemi. Natriuretic peptides stimulate steroidogenesis in the fetal rat testis. Biol Reprod. 2001; 65:595-600.

Evans, B.A., K. Griffiths, M.S. Morton, 1995. Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavanoids, J. Endocrinol. 147, 295-302.

Fritz, W.A., J. Wang, I.E. Eltoum, C.A. Lamartiniere, 2002. Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate, Mol. Cell. Endocrinol. 186, 89-99.

Gell, J.S., J., Oh, W.E., Rainey, B.R., Carr, 1998. Effect of estradiol on DHEAS production in the human adrenocortical cell line, H295R. J Soc Gynecol Invest. 5, 144-148.

Goldwyn, S., A., Lazinsky, H., Wei, 2000. Promotion of Health by Soy Isoflavones: Efficacy, Benefit and Safety Concerns. NY, NYC, Freund Publishing House Ltd.

Grotjan, H.E. Jr., E., Steinberger, 1978. On the relative biological and immunoreactive potencies of luteinizing hormone in the oestradiol benzoate-treated male rat. Acta Endocrinol (Copenh). 89, 538-550.

Gyles, S.L., C.J., Burns, B.J., Whitehouse, D., Sugden, P.J., Marsh, S.J., Persaud, P.M., Jones PM, 2001. ERKs regulate cyclic AMP-induced steroid synthesise through transcription of the steroidogenic acute regulatory protein (STAR) gene. The Journal of Biological Chemistry 276, 34888-34895.

Hales, D.B., 2002. Testicular macrophage modulation of Leydig cell steroidogenesis. Journal of Reproductive Immunology 57, 3-18.

Hall P.F., Testicular steroid synthesis: organization and regulation. In: Knobil E., Neill J.D., The physiology of Reproduction 2nd Edition. New York: Raven Press Ltd, 1994; 1335-1362.

Hauet, T., J., Liu, H., Li, M., Gazouli, M., Culty, V., Papadopoulos, 2002. PBR, StAR, and PKA: partners in cholesterol transport in steroidogenic cells. Endocr Res 28, 395-401.

Hawkins, C., C., Miaskowski, Testicular cancer: a review. Continuing Education 23, 1203-1213.

Hendrich, S., P.A., Murphy, 2001. Handbook of Nutraceuticals and Functional Foods. (Chapter 4 Isoflavones: A Source and Metabolism) CRC Press LLC.

Hodgert-Jury, H., T.R., Zacharewki, G.L., Hammond, 2000. Interactions between human plasma sex hormone-binding globulin and xenobiotic ligands. J. Steroid BCHM and M. Biol. 75, 167-176.

Hoeben E., J.V., Swinnen, W., Heyns, G., Verhoeven, 1999. Heregulins or Neu differentiation factors and the interactions between Peritubular Myoid cells and Sertoli cells. Endocrinology, 140, 2216-2223.

Huhtaniemi, I., 1995. Molecular aspects of the ontogeny of the pituitary-gonadal axis. Reprod Fertil Dev. 7, 1025-1035.

Humfrey, C.D.N., 1998. Phytoestrogens and human health effects: weighing up the current evidence. Natural Toxins 6, 51-59.
Irvine, C.H.G., M.G., Fitzpatrick, S.L., Alexander, 1998. Phytoestrogens in soy-based infant foods: concentrations, daily intake, and possible biological effects. Proc. Soc. Exp. Biol. Med 217, 247-253.

Jury H.H., T.R., Zacharewski, G.L., Hammond, 2000. Interactions between human plasma sex hormone-binding globulin and xenobiotic ligands. Journal of Steroid Biochemistry and Molecular Biology 75, 167-176.

Kawabe, K., T., Shikayama, H., Tsuboi, S., Oka, K., Oba, T., Yanase, H., Nawata, K.I., Morohashi, 1999. DAX-1 as one of the target genes of Ad4BP/SF-1. Molecular Endocrinology. 13, 1267-1284.

Keung, W.M., Dietary estrogenic isoflavones are potent inhibitors of ß-hydroxysteroid dehydrogenase of *P. testosteronii*, Biochem. Biophys. Res. Comm. 215, 1137-1144
Kirk, P., R.E., Patterson, J., Lampe, 1999. Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults. J. Am. Diet. Assoc. 99, 558-563.

Knorr, D.W., Vanha-Perttula, T., 1970. Lipsett, M.B., Structure and function of rat testis through pubescence. Endocrinology. 86, 1298-1304.

Konrad, L., M-A., Weber, S., Groos, M., Albrecht, G., Aumuller, 1998. Paracrine interaction in testiucular somatic cells. It. J. Anat. Embriol. 103, 139-152.

Krazeisen, A., R. Breitling, G. Moller, J. Adamski, 2001. Phytoestrogens inhibit human 17ß-hydroxysteroid dehydrogenase type 5, Molecular and Cellular Endocrinology 171, 151-162.

Kuiper G.G.J.M., J.G., Lemmen, B., Carlsson, J.C., Corton, S.H., Safe, P.T., van der Saag, B., van der Burg, J., Gustafsson, 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor ß. Endocrinology 139, 4252-4263.

Kula, K., R., Walczak-Jedrzejowska, J., Slowikowska-Hilczer, E., Oszukowska, 2001. Estradiol enhances the stimulatory effect of FSH on testicular maturation and contributes to precocious initiation of spermatogenesis. Molecular and Cellular Endocrinology 178, 89-97.

Kumi-Daika, J., R., Rodriguez, G., Goudaze, 1998. Influence of genistein (4',5,7-trihydroxy-isoflavone) on the growth and proliferation of testicular cell lines. Biol Cell 90, 349-354.

Kurzer, M.S., X., Xu, 1997. Dietary Phytoestrogens. Annu Revs Nutr. 17, 353-381.

Lamartiniere, C.A., 2000. Protection against breast cancer with genistein: a component of soy. Am J Clin Nutr. 71, 1705S-7S

Laurenzana, E.M., C.C., Weis, C.W., Bryant, R., Newbold, K.B., Delclos, 2002. Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P450 enzymes, and estrogen receptor alpha expression. Food Chem. Toxicol. 40, 53-63.

Le Bail, J-C., Y., Champavier, A-J., Chulia, G., Habrioux, 2000. Effects of phytoestrogens on aromatase, 3ß and 17ß-hydroxysteroid dehydrogenase activities and human breast cancer cells, Life Sciences 66, 1281-1291.

Lehtonen, J.Y.A., H. Adlercreutz, P.K.J. Kinnunen, 1996. Binding of daidzein to liposomes, Biochimica et Biophysica Acta 1285, 91-100.

Leopold, A.S., M., Erwin, J., Oh, B., Browning, 1976. Phytoestrogens: adverse effects on reproduction in California quail. Science 191, 98-100.

Lephart, E.D., J.M., Thompson, K., Setchell, H., Adlercrcutz, K.S., Weber, 2000. Phytoestrogens decrease brain calcium-binding proteins but do not alter hypothalamic androgen metabolizing enzymes in adult male rats. Brain Res 859, 123-131.

Leung, P.S., T.P., Wong, Y.W., Chung, H.C., Chan, 2002. Androgen dependent expression of AT1 receptor and its regulation of anion secretion in rat epididymis. Cell Biol. Int. 26, 117-122.

Lewis, R.W., N., Brooks, G.M., Milburn, A., Soames, S., Stone, M., Hall, J., Ashby, 2003. The effects of the phytoestrogen genistein on the postnatal development of the rat. Toxicol. Sci. 71, 74-83.

Li CH, Evans HM. Chemistry of anterior pituitary hormones. In: Pincus G., Thimann K., Eds The hormones. Vol. 1 New York: Academic Press, 1948; 631.

Lopez, D, T.W., Sandhoff, M.P., McLean, 1999. Steroidogenic factor-1 mediates 3'5'-cyclic adenosine monophosphate regulation of the high-density lipoprotein receptor. Endocrinology 140, 3034-3044.

Lopez, D., W., Shea-Eaton, M.D., Sanchez, M.P., McLean, 2001. DAX-1 represses the high-density lipoprotein receptor through interaction with positive regulators sterol regulatory element-binding protein-1a and steroidogenic factor-1. Endocrinology 142, 5097-5106.

Lund T.D. D.J., Munson, H., Adlercreutz, R., Handa, E.D., Lephart, 2004. Androgen receptor expression in the rat prostate is down-regulated by dietary phytoestrogens. Reproductive Biology and Endocrinology 2, 5.

Lund, T.D., D.J., Munson, M.E., Haldy, K.D.R., Setchell, E.D., Lephart, R.J., Handa, 2003. Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback, Biol. Reprod. Papers in Press. (BOR).

Machino, A., H., Nakano, B.I., Tamaoki, 1969. Influence of physical and chemical treatments upon the microsomal enzymes of the testes related to androgen biosynthesis, Endocrinol. Jpn. 16, 37-46.

Majdic G., R.M., Sharpe, P.T.K., Saunders, 1997. Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor-1 (SF-1/Ad4BP) in the fetal rat testis. Molecular and Cellular Endocrinology 127, 91-98.

Makela, S., M., Poutanen, M.L., Kostian, N., Lethimaki, L., Strauss, R., Santti, R., Vihko, 1998. Inhibition of 17beta-Hydroxysteroid Oxidoreductase by Flavanoids in Breast and Prostate Cancer Cells. PSEBM 217, 310-316.

Martin M.F., M., Haourigui, C., Pelissero, F.A., Benassayag, F.A., Nunez, 1996. Interactions between phytoestrogens and human sex steroid binding protein. Life Sci. 58, 429-436.

Mason, H., S., Franks, 1997. Local control of ovarian steroidogenesis. Baillieres Clin Obstet Gynaecol. 11, 261-279.

Matrone, G., W.W.G., Smart, M.W., Cater, V.W., Smart, 1955. Effect of genistein on growth and development of the male mouse. J. Nutr. 59, 235-241.

Merritt R.J., B.H., Jenks, 2004. Safety of soy-based infant formulas containing isoflavones: the clinical evidence. J Nutr. 134, 1220S-1224S.

McLachlan, R.I., L., O'Donnell, S.J., Meachem, P.G., Stanton, D.M., Krester, K., Pratis, D.M., Robertson, 2002. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys and man. Recent Prog Horm Res. 57, 149-179.

[A]McVey, M.J., G.M., Cooke, I.H.A., Curran, 2004. Increased serum and testicular androgen levels in F1 rats with lifetime exposure to soy isoflavones. Reproductive Toxicology , in press.

[B]McVey, M.J., I.H.A., Curran, G.M., Cooke, 2004. Altered testicular steroidogenic enzyme activities in rats with lifetime exposure to soy isoflavones. Journal of Steroid Biochemistry and Molecular Biology, submitted.

[C]McVey, M.J., I.H.A., Curran, G.M., Cooke, 2004. Altered developmental gene expression in testes of rats with lifetime exposure to soy isoflavones. Biology of Reproduction, submitted.

Mesiano, S., S.L., Katz, J.Y., Lee, R.B., Jaffe,1999. Phytoestrogens Alter Adrenocortical Function: Genistein and Daidzein Suppress Glucocorticoid and Stimulate Androgen Production by Cultured Adrenal Cortical Cells. J Clin Endocrin Metab 84, 2443-2448.

Messina, M., E.T., Gugger, D.L., Alekel, 2001. Handbook of Neutraceuticals and Functional Foods. (Chapter 5: Soy Protein, Soybean Isoflavones, and Bone Health: A Review of The Animal and Human Data.) CRC Press LLC.

Miller W.L., J.F., Strauss, 1999. Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein STAR. Journal of Steroid Biochemistry and Molecular Biology 69, 131-141.

Miyachi, Y., E., Nieschlag, M.B., Lipsett 1973. The secretion of gonadotropin and testosterone by the neonatal male rat. Endocrinology 92, 1-5.

Morito, K., T., Aomori, T., Hirose, J., Kinjo, J., Hasegawa, S., Ogawa, S., Inoue, M., Muramatsu, Y., Masamune, 2002. Interaction of phytoestrogens with estrogen receptors alpha and beta (II). Biol. Pharm. Bull. 25, 48-52.

Morohashi, K.I., T., Omura, 1996. Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J. 10, 1569-1577.

Morohashi, K.I., 1999. Gondal and extragonadal functions of Ad4BP/SF-1: Developmental aspects. TEM. 10,169-173.

Mueller, S.O., S., Simon, K., Chae, M., Metzler, K.S., Korach, 2004. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor (ER) alpha and ER beta in human cells. Tox. Sci. in press.

Murphy, P.A., 1982. Phytoestrogen content of processed soybean products. Food Technol 34, 60-64.

Nagao, T., S., Yoshimura, Y., Saito, M., Nakagomi, K., Usumi, H., Ono, 2001. Reproductive effects in male and female rats of neonatal exposure to genistein. Reprod. Toxicol.15, 399-411.

Naim, M., B., Gestetner, S., Zikah, Y., Birk, A., Bondi, 1974. Soybean Isoflavones. Characterization; Determination, and Antifungal Activity. J Agr Food Chem 22, 806-810.

Nalbant D., S.C., Williams, D.M., Stocco, S.A., Khan, 1998. Luteinizing hormone-dependent gene regulation in Leydig cells may be mediated by CCAAT/enhancer-binding protein-beta. Endocrinology. 139, 272-9.

Nomura M., K., Kawabe, S., Matsushita, S., Oka, O., Hatano, N., Harada, H., Nawata, K.I., Morohashi , 1998. Adrenocortical and gonadal expression of the mammalian Ftz-F1 gene encoding Ad4BP/SF-1 is independent of pituitary control. J Biochem 124, 217-224.

Odum, J., H., Tinwell, K., Jones, J.P, Van Miller, R.L., Joiner, G., Tobin, H., Kawasaki, R., Deghenghi, J., Ashby, 2001. Effect of rodent diets on the sexual development of the rat. Toxicology Sciences 61, 115-127.

Oesch-Bartlomowicz, B., F., Oesch, 2003. Cytochrome-P450 phosphorylation as a functional switch. Archives of Biochemistry and Biophysics. 409, 228-234.

Ohno S., S., Shinoda, S., Toyoshima., H., Nakazawa, T., Makino, S., Nakajin, 2002. Effect of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells, Journal of Steroid Biochemistry and Molecular Biology 80, 355-363.

Ojeda, S.R., Urbanski, H.F., 1998. The Physiology of Reproduction: Vol 1, NYC, NY, Raven Press. 1699-1762.

Paillart, C., E., Carlier, D., Guedin, B., Dargent, F., Couraud, 1996. Direct Block of Voltage-Sensitive Sodium Channels by Genistein, A Tyrosine Kinase Inhibitor. J Pharm Exp Therap 280, 521-526

Parker, K.L., A., Schedl, B.P., Schimmer, 1999. Gene interactions in gonadal development. Annu Rev. Physiol 61, 417-33.

Parker, K.L., B.P., Schimmer, 2002. Genes essential for early events in gonadal development. Ann Med 34, 171-178.

Patisaul H.B., M., Dindo, P.L., Whitten, I.J., Young , 2001. Soy isoflavones supplements antagonize reproductive behavior and estrogen receptor α- and ß-dependent gene expression in the brain. Endocrinology. 142, 2946-2952.

Payne, A., G., Youngblood, 1995. Regulation of expression of steroidogenic enzymes in Leydig cells, Biol Rep. 52, 217-225.

Pino, A.M., L.E., Valladares, M.A., Palma, M., Mancilla, M., Yanez, C., Albala, 2000. Dietary Isoflavones Affect Sex Hormone-Binding Globulin Levels in Postmenopausal Women. J Clin Endocrin Metab 85, 2797-2800.

Podesta, E.J., Rivarola, M.A., 1974. Concentration of androgens in whole testis, seminiferous tubules and interstitial tissue of rats at different stages of development. Endocrinology 95, 455-461.

Price, K.R., G.R., Fenwick, 1985. Naturally occurring oestrogens in foods-a review. Food Additives and Contaminants 2, 73-106.

Randall,S.F., L.K., Jiyuan, W., Singletary, 2001. Effect of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: involvement of CDC2, p21,p27 and CDC25C expression. BCHM Pharm 61, 979-989.
Resko, J.A., Feder, H.H., Goy, R.W., 1968. Androgen concentrations in plasma and testis of developing rats. J. Endocrinology 40, 485-491.

Rivas, A., J.S., Fisher, C., McKinnell, N., Atanassova, R.M., Sharpe, 2002. Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. Endocrinology 143, 4797-4808.

Roberts, D., D.N.R. Veeramachaneni, W.D. Schlaff, C.A. Awoniyi, 2000. Effects of chronic dietary exposure to genistein, a phytoestrogen, during various stages of development on reproductive hormones and spermatogenesis in rats, Endocrine 13, 281-286.

Saksena S.K., I.F., Lau, 1979. Variations in serum androgens, estrogens, progestins, gonadotropins and prolactin levels in male rats from prepubertal to advanced age. Exp. Aging Res. 5, 179-194.

Scherrer S.P., R.A., Rice, L.L., Heckert, 2002. Expression of steroidogenic factor 1 in the testis requires an interactive array of elements within its proximal promoter. Biology of Reproduction 67, 1509-1521.

Setchell, B.P., Brooks, D.E., 1988. The Physiology of Reproduction: Vol 1, NYC, NY, Raven Press. 759-780.

Setchell, K., S.P., Borriello, P., Hulme, D.N., Kirk, M., Axelson, 1984. Non-steroidal oestrogens of dietary origin: possible roles in hormone-dependent disease. Am J Clin Nutr 40, 569-578.

Setchell, K, M.B., Nadine, P., Desai, L., Zimmer-Nechemias, B.E., Wolfe, W.T., Brashear, A.S., Kirschner, A., Cassidy, J.E., Heubi, 2001. Bioavailability of Pure Isoflavones in Healthy Humans and Analysis of Commercial Soy Isoflavone Supplements. J Nutr 131, 1362S-1375S.

Sfakianos, J., L., Coward, M., Kirk, S., Barnes, 1997. Intestinal Uptake and Biliary Excretion of the Isoflavone Genistein in Rats. J. Nutr. 127, 1260-1268.

Sharpe, R.M., B., Martin, K., Morris, I., Greig, C., McKinnell, A.S., McNeilly, M., Walker, 2002. Infant feeding with soy formula milk: effect on the testis and on blood testosterone levels in marmoset monkeys during the period of neonatal testicular activity, Human Reprod. 17, 1692-1703.

Sharpe, R.M., N.E., Skakkebaek, 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341, 1392-1395.

Shea-Eaton W., T.W., Sandhoff, D., Lopez, D.B., Hales, M.P., McLean, 2002. Transcriptional repression of the rat steroidogenic acute regulatory (STAR) protein gene by the AP-1 family member c-FOS. Molecular and Cellular Endocrinology. 188,161-170.

Shutt, D.A., 1976. The effects of plant oestrogens of dietary origin. Endeavour 35, 110-113.

Skinner, M.K., 1991. Cell-cell interactions in the testis. Endocrinological review 12, 45-47.

Skinner, M.K., J.N., Norton, B.P., Mullaney, M., Rosselli, P.D., Whaley, C.T., Anthony, 1991. Cell-cell interactions and the regulation of testis function. Ann NY Acad. Sci. 637, 354-363.

Sprando, R.L., B.R., Zirkin, 1997. Is Leydig cell steroidogenic function affected by the germ cell content of the seminiferous tubules? Journal of Andrology 18, 424-430.

Sriraman V., E., Niu, J.R., Matias, P.K., Donahoe, D.T., MacLaughlin, M.P., Hardy, M.M., Lee, 2001. Mullerian inhibiting substance inhibits testosterone synthesis in adult rats. Journal of Andrology 22, 750-758.

Steinberger, E., M., Chowdhury, R., Tcholakian, 1977. Effect of estradiol benzoate on the pituitary-gonadal axis in the intact male rat. Andrologia. 9, 307-312.

Strauss, L., S., Makela, S., Joshi, I., Huhtaniemi, R., Santti, 1998. Genistein exerts estrogen-like effects in male mouse reproductive tract. Mol.Cell. Endocrinol. 144, 83-93.

Strom, S.S., Y., Yamamura, C.M., Duphorne, M.R., Spitz, R.J., Babaian, P.C., Pillow, S.D., Hursting, 1999. Phytoestrogen intake and prostate cancer: a case-control study using a new database. Nutr. Cancer 33, 20-25.

Suzuki, M., M., Nishiahara, 2002. Granulin Precursor gene: a sex-steroid-inducible gene involved in sexual differentiation of the rat brain. Mol. Genet. Metab. 75, 31-37.

Tajima, K., A., Dantes, Z., Yao, K., Sorokina, F., Kotsuji, R., Seger, A., Amsterdam, 2003. Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. J Clin Endocrinol Metab. 88, 2288-2299.

Tansey, G., C., Hughes, M., Cline, A., Krummer, D.K., Walmer, S., Schmoltzer, Effects of Dietary Soybean Estrogens on the Reproductive Tract in Female Rats. PSEBM 217, 340-344.

Tarsounas M., R.E., Pearlman, P.B., Moens, 1999. Meiotic activation of rat pachytene spermatocytes with okadaic acid: the behaviour of synaptonemal complex components SYN1/SCP1 and COR1/SCP3, J. Cell Sci. 112, 423-434.

Tena-empere, M., M.L., Barreiro, 2002. At the cutting edge Leptin in male reproduction: the testis paradigm. Molecular and Cellular Endocrinol. 188, 9-13.

Tikkanen, M.J., H., Adlercreutz, 2000. Dietary soy-derived isoflavone phytoestrogens: could they have a role in coronary heart disease prevention? Biochemcial Pharmacology 60, 1-5.

Trbovich A.M., P.M., Sluss, V.M., Laurich, F.H., O'Neill, D.T., MacLaughlin, P.K., Donahoe, J., Teixeira, 2001. Mullerian inhibiting substance lowers testosterone in Luteinizing hormone-stimulated rodents. PNAS 98, 3393-3397.

Tremblay J.J., R.S., Viger, 2001. GATA factors differentially activate multiple gonadal promoters through convserved GATA regulatory elements. Endocrinology 142, 977-986.

Tremblay, J.J., R.S., Viger, 2001. GATA factors differentially activate multiple gonadal promoters through convserved GATA regulatory elements. Endocrinology 140, 977-986.

Wade, M.G., A., Lee, A., McMahon, G., Cooke, I., Curran, 2003. The influence of dietary isoflavone on the uterotrophic response in juvenile rats. Food Chem. Toxicol. 41, 1517-1525.

Wakai, K., I., Egami, K., Kato, T., Kawamua, A., Tamakoshi, Y., Lin, T., Nakayama, M., Wada, T., Ohno, 1999. Dietary intake and sources of isoflavones among Japanese. Nutr Cancer 33, 139-145.

Walter, E.D., 1941. Genistin (an isoflavone glucoside) and its aglucone, genistein, from soybeans. J Am Oil Chem Soc 63, 3273-3276.

Walz, E. 1931. Isoflavone und saponin-glicoside in Soja-Hispida. Justus Liebigs Ann Chem. 489, 118-155.

Wang, J., I.E., Eltoum, C.A., Lamartiniere, 2004. Genistein alters growth factor signalling in transgenic prostate model (TRAMP). Mol. Cell. Endocrinol. 219:171-180.

Wangen, K.E., A.M., Duncan, B.E., Merz-Demlow, X., Xu, R., Marcus, W.R., Phipps, M.S., Kurzer, 2000. Effects of Soy Isoflavones on Markers of Bone Turnover in Premenopausal and Postmenopausal Women. J Clin Endocrinol Metab 85, 3043-3048.

Watt, M.J., G.L., Forster, J.M., Joss 2003. Steroid correlates of territorial behavior in male jacky dragons, Amphibolurus muricatus. Brain Behav Evol. 61, 184-94.

Weber, M.A., S., Groos, G., Aumuller, L., Konrad, 2002. Post-natal development of the rat testis: steroid hormone receptor distribution and extracellular matrix deposition. Andrologia 34, 41-45.

Weber, K.S., K.D.R., Setchell, D.M., Stocco, E.D., Lephart, 2001. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5 alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J. Endocrinol. 170, 591-599.

Weber, K.S., N.A. Jacobson, K.D.R. Setchell, E.D. Lephart, 1999. Brain aromatase and 5alpha-reductase, regulatory behaviors and testosterone levels in adult rats on phytoestrogen diets, Proc Soc Exp Biol Med. 221, 131-135.

Wei, H., R., Bowen, Q., Cai, S., Barnes, Y., Wang, 1995. Antioxidant and antipromotional effects of the soybean isoflavone genistein. P.S.E.B.M. 208, 124-130.

Whitten, P.L., C., Lewis, E., Russell, F., Naftolin, 1995. Potential Adverse Effects of Phytoestrogens. J Nutr 125, 771s-776s.

Whitehead, S.A., J.E. Cross, C. Burden, M. Lacey, 2002. Acute and chronic effects of genistein, tyrphostin and lavendustin A on steroid synthesis in luteinized human granulosa cells, Human Reproduction. 17, 589-594.

Wisniewski, A.B., S.L., Klein, Y., Lakshmanan, J.P., Gearhart, 2003. Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. The Journal of Urology 169, 1582-1586.

Wong, C.K., W.M. Keung, 1999. Bovine adrenal 3ß-hydroxysteroid dehydrogenase (E.C. 1.1.1.145)/5-ene-4-ene isomerase (E.C. 5.3.3.1): characterization and its inhibition by isoflavones, J. Steroid Biochemistry. 71, 191-202.

Wu, F.C., A., von Eckardstein, 2003. Androgens and coronary artery disease. Endocr Rev. 24, 183-217.

Yasuda, T., Y., Kano, K.I., Saito, K., Ohsawa, 1994. Urinary and biliary metabolites of daidzin an daidzein. Biol Pharm. 17, 1369-1374.

Ye R., A., Bodero, B-B., Zhou, K.K., Khanna, M.F., Lavin, S.P., Lees-Miller, 2001. The plant isoflavanoid genistein activates p53 and Chk2 in an ATM-dependent manner. The Journal of Biological Chemistry 276, 4828-4833.

Yi, M.A., H.M., Son, J-S., Lee, C-S., Kwon, J.K., Lim, Y.K., Yeo, Y.S., Park, J-S., Kim, 2002. Regulation of male sex hormone levels by soy isoflavones in rats. Nutrition and Cancer 42, 206-210.

Zhang F.P., T., Pakarainen, F., Zhu, M., Poutanen, I., Huhtaniemi. Molecular characterization of postnatal development of testicular steroidogenesis in luteinizing hormone receptor knockout mice. Endocrinology 2004; 145:1453-1463.

[A]Zhang, L., I.A., Khan, C.M., Foran, 2002. Characterization of the estrogenic response to genistein in Japanese medaka (*Oryzias latipes*). Comparative Biochemistry and Physiology. 132, 203-211.

[B]Zhang, Y., D.P., Kiel, R.C., Ellison, A., Schatzkin, J.F., Dorgan, B.E., Kreger, L.A., Cupples, D.T., Felson 2002. Bone mass and the risk of prostate cancer: the Framingham Study. Am J Med. 113, 734-739.

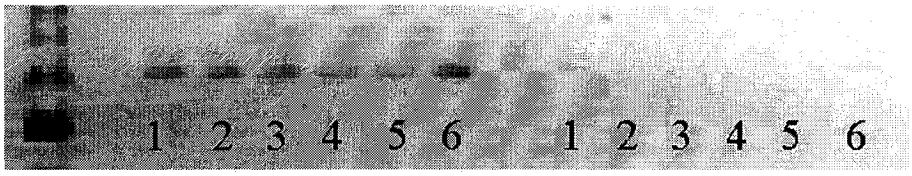
Zhao, L., M., Bakke, K.L., Parker, 2001. Pituitary-specific knockout of steroidogenic factor 1. Mol. Cell. Endocrinol. 185, 27-32.

Zung, A., R., Reifen, Z., Kerem, Z., Zadik, 2001. Phytoestrogens: the pediatric perspective. Journal of. Pediatric Castroenterology and Nutrition 33, 112-118.

Zwiller J, Sassone-Corsi P, Kakazu K, Boyton AL. Inhibition of PDGF-induced c-jun and c-fos expression by a tyrosine protein kinase inhibitor. Oncogene 1991; 6:219-221.

Appendix

Appendix 1



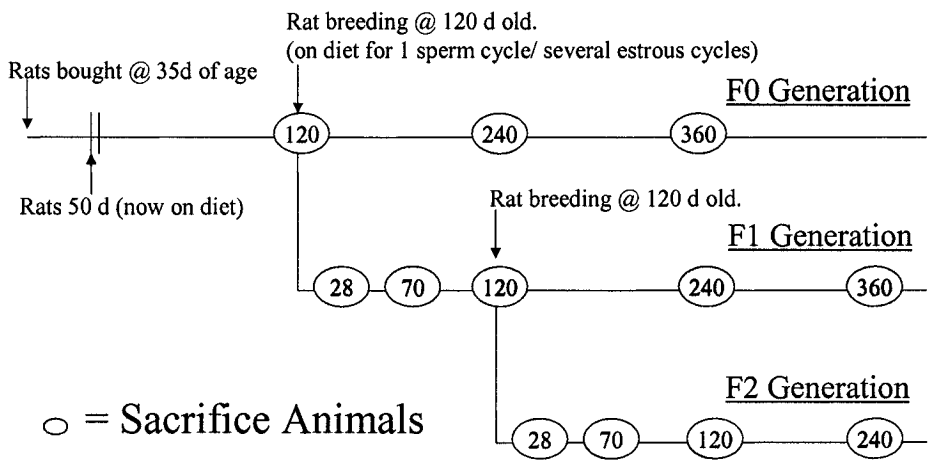
RT-PCR reactions of SF-1 with (left 1-6) and without (right 1-6) reverse transcriptase enzyme added to the reaction mixture. Results show no significant genomic DNA contamination, which may have led to false positives by acting as a template for the RT-PCR primers used.

Additionally G3PDH, DAX-1, SF-1 and CYPP17 RT-PCR products were isolated and sequenced (courtesy of Dr. Bill Casley, Health Canada). Sequences obtained were compared via NCBI nucleotide BLASTn homology search and revealed shared sequence with the rat genes: G3PDH (X02231, Rattus norvegicus mRNA for glyceraldehyde 3-phosphate-dehydrogenase), DAX-1 (X99470, R.norvegicus mRNA for DAX-1 protein.), SF-1(AB012960 Rattus norvegicus mRNA for FTZ-F1 beta1, complete cds.)and CYP17 (NM_012753, Rattus norvegicus cytochrome P450, subfamily 17 (Cyp17), mRNA).

Appendix 2

Multigenerational rat study dosing

Two Generation Breeding Study based on OECD TG 416



Pup body weights

Mean F1 male pup body weights ranged from (mean ± SE) 6.64 ± 0.1 g (diet 6) to 7.03 ± 0.1 g (diet 3) at PND 1. At PND 7,14 and 21 body weights ranged from 15.45 ± 0.2 g (diet 6) to 16.39 ± 0.4 g (diet 3), 30.72 ± 0.6 g (diet 1) to 32.84 ± 0.6 g (diet 2) and 55.45 ± 1.2 g (diet 6) to 59.89 ± 1.1 g (diet 5) respectively. One-way analysis of variance revealed no statistically significant differences in body weights between diet groups ($P > 0.05$).

Crown rump lengths

Mean F1 male crown rump lengths (CRL) at PND1 ranged from 4.95 ± 0.03 cm (diet 6) to 4.98 ± 0.06 cm (diet 5) and at PND 7 from 6.81 ± 0.04 cm (diet 1) to 6.99 ± 0.07 cm (diet 3). One-way ANOVA revealed no significant differences of CRL of rats of different diet groups ($P > 0.05$).

Anogenital distances

Mean F1 anogenital distances for male pups ranged from 3.97 ± 0.1 mm (diet 5) to 4.33 ± 0.07 mm (diet 3) for PND 1 rats. Statistical analysis with 1 way ANOVA revealed no significant differences between groups ($P > 0.05$).

# of F2 offspring per F1 mating pair per diet.

The number of F2 offspring per litter per mating pair (F1) ranged from 11.7 ± 1 pups (diet 1) to 14.5 ± 0.9 pups (diet 2). There were no statistical differences seen between groups ($P > 0.05$). The ratio of male to female pups did not vary significantly between diet groups ($P > 0.05$).

Appendix 3

Parental generation (F0) PND 240 and 360 serum isoflavone level estimates.

AGE	DIET	DAIDZEIN	GLYCITEIN	EQUOL	GENISTEIN	BIOCHAMIN A
PND 240	Diet 1	ND	ND	ND	ND	ND
PND 240	Diet 2	ND	ND	0.124	0.034	ND
PND 240	Diet 3	ND	0.015	0.164	0.043	ND
PND 240	Diet 4	0.034	0.009	0.188	0.053	ND
PND 240	Diet 5	0.269	0.034	0.855	0.294	ND
PND 240	Diet 6	1.246	0.158	3.189	1.214	ND

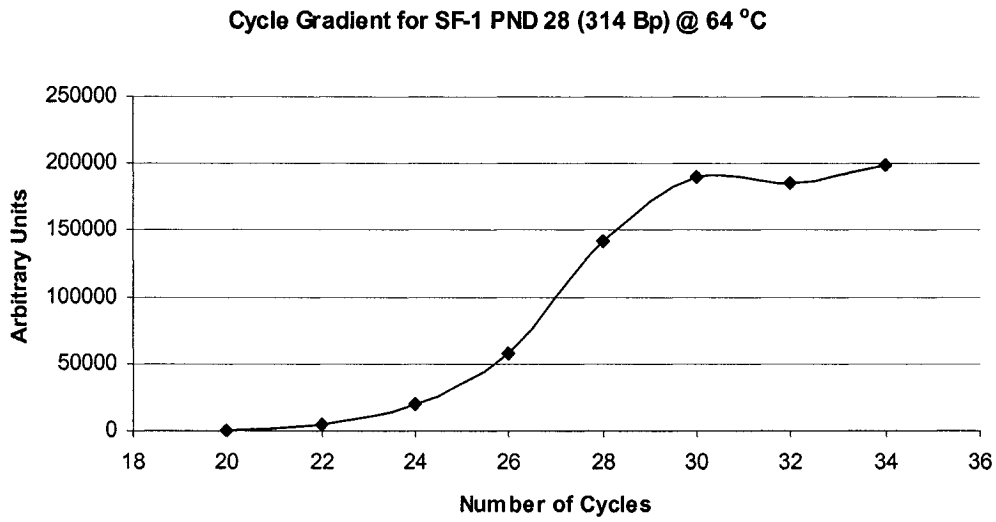
AGE	DIET	DAIDZEIN	GLYCITEIN	EQUOL	GENISTEIN	BIOCHAMIN A
PND 360	Diet 1	ND	ND	ND	ND	ND
PND 360	Diet 2	ND	ND	ND	ND	ND
PND 360	Diet 3	0.009	ND	ND	0.002	ND
PND 360	Diet 4	0.032	0.004	0.032	0.026	ND
PND 360	Diet 5	0.235	0.023	0.493	0.254	ND
PND 360	Diet 6	1.54	0.19	2.933	1.711	ND

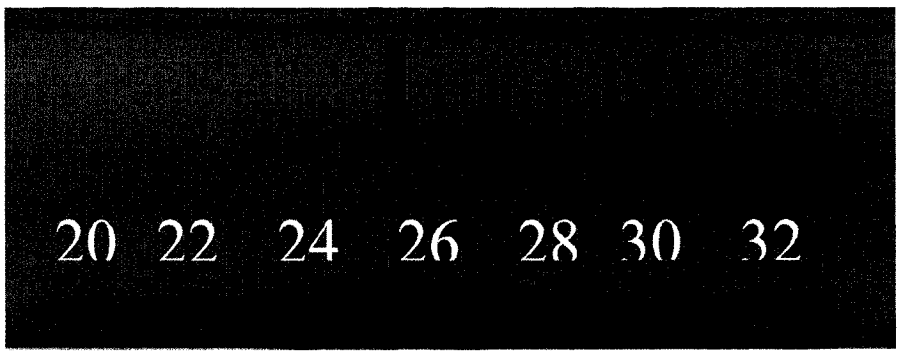
These preliminary F0 generation serum isoflavone data are courtesy of Estatira Sep'hr, Gerard Cooke, Ivan Curran, Pui-Yan Lau, Patrick Robertson and Sarwar Gilani of Health Canada.

These levels were generated by simple liquid chromatography combined with mass spectrometry (LC-MS). The instrument was a Waters Alliance 2695 HPLC with a Waters Micromass ZQ 4000 MS detector.

Appendix 4

Example depicting the PCR cycle gradient conducted for PND 28 SF-1 mRNA. Image shown is an ethidium bromide stained 1% agarose gel run with TBE buffer with cycle numbers shown beneath bands (cycle optimized to 27 cycles).

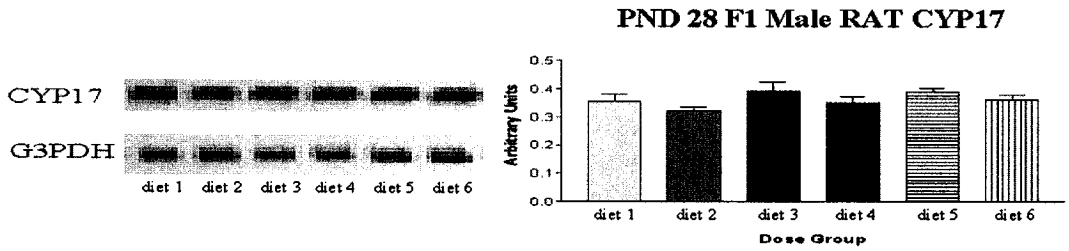




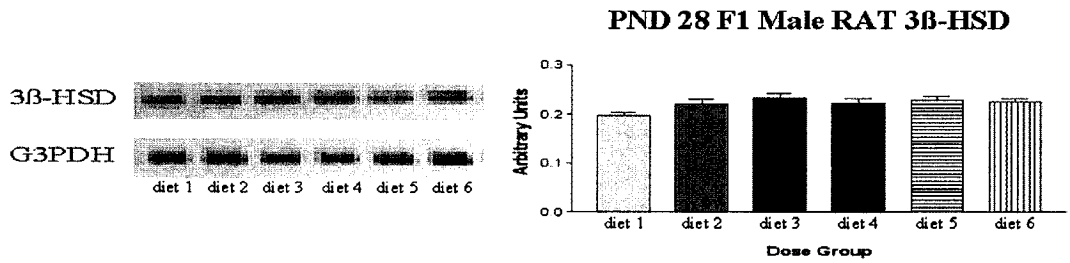
Appendix 5

(A) CYP17 and (B) 3ß-HSD mRNA levels and Western Blots from testes of rats of each diet level at PND 28. mRNA depicted are a single representative (left) of each diet group, followed by a statistical analysis of semi-quantitative PCR based on CYP17 or 3ß-HSD ratioed with G3PHD a consituative control. There were 6 rats sampled per diet with the exception of diets 2 and 3 which had 5 rats examined. Western blots (C) CYP17 and (D) 3ß-HSD show 2 different rats per diet at PND 28.

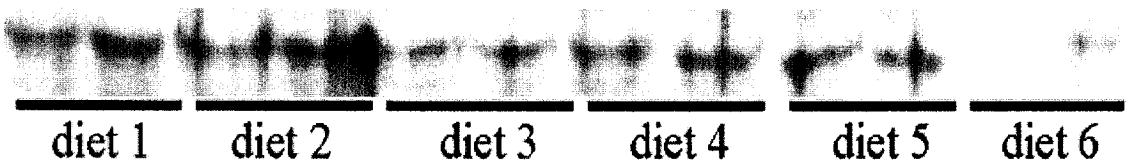
A



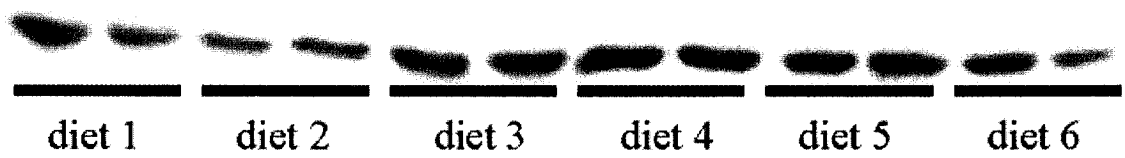
B



C



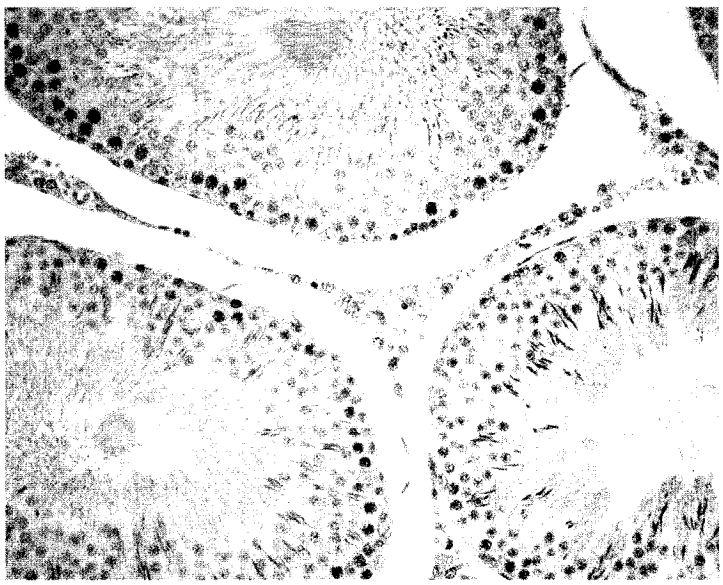
D



Appendix 6

Immunohistochemical staining from rat testis tissue of PND 120 (A) diet 1 and (B) diet 6 DAX-1 stained testis slides shown at 400X magnification.

A



B