EXAMINATION OF EXOGENOUS ESTROGENIC CHEMICAL EXPOSURE AND ALTERED FETAL NUTRITION IN THE CD-1 MOUSE FETUS

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Doctor of Philosophy

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by

JAMES RAYMOND KIRKPATRICK

Dr. Frederick S. vom Saal, Dissertation Supervisor

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

EXAMINATION OF EXOGENOUS ESTROGENIC CHEMICAL EXPOSURE AND ALTERED FETAL NUTRITION IN THE CD-1 MOUSE FETUS

presented by James R. Kirkpatrick

a candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance

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Professor Frederick vom Saal

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Professor Joel Maruniak

_____

Professor Susan Nagel

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Professor Steve Yang

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LIST OF ABBREVIATIONS

bisphenol-A (BPA)

developmental origins of health and disease (DOHaD)

diethylstilbestrol (DES)

fetal estrogenization syndrome (FES)

gestation day (GD)

intrauterine growth restriction (IUGR)

methylaminoisobutyric acid (MeAIB)

postnatal day (PND)

selective estrogen receptor modulator (SERM)

small for gestational age (SGA)

ABSTRACT

The "developmental origins of health and disease" (DOHAD) hypothesis is a growing theory that concerns how disruption of early development of an individual can result in disease later in life. One marker of disrupted fetal development is altered fetal growth. My dissertation examines two issues related to fetal growth: exposure to exogenous estrogenic chemicals and altered fetal nutrition. My first set of studies were aimed at examining the effects of manipulating isoflavones in feed on serum estradiol levels in pregnant female CD-1 mice and their fetuses on gestation day 18. In my first experiment, I compared feeds with and without isoflavones (using feed with casein or soy protein) fed to pregnant mice. The second experiment used a soy-based, isoflavone-extracted feed with increasing amounts of genistein added to the feed. In our third experiment, pregnant mice were fed a normal soy diet and orally administered a low or a high dose of the estrogenic drug diethylstilbestrol (DES) from gestation day 11-17. The results were that fetal serum estradiol concentrations were elevated due to the absence of either isoflavones or soy protein in feed. Also, genistein gave a U-shaped dose-response curve, while DES gave an inverted-U dose-response curve in relation to fetal serum estradiol levels. This suggests that low doses of natural and manmade estrogens can have dramatically different effects on endogenous hormone levels in fetuses. My next study examined how maternal consumption of different concentrations of isoflavones in a soy-based feed affected fetuses during development and their resulting adult phenotype. Isoflavones gave a nonmonotonic dose-response curve in that low levels of isoflavones

elevated fetal estradiol levels and higher doses decreased fetal estradiol levels. The data show that the feed groups that had earlier onset of puberty in females were the same feed groups with higher estradiol during fetal life. These findings stress the importance of controlling, but not removing, the amount of isoflavones in the diet when performing endocrine studies. My last study involved examining placental transport in a crowded uterine horn. Intrauterine growth restriction (IUGR) is a disorder that is believed to be due to a decrease in fetal nutrition. Existing animal models of IUGR employ nutrient deprived diets or gene/chemical manipulations, but these models may not be representative of the basis of IUGR in humans. In the model used here, uterine crowding causes differential blood flow to fetuses. The fetuses with decreased blood flow relative to their siblings show decreased growth. The placenta is thought to be a nutrient sensor that influences fetal growth by regulating the transport of nutrients. I report here that infusion of radiolabeled MeAIB, an amino acid transporter substrate, on gestation day 18 is seen to have differential maternal-fetal transport in fetuses in the crowded uterine horn. My findings also show that placental weight is a significant factor in the transport of amino acids to the fetus. Another interesting finding is a trend for a difference between male and female placentas in the uptake of amino acids. The importance of the crowded uterine model for the study of the effects of fetal nutrition on fetal growth is that this model compares siblings that receive differential placental blood flow, thus controlling for genetic background.

CHAPTER 1

GENERAL INTRODUCTION

Developmental Origins of Health and Disease

The DOHaD paradigm states that when development of an individual is altered, permanent physiological changes occur if these disruptions occur during the "critical period" for gene "programming" [1]. This was formerly known as the "fetal basis of adult disease," but since mammals have "critical periods" that span both fetal and early postnatal life the name was changed. This dissertation focuses on examining factors that can cause alterations in the fetal environment, specifically fetal exposure to either exogenous estrogenic chemicals or a reduced blood flow.

Exogenous estrogenic chemicals cause alterations in development that result in differences in postnatal phenotypes, which include differences in the rate of postnatal growth, timing of sexual maturation, adult reproductive organ function, reproductive fitness, as well as increased predisposition to obesity and certain types of cancers [2-7]. For specific outcomes, studies have characterized some of the mechanisms by which changes in fetal/neonatal development produce differences in postnatal phenotype [8, 9]. However, for many outcomes, such as differential rates of postnatal growth and differential reproductive fitness, mechanisms are only now beginning to be elucidated [7].

There is increasing animal research and extensive epidemiological evidence showing that babies with IUGR resulting in low body weight at birth are at increased risk for subsequent obesity [10], which is described as part of the DOHaD paradigm [11]. Alterations in nutrition that disrupt development have also been linked to cardiovascular disease, hypertension, impaired glucose tolerance, non-insulin-dependent or type II diabetes, which are the complex diseases referred to as metabolic syndrome in adult life [11]. The DOHaD hypothesis suggests that the increase in occurrence of certain reproductive cancers, obesity, and diabetes is due to disruption of normal development during early life [12-14]. The "thrifty phenotype" hypothesis proposes that a biological state adapted for subsistence conditions is created during fetal life by IUGR. However, we are faced with the seeming paradox of increased adult adiposity at both ends of the birth weight spectrum, since fetal over-nutrition leading to high birth weight, or macrosomia, is also associated with a higher attained adult body mass index (BMI)[15]. There thus appears to be a markedly different etiology of obesity in these two sub-populations relating to differences in fetal growth.

Components of nutrients during pregnancy, lactation, and after weaning impact phenotype [16]. A recent report showed that, relative to soy-based mouse feed containing phytoestrogens (Purina 5008 and 5001), casein-based feed (Purina 5K96C) that does not contain phytoestrogens dramatically increases postnatal rate of growth, accelerates the onset of sexual maturation, increases adult body fat, and disrupts normal reproductive system development associated with abnormal hormone levels [6]. A particularly interesting finding is that fetuses carried by pregnant females maintained on the casein-

based feed have significantly elevated serum estradiol levels relative to fetuses whose mothers are maintained on the soy-based feed. Elevated estrogen levels during "critical periods" in development accelerate postnatal growth [16, 17] and disrupt development of the reproductive system in males [18].

Obesity

The epidemic of obesity has increased to such a degree that recent global estimates by the World Heath Organization indicated approximately 2 billion adults are either overweight or obese and that at least 20 million children under 5 years old are overweight. Other examples of how much obesity has increased in the world are shown by the Federal Aviation Administration instructing airlines to increase the estimated average passenger weight because of safety concerns and by doctors needing longer needles to penetrate the thicker layers of fat when administering vaccines and drawing blood [19, 20].

Individuals who are obese are at risk for a number of other serious medical complications such as type II diabetes, cardiovascular disease, endometrial, prostate, breast, and colon cancers, and reproductive, liver, gallbladder, and respiratory diseases [21]. Research has also shown that an obese individual's own assessment of their quality of life is similar to an individual's with cancer [22]. We now know that it is incorrect to say that the cause of obesity solely resides in personal choices that produce a positive energy balance. Instead, this disease stems from behavioral choices, as well as genetic and environment factors.

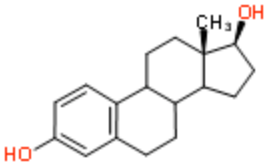
Baillie-Hamilton showed in 2002 that the current obesity epidemic coincided with the increased use of industrial chemicals in the environment [13]. A plethora of research now shows that exposure to these environmental chemicals early in life creates detrimental effects later in adulthood. Estrogenic environmental compounds have garnered much attention due their wide range of effects on an individual's physiology [23-26].

It has been reported that fetal nutrition plays a strong role in the programming of obesity in that either an abundance of nutrients or a restricted amount are linked with increased disposition to obesity [27]. It is still unclear as to how fetal nutrition "programs" the fetus for obesity later in life, since two different fetal phenotypes (IUGR and macrosomia) result in an increased predisposition to obesity. However, it is clear that fetal nutrition does program the body for obesity differently than postnatal nutrition [28, 29].

Intrauterine growth restriction (IUGR)

IUGR is a disorder characterized by fetuses whose weight falls below 5.5 lbs after gestation week 37. This disorder is believed to be due to a decrease in fetal nutrition. The WHO currently estimates the incidence of IUGR to be 15% worldwide, affecting approximately 30 million newborns. The WHO also reports these incidences to be more prevalent in developed countries. IUGR is a major cause of perinatal mortality and morbidity [30]. Existing animal models of IUGR employ nutrient-deprived diets or gene or chemical manipulations, but these models may not be representative of the basis of

IUGR in humans [31]. The concern arises when an animal model manipulates a specific factor (e.g., protein restriction) and applies the results to human IUGR when that factor does not appear to be the cause of the IUGR, at least for most IUGR babies born in developed countries. The general problem with animal models that use diet restriction methods to induce IUGR is comparing that data to incidences of IUGR in developed countries where maternal undernutrition is very unlikely to occur. Human data from developed countries seem to point towards decreased uterine-placental blood flow as major factor in IUGR as opposed to maternal caloric or specific nutrient deprivation [32, 33]. The role of environmental estrogen exposure in IUGR remains to be examined in epidemiological studies, but the ongoing National Children's Study should address this data gap.



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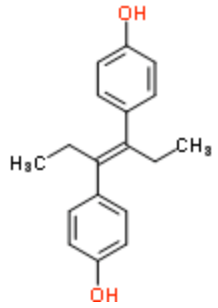

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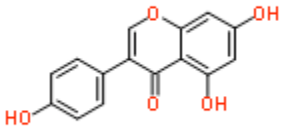

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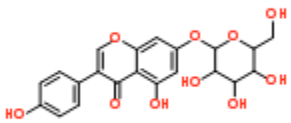

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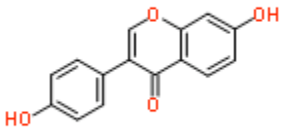
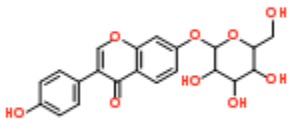
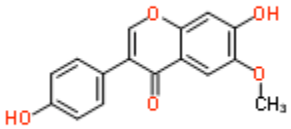


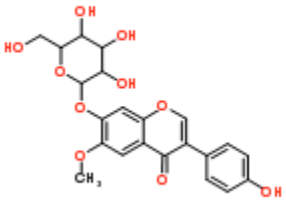
Daidzein



Daidzin



Glycitein



Glycitin

Figure 1 Chemical structure of estradiol and related compounds obtained from http://www.chemspider.com.

Dietary soy phytoestrogens

Dietary disruption of the endocrine system by phytoestrogens was first examined in 1946 due to reproduction problems with sheep grazing on red clover on Australian pastures [34]. This phenomenon was later termed "clover disease". This disruption in fertility can be permanent. In a prior study, there were more than one million sheep in Australia that have permanent disruptions in fertility due to consumption of phytoestrogens [35].

A major class of phytoestrogen is the isoflavones, which are found abundantly in soy and red clover, with the major isoflavones being genistein, daidzein, and glycitein [36]. Soy products receive increasing attention due to reports that a diet supplemented with soy products can have numerous health benefits, such as the treatment of menopause symptoms and prevention of cancer, osteoporosis, and heart disease [36]. Many of the benefits discussed are thought to arise from the isoflavones. There is concern, however, about whether isoflavones are beneficial due to reports of unfavorable outcomes in response to isoflavone exposure. In 2001, Newbold et al. showed that neonatal exposure to genistein induced an increased incidence of uterine adenocarcinoma similar to the effect of neonatal exposure to the estrogenic drug DES [37]. In 2004, Allred et al. reported diets containing highly processed soy-based products increased breast cancer tumor growth to a higher degree than diets containing minimally processed soy-based products [38].

Even though genistein and daidzein bind to and activate the estrogen receptor, it is difficult to predict their effects in tissues because they are SERMs and have dose-

dependent and tissue-dependent effects [39, 40]. Also complicating this issue is that when endogenous estrogen is present in a premenopausal concentration (10^{-9} M), genistein and daidzein are antagonistic to estrogen action, but in a postmenopausal estrogen concentration (10^{-11} M), genistein and daidzein are estrogen receptor agonists [40]. This suggests that accounting for background circulating estradiol levels will allow a better prediction as to what role phytoestrogens may play in health and disease.

Bioavailability of these compounds is difficult to predict as well. The majority of isoflavones are in the inactive, glycoside form (genistin, daidzin, and glycitin) while present in soy-based food. The concentrations of different isoflavone forms can change with increased food processing, since high temperatures and low pH have been reported to change isoflavone profiles in soy-based food [41, 42]. In the digestive system, isoflavones are deconjugated, beginning in the mouth and continuing in the small intestine, to the active, aglycone forms (genistein, daidzein, and glycitein), and the proportion of individuals in which this deconjugation occurs is species specific [43-46]. Deconjugation of isoflavones must occur for absorption in the digestive system [47]. Difficulty arises in predicting bioavailability due to conflicting reports. Izumi et al. reported that aglycones, rather than glycosides, are more bioavailable, but Setchell et al. demonstrated that the glycosides are more bioavailable [48, 49]. Adding to this, Zubik et al. showed that there is no difference between the two forms [50]. There are also data showing differences in pharmacokinetics of glycosides and aglycones between men and women, and only 30% of subjects were able to convert daidzein to its other active metabolites [45].

Isoflavones, as well as other endogenous estrogenic chemicals, currently are being examined as a possible factor in adult disease [51]. A problem that arises in studying these chemicals in experimental animals is the possibility that the estrogenic components of research animal feed, such as isoflavones in soy-based feeds, can disrupt experimental endpoints [52]. Some biologists believe that it is important to eliminate isoflavones from the diet of research animals because of potential developmental interference by the phytoestrogens [53]. The alternative could be to use milk protein, casein, instead of soy protein in the feed. However, two recent reports have shown that when soy protein was removed from feed and replaced with casein protein, experimental animals were significantly heavier with disruption of fat and glucose homeostasis [6, 54].

Diethylstilbestrol (DES)

DES was first used as treatment for spontaneous abortions in pregnant women, but sadly, was found to cause great disruption to the adult female reproductive system [55]. Studies using DES have shown it to be a great example of how exogenous estrogenic chemicals have the potential to disrupt the homeostasis of the endocrine system, which is why it has been used as a positive control in numerous studies examining estrogenic effects of various chemicals. Exposure to low doses of DES while *in utero* has also been associated with disruptions in male prostate development [56]. What is interesting is that at high doses this outcome is reversed in that low doses of DES increased prostate weight and high doses decreased prostate weight. DES is now being used as a way to study how developmental exposure to exogenous estrogenic chemicals

results in obesity. It was reported that fetal exposure to a high dose of DES decreased fetal body weight *in utero*, while a low dose has no effect on fetal body weight. When these offspring reached adulthood, both dose groups had increased body weight and fat in adulthood relative to controls [51, 57]. Taken together, these findings suggest that while a low and high dose of DES can bring about the same outcome for body weight in the adult, they do so through different mechanisms.

Effects of differences in serum estradiol in mouse fetuses

I describe below studies that focus on FES by relating serum estradiol levels in the fetus to phenotypic outcomes later in life. Of importance is understanding that there have been many studies, in vivo and in vitro, relating estradiol differences early in life to detrimental adult outcomes in terms of what is actually the active, or relevant, concentration of estradiol in the blood [56, 58-60], what this active concentration means in terms of stimulating effects in specific tissues [61, 62], and characterizing the responses that occur in those specific tissues [56, 60, 63, 64].

An important aspect of this issue for research with mice or rats is that the neonatal mouse and rat are appropriate models for studying the effects of chemicals on organogenesis, which is still occurring in the neonatal mouse and rat, a developmental period which is equivalent roughly to the fifth through ninth month in human pregnancy. However, the fact that the neonatal mouse and rat suddenly experience a dramatic drop in serum estradiol relative to human fetuses needs to be considered when using the neonatal mouse and rat as a model. The potential for markedly different interactive effects

between components of feed and administered estrogenic chemicals in toxicological studies is an important issue that needs to be investigated.

Crowded uterine horn model

We have developed a novel model of IUGR using a "crowded uterine horn", which allows us to compare siblings showing normal fetal growth, IUGR and macrosomia within a crowded litter produced by hemi-ovariectomized female mice, in which the one remaining ovary ovulates ~12 oocytes. Differential fetal growth is due to the unique vascular anatomy of the independent uterine horns. Preliminary data show elevated fetal growth over normal occurs for fetuses at each end of the crowded uterine horn, while fetuses in the middle are growth restricted compared to normal siblings [32]. Specifically, a series of studies showed that in rats and mice there is a loop uterine arterial and venous system. These studies dealt with uterine blood flow in both rats [65] and mice [63], where blood flow to each uterine horn was shown to come from both the descending aorta (on the cranial end) and internal iliac (on the caudal end). This resulted in reduced blood flow and thus reduced growth for the fetuses in the middle of the uterine horn. The effect of location in relation to uterine blood flow on fetal growth was previously shown to increase as a function of the number of fetuses in the uterine horn [66].

Transport systems – amino acids

Amino acids have many different transport systems, with specific systems

generally transporting specific amino acids, although some overlap between systems may occur. For example, System A transports alanine, serine, and glycine, but System ASC also transports alanine [67]. System A is a sodium-dependent, neutral amino acid transporter that has shown reduced activity when there is reduced fetal growth [68]. MeAIB is a substrate of System A that is unmetabolizable [69]. Because of these properties, it is an appropriate chemical to use to examine placental transport of nutrients to the fetus facilitated by System A. In 2006, Jansson et al. reported that the reduction in System A transport, shown by transport of MeAIB, was seen before there was a reduction in fetal growth, providing evidence to support that the placenta has some control of fetal growth by controlling fetal nutrition [70].

CHAPTER 2

GENERAL METHODS

Animal Care and Maintenance

Animals used in this study were CD-1 mice (Mus musculus domesticus) from the vom Saal lab colony in the Lefevre Hall animal facility, derived from animals originally purchased from Charles River (animals in the breeding colony are replaced ever two years). The CD-1 mouse is a primary model animal used in developmental and toxicological research. In numerous previous experiments described in detail below, differences in morphological and physiological characteristics have been identified in CD-1 mice exposed during development to estrogenic chemicals and to abnormal fetal nutrition levels.

Mice were maintained on a 12-hour light/dark cycle, and the rooms were maintained at 25±2°C. Mice were housed on corncob bedding in standard (11.5 x 7.5 x 5 in) polypropylene cages. Water was purified by ion exchange followed by a series of carbon filters and provided *ad libitum* in glass bottles. Purina 5008 (soy-based; Ralston-Purina, St. Louis, MO) was used during pregnancy and lactation and then switched to Purina 5001 (soy-based; Ralston-Purina, St. Louis, MO) after mice were weaned. Both feeds were fed *ad libitum*. Breeder females (F0) were paired with males and then singly housed when visibly pregnant. Litters were reduced to 12 pups on the day of birth. Pups

(F1) were weaned at 21 days of age, and 3-5 littermates of the same sex were housed per cage until one animal per litter was randomly assigned to each specific experiment. For all experiments, adult females (F0) were paired with stud males between 0800 and 1100 h and then singly housed when determined to be pregnant, which is gestation day (GD) 0 based on examination for a vaginal plug. This animal facility is housed in Lefevre Hall, Division of Biological Sciences, University of Missouri and is AALAC accredited. All procedures were approved by the University of Missouri Animal Care and Use Committee and conform to the NIH guide. Periodic (quarterly) examination (serology, microbiology, histopathology) of animals was a routine procedure in the laboratory during these experiments. Examinations were conducted in the pathology laboratory at The Research Animal Diagnostic and Investigative Laboratory in the College of Veterinary Medicine. Euthanasia at the end of an experiment conformed to recommendations by the Panel on Euthanasia of the American Veterinary Medical Association. Euthanasia occurred by CO2 asphyxiation and subsequent cervical dislocation.

AIN-93G feeds

Some of these experiments used purified feeds based on the AIN-93G formulation in order to control the estrogenic activity within the different diets. I manipulated the estrogenic activity of the feeds while maintaining a constant caloric value of 3.89 kcal/gm (Table 1). The extraction of phytoestrogens from feeds was approximated ~90 %, to yield a phytoestrogen-reduced feed. These diets were pelleted by dry extrusion and

color-coded for identification in the Food Science and Human Nutrition Department at the University of Missouri-Columbia. Females from our breeder colony were randomly assigned to one of these feed groups 2 weeks before mating and remained on the given feed throughout pregnancy.

Table 1 Differences in components of 5008 and AIN-93G feeds used in this dissertation.

Components	Purina 5008	Purina 5001	AIN-93G (soy)	AIN-93G (casein)
Protein (%)	23.5	23.4	17.9	17.9
Fat (%)	6.5	4.5	7.1	7.1
Fiber (%)	3.8	5.3	5.0	5.0
Carbohydrates (%)	38.32	37.78	63.2	63.2
Metabolizable Energy (kcal/gm)	3.31	3.04	3.89	3.89

Assays

Measurement of feed intake and stabilization prior to mating. When mice were switched to a different feed before an experiment, daily food intake and body weight was monitored during the 2 weeks prior to mating and during pregnancy to ensure there were no gross differences between feeds. We used a feed holder that attaches to the front of the polypropylene cages that retains spillage to measure the amount of feed consumed. The feed holder can be removed and was weighed daily, and intake of feed in relation to body weight was measured. Energy intake was obtained by multiplying food intake by the caloric value of the diet (kcal/g).

Organ, blood, and fat collection. Adult mice and fetuses were weighed and then killed by either CO_2 asphyxiation and cervical dislocation (adult), or CO_2 asphyxiation and decapitation (fetus). Organs were dissected, weighed, frozen in liquid nitrogen, and stored at -70°C for RT-PCR analyses. Maternal and fetal blood was also collected, and serum was stored at -30°C.

Estradiol Radioimmunoassay. Serum estradiol was measured using assays that have been described and conducted in previous studies [71]. [^{125}I]Estradiol and antisera were obtained from MP Biomedicals (Costa Mesa, CA), and unlabeled estradiol were obtained from Steraloids (Wilton, NH). Sensitivity of the assay was 0.5 pg per tube. Intra- and inter-assay coefficients of variation were 5% and 4%, respectively. We determined the percent cross-reactivity of the estradiol antiserum with estrone to be 0.6%. Cross-

reactivity with other steroids was reported by MP Biomedicals to be negligible.

Statistical Analyses. ANOVA (GLM procedure in SAS) was used, and for planned comparisons the SAS LSmeans Test was used when justified by statistical significance on the overall ANOVA. Since more than one mouse per litter was examined for these endpoints, litter was included as a main variable, and the F value for feed group was divided by the F value for litter to adjust for variance due to maternal effects. Differences were considered statistically significant when $P < 0.05$.

CHAPTER 3

ISOFLAVONE AND DES EXPOSURE IN RELATION TO ESTRADIOL LEVELS IN THE CD-1 MOUSE FETUS

Introduction

The Developmental Origins of Health and Disease (DOHaD) paradigm is based on the prediction that disruption of normal development can result in increased risk of disease in adulthood [72]. Estrogenic compounds such as diethylstilbestrol (DES), bisphenol A (BPA), and phytoestrogens in soy have been shown to disrupt development [6, 73, 74]. A problem with animal experiments designed to examine the effects of these estrogenic compounds is controlling the estrogenic activity in the feed the animals are consuming [52, 75, 76].

Since most of the endpoints that are altered by exogenous estrogenic chemicals are the same as those altered by estrogenic components in feed, it is difficult to replicate experimental results if the estrogenic chemicals in feed are highly variable. It is evident that even if the same type of feed is used for an entire experiment, variability in estrogenic activity may still arise due to batch-to-batch differences of the same feed [52]. Also, measuring a soy-based diet for phytoestrogens before use may not solve the problem, since there are other sources of estrogenic activity in feeds such as mycotoxins [77]. This is an issue that needs to be resolved, since feeds with different estrogenic

components produce alternate phenotypes [6, 54, 78, 79].

A common protein source for animal feed is soy protein, which contains a class of phytoestrogens called isoflavones. The major sources of estrogenic activity from these feeds are the isoflavones genistein, daidzein, and glycitein [80]. When present in the feed, the majority of isoflavones are in their inactive glycoside form (genistin/daidzin/glycitin) and then are converted upon consumption to the active, aglycone form (genistein/daidzein/glycitein) to be absorbed in the digestive system [39, 43]. In rats and humans, this deconjugation event must occur in order for the isoflavones to be absorbed across the intestinal wall [44, 47].

Genistein is the most potent estrogenic compound in soy and has a higher concentration in soy-based feed than daidzein. Genistein also has a higher affinity for estrogen receptor beta than estrogen receptor alpha [39, 80]. However, conflicting studies make it unclear as to how it affects transcription [39, 81, 82]. In addition to this, genistein acts via other non-hormonal pathways [83]. All of these findings make it very difficult to sort out genistein's effects, since it is a selective estrogen receptor modulator (SERM) in that it can be both an estrogen agonist and antagonist depending on the form of estrogen receptor present in a given tissue and the concentration of background estradiol [39, 40].

Here I examined whether exposure to exogenous estrogen affected circulating estradiol levels in pregnant mice and fetuses. Specifically, maternal and fetal estradiol levels were measured in relation to the presence or absence of phytoestrogens on a background of soy protein or casein protein in the maternal diet, as well as against

increasing amounts of either genistein or DES on a soy protein background.

Materials and Methods

Four-month old virgin females that had been fed Purina 5001 feed beginning at weaning were randomly assigned to experimental feed groups. Females in the different feed groups were monitored to ensure stabilization of food intake on the different feeds by measuring daily food intake and body weight during the 2 weeks prior to mating and during pregnancy. Animals were time mated, and pregnancy was confirmed by vaginal plug (gestation day 0).

On day 18 of gestation, pregnant females were euthanized by CO_2 asphyxiation and cervical dislocation, and maternal blood was collected. Cesareans sections were performed to collect fetuses. Fetal sex was determined, fetal body weight was measured, and fetal blood was collected by decapitation. Blood from fetuses of the same sex within a litter was pooled. Maternal and fetal blood samples were centrifuged, and serum was frozen prior to hormone analysis. These experiments used AIN-93G purified diets as described above.

Experiment 1 - Soy vs. casein protein, with and without isoflavones. The purified AIN-93G formulated soy minus isoflavones feed was made by extracting the isoflavones from the soy in the feed, and the purified AIN-93G casein or soy plus isoflavones feed was made by the addition of Prevastein (Solae, St. Louis, MO). Prevastein is an aglycone isoflavone supplement that has been used in other experiments to examine isoflavone

exposure [84, 85]. Female mice were divided into five different feed groups: Purina soy-based 5008 (soy-based; Ralston-Purina, St. Louis, MO), which is the standard feed used in the mouse colony, AIN-93G soy with natural isoflavones (Soy +), AIN-93G soy minus isoflavones (Soy -), AIN-93G casein plus Prevastein (Casein +), and AIN-93G casein no isoflavones added (Casein -). HPLC analyses were used to measure genistein and daidzein concentration in the Soy -, Soy +, and Casein + feeds (Table 2). Estrogenic activity was verified by MCF-7 bioassay as described previously [86]. Casein – was not measured due to previous reports of negligible isoflavone content in these casein-based feeds [87].

Experiment 2 - Genistein added to AIN-93G feed minus isoflavones. In this study, a base feed was made as described above for soy with isoflavones being removed, and different doses of genistein (LC Laboratories, Woburn, MA) were added to the feed. Female mice were divided into four different genistein dose groups: 0, 225, 450, and 900 ppm genistein added to the feed. The genistein concentrations that were added in feed reflect HPLC analyses of the diets used in Experiment 1 (Table 1). Our objective was to administer a dose similar to the total genistein equivalence of the Soy + feed from Experiment 1 and have a concentration on either side. Estrogenic activity was verified (Table 3) by an MCF-7 cell bioassay.

Experiment 3 - DES exposure on Purina 5008 feed. All female mice for this experiment were fed Purina 5008 (soy-based; Ralston-Purina, St. Louis, MO) breeder chow

throughout gestation. The females in each group were time mated, and blood was collected as described above. Pregnant dams were fed p.o. DES once per day from GD 11-17 using an electronic micropipetter by picking up the mouse and gently placing the tip of the pipette into the animal's mouth. DES was dissolved in tocopherol-stripped corn oil (Fisher. Pittsburgh, PA), and the volume administered was adjusted to maintain a constant dose per kg body weight. Mice readily consume these solutions, and this method of administering a drug is less stressful than gavage. The doses of DES used were 0, 0.1 (low dose), and 50 (high dose) μg/kg/day.

Table 2 Mean parts per million (ppm) of the three major isoflavones in the modified feeds used in Experiment 1. Glycitein, daidzein, and genistein were measured using HPLC. Gen Eq = genistein equivalence, which was measured using the MCF-7 bioassay.

	HPLC Analysis			MCF-7 Bioassay
Feed	Glycitein (ppm)	Daidzein (ppm)	Genistein (ppm)	Gen Eq (ppm) *
Soy –	0	0	2.13	7.7
Soy +	36.81	262.67	434.30	70.4
Casein +	3.19	194.65	556.16	303.0

Table 3 Mean ppm genistein equivalents measured from feeds in Experiment 2 by MCF-7 cell bioassay.

ppm Genistein Added to Feed	ppm Genistein Equivalence	SEM
0	0.00	± 0.00
225	197.25	± 33.71
450	371.50	± 41.50
900	769.50	± 65.50

Results

Experiment 1. Maternal serum estradiol concentrations for the Soy + and Purina 5008 diet were significantly lower than the Soy – ($P < 0.01$) and Casein – ($P < 0.01$). Soy + feed resulted in significantly lower serum estradiol than Casein + feed ($P < 0.05$) (Figure 2). Estradiol in the maternal sera for the casein diets did not differ whether or not isoflavones were present. The effect of the feeds on fetal serum estradiol concentrations was similar to the effect on maternal estradiol levels. Fetal serum estradiol levels for the Soy + and Purina 5008 diet were significantly lower than the Soy – ($P < 0.01$), Casein – ($P < 0.01$), and Casein + ($P < 0.01$) diets. The fetuses whose mothers were fed the casein-based feeds had elevated estradiol levels regardless of the presence or absence of isoflavones. Thus, for this experiment, only pregnant mothers and their fetuses that were fed a soy-based feed containing isoflavones showed a reduced serum estradiol level. For all feed groups, there was no significant difference between male and female fetuses. Also, when maternal serum estradiol changed, fetal levels responded the same way (i.e. a reduction in maternal serum estradiol was matched by a reduction in fetal serum estradiol).

Experiment 2. There was no significant difference in maternal serum estradiol levels for all feeds in this experiment (Figure 3). Fetal serum estradiol was elevated when

isoflavones were removed from the feed (Soy 0, S0). Addition of increasing doses of genistein reduced fetal serum estradiol (S225 and S450), while the highest genistein dose (S900) increased fetal serum estradiol relative to the S450 dose, forming a U-shaped dose-response relationship. These differences were only significant between S0 and S450 for all fetuses regardless of sex ($P < 0.05$). An interesting finding was pregnant dams that consumed the 450 ppm genistein feed did not have significantly altered serum estradiol levels, but their fetuses had reduced serum estradiol levels. There was a fetal sex difference with S900 in that males had higher serum estradiol levels than female fetuses ($P < 0.05$). Also in the S900, fetal body weight differed with males being heavier than females ($P < 0.05$) (Figure 4). Fetuses showed a significant difference in weight between S0 and S225 feeds ($P < 0.05$). A regression analysis between fetal serum estradiol concentration and fetal body weight showed an r^2 value of 0.09 ($P < 0.01$) (Figure 5).

Experiment 3. As expected, pregnant dams fed 5008 showed a decrease in serum estradiol with increased exposure to DES. Difference was significant between controls (no DES) and animals that received 50 µg/kg/day of DES ($P < 0.01$) (Figure 6). However, a low dose of DES (0.1 µg/kg) stimulated an increase in fetal serum estradiol relative to controls, while a high dose of DES (50 µg/kg) reduced fetal serum estradiol relative to controls, forming an inverted-U dose-response relationship. Both of these findings were significant ($P < 0.01$). Although fetal estradiol levels were lower in male fetuses relative to female fetuses across all dose groups, this difference was not

statistically significant (P = 0.12).

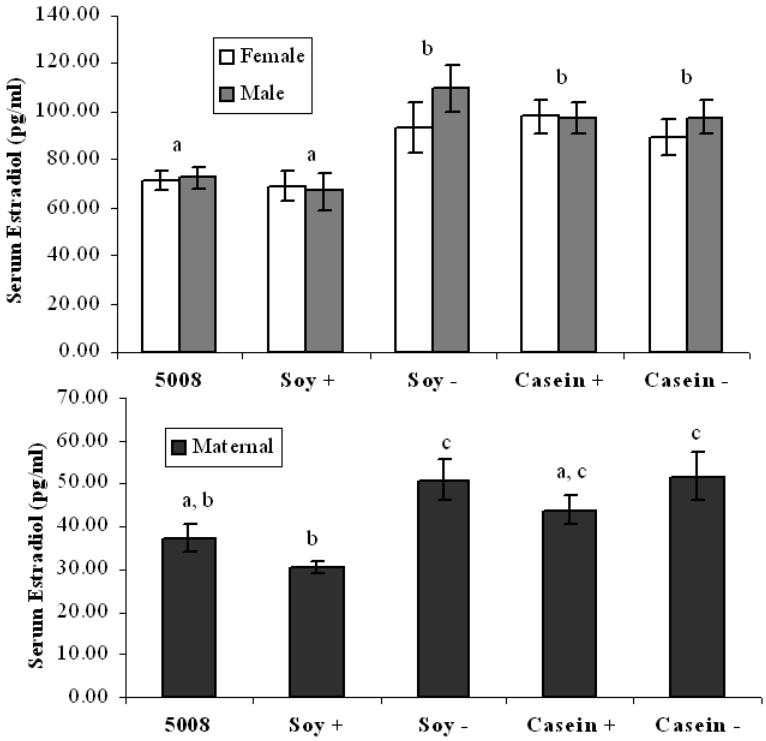


Figure 2 Mean (±SEM) serum estradiol levels (pg/ml) on gestation day 18 in pregnant females and their pooled male and pooled female fetuses from each litter fed 5008 (n = 14) or 4 different AIN-93G purified diets: soy with isoflavones (Soy +; n = 9), soy without isoflavones (Soy -; n = 10), casein with isoflavones (Casein +; n = 9) or casein without isoflavones (Casein -; n = 11). Different letters designate significant differences with $P < 0.05$. Female and male fetal estradiol levels were combined in each feed group for statistical analysis since there were no significant sex differences.

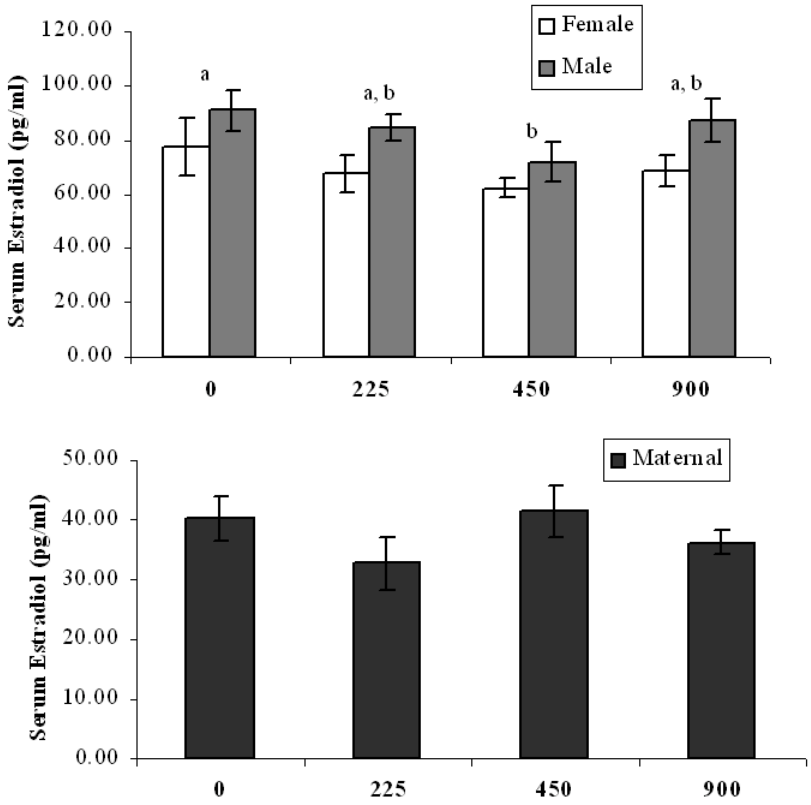


Figure 3 Mean (± SEM) serum estradiol levels (pg/ml) on gestation day 18 (mating = GD 0) in mothers (and their pooled male and pooled female fetuses from each litter) fed AIN-93G purified diets with phytoestrogens removed and replaced with genistein at 0 (n = 9), 225 ppm (n = 8), 450 ppm (n = 7), and 900 ppm (n = 11). Female and male fetal estradiol levels were combined in each feed group for statistical analysis since there were no significant sex differences in each feed group. Different letters designate significant differences with $P < 0.05$. Fetal serum estradiol was significantly heavier in males compared to females only in the 900 ppm group.

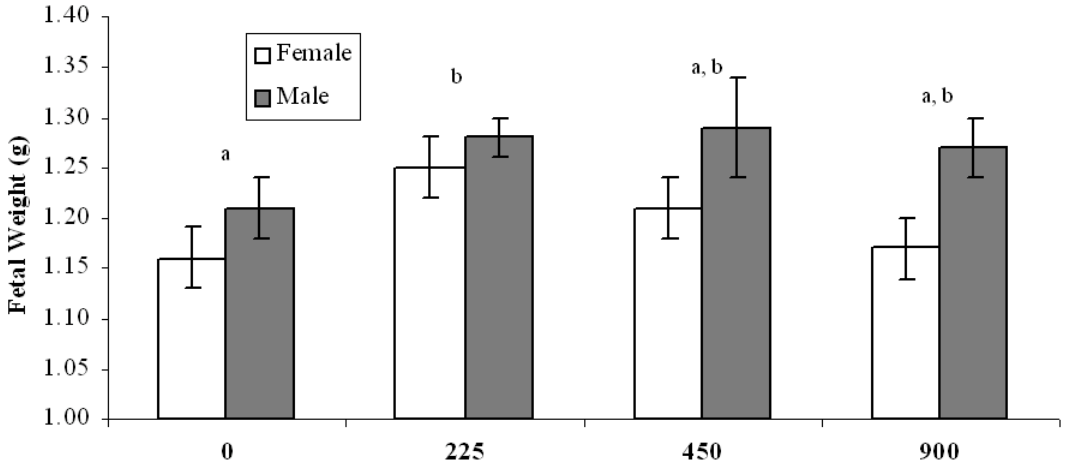


Figure 4 Mean (± SEM) fetal body weights (g) on gestation day 18 (mating = GD 0) in mothers fed AIN-93G purified diets with phytoestrogens removed and replaced with genistein at 0 (n = 9), 225 ppm (n = 8), 450 ppm (n = 7), and 900 ppm (n = 11). Letters designate significant differences with $P < 0.05$. Female and male fetal weights were combined in each feed group for statistical analysis since there were no significant sex differences in each feed group. Fetal body weight was significantly heavier in males compared to females only in the 900 ppm group.

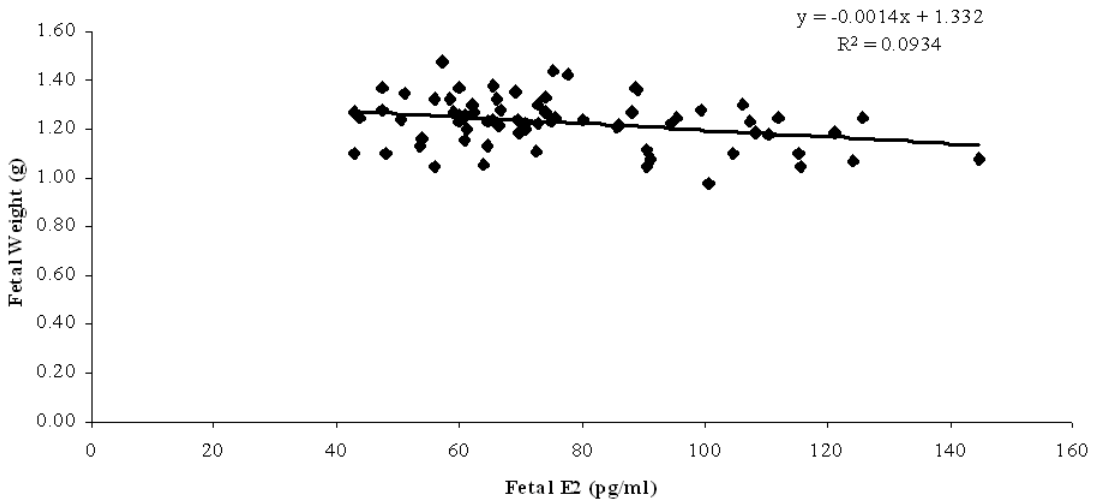


Figure 5 Regression analysis of fetal serum estradiol levels to fetal body weight (g) on gestation day 18 (mating = GD 0) in mothers fed AIN-93G purified diets with phytoestrogens removed and replaced with genistein at 0, 225 ppm, 450 ppm, and 900 ppm. $P < 0.01$.

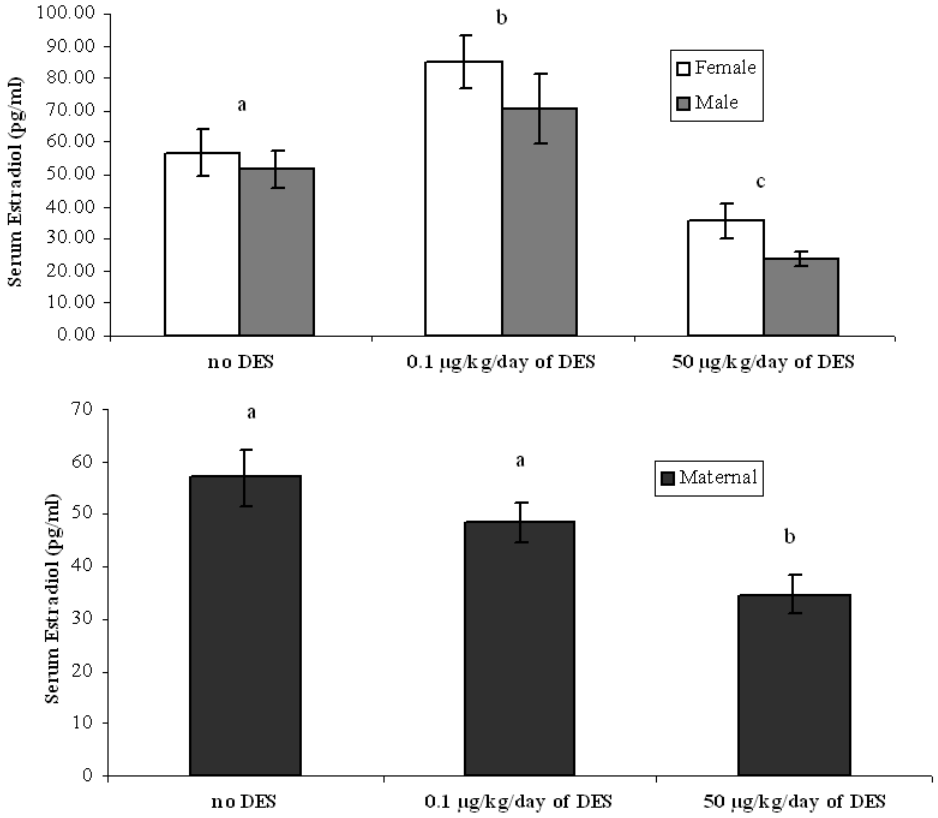


Figure 6 Mean (± SEM) serum estradiol levels (pg/ml) on gestation day 18 in pregnant dams fed Purina 5008 and their pooled male and pooled female fetuses from each litter. DES was administered via a micropipettor in tocopherol-stripped corn oil to pregnant dams on gestation days 11-17 in doses of 0 (n = 10), 0.1 (n =9), and 50 (n =8) µg/kg/day. Different letters designate significant differences with $P < 0.05$. Female and male fetal estradiol levels were combined in each feed group for statistical analysis since there was no significant sex difference.

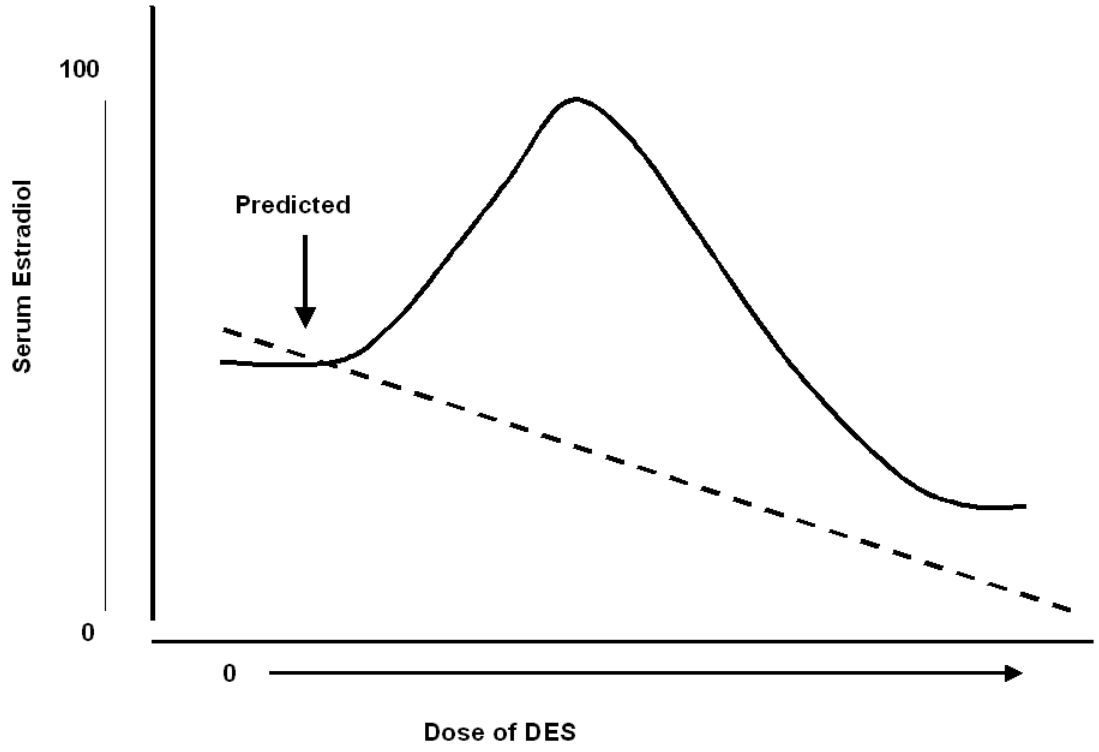


Figure 7 Schematic representing the different dose response curves for serum estradiol in response to low and high doses of DES in pregnant females and their fetuses. A linear trend was expected and found in the maternal response to increasing doses of DES. The fetal response, however, showed an inverted-U dose response curve with low doses of DES increasing fetal estradiol from controls and high doses of DES decreasing fetal estradiol relative to controls.

Discussion

I report here that exogenous estrogenic chemicals alter endogenous estradiol levels in the fetal mouse. Specifically, mouse fetuses whose mothers were fed a soy-based feed containing estrogenic isoflavones had a lower circulating estradiol level, and removal of the isoflavones from soy-based feed or feeding the mothers a casein-based feed resulted in elevated estradiol levels. In addition to this, a concentration of isoflavones in maternal feed around 225-450 ppm resulted in lowered fetal estradiol levels, while an increased dose of 900 ppm elevated fetal estradiol levels. We also report that exposure to increasing concentrations of DES resulted in an inverted-U dose response curve in fetal estradiol levels.

It has previously been reported that the diet of research animals needs to be taken into consideration because it has the potential to alter experimental outcomes [52]. To avoid dealing with variation in phytoestrogen levels in feeds, some researchers have suggested switching from a soy-based feed to a casein-based feed [53, 76, 88]. I show here that phytoestrogens play a major role in regulating fetal estradiol levels in the mouse and that care needs to be taken when considering alternative protein sources. These data agree with previous reports that switching from a soy-based to a casein-based feed is not an appropriate option [6, 54]. Specifically, using a casein-based feed rather than a soy-based feed causes an elevation of fetal estradiol levels, which has been shown to alter adult phenotype [6, 56].

These data also show that phytoestrogens themselves do not have the capability to reduce fetal estradiol levels when given in the presence of an inappropriate background

protein. Specifically, adding phytoestrogen to soy protein (Soy + feed) resulted in a reduction in fetal estradiol levels, but the phytoestrogens alone did not induce the same response when the soy extract containing phytoestrogens, Prevastein, was added to casein-based feed (Casein + feed). Thus, when casein was the background protein there was no difference in estradiol levels regardless of the phytoestrogen content (Casein + vs. Casein -). This finding suggests that in order for the phytoestrogens to have an effect on fetal serum estradiol, they must be present in their original soy matrix. These data show that care must be taken when removing nutrients from their original matrix and suggest that if supplements are taken with an improper background of nutrients, they may not produce the desired effects. More data need to be generated on phytoestrogen supplements, since they are currently being used for many ailments such as alleviation of post-menopausal symptoms and improvement of cognitive function [89].

This study further demonstrates the need for feed manufacturers to provide information to researchers about the estrogenic activity in different batches of feed so they can make informed decisions about whether the feed being used meets the needs of the experiment. Researchers have reported that their *in vivo* assays have been rendered insensitive to exogenous estrogen due to uncontrolled phytoestrogen levels [75, 76].

Isoflavone levels in different soy-based feeds range from below 100 ppm to above 500 ppm, and effects on experimental endpoints have been reported from isoflavone concentrations of 350 ppm and above [87]. In experiment 2 I chose to focus on the effects of genistein, since it is the major estrogenic isoflavone. However, care should be used in extrapolation of this dose-response curve since genistein, despite being more

estrogenic, is not the only isoflavone contributing estrogenic activity to a soy-based feed. When determining the concentrations of genistein to use, I wanted to have one dose that matched the Soy + feed in Experiment 1, but I also wanted to examine doses that spanned the range of isoflavone concentrations reported elsewhere in commercial feeds [87]. The data from experiment 2 show that CD-1 mice are sensitive to a wide range of concentrations of isoflavones. This data also show that fetal estradiol levels display a nonmonotonic dose response curve when exposed to increasing levels genistein, which is a typical response to estrogens [62]. Nonmonotonic dose curves have made establishing safe exposure levels of estrogenic chemicals for the public very difficult. A wide and sufficient dose range needs to be established for these hormonally active chemicals so informed decisions can be made regarding the hazards of human exposure to low doses of chemicals.

My findings show that maternal exposure to both DES and genistein produce nonmonotonic dose curves in fetal serum estradiol. However, these curves are very different in that exposure to high and low doses of genistein on a zero isoflavone background results in a U-shaped dose-response curve and exposure to high and low doses of DES on a relatively high isoflavone background results in an inverted U-shaped dose-response curve. Findings such as these make it difficult to predict effects of estrogenic chemicals because it is unclear how these chemicals work when they are both present. The data from this report suggest that the low-dose effect of DES on fetal estradiol levels could be masked or reduced if the animals were given a casein-based feed or a soy-based feed with very low phytoestrogens because their estradiol levels would

already be elevated. This again points out that researchers need to consider feed composition in their experimental design so their experiments are not disrupted.

The finding that genistein has opposite effects from DES seems appropriate due to previous reports. However, interpreting this data is complicated due to differences in background isoflavone levels. In 2000, Gupta reported that both DES and BPA showed similar effects in mouse prostate cultures by increasing EGF mRNA [90]. In 2007, Dolinoy et al. showed that exposure to the estrogenic chemical, BPA, caused hypomethylation events in the viable yellow agouti mouse, but that genistein caused hypermethylation events [91]. Future studies will need to be conducted to determine whether show similar or different against the same background of isoflavones

CHAPTER 4

ISOFLAVONE EXPOSURE IN RELATION TO FETAL ESTRADIOL LEVELS AND ADULT PHENOTYPE IN THE CD-1 MOUSE

Introduction

A recent report by Heindel and vom Saal stated that researchers should be aware that estrogenic components in animal feeds have the potential to disrupt experimental endpoints [52]. The problem is that variability in estrogenic activity of feed has disrupted experiments even when researchers have used different batches of the same feed from experiment to experiment [75, 76]. The issue of uncontrolled estrogenic activity in feed is of great concern due to finding from research that environmental estrogens are causal factors for adult disease, such as obesity [51].

The estrogenic activity in feeds comes from many different sources, but of particular interest are the phytoestrogens. Phytoestrogens are components of soy-based feeds that can vary from batch-to-batch of the same feed [52, 87]. These differences in phytoestrogen concentrations can cause a decrease in the ability of sensitive *in vivo* assays to detect estrogenic activity [88]. Researchers have called for removal of the phytoestrogens from feed used in experiments examining estrogenic chemicals [53, 88], although this problem calls for a more complex answer than simply removal of the phytoestrogens, since reports have shown that phytoestrogens are important to some

animals used in research, specifically the CD-1 mouse strain.

In 2007, Cederroth et al. showed that a diet with a total genistein equivalence of 340 ppm resulted in CD-1 mice that were lighter with an increase in lipid oxidation and locomotor activity than those mice that were fed a feed devoid of phytoestrogens [54]. In 2008, Ruhlen et al. showed that removal of phytoestrogens from feed produced mice that were heavier and showed disruptions to reproductive endpoints similar to mice that were developmentally exposed to the estrogenic chemical BPA [6]. Both of these studies show that not only are phytoestrogens important to the development of CD-1 mice, but that removal of phytoestrogens from feed causes detrimental effects.

The major class of phytoestrogens is the isoflavones. The majority of isoflavones in unprocessed soy foods are in the glycosylated form, but upon food processing or consumption, they are deconjugated [41-44]. The three major isoflavones, in their aglycone form, are daidzein, glycitein, and genistein. They are known as daidzin, glycitin, and genistin when they are present in their glycoside form. These compounds vary in their estrogenic activity, concentration in soy-based food, and their metabolism in the body [39, 83, 87].

The goal of this study was to examine the effect of maternal consumption of increasing concentrations of a mix of isoflavones on fetal hormone levels and adult phenotype. In Chapter 3 of this dissertation, I showed that exposure to genistein altered fetal estradiol in a nonmonotonic dose-response curve. The question is whether or not this is relevant to diets with different levels of isoflavones in addition to genistein, since humans and animals are exposed to a mix of isoflavones when consuming soy-based

foods. This is an important question since the addition of other isoflavones will also add metabolites such as equol, which is an estrogenic metabolite of daidzein.

Materials and Methods

For this experiment, I examined the effects of an extract that contained a mix of isoflavones called Prevastein, instead of just one isoflavone. Using Prevastein is more relevant to human exposure to isoflavones since soy-based foods contain a mix of isoflavones. I used purified, soy-based AIN-93G feeds that had the isoflavones removed in the same way as the feeds in Chapter 1, but here I added back Prevastein in different amounts to obtain feeds with a range of isoflavone concentrations. Four different feeds were made with 0, 10, 15, or 20 grams of Prevastein added to the feed, making the total isoflavone concentrations in the feeds 0 ppm, 290 ppm, 434 ppm, and 579 ppm, respectively. One additional diet was made in which the isoflavones were removed, but the extract that was obtained was simply put back in the feed (ST). I did this to control for the extraction process so that I could see whether this had an effect on the experimental endpoints I examined.

Randomly selected females from the colony were separated into feed groups for two weeks prior to time mating to insure no gross differences in energy intake across diets. Females were then time mated and kept on their respective feed throughout pregnancy and lactation. On GD 18, approximately 10 pregnant mice from each feed group were selected for measurement of maternal and fetal serum estradiol levels as well as fetal body weight. For fetal serum collection, fetuses of the same sex were pooled

within each litter.

At birth, the sex of pups was determined, and the number and weight of pups in each litter was recorded. On PND 21, mice were weaned and separated into cages with no more than 5 to a cage. At this time, approximately 25 female weanlings from the S0, S10, S20, and ST were used to measure day of first ovulation. Two immature females from each litter were placed with a stud male. When the females were visibly pregnant the stud male was removed, and the pregnant female mice were observed daily for presence of pups. First ovulation was measured by subtracting 19 days (gestation length in mice) from when pups were first observed. Since more than one mouse per litter was examined for these endpoints, litter was included as a main variable, and the F value for feed group was divided by the F value for litter to adjust for variance due to maternal effects.

At PND 120 (four months of age), six animals from S0, S10, S20, and ST were checked for glucose tolerance as described previously [92]. These animals were fasted over night (12 hours) and given 2mg of glucose per gram of body weight in 0.9% saline solution by intraperitoneal injection. Blood glucose was measured by tail nick with an Accu-Check Glucometer (Roche, Indianapolis, IN). All other animals were left until approximately PND 180 (6 months of age) for measurement of adult body weight, fat pad, and reproductive organ weight.

Results

On GD 18, there was no significant difference in maternal serum estradiol levels across all diets (Figure 8). Fetal serum estradiol levels did differ between groups. Female fetal estradiol levels were the lowest in the S20 feed, which was significantly lower than S10 and S15 ($P < 0.001$ and $P < 0.05$, respectively). Fetuses whose mothers were fed Purina 5008 had significantly lower serum estradiol levels than those fetuses whose mothers were fed the S10 feed ($P < 0.01$). Male fetuses had a similar response in that the serum estradiol levels were significantly lower in S20 and 5008 compared to S10 and S15 (S20 – S10, $P < 0.05$; S20 – S15, $P < 0.05$; 5008 – S10, $P < 0.01$; 5008 – S15, $P < 0.05$).

Female fetal weight was significantly lower in S10 when compared to females in 5008 ($P < 0.05$), S0 ($P < 0.05$), S20 ($P < 0.01$), and ST ($P < 0.05$) (Figure 9). Male fetal weights in S10 were significantly lower than males in S0 ($P < 0.05$) and S20 ($P < 0.01$). Male fetuses in S15 had the lowest weights, being significantly different when compared to S0 ($P < 0.05$) and S20 ($P < 0.01$). Fetuses did not differ in serum estradiol levels or body weight according to gender. A regression analysis on fetal serum estradiol levels and fetal body weight gave an r^2 value of 0.3623 ($P < 0.001$).

On PND 1, there was no difference in body weights between diet groups. There was a in ST with males being heavier than females ($P < 0.01$) (Figure 11). On PND 21 (weaning), mean body weight of all males was heavier than females ($P = 0.05$). Males in the S20 group had significantly lower body weight than S10 ($P < 0.05$) and showed a trend when compared to S15 ($P = 0.08$)(Figure 12).

Day of first ovulation occurred significantly earlier in females in S10 and ST when compared to S20 ($P < 0.01$) and with a trend when compared to S0 ($P = 0.06$) (Figure 13). Across all diets used there was no significant difference in number males and females produced and no significant difference in litter size in the F2 generation. There were no differences in number of offspring or PND 1 weights in the F2 generation between diets, although mean body weight of all males was heavier than females ($P < 0.01$) (Figure 14).

At PND 120 females showed no signs of glucose intolerance across all feed groups (Figure 15). At PND 180, female adult body weight did not differ significantly across feed groups (Figure 16). There was also no difference in female gonadal fat pad weight (Figure 17) or uterine weight across feed groups (Figure 18).

There were no signs of glucose intolerance for males at PND 120 (Figure 19). Males at PND 180 did not show a significant difference in body weight (Figure 20). The male gonadal fat pad weight did not differ across diet groups as well (Figure 21). All male reproductive organs, except the coagulating gland, did not differ significantly in weight across diet groups (Figure 22). The coagulating gland was the biggest in S0 with significance when compared to S10 ($P < 0.05$) and with a trend when compared to S20 ($P < 0.07$) (Figure 23).

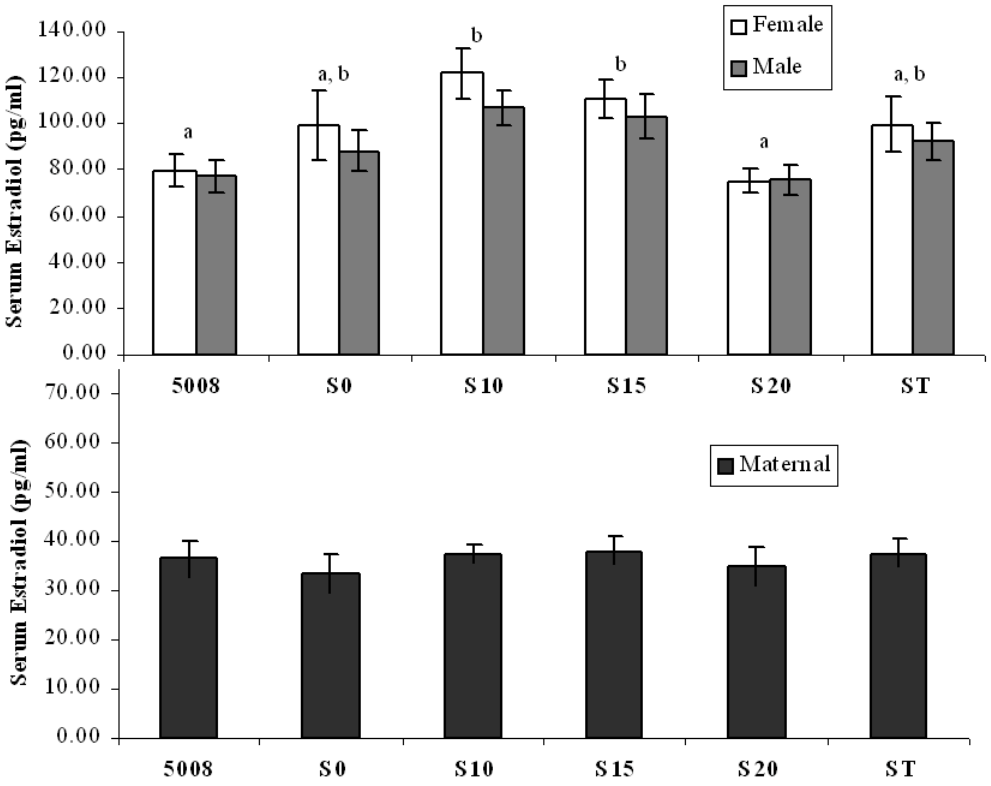


Figure 8 Mean (±SEM) serum estradiol levels (pg/ml) on gestation day 18 in pregnant females and their pooled male and pooled female fetuses from each litter fed Purina 5008 (n = 9) and 5 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 10, S10 n = 10, S15 n = 9, and S20 n = 8) or put back in (ST n = 10). Different letters designate significant differences with $P < 0.05$. Data from male and female fetuses were combined for statistical analysis, since there were no significant sex differences.

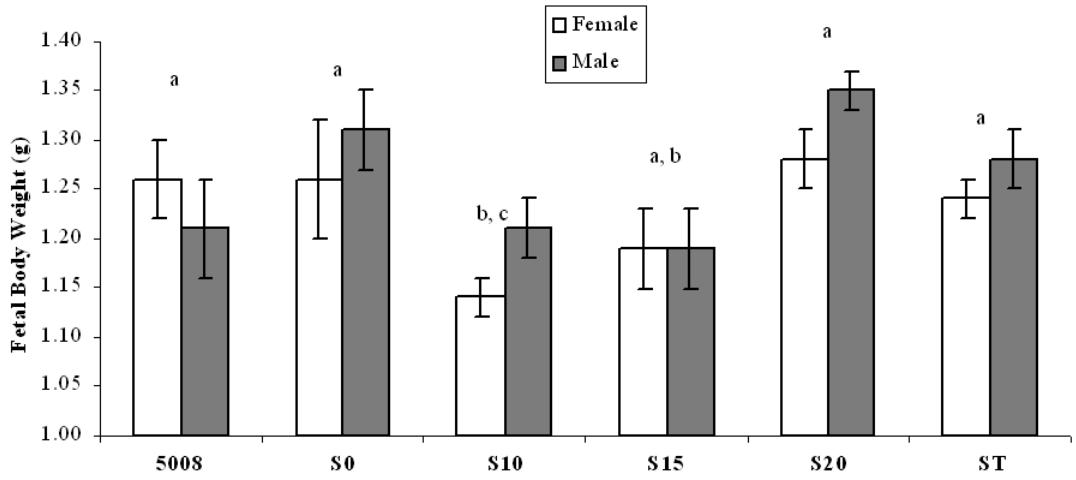


Figure 9 Mean (± SEM) fetal body weights (g) on gestation day 18 (mating = GD 0) whose mothers were fed Purina 5008 (n = 9) and 5 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 10, S10 n = 10, S15 n = 9, and S20 n = 8) or put back in (ST n = 10). Different letters designate significant differences with $P < 0.05$. Data from male and female fetuses were combined for statistical analysis, since there were no significant sex differences.

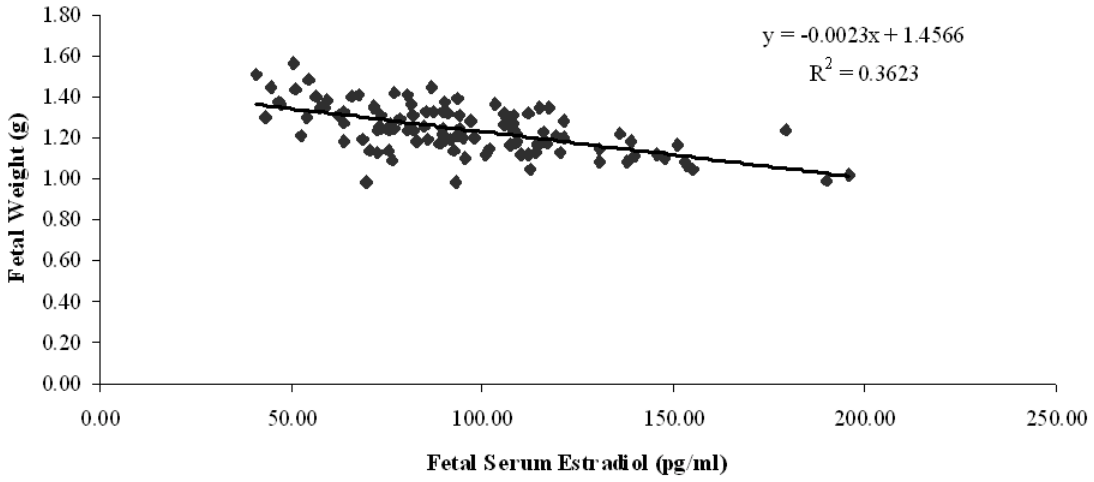


Figure 10 Regression analysis of fetal serum estradiol levels (pg/ml) to fetal body weight (g) on gestation day 18 (mating = GD 0) in fetuses whose mothers were fed Purina 5008 and 5 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0, S10, S15, and S20) or put back in (ST). $P < 0.001$.

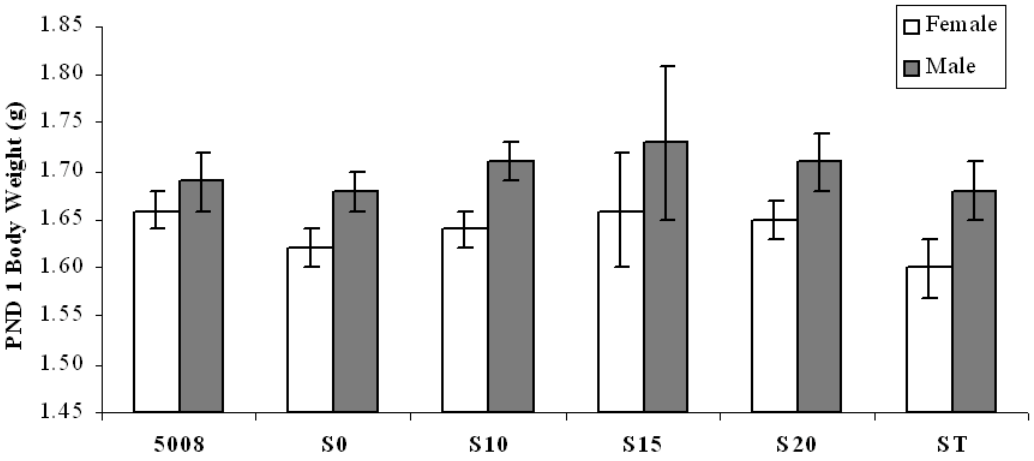


Figure 11 Mean (± SEM) postnatal body weights (g) on PND 1 of offspring whose mothers were fed Purina 5008 (n = 7) and 5 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 17, S10 n = 12, S15 n = 5, and S20 n = 18) or put back in (ST n = 14). Males in the ST group were significantly heavier than females in the ST group.

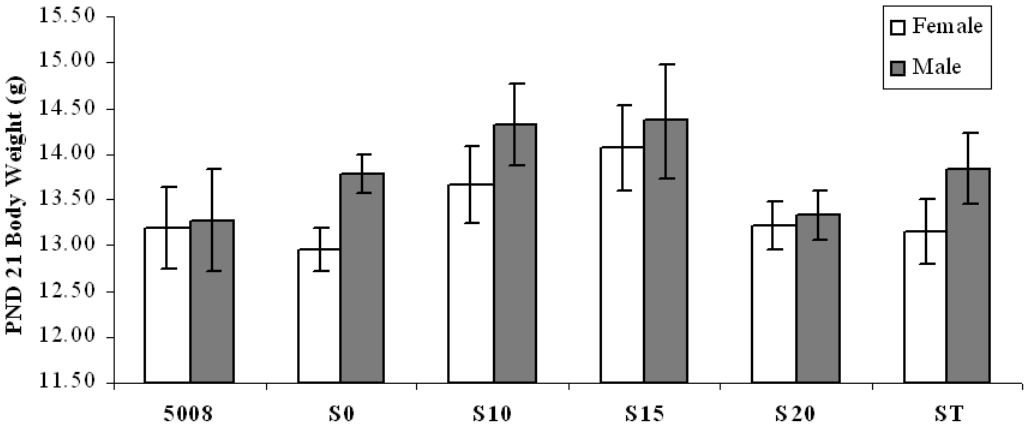


Figure 12 Mean (± SEM) weaning body weights (g) on PND 21 of offspring whose mothers were fed Purina 5008 (n = 6) and 5 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 16, S10 n = 12, S15 n = 6, and S20 n = 17) or put back in (ST n =13). Males in the S20 group had significantly lower body weight than S10 ($P < 0.05$) and showed a trend when compared to S15 ($P = 0.08$)(Figure 12)

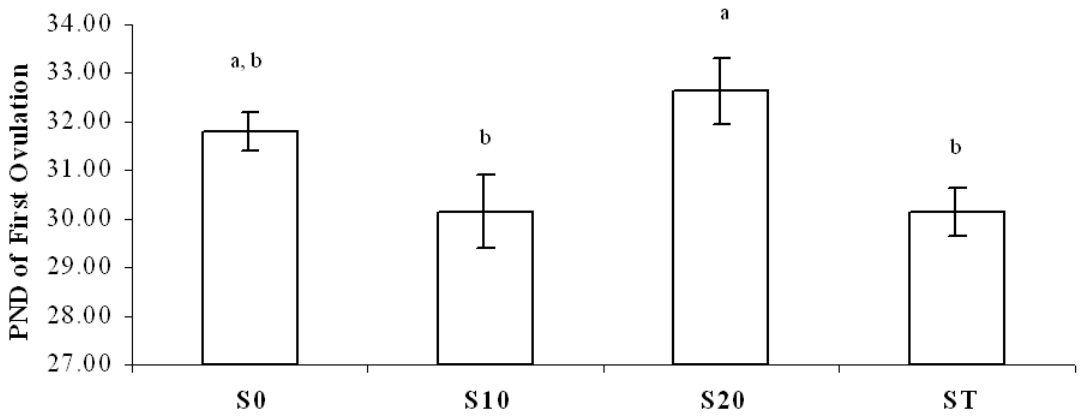


Figure 13 Mean (± SEM) PND of first ovulation of female offspring whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 29, S10 n = 20, and S20 n = 32) or put back in (ST n = 20). Different letters designate significant differences with $P < 0.05$ based on correcting for litter due to more than one female per litter was used in this experiment.

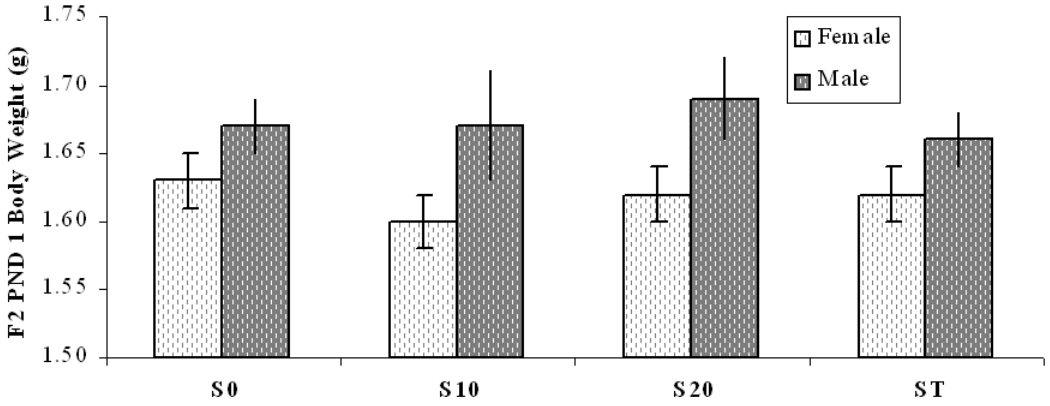


Figure 14 Mean (± SEM) postnatal body weights (g) on PND 1 of F2 offspring whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 29, S10 n = 20, and S20 n = 32) or put back in (ST n = 20). Mean body weight of all males was heavier than all females.

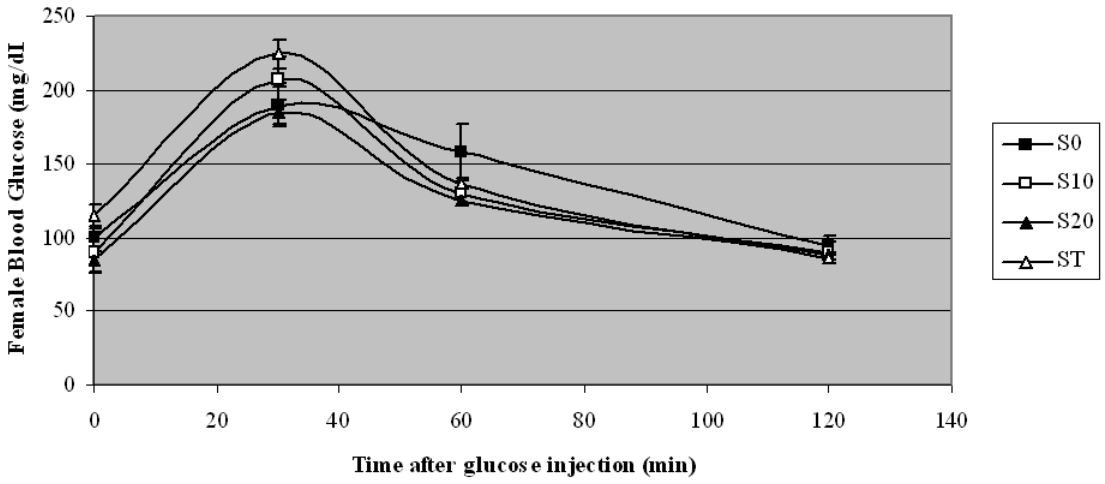


Figure 15 Mean (± SEM) adult blood glucose levels (mg/dL) of female offspring at approximately PND 180 (n = 6 per feed group) after intraperitoneal injection of 2 mg/g of glucose whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0, S10, and S20) or put back in (ST).

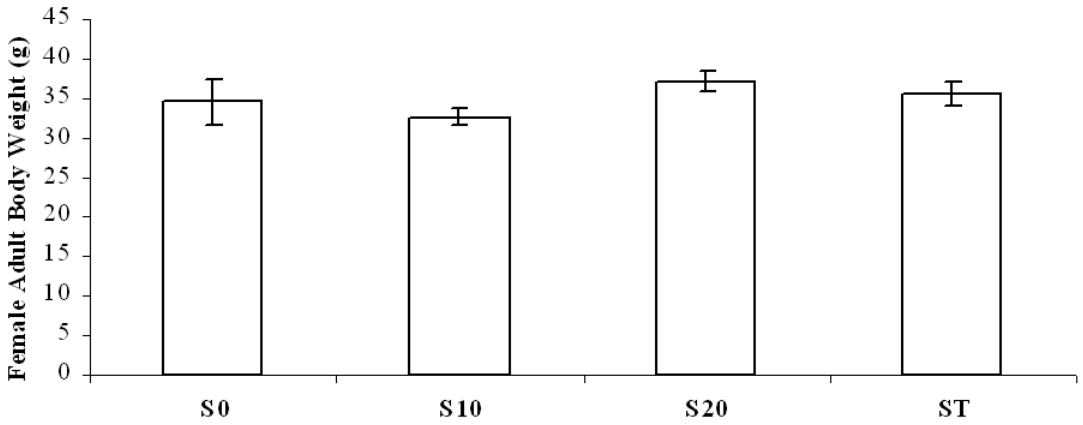


Figure 16 Mean (± SEM) adult body weights (g) of female offspring at approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 6, S10 n = 6, and S20 n = 6) or put back in (ST n = 6).

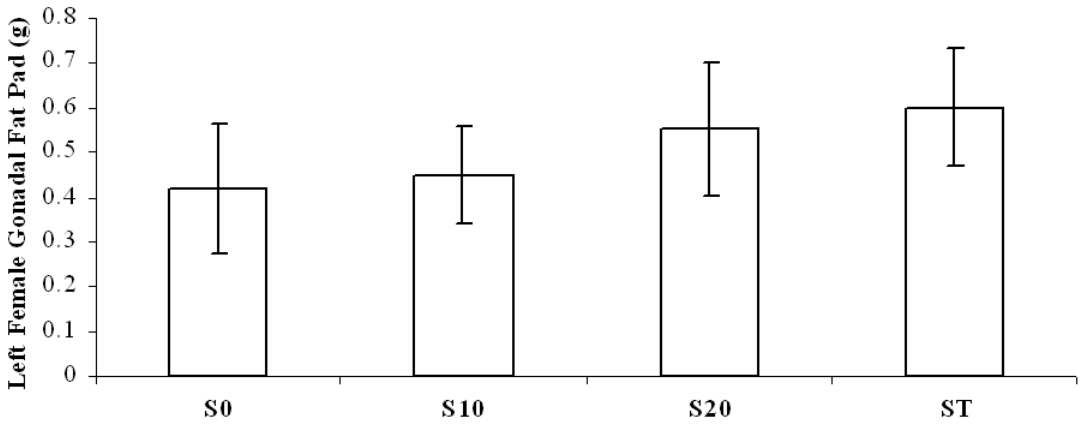


Figure 17 Mean (± SEM) left gonad fat pad weights (g) of female offspring approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 6, S10 n = 6, and S20 n = 6) or put back in (ST n = 6).

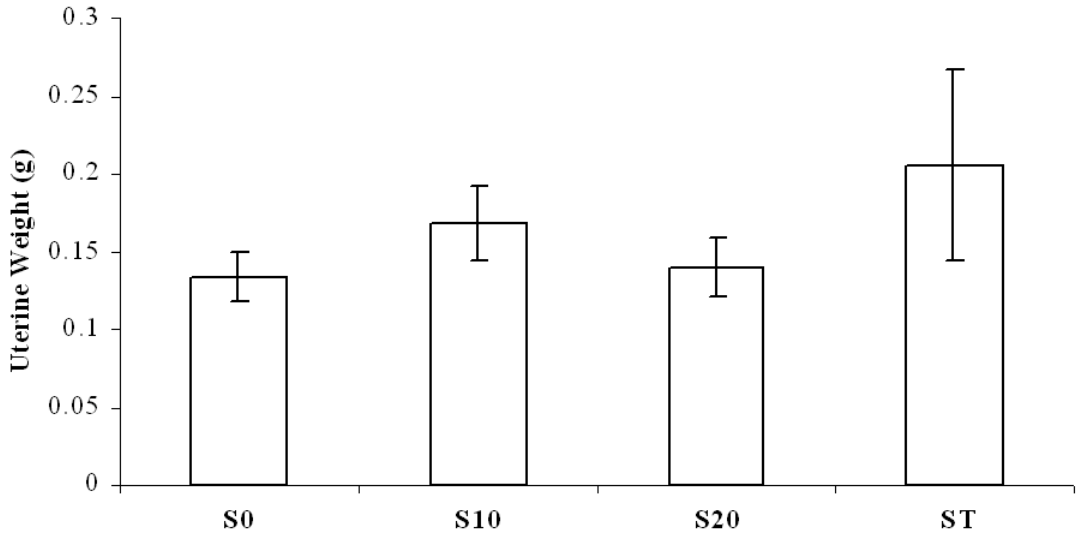


Figure 18 Mean (± SEM) uterine weights (g) of female offspring approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 6, S10 n = 6, and S20 n = 6) or put back in (ST n = 6).

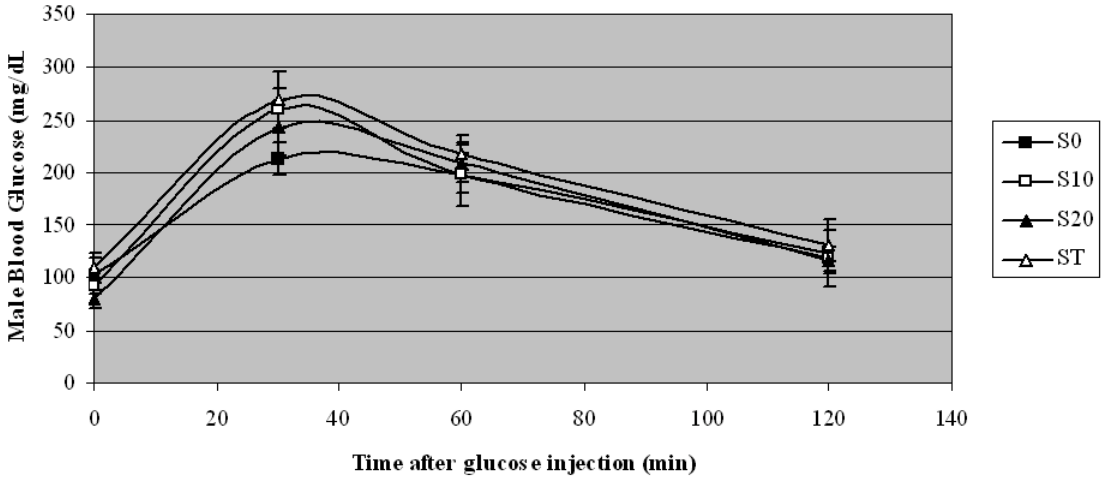


Figure 19 Mean (± SEM) adult blood glucose levels (mg/dL) of male offspring at approximately PND 180 (n = 6 per feed group) after intraperitoneal injection of 2 mg/g of glucose whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0, S10, and S20) or put back in (ST).

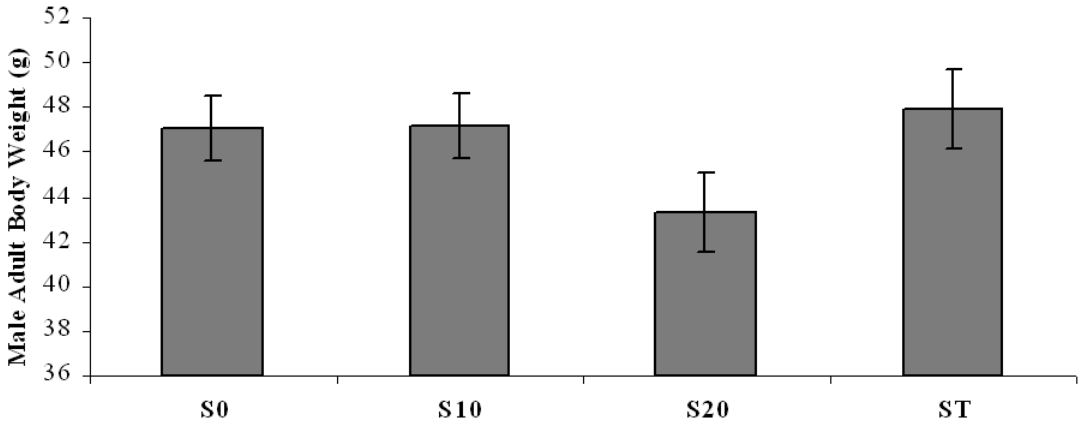


Figure 20 Mean (± SEM) adult body weights (g) of male offspring approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 10, S10 n = 10, and S20 n = 10) or put back in (ST n = 10).

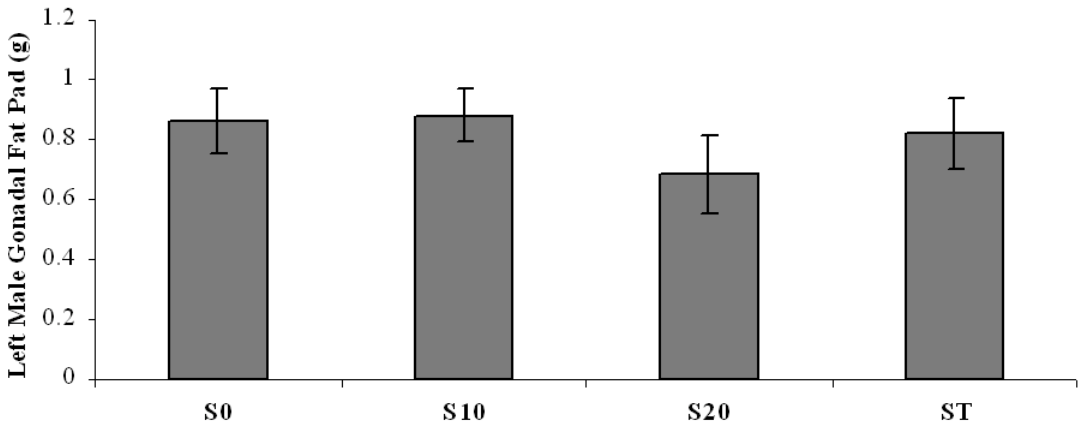


Figure 21 Mean (± SEM) left gonad fat pad weights (g) of male offspring approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 10, S10 n = 10, and S20 n = 10) or put back in (ST n = 10).

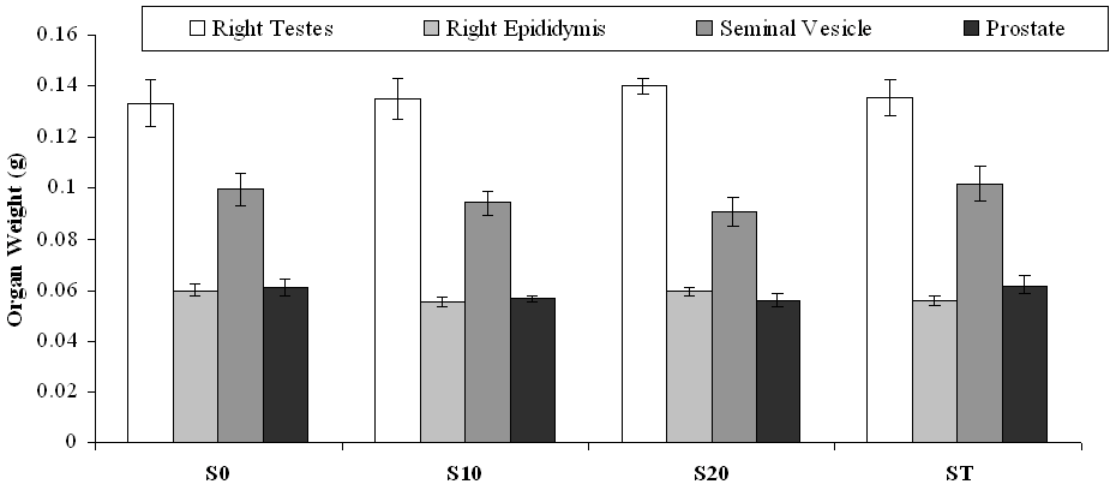


Figure 22 Mean (± SEM) organ weights (g) of male offspring approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 10, S10 n = 10, and S20 n = 10) or put back in (ST n = 10).

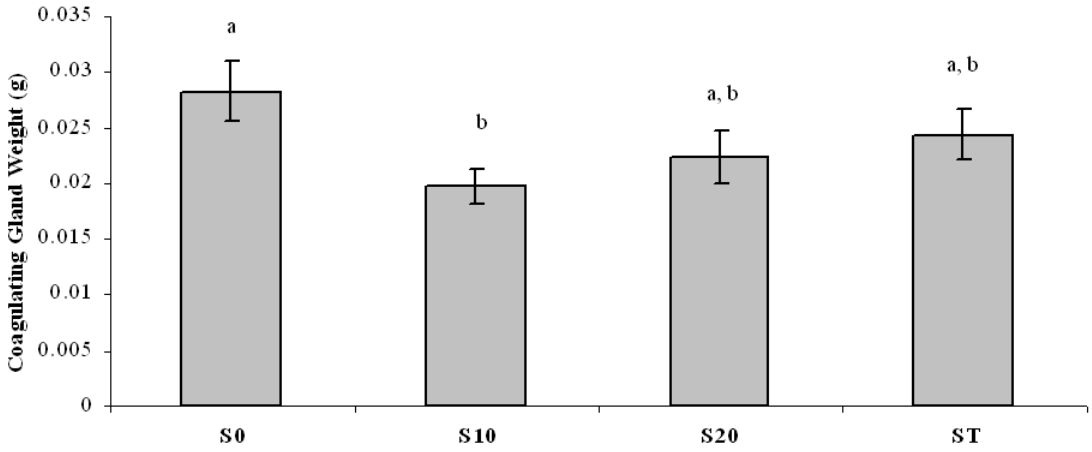


Figure 23 Mean (± SEM) coagulating gland weights (g) of male offspring approximately 6 months of age whose mothers were fed 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0 n = 10, S10 n = 10, and S20 n = 10) or put back in (ST n = 10). Different letters designate significant differences with $P < 0.05$. One male per litter was examined to control for litter effects.

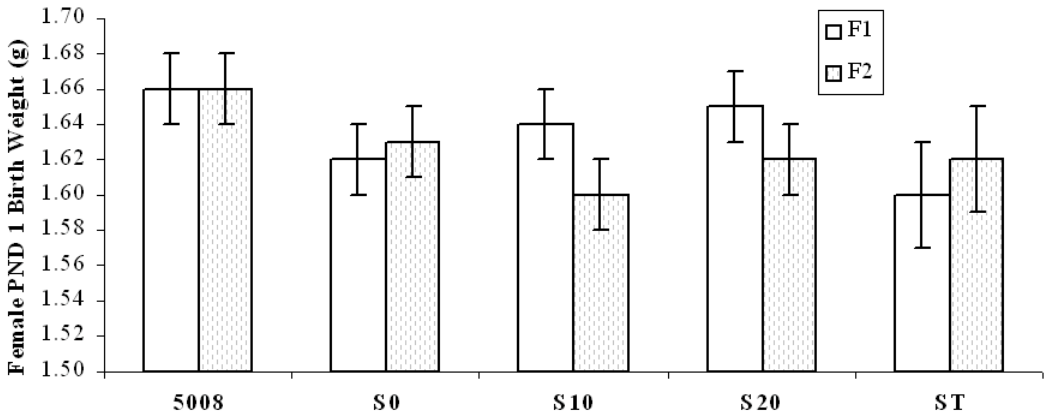


Figure 24 Mean (± SEM) postnatal body weights (g) on PND 1 of F1 and F2 female offspring whose mothers were fed Purina 5008 or 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0, S10, and S20) or put back in (ST).

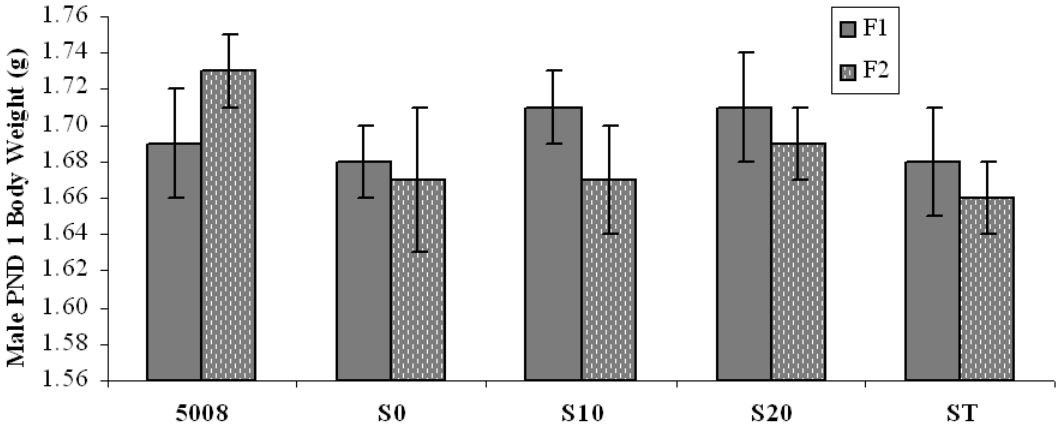


Figure 25 Mean (± SEM) postnatal body weights (g) on PND 1 of F1 and F2 male offspring whose mothers were fed Purina 5008 or 4 AIN-93G purified diets with phytoestrogens removed and either replaced with Prevastein at increasing concentrations (S0, S10, and S20) or put back in (ST).

Discussion

I report here that different concentrations of an isoflavone extract in maternal feed can result in offspring with different phenotypes. Specifically, maternal consumption of higher concentrations of Prevastein significantly reduces fetal serum estradiol levels relative to consumption of lower concentrations of Prevastein. I show that elevated fetal serum estradiol is related to reduced fetal growth regardless of maternal diet. These data also show that feeds supplemented with extracts containing different concentrations of isoflavones alter body weight at time of weaning and time of first ovulation. However, these feeds do not affect adult body weight, gonadal fat pad weight, or glucose intolerance.

These data agree with my findings from Experiment 2 in Chapter 3 in that maternal consumption of increasing isoflavone concentrations does not alter maternal serum estradiol but does alter fetal serum estradiol. However, the data presented here do not show the same shaped curve as the data reported previously. In Chapter 3, maternal consumption of increasing concentrations of genistein resulted in a U-shaped dose-response curve, while here, maternal consumption of increasing concentrations of the isoflavone extract resulted in an inverted-U dose-response curve. The difference in dose-response curves is not surprising since only genistein was used before, but the extract used in this experiment contained a mix of isoflavones, such as daidzein and glycitein. Even though daidzein and glycitein are isoflavones, they bind to the estrogen receptor with less affinity and show reduced transcriptional activation than genistein [39]. Also, daidzein and glycitein are broken down into different metabolites than genistein. This

shows that researchers need to be cautious in estimating possible outcomes of exposure from multiple isoflavones from data with feeds supplemented with only genistein.

An interesting finding from these data is there is evidence that the protein source, independent of isoflavone concentration, can disrupt experimental endpoints. Shown earlier in this dissertation and by Ruhlen et al., pregnant mice consuming a casein-based feed causes elevated fetal serum estradiol levels as well as removal of isoflavones from soy-based feed [6]. What is interesting is that adult male mice whose mothers were fed a casein-based feed were heavier, showed glucose intolerance, and had disruptions in their reproductive organ weights compared to males whose mothers were fed a soy-based diet [6]. Here I show that the adult outcomes reported by Ruhlen et al. may not be due to differences in isoflavone content but in protein source, since the soy-based feed with no isoflavones did not produce adults that were different in weight and glucose tolerance from a soy-based feed with an isoflavone extract. This suggests that the differences presented by Ruhlen et al. in the casein-based and soy-based feed were not solely due to differences in isoflavone concentrations, but rather disruption caused by some component that was present in the casein-based feed and not the soy-based feed.

This study showed that day of first ovulation of female offspring was significantly affected due to consumption of specific doses of isoflavones. The pubertal measures are a sensitive endpoint that is used to measure the estrogenic activity of exogenous compounds, since increased estrogenic exposure is related to earlier onset of puberty. In 1999, Howdeshell et al. showed that fetuses exposed *in utero* to BPA had accelerated growth and earlier onset of puberty [17]. Ruhlen et al. reported that the casein diet used

in their study caused increased fetal estradiol levels and after birth, accelerated growth and earlier onset of puberty [6]. Taken together, it seems that the changes in growth rate and pubertal timing seen in these experiments are due the alterations induced by estrogenic exposure during fetal life.

In 2007, Thigpen et al. showed that a feed spiked with increasing levels of genistein (up to 450 ppm) caused earlier day of vaginal opening in CD-1 mice [88]. Their conclusion was that isoflavones should be removed from the feed of research animals so that their assay could remain sensitive when testing other estrogenic chemicals. The idea behind this is that if the control animals begin puberty later in life, then they are more sensitive to estrogenic exposure. This conclusion is inappropriate since they exposed mice beginning at 8 days old and not throughout the life of the animal. I show here that day of first ovulation was only induced earlier from feed given throughout the animal's life containing a lower dose of the isoflavone extract (290 ppm of total isoflavones), and that puberty was delayed significantly later at a higher dose (579 ppm of total isoflavones). This shows that researchers need to be careful when extrapolating data due to significant differences in effects from exposures during different developmental time periods.

My data also show that researchers need to use caution in comparing isoflavone exposure outcomes when the protein sources are different. In Chapter 1 of this dissertation, I showed that supplementation of isoflavones on a casein-based feed does not result in the same outcome as isoflavone exposure on a soy-based feed. In 2009, Rando et al. showed that administration of soymilk caused activation of ER

transcriptional activity, but that supplementation of pure isoflavones on a casein-based feed did not induce the same response [93]. Many researchers when studying isoflavone exposure will use a casein-based feed to reduce the amount of isoflavone variability from feed and then supplement the diet with pure genistein to examine isoflavone effects. The problem with this is that, as discussed earlier, caution should be used in extrapolating results found from genistein exposure to predicted outcomes from exposure to a mix of isoflavones.

CHAPTER 5

PLACENTAL AMINO ACID TRANSPORT IN CD-1 MOUSE FETUSES USING A NOVEL CROWDED UTERINE HORN MODEL

Introduction

Intrauterine growth restriction (IUGR) is a disorder characterized by having a severely reduced fetal growth rate (weight < 5.5 lbs in gestation week 37 or after). It is a major cause of infant mortality [30]. Babies with significantly reduced birth weight are at increased risk for many adult diseases such as cardiovascular disease, type 2 diabetes, and obesity [32, 94, 95].

In discussing restricted fetal growth, it is important to distinguish incidences of IUGR from incidences of small for gestational age (SGA). These two scenarios are related since they define a fetus or newborn as significantly smaller than the majority of other fetuses or newborns. However, SGA is merely a term used to describe a fetus or newborn that is in the lower range of weights, meaning that a fetus that is SGA does not have a disturbed growth rate. IUGR is used to describe a fetus or newborn that is lower in weight due to a disruption of growth rate from any number of factors.

Factors influencing induction of IUGR are maternal body composition, maternal dietary intake, uteroplacental blood flow, placental transfer, fetal genome, and stress hormone levels [32]. In some cases the cause of altered fetal growth may seem clear

because of known predisposing factors such as maternal cigarette smoke inhalation, maternal consumption of alcohol, and maternal undernutrition [96-98]. However, for many cases, the source and mechanism of disruption are not clear. It appears that disruptions in uterine-placental blood flow are more relevant to incidences of IUGR in developed countries [31].

There are many animal models designed to induce IUGR [31]. Reviewing all of these models shows that induction of IUGR is mainly the result of fetal undernutrition. Many of the animal models restrict nutrients to the mother and thus restrict nutrients to the fetus. While this approach may induce IUGR, it seems unlikely that the mechanisms governing the induction of IUGR by maternal diet restriction are the same as the mechanisms responsible for induction of IUGR in developed countries where maternal undernutrition is rare. Other models utilize surgical manipulations, but these models use either suture or cuffs to restrict uterine or placental blood flow after implantation has occurred. While these models are very successful in inducing IUGR, caution should be used since other mechanisms may be induced by surgical manipulations during gestation.

The "crowded uterine horn" model used here induces IUGR in fetuses by causing a decrease in fetal nutrition by way of uterine-placental blood flow. In a normal pregnant mouse, there is differential uterine-placental blood flow throughout the uterus causing differential fetal weight in siblings. This is due to the bi-direction uterine artery loop [65]. When the uterus is crowded the difference in uterine-placental blood flow is amplified relative to differences in a non-crowded uterus.

Crowding of the uterus is accomplished by hemi-ovariectomizing females, which

causes the remaining ovary to super-ovulate. The same number of fetuses that would have been in both uterine horns now occurs in one, causing crowding and reduced blood flow to each placenta. The importance of the crowded uterine model for the study of the effects of fetal nutrition on fetal growth is that this model compares siblings that receive differential placental blood flow, thus controlling for genetic background. In addition, this model induces IUGR without maternal undernutrition and without post-implantation interventions.

Here I examined whether there was variation in placental amino acid transport in fetuses in a crowded uterine horn. Incidences of IUGR have been linked with a reduction in placental amino acid transport, which is an active process [68]. Relating amino acid transport to fetal weight helps give insight into the placenta's role in regulating nutrient delivery in fetuses that show restricted growth.

Methods

The "crowded uterine horn" model was used to examine whether reduced uterine-placental blood flow would affect placental transport of neutral amino acids to the fetus. Randomly selected females from the colony were paired with stud males. After females were visibly pregnant, stud males were removed. Approximately two days after giving birth, pups were removed and hemi-ovariectomy of the left ovary was performed on mothers. For anesthesia, a ketamine, xylazine, acepromazine cocktail was used. The skin was shaved, and a 1-cm incision was made over the ovary. The fallopian tube was ligated, and the ovary then removed. The surgery took approximately 2 minutes. Wounds

were closed with a wound clip, and the animals kept warm under heat lamps until fully active. After their recovery period, hemi-ovariectomized female mice were time mated with stud males, and pregnancy was confirmed by vaginal plug (gestation day 0).

On gestation day 18, pregnant dams were anesthetized with ketamine and acepromazine (12.0 mg, 0.05 mg/100g body weight) via intraperitonial injection. A polyethylene-10 catheter tempered on the inserted end was inserted into the left carotid artery and advanced to the level of aortic arch for monitoring blood pressure, heart rate, and infusing radioactive molecules. The catheter was filled with heparinized saline (100 IU/ml). After obtaining aortic pressure, I infused the radiolabeled amino acid transport substrate, MeAIB. This radiolabeled nutrient cannot be metabolized so HPLC verification of intact nutrient is not necessary for this study [69]. Briefly, a solution of the molecules were infused into the mouse through the carotid catheter over ~20 s. After a period of ~10 min to allow transport of the MeAIB, females were euthanized by cervical dislocation for dissection. Maternal blood, fetal blood, placenta, and fetal body were collected. Sera were obtained from blood samples and added to scintillation fluid for measurement of radioactivity. The placenta and fetal body needed to be solubilized with Solvable (PerkinElmer, Waltham, MA). After solubilization of samples, hydrogen peroxide was added to prevent quenching of samples. Beta emissions of samples were counted using a Coulter beta counter to determine amino acid transport.

Results

In order to compare radioactive measures between litters, data were expressed as relative to the fetus with the smallest placenta. Table 4 shows an example of how this was done for placental weight and placental DPM. As expected, regression analysis showed a significant relationship between fetal weight and fetal DPM with an r^2 value of 0.70 ($P < 0.001$). A statistically significant relationship was observed between placental weight and amount of labeled nutrient transported across the placenta to the fetus with an r^2 value of 0.24 ($P < 0.001$). There was also a statistically significant relationship observed between placental weight and the ratio of labeled nutrient concentration in the fetal blood to that of the maternal blood with an r^2 value of 0.11 ($P < 0.05$). Regression analysis significantly related fetal weight and placental DPM with an r^2 value of 0.54 ($P < 0.001$). Regression analysis showed no relationship between placental weight and fetal body weight. There was also no relationship between fetal position and fetal body weight, placental weight, fetal DPM, or placental DPM. There was no significant difference between male and female fetuses in body weight or placental weight. However, fetal DPM and placental DPM differed in that female fetuses had a slightly higher mean than males in both measurements, although this was not significant. There was a trend found between male and female fetuses in the placental DPM / placental weight ($P = 0.08$).

Table 4 Example of data transformation from absolute values to relative values. The data for each litter were sorted from the fetus with the lowest placental weight to the highest placental weight. Transformation was done for each endpoint by taking a measurement from a fetus and dividing it by the measurement from the fetus with the smallest placenta.

Fetal Wt. (g)		F Wt. Norm	Placenta Wt. (g)		P Wt Norm
1.24	/ 1.24 =	1.00	0.0627	/ 0.0627 =	1.00
1.33	/ 1.24 =	1.07	0.0733	/ 0.0627 =	1.17
1.37	/ 1.24 =	1.10	0.0771	/ 0.0627 =	1.23
1.31	/ 1.24 =	1.06	0.0788	/ 0.0627 =	1.26
1.46	/ 1.24 =	1.18	0.0803	/ 0.0627 =	1.28
1.39	/ 1.24 =	1.12	0.0889	/ 0.0627 =	1.42
1.35	/ 1.24 =	1.09	0.0901	/ 0.0627 =	1.44
1.54	/ 1.24 =	1.24	0.0910	/ 0.0627 =	1.45
1.52	/ 1.24 =	1.22	0.0956	/ 0.0627 =	1.52
1.53	/ 1.24 =	1.23	0.0972	/ 0.0627 =	1.55
1.23	/ 1.24 =	0.99	0.1010	/ 0.0627 =	1.61
1.38	/ 1.24 =	1.11	0.1026	/ 0.0627 =	1.64
1.47	/ 1.24 =	1.18	0.1046	/ 0.0627 =	1.67
1.54	/ 1.24 =	1.24	0.1056	/ 0.0627 =	1.68
1.54	/ 1.24 =	1.24	0.1084	/ 0.0627 =	1.73
1.48	/ 1.24 =	1.19	0.1458	/ 0.0627 =	2.33

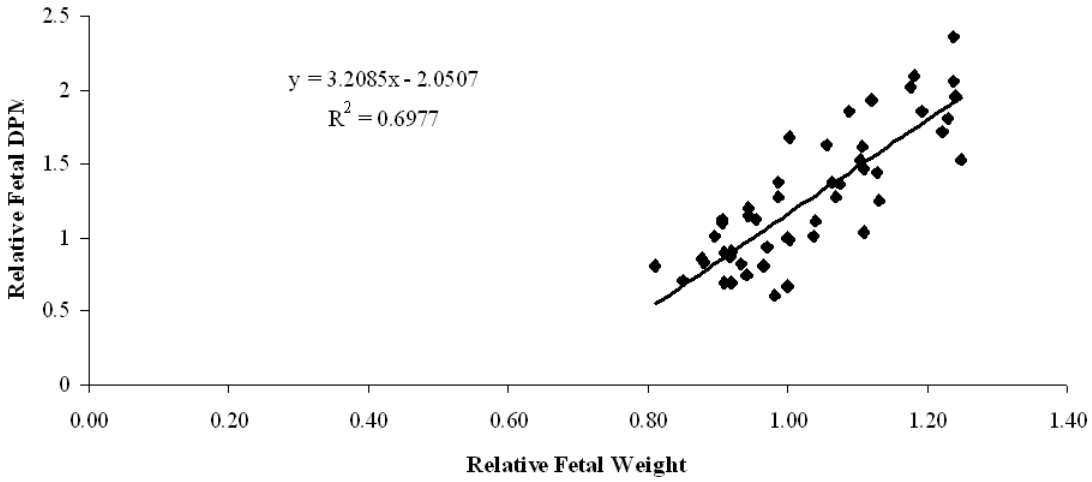


Figure 26 Regression analysis on relative fetal weight in relation to relative fetal DPM on gestation day 18 (mating = GD 0) in fetuses from a crowded uterine horn. This relationship was significant with an r^2 value of 0.70 ($P < 0.001$).

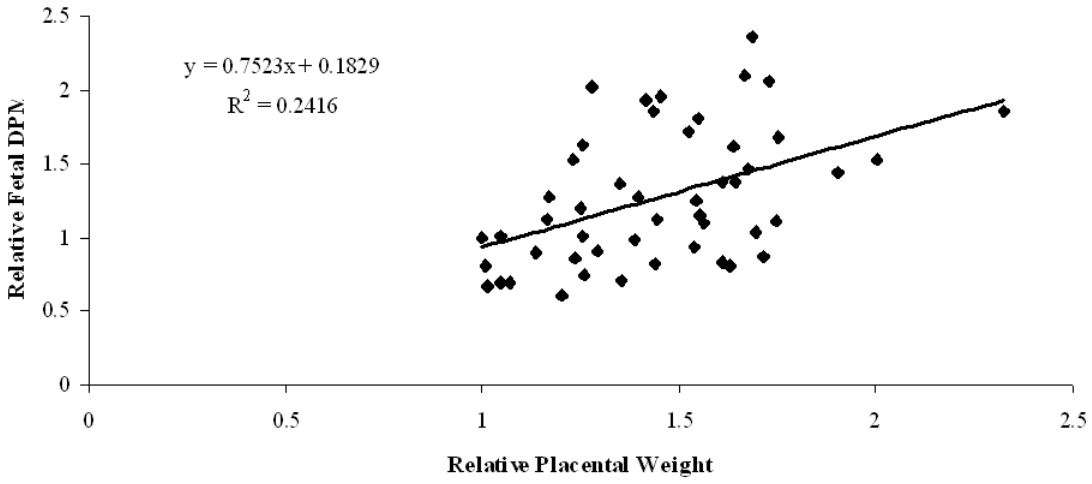


Figure 27 Regression analysis on relative placental weight in relation to relative fetal DPM on gestation day 18 (mating = GD 0) in fetuses from a crowded uterine horn. This relationship was significant with an r^2 value of 0.24 ($P < 0.001$).

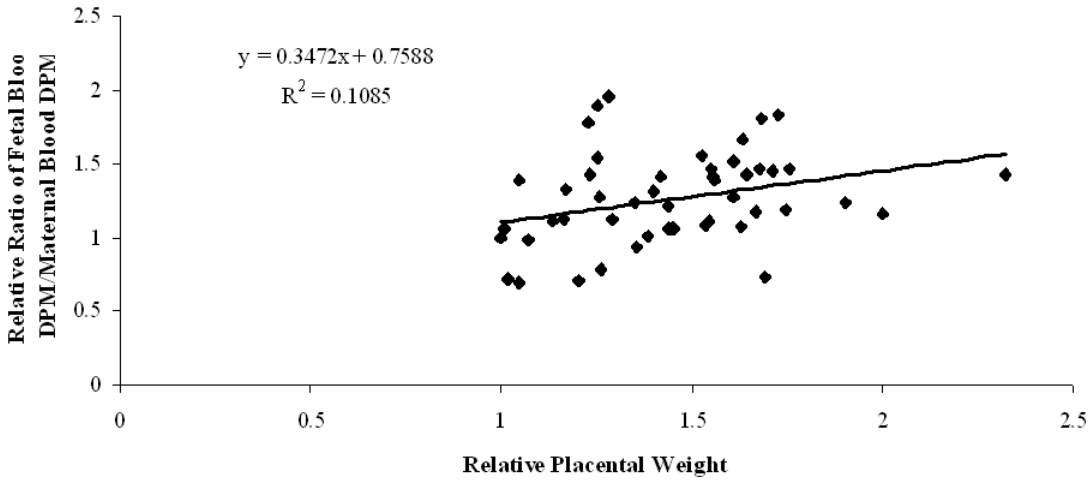


Figure 28 Regression analysis on relative placental weight in relation to relative ratio of fetal blood DPM / maternal blood DPM on gestation day 18 (mating = GD 0) in fetuses from a crowded uterine horn. This relationship was significant with an r^2 value of 0.11 ($P < 0.05$).

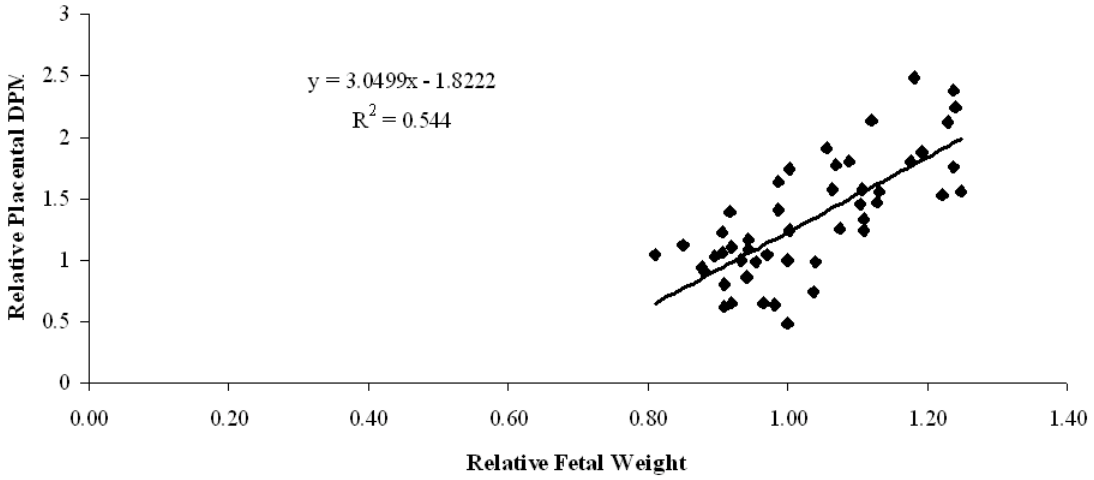


Figure 29 Regression analysis on relative fetal weight in relation to relative placental DPM on gestation day 18 (mating = GD 0) in fetuses from a crowded uterine horn. This relationship was significant with an r^2 value of 0.54 ($P < 0.001$).

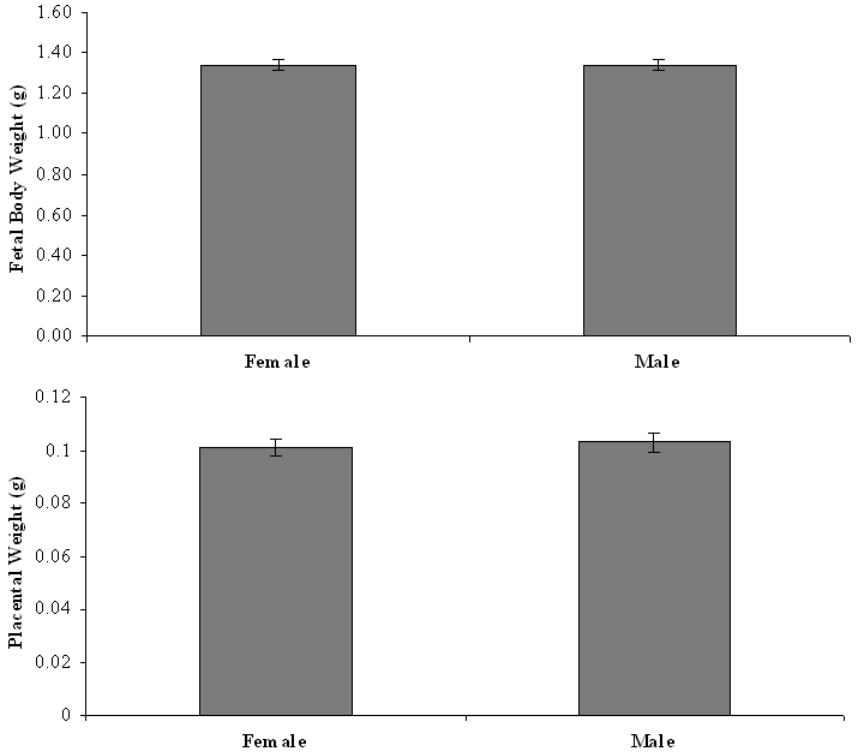


Figure 30 Mean (± SEM) fetal body weights (g) and mean (± SEM) placental weights (g) on gestation day 18 (mating = GD 0) in male (n = 31) and female (n = 21) fetuses from a crowded uterine horn.

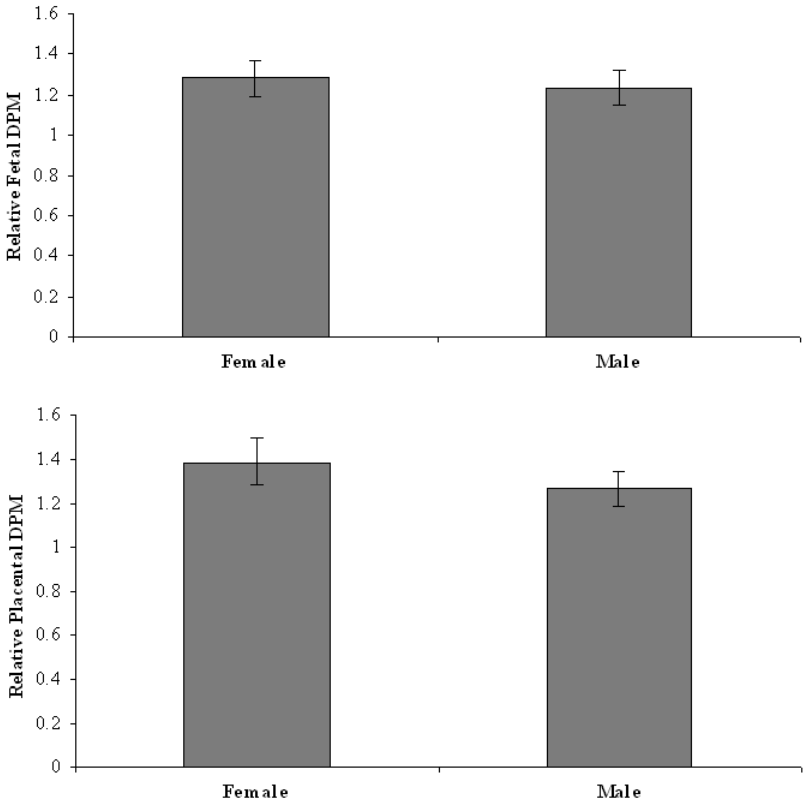


Figure 31 Mean (± SEM) relative fetal DPM measurements and mean (± SEM) relative placental DPM measurements on gestation day 18 (mating = GD 0) in male (n = 31) and female (n = 21) fetuses from a crowded uterine horn.

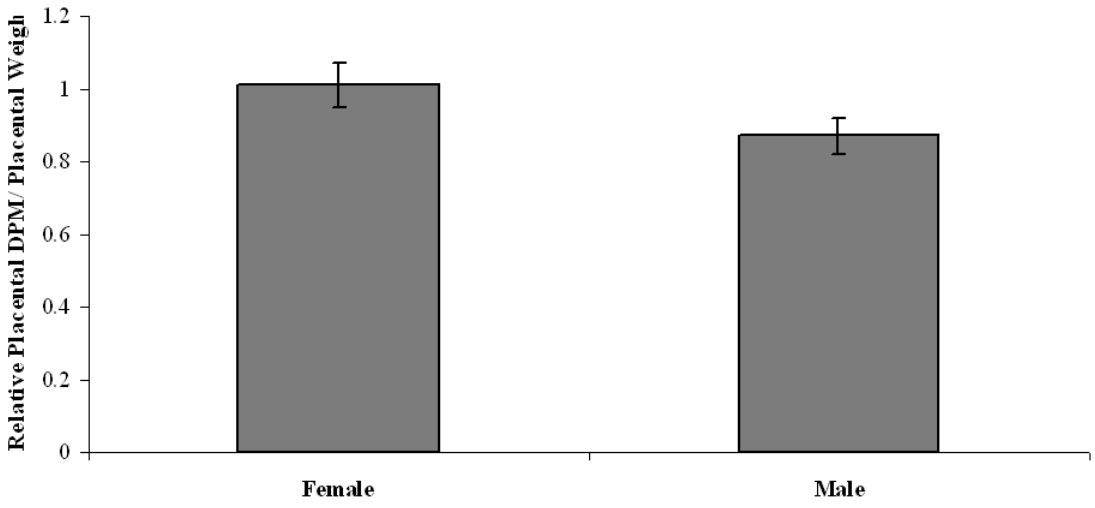


Figure 32 Mean (± SEM) relative placental DPM / placental weight on gestation day 18 (mating = GD 0) in male (n = 31) and female (n = 21) from a crowded uterine horn (P = 0.08).

Discussion

The data presented here show that there is differential transport of amino acids to fetuses within a crowded uterine horn. These data also show that the placenta influences transport of amino acids to the fetus. There was also a trend in the placenta of females showing a slightly elevated amino acid uptake over placenta of male fetuses.

It has been shown that reducing maternal nutrition, and thus fetal nutrition, by feeding low protein or low calorie diets disrupts fetal growth [31]. These studies also linked disruption of fetal growth to altered placental transport of nutrients [99]. However, these studies did not address the problem of IUGR occurrence in nutritionally sound mothers, which is typically the case in developed countries. There are many ways to alter fetal nutrition [31], but our lab has developed a novel mouse model that restricts fetal nutrition by over-crowding the uterus, causing altered uterine-placental blood flow and resulting in growth restriction. My observations show that placental transport of amino acids is not the same for every fetus in a crowded uterine horn. These data support that this model allows for modification of fetal nutrition while still having a nutritionally sound dam.

The data showing reduced placental transport of amino acids to growth-restricted fetuses are in agreement with previous reports [100]. It is important to note the range of placental weights within a litter. Some litters showed placenta with 2 fold differences in weight. This is could mean an increase in the placenta's capacity to transport nutrients to the fetus. This seems an appropriate conclusion, since I show here that an increase in placental weight is related to an increase in the fetal/maternal ratio of amino acids in fetal

blood. However, placental weight may not be the most accurate measurement of the placenta's capacity to transport amino acids due to evidence that an increase in placental surface area does not account for the increase in fetal growth late in gestation [101].

For this study, I used MeAIB, an unmetabolizable substrate of the System A transporter, to examine how amino acids were transported across the placenta to the fetus. It is important to note that there are many other amino acid transporter systems that actively transport amino acids to the fetus, each with their own specific amino acid ligands and location within the trophoblast [68]. More studies need to be conducted with this model examining other transport systems since not all of them have shown differences in relation to IUGR [101].

CHAPTER 6

GENERAL DISCUSSION

I report here that isoflavones are an important component of the CD-1 mouse diet and that removal of the isoflavones from feed or switching the feed protein source to casein causes detrimental effects. Many studies have reported that the isoflavones in animal research feed can cause disruption to experimental endpoints [52, 75, 77, 80, 87, 88]. Some animal researchers have proposed that switching from soy-based feed (high isoflavone concentrations) to a casein-based feed (low isoflavone concentrations) will eliminate any potential disruption caused by isoflavone variation in feed [53, 88].

My data show that the variability in feeds previously reported [87] has the capacity to alter fetal estradiol levels and disrupt the adult phenotype in the CD-1 mouse. However, my data show that removal of isoflavones also disrupts fetal estradiol. Elevated fetal estradiol levels results in FES [6]. The only scenario that resulted in reduced fetal estradiol levels and unaffected adult phenotype was maternal consumption of a soy-based feed with isoflavones. This means that soy-based foods should be used, but that researchers need to monitor components of these diets to avoid potential disruption.

Monitoring of feed prior to use can be done and requires performing two different analyses on the diet [52]. It involves HPLC separation of compounds and analysis of

each fraction using MCF-7 cell proliferation to determine concentrations of known estrogenic components, such as the isoflavones. The next analysis will be to use a sensitive bioassay that measures total estrogenic activity of feed, such as the MCF-7 breast cancer cell bioassay used in this dissertation. This second step is used for monitoring for unknown estrogenic components. Performing both of these analyses will help researchers use diets that are appropriate for their experiments.

It is understandable that researchers would want to avoid having to perform these analyses for every bag of feed that is used. These analyses take time for research labs to perform. Also with this approach, researchers may be stuck with unusable bags of feed if the analyses show contamination. This is why it makes sense for diet manufacturers to set up in-house analyses to measure the estrogenic components of the feeds. This way feeds can be measured, and the results reported to researchers before the feeds are purchased.

Although, with reporting concentrations of estrogenic compounds in feed comes the fact that researchers will then have a range of concentrations to pick from when ordering feed. This raises the issue of whether all animal models should have a universal concentration of estrogenic components or if certain concentrations are acceptable for specific animal models and others not. It has been suggested that an isoflavone free diet be used in animal models used to measure effects of exogenous estrogenic chemicals [53, 88]. The reason for this is that both Kanno and Thigpen reported that when isoflavones were removed, their *in vivo* assays were more sensitive. The two assays used in these two reports were measuring uterine weight gain [53] or measuring time of vaginal

opening [88] in response to concurrent exogenous estrogenic chemical exposure. One could argue that if the only endpoint being measured was either uterine weight gain or time of vaginal opening then feeds with no isoflavones could be used at the time of testing. However, my data and others show that these and other endpoints are significantly disrupted when isoflavones are removed from feed throughout an animal's life [6, 57].

Although researchers need to consider estrogenic components, my data and others show that other factors need to be considered as well. In 2007, a report showed that, compared to a soy-based feed, maternal consumption of a casein-based feed resulted in offspring that had decreased energy expenditure and increased body weight and fat percentage [54]. In 2008, Ruhlen et al. showed that switching from a soy-based feed to a casein-based feed resulted in elevated fetal estradiol levels with subsequent disruptions in reproductive organs and fat and glucose regulation in the adult [6]. My data show that removal of isoflavones from a soy-based feed results in elevated fetal estradiol levels, but that it does not cause the same disruptions in adult weight or reproductive organs as the two studies above. This suggests that it is not just the elevated fetal estradiol levels that are influencing the disruptions found in the adult phenotype, but that there is another component of the casein-diet that is disruptive to the CD-1 mouse.

Another factor that researchers need to consider in both designing their own experiments and interpreting others is whether the isoflavones that are in feeds used in an experiment were put in from natural sources or supplemented artificially. In 2009, Rando et al. showed that supplementation of pure genistein on a casein-based feed did not have

the same effect on ER transcriptional activity as consumption of soymilk on a casein-based feed. I showed in Chapter 1 that supplementation of isoflavones on a casein-based feed resulted in the same elevated fetal estradiol levels that were obtained from a casein-based feed with no isoflavones. This may also explain the differences in my data and those reported by both Cederroth and Ruhlen [6, 54]. Ruhlen found weight differences at birth, while I did not (Cederroth did not report birth weights). Neither of these reports found differences in weaning weight, but they did find a significant difference in adult body weight and fat weight. I found significant differences in weaning weights, but I found no differences in adult body weight and fat weight. The difference in the soy-based feeds used in these experiments was that theirs contained isoflavones in their natural matrix while my feeds contained Prevastein, an isoflavone extract. This is an important issue that researchers need to be aware of in designing experiments since isoflavone activity could change whether or not the isoflavones are present in their original matrix.

I report that supplementation of feed with genistein produces a different dose-response curve in fetal estradiol levels than supplementation of feed with an isoflavone extract. Isoflavones are a class of chemicals that include several different compounds, each with its own ER binding affinity and ER transcription activation ability [39]. These compounds are weakly estrogenic with genistein having the strongest binding affinity, following by daidzein and then glycitein. These compounds are SERMs due to stronger binding to estrogen receptor beta, with genistein having similar binding affinity to estrogen receptor beta as estradiol [39]. Isoflavones, upon consumption, are converted to

metabolites that are also estrogenic, with some metabolites being more potent in estrogenic activity than their precursors [39]. This is the case with daidzein's metabolite equol. These findings give insight as to why I found a difference in the dose-response curve in fetal estradiol levels resulting from maternal consumption of genistein versus an isoflavone extract.

Differences in the nonmonotonic dose-response curves were also seen between DES and genistein. Maternal exposure to a low dose of genistein reduced fetal estradiol levels, but as the concentration of genistein increased, fetal estradiol levels became elevated. In contrast, a low dose of DES stimulated fetal estradiol levels while a high dose decreased estradiol levels. In 2007, Dolinoy et al. showed genistein and BPA had opposite effects in the viable yellow agouti mouse [91]. Taken together, these studies support my finding that DES and genistein have opposite effects on fetal estradiol levels. The issue that different estrogenic chemicals have different effects needs to be addressed. There is still debate on how estrogenic chemicals behave when present together, especially when the dose-response curves are different from each other.

I report in this dissertation that fetal exposure to increasing concentrations of isoflavones causes an alteration in timing of puberty. Puberty was measured by observing when the first mating occurred instead of vaginal opening since, in mice, vaginal opening and first ovulation are not highly correlated [17]. The timing of puberty is sensitive to estrogenic influence, which is why it is used to measure estrogenic activity of exogenous compounds [88]. The sensitivity is evident in that a female that is exposed *in utero* to a low dose of BPA (2.4μg/kg/day) begins puberty much earlier than controls

[17].

There have been studies concerning whether the negative feedback system for gonadotropin regulation of gonadal steroids is operational in the fetus. In 1974, Stiff et al. showed LH concentrations indicative of negative feedback loop control in male, but not female, fetal mice [102]. In 1985, Resko et al. showed gonadotropin secretion was under negative feedback control in rhesus monkeys [103]. In 1998, Miller et al. showed that GnRH immunoneutralization caused an increase in FSH secretion in male fetuses relative to controls, and there was no difference in female FSH secretion relative to controls [104]. These studies support that negative feedback control of steroid hormones is active in the male fetus but not in the female fetus. In my experiments estrogenic exposure caused alterations in fetal estradiol levels similarly in male and female fetuses. Since negative feedback is active in one gender and not the other, it seems unlikely that the elevation in fetal estradiol levels seen here is achieved through disruption of the negative feedback loop.

A more probable pathway that exogenous estrogenic chemicals could take to alter fetal estradiol levels is through the altered production or degradation of estradiol in the fetus. Aromatase is the enzyme that converts testosterone to estradiol. Alpha fetoprotein is the serum binding molecule for estradiol that binds will high affinity. Alpha fetoprotein is thus the major binding molecule of estradiol in the fetus. Investigating aromatase and alpha fetoprotein activity will give more information about how the serum estradiol levels being altered by either production (aromatase) or decreasing degradation (alpha fetoprotein) of estradiol.

The last part of this dissertation focused on examining whether amino acid transport varies in different placenta within a crowded uterine horn. The crowding is a result of superovulation of the hemi-ovarectomized mother. The "crowded uterine horn" model is meant to study mechanisms of IUGR since it induces growth restriction in some fetuses and not others within the same uterine horn. This model induces IUGR by reducing fetal nutrition while still keeping maternal nutrition intact. There are many models of IUGR [31], but the "crowded uterine horn" model is more relevant to incidences in developed countries since maternal undernutrition is unlikely to happen.

IUGR is a clinical term used to describe fetuses that do not realize their full growth potential. One-third of IUGR births are related to genetic factors and the other two-thirds are environmental factors [105]. Placenta transport of some nutrients is altered in fetuses with IUGR, such as amino acid transport [68]. It has been suggested that the placenta acts as a nutrient sensor for the fetus by regulating the transport of nutrients in IUGR fetuses [70]. There seems to be evidence for this since reduced transport of amino acids was observed before a reduction in fetal growth [99].

Since both exposure to exogenous estrogenic compounds and reduced fetal nutrition are factors that influence fetal growth, it is important to examine their effects when combined due to them possibly acting through different mechanisms. This can be accomplished by using the "crowded uterine horn" mouse model to examine how exposure to exogenous estrogenic chemicals, such as isoflavones and DES, affects fetal growth in sibling fetuses with IUGR, macrosomia and normal growth. It will be important to also measure uterine-placental blood flow and nutrient transport in relation

to exogenous estrogenic chemical exposure to investigate whether there is an overlap in each factor's influence on fetal growth. This is critical since humans are typically exposed to multiple factors throughout life that have, individually, been related to adult obesity. The worry is that a fetus with reduced fetal growth due to nutrition deprivation will be even more susceptible to fetal growth restriction if it is exposed to increased estrogen.

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VITA

James Kirkpatrick was born on June 18, 1983. He has lived in Imperial, MO through the majority of his adolescent life. Upon graduating from Seckman High School in May 2001, he began attending the University of Missouri – Columbia in the fall of that same year. In 2003, he began working with Dr. Frederick vom Saal as an undergraduate research assistant. He graduated with a B.S. in Biological Sciences in May 2005 and continued his work in Dr. vom Saal's laboratory by beginning a Ph.D. project immediately following. Also in May 2005, James became a husband to his wife, Sarah Kirkpatrick. In January 2009, he became a father with the birth of his daughter, Grace Elizabeth Kirkpatrick. After completing his dissertation, James plans on attending the Saint Louis University to attend an M.D.