EFFECTS AND INTERACTIONS OF ENDOCRINE DISTRUPTING CHEMICALS AND DIET ON THE MOUSE REPRODUCTIVE SYSTEM

A Thesis Presented to the Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment
Of the Requirements For the Degree

Master of Arts

By
MAREN BELL JONES

Dr. Frederick vom Saal, Thesis Supervisor

MAY 2007

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

Effects and Interactions of Diet and Endocrine Disrupting Chemicals
On the Mouse Reproductive System

Presented by Maren Bell Jones
A candidate for the degree of Master of Arts

And hereby certify that in their opinion it is worthy of acceptance.

Dr. Frederick vom Saal

Dr. Joel Maruniak

Dr. Donald Tillitt

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Dr. Fred vom Saal for his infinite patience, knowledge, and trust in my work. Many thanks also go to my fellow graduate students Ben Coe, James Kirkpatrick, Brittany Angle, Davide Ponzi, and Katie Hurrelmeyer who were not just colleagues in the lab, classes, and teaching, but also great friends. My work would not have been possible without the technical expertise, advice, and assistance from Drs. Julia Taylor, Jiude Mao, Cathy Richter, Susan Nagel, Wade Welshons, and Cindy Besch-Williford. I also thank my committee members Dr. Joel Maruniak and Dr. Don Tillitt for their interest in my academic progress and research.

TABLE OF CONTENTS

Acknowledgements ii
List of Tables iv
List of Illustrations v
Chapter One: Introduction and Background 1
Chapter Two: General Procedure
- **Animals, Husbandry, Mating 5**
- **Assignment to Diet Groups 6**
- **Timed Mating & Fetal Blood Collection 6**
- **Production of F_1 Animals 7**
- **Statistical Analysis 7**

Chapter Three: Purina 5001/5008 vs 5K96C High/Low
- **MCF-7 Breast Cancer Cell Proliferation Assay 8**
- **Fetal Serum Estradiol Levels 9**
- **Pre-pubertal Uterotrophic Response Assay 9**
- **Age of Onset of Fertility 10**
- **Body and Organ Weights of Adult Males and Females 11**
- **Glucose Challenge of Adult Males and Females 12**

Chapter Four: Purina 5008 vs Harlan 2018 and 2016
- **Animals, Husbandry, Mating 13**
- **Assignment to Diet Groups 13**
- **Timed Mating & Administration of DES 14**
- **Fetal Serum Estradiol Levels 14**
- **Production of F_1 Animals 15**
- **Adult Uterotrophic Response Assay 15**

Discussion 16
Appendix
- **Tables 21**
- **Figures 27**

Works Cited 38
Vita 41

LIST OF TABLES

Table **Page**

1. **Ingredients and Components of Purina 5001, 5008, and 5K96C Diets..........20**
2. **Average Genistein Equivalents of the Diets...22**
3. **Average Body and Organ Weights of F1 Females...23**
4. **Average Body and Organ Weights of F1 Males...24**
5. **Blood Glucose Levels Following a Glucose Challenge....................................25**

LIST OF ILLUSTRATIONS

Figure **Page**

1. **Chemical Structure of Isoflavones ... 26**
2. **Average Genistein Equivalencies of the Purina Diets ... 27**
3. **Fetal Serum Estradiol Levels on Purina Diets ... 28**
4. **Pre-pubertal Uterotrophic Response ... 29**
5. **Timing of the Onset of Puberty in F1 Females ... 30**
6. **Female Gonadal Fat Weights at 2 Months of Age ... 31**
7. **Female Serum Leptin Levels at 2 Months of Age ... 32**
8. **Fetal Serum Estradiol Levels on Purina and Harlan Diets ... 33**
9. **Adult Uterotrophic Response ... 34**
10. **Female Gonadal Fat Weight at 5 Months of Age ... 35**
11. **Female Body Weights at 5 Months of Age ... 36**

CHAPTER 1

Introduction and Background

Recent studies have indicated that a number of ubiquitous chemicals found in the environment (and subsequently in the tissues of humans and wildlife) are linked to a myriad of health concerns, including reproductive abnormalities and increases in infertility, cancer, and obesity via their actions as exogenous hormones (Simonich and Hites 1995, Sasco 2001, Swan 2003, Newbold 2005, Lee 2006). Because of the importance of evaluating the effects of these chemicals in living model organisms, maintaining the sensitivity of various bioassays as indicators of hormone action is critical.

Controlling variability within any experiment is always important, but it is especially important when trying to determine very sensitive physiological endpoints. The diet of the model organism is a vital part of controlling variability in experiments evaluating these endpoints. Diets fed to laboratory research animals often have naturally occurring hormonally active compounds such as phytoestrogens that can act as estrogen agonists. Therefore, if there is interference due to varying background levels of phytoestrogens in the ingredients of the diets, then they may have the potential to alter the body's response to the chemical of interest and possibly decrease the sensitivity of the response (Thigpen 2003).

The studies described here concern the effects caused by differences in levels of soy-based phytoestrogens in diets of laboratory animals. Much research and public interest have been recently focused on the possible health benefits and drawbacks of a diet supplemented with soy protein in humans, although other hormonally active compounds exist in the diets as well. These include, but are not limited to, coumestrol in alfalfa, lignans in flaxseed, and certain fungal mycotoxins (Adams 1995, Setchell 1998). The phytoestrogens as a class are often described as "weakly estrogenic," since while they are structurally similar to 17ß-estradiol (Figure 1) and they have a lower binding affinity for the estrogen receptor (Milligan 1998). Nonetheless, there is strong evidence for their estrogenic action *in vivo* and *in vitro* (Mäkelä 1995, Mäkelä 1994).

A classic example of the potency of phytoestrogens in plants being consumed by animals with profound reproductive effects is "clover disease" that presented in female sheep from Perth, Australia in the 1940s that grazed on pasture with red clover (Bennetts 1946). In permanent clover disease, ewes have difficulty conceiving, maintaining their pregnancies, and experience dystocia during lambing due to permanent changes in the reproductive tract and the neuroendocrine system from the influence of the phytoestrogens in the red clover. Since first identified more than 60 years ago, more than one million sheep in Australia are estimated to be permanently infertile due to the estrogenic effects of the phytoestrogens in the red clover (Adams 1990). This naturally occurring phenomenon emphasizes the importance of understanding the interactions between hormonally active dietary components and the reproductive system in animals and humans.

Although the soybean plant originated in Asia, its widespread cultivation in North America and elsewhere has lead to its incorporation as an inexpensive source of protein in feed designed for food production, companion, and laboratory research animals. However, because of the known estrogenicity of the phytoestrogens in soy and other common components in research laboratory animal diets, there is growing concern that the diets fed to laboratory research animals in studies in the fields of environmental toxicology and endocrine disruption may be affecting the sensitivity and results of various endpoints of commonly used bioassays. For example, in studies conducted with mice and rats, diets formulated with soy and other phytoestrogen containing ingredients have been reported to influence results in toxicology and endocrine disruption research (Thigpen 2002, Ashby 2000, Thigpen 1999, Boettger-Tong 1998).

Most commercially available rodent diets from manufacturers, such as those made by Purina Mills and Harlan Teklad used in the experiments described in this paper, are often referred to as "closed" since their ingredients are released to the public, but the exact components in the diets are either proprietary trade secrets or unknown. Conversely, the exact components of "open" diets are known and available to researchers and the public. The nutrients in the diets the animals consume are typically assumed to be stable in a "constant nutrition" formula with presumably the same amount of calories, fat, protein, fiber, and other relevant nutritional levels but different levels of the various ingredients from lot to lot. Alternatively, "fixed formula" diets should not vary from lot to lot in terms of ingredients used. Soy protein is used in most laboratory animal diets designed for rodents, as it is typically inexpensive and considered nutritionally complete. More recently, because of concerns over phytoestrogen levels in feed causing potential

interference with the results of estrogen sensitive bioassays, milk-derived casein is being marketed as an appropriate protein source for diets conducted in toxicology and endocrine disruption research, as it lacks phytoestrogens such as genistein and daidzein in soy-based diets. In theory, a casein diet should mitigate any interference from varying background levels of phytoestrogens in soy.

In a previous study conducted in our laboratory, two types of commercial rodent diets manufactured by Purina Mills (soy-based PMI 5008 and 5001 and casein-based PMI 5K96C) were compared with particular attention to effects of body and gonadal fat weight, uterotrophic response in pre-pubertal females, response to a glucose challenge, and reproductive organ weights in the males. In the follow-up study described below, two batches of PMI 5K96C were found to have markedly different profiles in terms of estrogenic activity as determined by the MCF-7 breast cancer cell bioassay. The goal of this follow-up study was to evaluate if a diet that was assumed to be stable in terms of nutrition levels would yield different results if the level of phytoestrogens were different.

In addition, our second aim was to examine differences in fetal and adult phenotype endpoints after a prenatal exposure to the synthetic estrogen diethylstilbestrol (DES) while the animals were maintained from conception through adulthood on different commercially available soy and casein based diets with varying levels of phytoestrogens (soy-based PMI 5008, Harlan Teklad 2018, and casein-based Harlan Teklad 2016). For this study, we focused mainly on the female offspring and the endpoints of interest were the fetal serum estradiol levels, body and gonadal fat weight, and adult female uterotrophic response to estradiol following ovariectomy.

CHAPTER 2

GENERAL METHODS

Animals and husbandry

The CD-1 outbred stock of mice (*Mus musculus domesticus)* was chosen for its widespread use in environmental toxicology research. CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) in 1998 and were maintained as a closed outbred colony with periodic replacement of animals used in the breeding colony. Animals were housed in standard (11.5 × 7.5 × 5 in) polypropylene mouse cages (Lab Products Inc., Maywood, NJ) on corncob bedding in an AAALAC accredited facility. Water was purified by ion exchange followed by a series of carbon filters and was supplied *ad libitum* in glass bottles. Rooms were maintained at 25 ± 2°C under a 12:12 light:dark (L:D) cycle. The University of Missouri Animal Care and Use Committee approved all procedures, which conformed to the NIH Guide for the Care and Use of Animals.

Assignment of Animals to Diet Groups: PMI 5008/5001, "5K96C High" and "5K96C Low" Phytoestrogen Feeds

Dams for this study were derived from a breeding colony where pregnant and lactating females were fed soy-based PMI 5008 breeder chow (Purina Mills Inc., St. Louis, MO). After weaning on postnatal day 21, these females (as well as males used as

colony studs), were fed soy-based PMI 5001 maintenance chow and housed up to 4 per cage until the beginning of the experiment. Adult females were randomly assigned to a diet group one week prior to being time mated. Diets examined were soy-based PMI 5008, soy-based PMI 5001, and casein-containing PMI 5K96C. From the beginning of this study to its conclusion, the diet groups were all maintained on the feed derived from the same lot from the manufacturer to decrease variability. A summary of the nutrients and ingredients in the feeds used in this study is in Table 1.

We examined two different batches of the PMI 5K96C diet. One batch used had very low estrogenic activity (referred to as 5K96C-L) and one lot had higher estrogenic activity (referred to as 5K96C-H) that had about 50% of the activity of the soy-based PMI 5001 as measured by the MCF-7 cancer cell assay (Table 2, Figure 2). All offspring from mothers fed 5K96C and 5001 remained on their respective diets throughout the study, while the PMI 5008 offspring were transitioned to PMI 5001 (with lower percent fat) after weaning.

Timed Mating and Maternal and Fetal Blood Collection

Timed mating of the females involved pairing females with a male of known reproductive competence for approximately four hours starting the last two hours of the twelve hour dark phase of the 24 hour light:dark cycle (approximately 0900 with lights on at 1100). Mating was considered gestation day (GD) 0 and was verified by the presence of a vaginal plug. Pregnant females were housed three per cage until GD 17 when they were housed individually in preparation for parturition. On GD 18, a subgroup of 7 litters from each diet group was collected for fetal serum hormone levels.

Pups were delivered via caesarean section, and blood was collected by decapitation. Serum from males and from females was pooled, so each litter contributed one hormone value per sex. Serum was then frozen for later analysis of estradiol.

Production of F_1 Animals for Postnatal Studies

Litters produced by mothers on different types of feed were reduced to 12 pups on the day of birth. Pups (F_1) were weaned at 21 days of age, and 3-4 littermates of the same sex were housed together until being randomly assigned to an experiment. With the exception of the study of the age at the first ovulation, in all other experiments, we controlled for maternal effects by randomly selecting only one animal from each litter.

Statistical Analyses

All analyses were conducted using the Statistical Analyzing System, Mixed Model procedure (SAS Institute Inc., Cary, NC). Analyses were made using proc GLM in SAS, and planned comparisons were made using the LS means test if the overall ANOVA was statistically significant, using $p < 0.05$ as the criterion for statistical significance.

CHAPTER 3

EXPERIMENTAL METHODS AND RESULTS

MCF-7 Human Breast Cancer Cell Bioassay to Determine Estrogenic Activity in Feed

We measured the estrogenic activity in the feed by solvent extraction and examination of the degree to which the extract stimulated proliferation of human MCF-7 breast cancer cells, since this bioassay provides a highly sensitive method for detecting estrogenic activity in feed (Welshons 1990, Welshons 2003). Use of this bioassay to screen feeds for estrogenic activity prior to use is required, since there can be significant batch-to-batch variability in soy isoflavones in soy-based feeds as well as variability in other estrogenic contaminants in feeds that do not contain soy. Briefly, ~10,000 cells were seeded per well of a 24-well plate on Day 0 in phenol red-free (estrogen-free) medium, fed on Day 1 with the same medium, and then treated on Days 3 through 6 with the test media, with daily medium changes. Test media for feeds contained methanol extracts from feed. On Day 7 the wells were washed twice with 1 ml HBSS, and each well was then assayed for DNA content. By comparing the levels of DNA with known levels for genistein, the genistein equivalency for the diet samples was calculated, since genistein is the primary phytoestrogen in soy-based feed. However, it is not assumed that all estrogenic activity is due to genistein as there are other isoflavones in soy, such as

daidzein. Table 2 and Figure 2 summarize the mean genistein equivalencies (in ppm) of samples of the diets used in this study.

Fetal Serum Estradiol Levels on Gestational Day 18

On GD 18, a subgroup from each diet and dose group was collected for fetal blood serum hormone levels. Pups were delivered via caesarean section, resuscitated, and blood was collected by decapitation. The level of estradiol in the serum was then measured by radioimmunoassay. As shown in Figure 3, there were no differences between the 5K96C-L and 5K96C-H diets in their effect on serum estradiol in either male or female fetuses in this experiment or between 5008 and the 5K96C feeds. However, the serum levels of estradiol for the 5008 males and females were much greater than values observed in prior experiments (Ruhlen 2002, unpublished observation).

Pre-pubertal Uterotrophic Assay

A uterine challenge was conducted on two female pups from each litter at weaning to determine uterine sensitivity to estrogen while remaining on their respective diets. Starting on PND 21, the two females were injected s.c. with 5 μg/day of the synthetic estrogen diethylstilbestrol (DES) once per day. On PND 23, the females were injected for the final time and were collected 5 hours later. The animals were euthanized via carbon dioxide inhalation followed by decapitation for blood collection. The uterus was removed and wet weight was recorded.

Pre-pubertal females injected with either oil vehicle on PND 21 through PND 23 showed no differences in uterine wet weight based on type of feed. However, 5K96C-H

females administered 5-μg/kg/day DES had significantly heavier uteri than either the 5008 or 5K96C-L that had been administered DES (Figure 4). No significant differences in body weights due to diet or DES treatment were observed. This finding suggests that the 5K96C-H feed resulted in uteri being more sensitive to the uterotrophic action of DES relative to either the 5008 or 5K96C-L feeds.

Age at Onset of Fertility

To determine the onset of puberty after weaning in the females, two F_1 females from each litter were each housed undisturbed with a male of known reproductive competence. When the female was visibly pregnant, the male was removed and the female was observed daily for the presence of pups. Date of birth, weight of the female after parturition, and weight and sex ratio of pups were recorded.

Pre-pubertal females from both the 5K96C-H and 5K96C-L paired with a stud male at weaning ovulated, mated, and produced their first litters of pups at a younger age ($p < 0.01$) relative to females on the 5008 diet (Figure 5). While the 5K96C-H and 5K96C-L diets did not differ from each other significantly, the 5K96C-H group entered puberty at the youngest age. On the day of parturition, the average body weight of the 5008 females were significantly greater ($p < 0.05$) than either of the females on the 5K96C-L and 5K96C-H diets (37.78 g ± 0.57 versus 36.27 g ± 0.57 and 35.72 g ± 0.58, respectively). No differences were observed in the diet groups with respect to number of pups, weight, or sex ratio of the pups at birth.

Adult Body Weights, Gonadal Fat Weights, and Serum Leptin Levels

There were no consistent differences in adult body weight due to diet in either males or females collected at 3 months of age, though animals on the 5K96C-H and 5K96C-L diets tended to be slightly heavier than those on the 5008/5001 diets (Table 3 and 4). However, there was a significant difference due to feed with respect to the weight of the gonadal fat pad in the females, especially the between the PMI 5008/5001 and the 5K96C-L group (Table 3, Figure 6). Female serum leptin levels from blood collected and measured by radioimmunoassay were likewise significantly different between the female 5001/5008 group and the 5K96C-L group (Figure 7). When comparing the females, there was a significant positive correlation between the weight of the gonadal fat pad and serum leptin levels, since leptin is produced by adipocytes ($n=27$, $r^2 = 0.87$, $P < 0.001$). Because leptin is designed to down regulate the production of more fat, this suggests a possible insensitivity to the hormone in the females on the 5K96C-L diet since they also had more gonadal fat. This relationship was not as strong in the males, as 5K96C-H males had the greatest amount of gonadal fat while 5K96C-L males had the highest leptin levels (Table 4).

Male Reproductive Organ Weights

F_1 male reproductive organs, kidney, liver, and gonadal fat were also collected at 3 months of age and are reported in Table 4. Males on the 5K96C-L diet had smaller testes, epididymides, and kidneys in relation to the 5008/5001 diet while the 5K96C-H males did not differ significantly from either of the other diets. The 5K96C-L males approached significance ($p = 0.07$) in difference between the weights of the right testis

compared with the 5008/5001 diet and were not different in relation to the 5K96C-H males. There were no significant differences in daily sperm production or weight of the prostate, seminal vesicles, coagulating glands, and liver in the groups with respect to diet.

Blood Glucose Levels Before and After a Glucose Challenge

Adult F_1 males and females on the different diets were fasted overnight for 12 hours with a clean cage, a paper towel for bedding, and a water bottle. The next morning, the mice were given an i.p. injection of approximately 200 μl (based on the mouse's body weight) of a 2 mg/kg body weight glucose solution. The tail was nicked and blood glucose levels were measured with an Accu-Chek glucometer (Roche Diagnostics, Indianapolis, IN) just before injection and then at 30, 60, and 120 minutes afterwards.

The males on the different diets used in the diet study did not differ significantly in body weight. 5K96C-L males differed from 5008/5001 males in that their baseline glucose levels were significantly lower. 5K96C-H males were intermediate in their blood glucose levels and not significantly different from either 5008/5001 or 5K96C-L males (Table 5A). Thirty minutes after the injection, the 5K96C-L males had significantly higher glucose levels than the 5K96C-H, but there was no significant difference at the later time points.

The females on the 5K96C-L were significantly heavier than 5008/5001 females, while the body weights of the 5K96C-L and 5K96C-H did not differ significantly (Table 5B). Similar to the males, the baseline blood glucose levels of the 5K96C-L females were significantly lower compared to the 5008/5001 females, with the 5K96C-H female

in between and not significantly different from either of the other. There were no differences in the females after the glucose injection based on the diet.

CHAPTER 4

Animals and husbandry

Breeding colony pregnant and lactating females were fed soy-based PMI 5008 breeder chow (Ralston- PMI, St. Louis, MO). After weaning on postnatal day 21, animals were fed soy-based PMI 5001 maintenance chow until the beginning of the experiment. Water was purified by ion exchange followed by a series of carbon filters and was supplied *ad libitum* in glass bottles. Rooms were maintained at 25 ± 2°C under a 12:12 light:dark (L:D) cycle.

Assignment of Animals to Diet Groups: PMI 5008 and Harlan Teklad 2018 and 2016

Commercial diets of interest were soy-based PMI 5008 (23% protein, 6% fat), soy-based Harlan Teklad 2018 (18% protein, 5% fat), and casein-based Harlan Teklad 2016 (16% protein, 3.5% fat) (Wilmington, DE). Specific information about components of these feeds can be found at http://www.teklad.com. Experimental virgin females between three and five months old were randomly assigned to a diet group and were

maintained on their respective diets throughout pregnancy and lactation. Animals were allowed a period of at least twelve days to adjust to the new diet before proceeding with timed mating.

Timed Mating and Administration of DES to Pregnant Females,

Timed mating of the females was as described for the prior study. Positively plugged females were housed three per cage until they were singly housed on GD 17-18 to deliver their pups. Beginning on GD 11 through GD 17, all pregnant females were orally dosed one time per day with 30 μL of tocopherol-stripped corn oil vehicle (control), 0.1 μg/kg body weight (low dose), or 100 μg/kg body (high dose) weight DES. A randomly selected group of females on each feed was not handled or administered the oil vehicle, and these groups were designated as unhandled.

Collection of Maternal and Fetal Serum Estradiol

On GD 18, a subgroup from each diet and dose group was collected for fetal blood serum hormone levels. Pups were delivered via caesarean section, resuscitated, and blood was collected by decapitation. The level of estradiol in the serum was then measured by radioimmunoassay. As shown in Figure 8, the female fetal blood serum estradiol concentration reaction to prenatal DES is dependent on diet. Purina 5008 showed a non-monotonic curve in response to DES, with fetal estradiol levels stimulated by a low dose but inhibited by a high dose. The two other diets did not demonstrate this type of dose response. Although all three diets displayed a suppression of estradiol levels with the high dose of DES, the suppression was not statistically significant on the 2018

diet. In addition, the 2016 diet produced significantly higher baseline levels of fetal serum estradiol in the control animals relative to the PMI 5008 feed. In this experiment, the serum estradiol levels on the 5008 feed were also lower than in the previous study described above, and were more similar to levels observed in prior studies (Ruhlen 2002, unpublished).

Production of F_1 Animals for Postnatal Studies.

The remaining litters were born on GD 19. If the dam was not able to deliver the pups vaginally on GD 19 (which occurred 6 out of the total 9 litters due to the high DES dose), they were delivered via caesarean section and fostered with another untreated dam on the same diet. The pups were weaned on day 21 and were housed 2-5 mice per cage by sex. Unlike the experiment described above, all offspring remained on their respective diets throughout the study. This included the PMI 5008 group in order to minimize variability from transitioning from PMI 5008 to 5001 at weaning.

Adult Uterotrophic Assay

At approximately 5 months of age, all females were bilaterally ovariectomied using a small incision over both ovaries. Mice were anesthetized with a cocktail (25 mg/ml ketamine, 2.5 mg/ml xylazine, 0.5 mg/ml acepromizine) at a dose of 0.1 ml injected s.c.. Incisions were closed with wound clips. Post-operative care was provided through heat lamps, and mice were returned to clean cages with no more than three per cage after regaining consciousness. Three days after surgery, the females were injected with either 50 μL of 40 μg/kg 17 β estradiol in corn oil or corn oil vehicle. Three hours

after injection, the animals were euthanized via carbon dioxide inhalation followed by decapitation for blood collection. Body weight, gonad fat, and wet uterine weight were measured.

In the adult females, the uterine weights did not significantly differ from each other in the control oil injection, which indicates that the baseline uterine weight following ovariectomy were the same irrespective of diet. However, estradiol stimulated a 50% increase in uterine size in the low DES animals fed 2018 and 5008, but not 2016 (Figure 9). The injection of estradiol failed to elicit a significant uterine response within three hours in the high DES dose group regardless of diets compared to the control oil injection.

As shown in Figure 10 and 11, adult female gonadal fat and body weights demonstrated the permanent effects of prenatal DES exposure as both endpoints were significantly increased by the high dose of DES compared to oil, with the greatest response by far occurring on the PMI 5008 diet.

Discussion

The consumption of phytoestrogens that occur naturally in soy and other plants and their effects on the reproductive system has attracted increasing attention from both members of the scientific community and the public. Because previous studies in our laboratory (Ruhlen *et al.*, unpublished) and reports from others (Thigpen 2004) have indicated that rodent diets can be highly variable over time in their level of phytoestrogens and can yield different results in terms of biological endpoints, some authors have suggested using a low phytoestrogen rodent diet with an alternate protein

source such as milk-based casein instead of soy as a way to eliminate this inherent variability (Brown and Setchell 2001). Other authors have recommended using diets with no or low phytoestrogen diets only with certain types of experiments (Jensen and Ritskes-Hoitinga 2007).

Our studies demonstrate that removal of soy isoflavones by using alternate protein sources like casein can present additional problems because the rodents used in laboratory experiments, such as the CD-1 mouse used here, have been bred for thousands of generations by commercial breeders on soy-based diets. These animals have therefore gone through a long selection process for tolerance to phytoestrogens in soy (http://www.labdiets.com). That there are consequences of removing most phytoestrogens from the feed is thus not unexpected. For example, the female F_1 mice raised on either of the batches of casein-based 5K96C diet entered into puberty significantly earlier than the females raised on the soy-based 5008 diet. Entering puberty and having a litter too early (as the female mouse itself is still growing at this age) could be detrimental to the well-being of both the dam and the pups if it has to divert resources away from its own growth and put them towards the demands of pregnancy and lactation (Wang and vom Saal 2000). This seems to be a possibility as our data showed that the dams on either batch of casein-based 5K96C were significantly lighter on the day of parturition than their soy-based 5008 counterparts.

In addition, because of genetic variations in populations of inbred and outbred mice, certain strains (such as the CD-1 mouse commonly used in toxicological studies) are significantly less susceptible to endocrine disruption by estradiol (Spearow 1999). Therefore, in addition to using strains that are resistant to endocrine disruption, selecting

a diet that further exacerbates potential insensitivity in the bioassays used to indicate a response to estrogenic chemicals may result in false negatives even in positive controls (Ashby 1999, Cagen 1999). This diet-induced insensitivity was demonstrated in our results by the non-monotonic dose response to DES administered prenatally in the PMI 5008 group where the low dose stimulated an elevated level of serum fetal estradiol and a high dose showed an inhibited level of estradiol. This low dose effect was not seen in either of the other diets to a significant degree and would have been reported as a false negative if the Harlan diets were the sole diets used. The basis for this lack of DES effect was that the Harlan diets significantly increased serum estradiol relative to the PMI 5008 feed.

As our findings in these two experiments and other studies demonstrate (Thigpen 2004, Thigpen 2003), varying background levels of phytoestrogens when compared brand to brand, lot to lot, and even from bag to bag do indeed cause significant differences in sensitive bioassays such as estradiol levels in the fetus, timing of the onset of puberty and vaginal opening in the females, and the female pre-pubertal and adult uterotrophic assays. This is highlighted in the two experiments described here that were conducted approximately one year apart and both measured the female fetal estradiol levels on gestational day 18 in pregnant dams fed PMI 5008. When comparing the PMI 5008 group in the first experiment and the PMI 5008 group with control dosing in the second experiment, the first group showed an elevated level of estradiol in the female fetuses compared with the group one year later. This further demonstrates the variability in commercial laboratory diets over time as well as the need to characterize the

phytoestrogen levels before beginning a study as they clearly can affect experimental results.

The issue of naturally occurring hormonally active components in laboratory diets is an area of controversy and opinions differ on the level of phytoestrogens that should or should not be in the diets. But there is consensus that this is a subject that no longer can be ignored by researchers. To help reduce the inherent variability in experimental results in using different rodent diets, the following steps should be taken. First, major manufacturers should move towards full disclosure with open diets instead of closed so researchers have a more complete understanding of what is in the diets before incorporating them into their experimental design (Thigpen 1999b). Second, using sensitive prescreening methods such as the MCF-7 breast cancer cell proliferation assay to test the estrogenic activity of the diets are needed (Welshons 1990, Soto 1995). Finally, the phytoestrogen levels of the diets should be reported along with the results in future publications to track the effects of the diets on the physiological endpoints over time (Jensen and Riskes-Hoitinga 2007, Brown and Setchell 2001, Ashby 2000, Thigpen 1999a).

The results of our experiments corroborate previous studies concerning the consequences for phenotype in mice due to variation in components of commercially available rodent diets and help to further characterize the profiles of diets, which in turn yield different phenotypes. Most importantly, they show that not all diets used in endocrine disruption research are appropriate if they render important physiological endpoints insensitive. The effects and interactions between diet and exposure to endocrine disrupting chemicals on the mouse reproductive system will be a continuing

area of research in our laboratory. Future studies will seek to further characterize an appropriate level of phytoestrogens in diets fed to rodents with highly sensitive physiological endpoints in mind. In addition, other environmental chemicals of interest such as the plastic monomer bisphenol A will also be tested to further investigate the interactions of naturally occurring phytoestrogens, endogenous estrogens, and synthetic xenoestrogens.

Table 1.
A. Percent components of soy-based Purina 5001 and 5008 and Purina 5K96C feeds.
B. Components of these feeds that are present (+) or absent (blank) in each type of feed.

A. PERCENT NUTRIENTS AND ENERGY VALUE

COMPONENTS OF FEEDS	5008	5001	5K96C
Percent Protein	23.5	23.4	18.9
Percent Fat (ether extract)	6.5	4.5	4.3
Percent Fiber (Crude)	3.8	5.3	3.6
Percent Starch	34.9	31.9	44.8
Percent Sucrose	2.6	3.7	0.4
Total Digestible Nutrients (%)	81.2	76	75.2
Gross Energy (kcal/gm)	4.15	4.00	4.05
Physiological Fuel Value (kcal/gm)	3.50	3.30	3.44
Metabolizable Energy (kcal/gm)	3.31	3.04	3.15

B INGREDIENTS THAT DIFFER IN PURINA FEEDS

COMPONENTS OF FEEDS	5008	5001	5K96C
Animal fat preserved with BHA	+	+	
Biotin			+
Cane molasses	+	+	
Casein			+
Corn gluten meal			+
Corn oil			+
Dehydrated alfalfa meal	+	+	
Dicalcium phosphate			+
Dried beet pulp,	+	+	
Dried whey	+	+	
Ferrous carbonate	+	+	
Ferrous sulfate0		+	
Ground wheat	+		+
Magnesium oxide			+
Nicotinic acid		+	
Porcine meat meal	+	+	
MSB (vitamin K)*	+		+
Nicotinic acid	+		+
Wheat germ	+	+	

Table 1. C.

Additional ingredients found in all diets: brewers dried yeast, calcium carbonate, calcium iodate, calcium pantothenate, cholecalciferol, choline chloride, cobalt carbonate, copper sulfate, cyanocobalamin, dehulled soybean meal, dl-alpha tocopheryl acetate, DL-methionine, fish meal, folic acid, ground corn, ground oats, manganous oxide, pyridoxine hydrochloride, riboflavin, salt, thiamin mononitrate, vitamin A acetate, wheat middlings, zinc oxide, and zinc sulfate.

***MSB = menadione sodium bisulfite (source of vitamin K).**

Table compiled from www.labdiet.com, 2003.

GENISTEIN EQ. (ppm or µg/gm)			ppm		
Assay date	**Lab Sample ID**	**Sample**	**Gen eq.**	**Avg. ppm Gen**	
9/26/03	030609JT01	5001-1	36.3	40.0	Mean
9/26/03	030609JT02	5001-2	31.9	± 4.1	sem (10.2%)
11/7/03	030609JT03	5001-3	40.6	20.5%	cv=sd%
11/7/03	030609JT04	**5001-4**	**51.0**	1.60	max/min
9/26/03	030609JT05	5008-1	27.3	25.8	Mean
9/26/03	030609JT06	5008-2	20.1	± 2.7	sem (10.3%)
11/7/03	030609JT07	5008-3	23.3	20.6%	cv=sd%
11/7/03	030609JT08	**5008-4**	**32.4**	1.61	max/min
9/26 & 10/10	030609JT09	5K96Lo-1	3.5	4.9	Mean
9/26 & 10/10	030609JT10	**5K96Lo-2**	**3.7**	± 1.2	sem (24%)
10/3 & 11/7	030609JT11	5K96Lo-3	3.6	53.7%	cv=sd%
10/3 & 11/7	030609JT12	5K96Lo-4	3.9	2.70	max/min
10/3 & 11/7	030609JT13	5K96Lo-5	9.5		
9/26 & 10/10	030609JT14	5K96Hi-1	7.4	15.2	Mean
9/26 & 10/10	030609JT15	5K96Hi-2	15.8	± 2.1	sem (14.2%)
10/3 & 11/7	030609JT16	5K96Hi-3	15.8	31.7%	cv=sd%
10/3 & 11/7	030609JT17	**5K96Hi-4**	**20.6**	2.80	max/min
10/3 & 11/7	030609JT18	5K96Hi-5	16.4		

Table 2. Summary of the mean ppm genistein equivalents measured in samples of the Purina diets.

	5008/5001	5K96C-Low	5K96C-High
Number	9	8	10
Body weight, g	27.0 ± 0.5	28.4 ± 0.7	27.2 ± 0.9
Gonad fat pad, mg	437.3 ± 42.3^a	1,023.8 ± 134.0^b	596.3 ± 98.3^{ab}
Fat/body wt (%)	16.2 ± 1.6^a	35.4 ± 4.1^b	21.1 ± 3.0^{ab}
Leptin, ng/ml	3.70 ± 0.61^a	7.62 ± 1.29^b	4.85 ± 0.67^a

Table 3. Mean (±SEM) F_1 female body weight, gonad fat and serum leptin concentrations when 2 months old. Different letters after the mean indicate that group means were significantly different ($P < 0.05$), while the same letter indicates that the means were not significantly different.

	5008/5001	5K96C-Low	5K96C-High
Number	9	10	11
Body weight, g	35.6±1.1	35.1±1.3	37.0±0.8
Gonad fat pad, mg	647.0±60.1[a]	748.1±68.0[ab]	827.7±67.3[b]
Fat/body (%)	18.0 ± 1.3	21.1 ± 1.5	22.3 ± 1.7*
Leptin, ng/ml	3.05±0.25	4.28±0.62*	3.77±0.34
Right Testis, mg	121.4±3.6	111.1±3.6*	112.5±3.9
DSP $\times 10^6$	8.12±0.51	8.16±0.48	8.56±0.46
DSP/gram testis	69.80±5.14	74.4±4.88	76.59±4.65
Epididymis, mg	42.9±3.9[a]	35.8±1.6[b]	46.7±1.8[a]
Seminal Vesicle, mg	52.0±3.3	47.1±2.0	47.3±2.0
Coagulating Glands, mg	17.5±1.2	16.1±0.7	18.8±2.1
Prostate, mg	29.9±1.5	27.8±1.6	30.7±0.9
Liver, g	2.27±0.12	2.18±0.12	2.33±0.08
Kidney, mg	344.9±13.6[a]	313.6±17.1[b]	348.6±13.9[a]

Table 4. F_1 male body weight and weight of the right testis, epididymis, paired seminal vesicles, prostate, liver and kidney, as well as serum leptin, daily sperm production per testis (DSP) and daily sperm production per gram testis. Different letters after the mean indicate that group means were significantly different ($P < 0.05$), while the same letter indicates that the means were not significantly different. * indicates that the group mean differed from 5008/5001 at $P < 0.07$.

Time	5008/5001	5K96C-Low	5K96C-High
Body weight (g)	30.43±0.94	31.39±0.62	31.72±0.89
0 min	69.11±6.24 [a]	53.90±3.18 [b]	65.09±3.76 [ab]
30 min	205.67±33.12 [ab]	255.60±24.40 [ab]	178.45±23.49 [a]
60 min	196.33±29.80	314.20±17.99	166.54±19.98
120 min	121.22±19.85	127.40±16.36	109.64±17.51

Table 5.A. Mean±SEM glucose concentration (mg/dl) before and after glucose challenge with an injection of 2 mg/kg glucose in F1 male mice fed 5008/5001 (n = 9), 5K96C-L (n = 10) or 5K96C-H (n = 11).

Time	5008/5001	5K96C-Low	5K96C-High
Body weight (g)	22.77±0.56 [a]	24.65±0.55 [b]	24.04±0.66 [ab]
0 min	61.22±3.87 [a]	48.22±3.49 [b]	53.36±2.46 [ab]
30 min	229.56±17.31	215.67±18.27	200.00±14.69
60 min	168.44±15.24	152.78±18.30	151.09±7.76
120 min	92.89±8.32	71.33±9.06	91.91±9.74

Table 5.B. Mean±SEM glucose concentration (mg/dl) before and after glucose challenge with an ip injection of 2 mg/kg glucose in F1 female mice fed 5008/5001 (n = 9), 5K96C-L (n = 9) or 5K96C-H (n = 11).

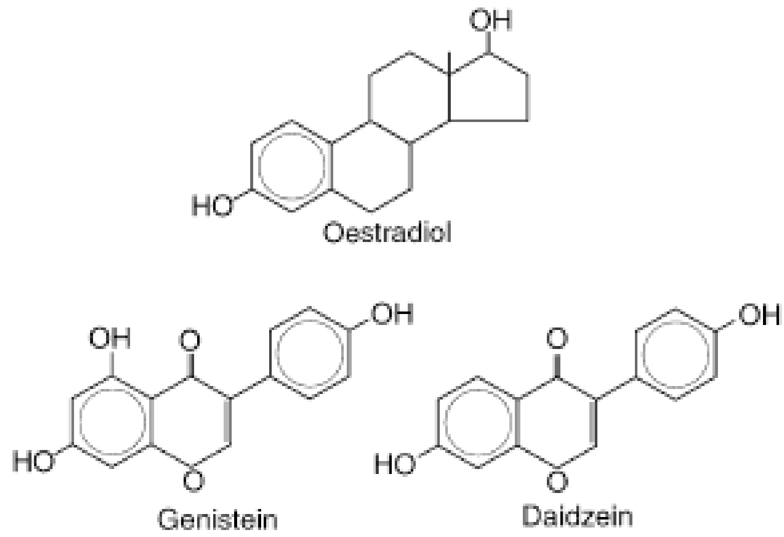

Figure 1. The structural similarity between 17-β estradiol and two types of soy isoflavones (genistein and daidzein) allow isoflavones to bind to the estrogen receptor (from Jansen and Riskes-Hointinga 2007).

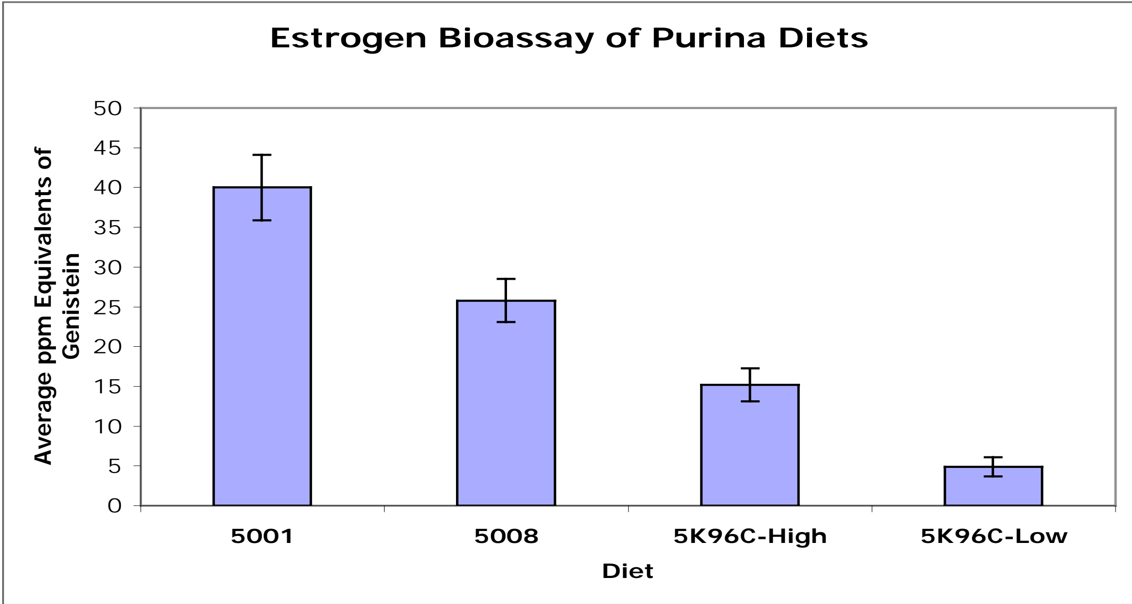

Figure 2. Summary of the mean (+/- SEM) ppm genistein equivalents measured in samples (n=4-5) of the Purina Diets

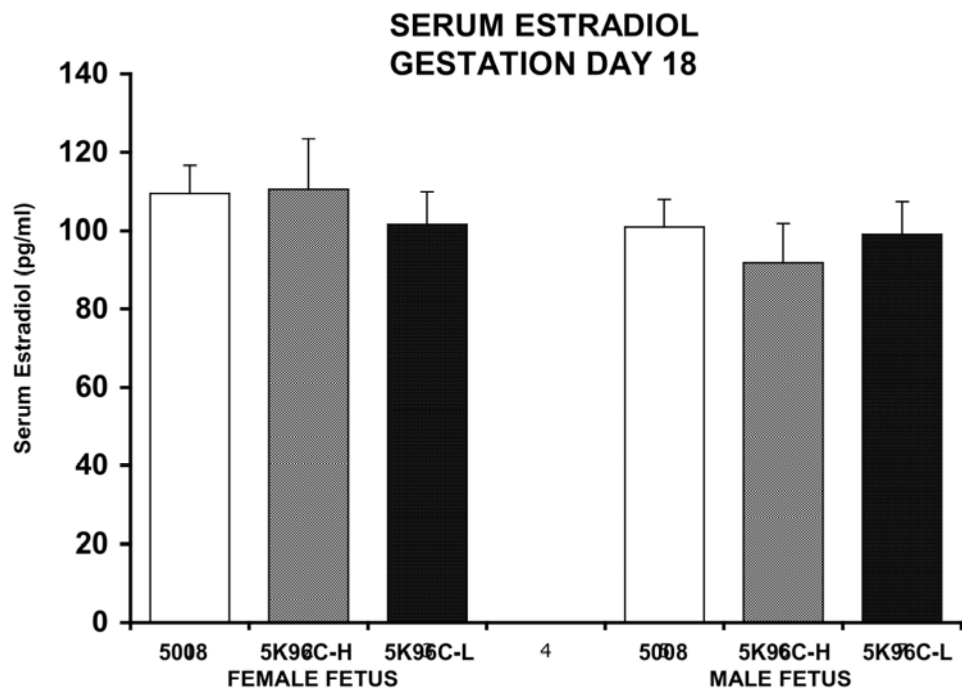

Figure 3. Mean (± SEM) fetal serum estradiol levels (measured in pg/ml) in gestational day 18 female and male fetuses

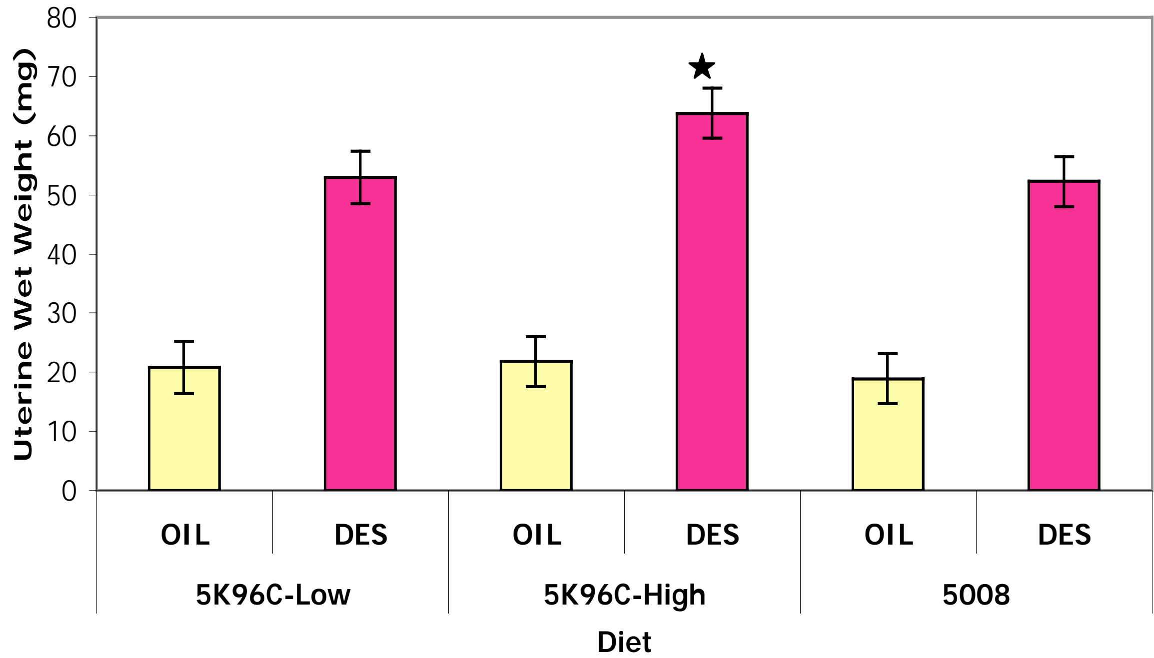

Figure 4. Mean (± SEM) of pre-pubertal female uterine weights after daily injection with either oil or 5 μg/kg/day diethylstilbestrol (DES) on postnatal day 21 through 23 by diet.
★ indicates $p < 0.05$ respective to the other diets for corresponding DES dose.

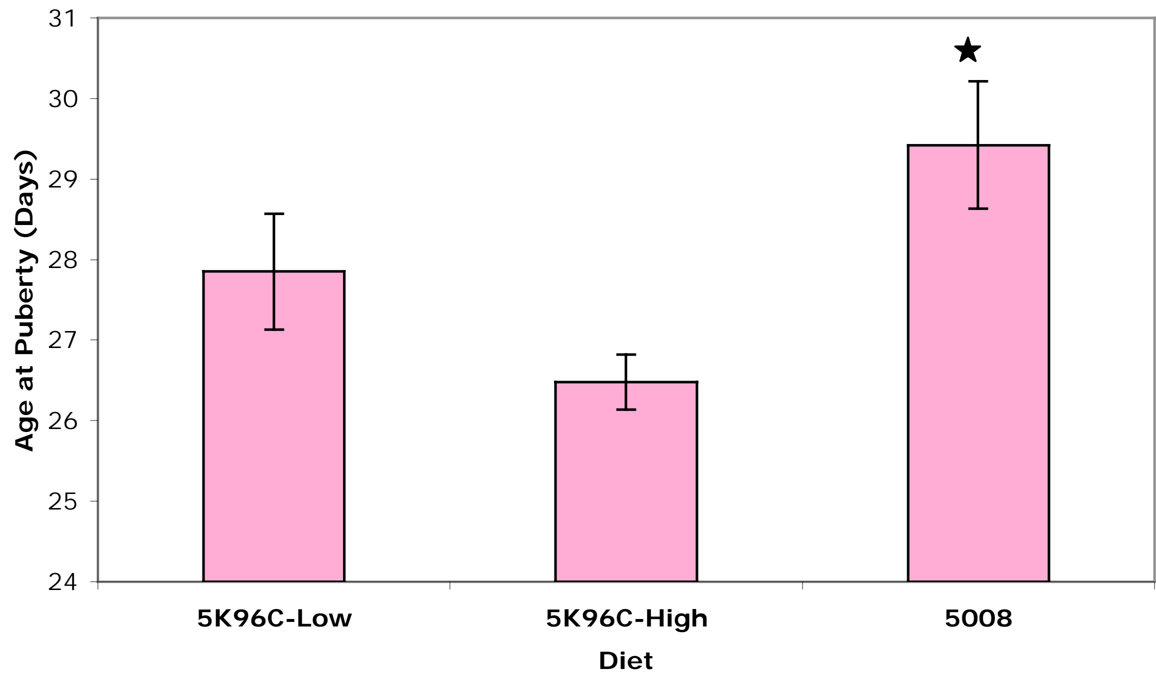

Figure 5. Mean (± SEM) of age of females at puberty when paired with a stud male at weaning. ★ indicates $p < 0.05$ relative to the 5K96C-Low and 5K96C-High diet.

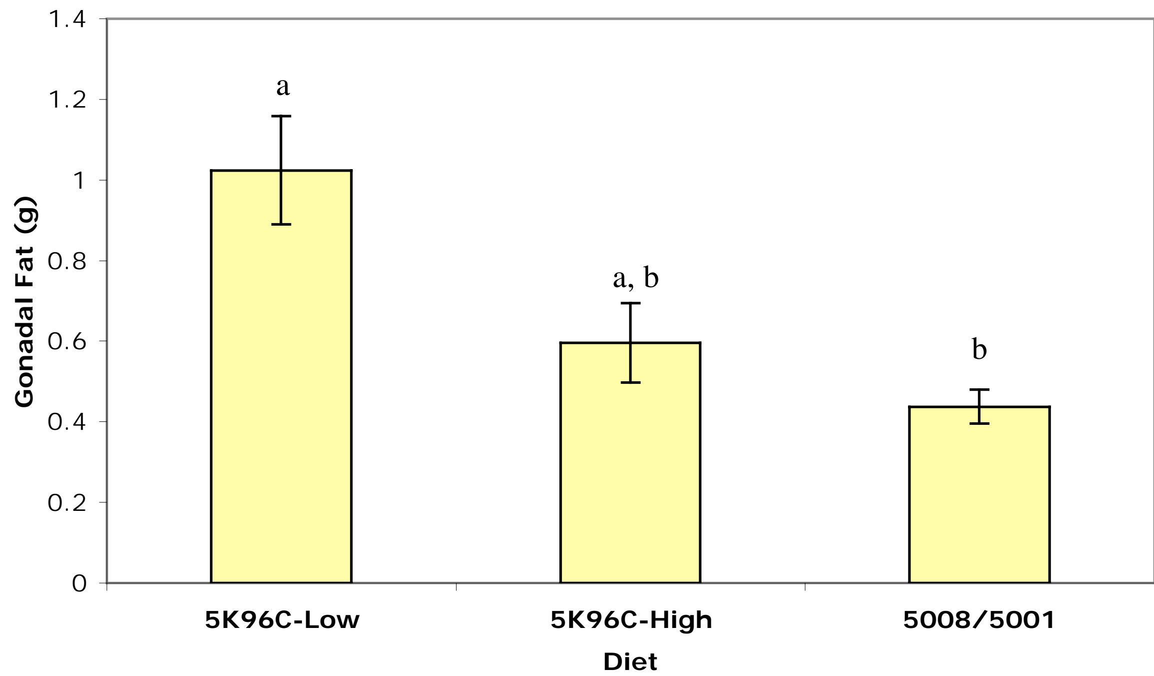

Figure 6. Mean (± SEM) gonadal fat weights in 2 month old F1 female mice maintained on the different diets. Different letters indicate that group means were significantly different ($p < 0.05$), while the same letter indicates that the means were not significantly different. $n \geq 8$ animals in all groups.

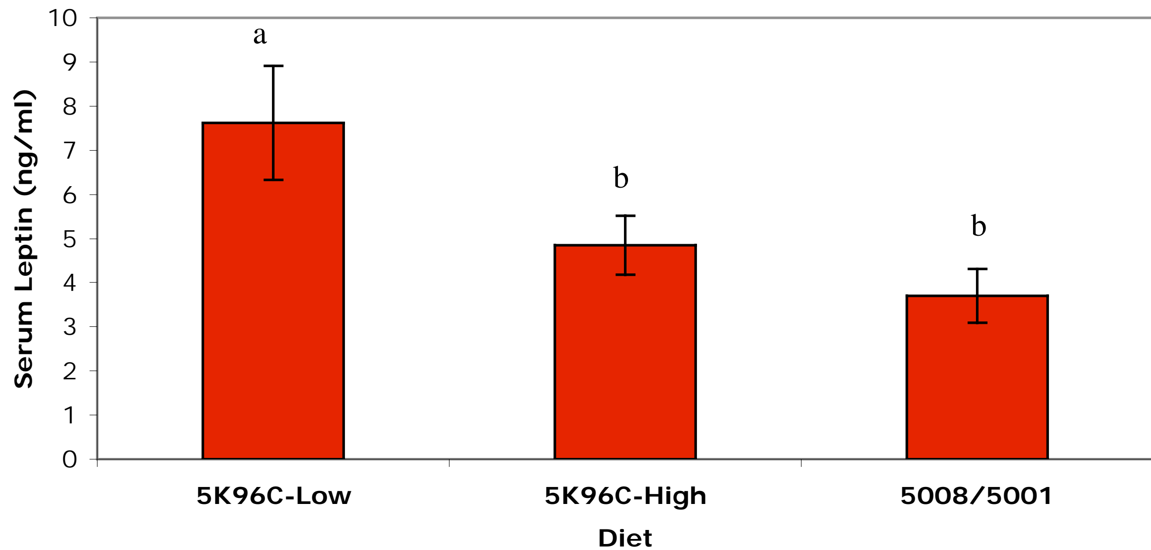

Figure 7. Mean (± SEM) serum leptin levels in 2 month old F1 female mice maintained on the different diets. n ≥ 8 animals in all groups. Different indicate that group means were significantly different ($p < 0.05$), while the same letter indicates that the means were not significantly different.

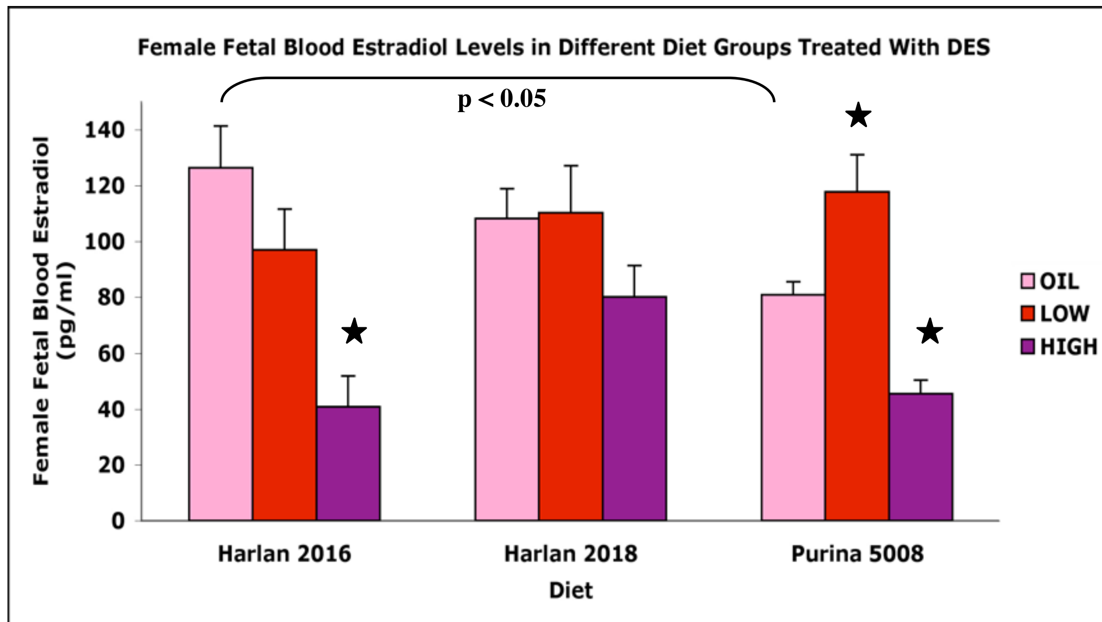

Figure 8. Mean (± SEM) fetal blood estradiol levels (measured in pg/ml) in gestational day 18 female mouse pups. ★ indicates $p < 0.05$ relative to oil-treated fetuses in their respective diet group.

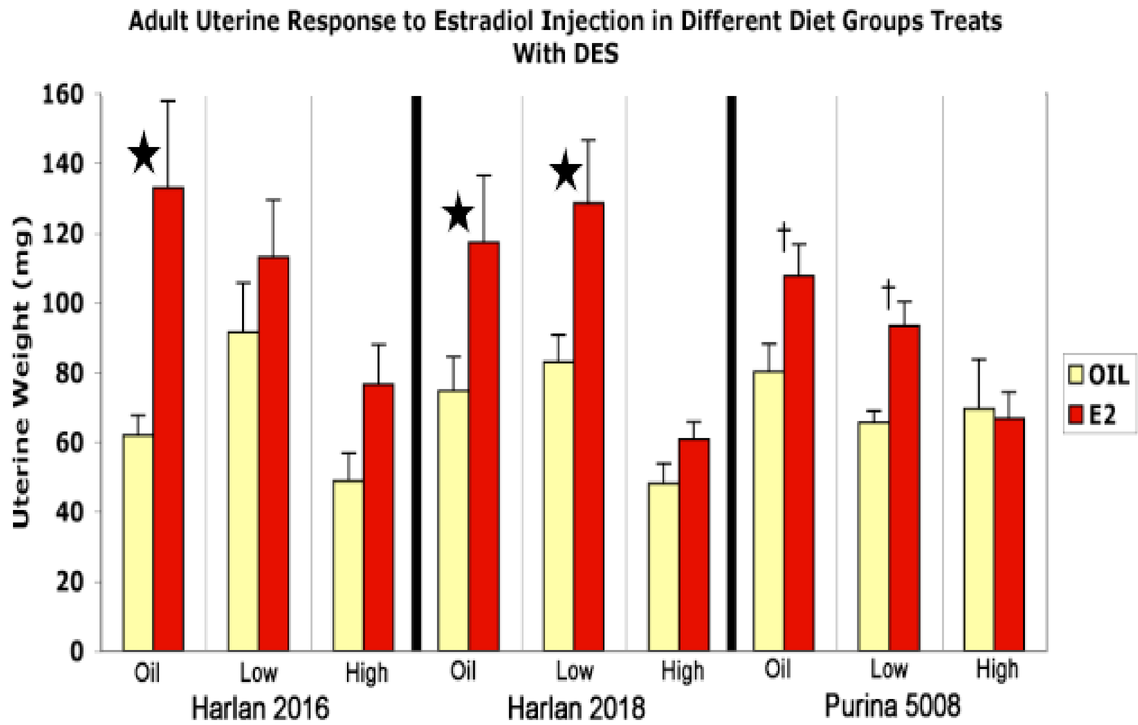

Figure 9. Mean (± SEM) adult female uterine weights 3 hours after an injection with either oil or 40 μg/kg estradiol by diet after prenatal doing of oil, low, or high DES. † indicates $p < 0.1$; ★ indicates $p < 0.05$ relative the respective oil treatment.

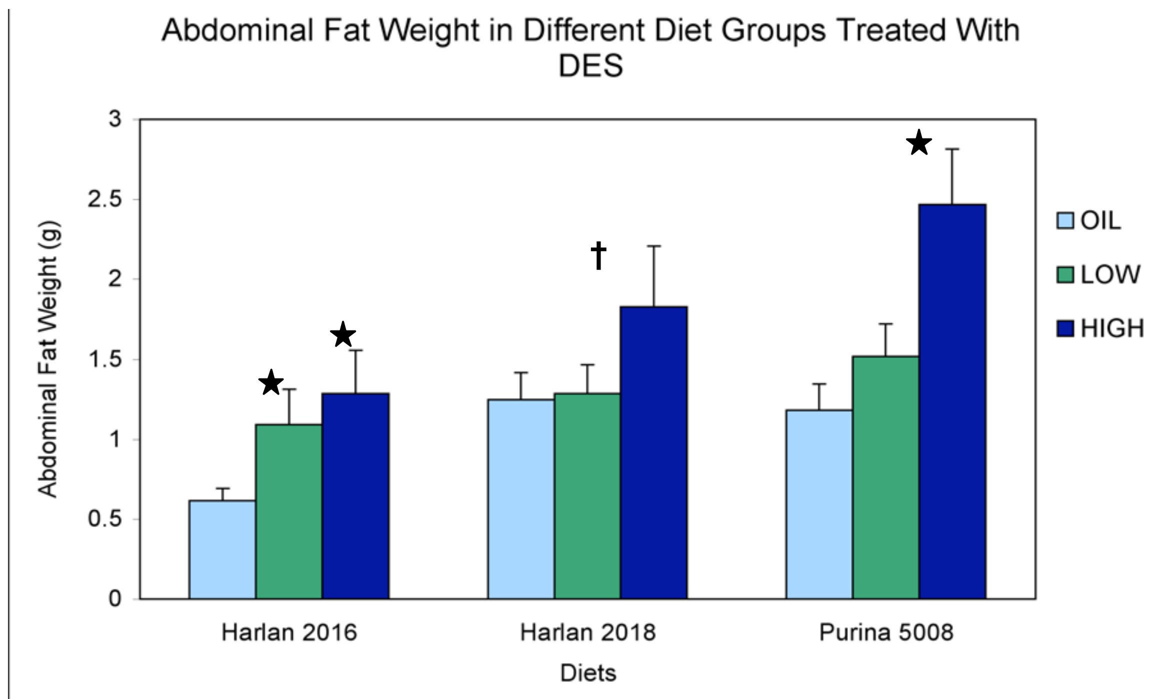

Figure 10. Mean (± SEM) gonadal fat weights collected from females 5 months old maintained on either Harlan 2016, Harlan 2018, or Purina 5008 diets after prenatal dosing with 0, low, or high DES. † indicates $p < 0.1$; ★ indicates $p < 0.05$ respective to the corresponding oil treatment.

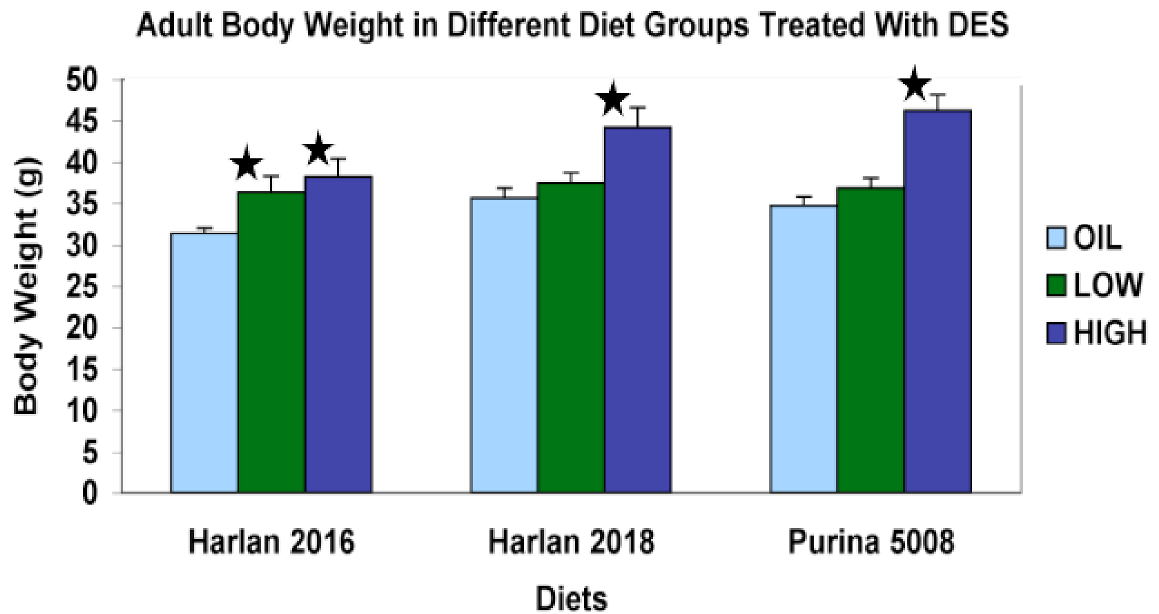

Figure 11. Mean (± SEM) body weights collected from females 5 months old maintained on either Harlan 2016, Harlan 2018, or Purina 5008 diets after prenatal dosing with 0, low, or high DES. ★ indicates $p < 0.05$ respective to the corresponding oil treatment.

WORKS CITED

Adams, NR. 1995. Detection of the Effects of Phytoestrogens on Sheep and Cattle. Journal of Animal Science. 73:1509-1515.

Adams, NR. 1990. Permanent infertility in ewes exposed to plant oestrogens. Australian Veterinary Journal. 67:197-201.

Ashby J, Tinwell H, Odum J. 2000. Uterotrophic activity of a "phytoestrogen-free" rat diet [letter]. Environmental Health Perspectives. 108: A12-A13.

Ashby J, Tinwell H, Haseman. 1999. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF-1 mice exposed in utero. Regulatory Toxicology and Pharmacology. 30:156-166.

Bennetts HW, Underwood EJ, Shier FL. 1946. A specific breeding problem of sheep on subterranean clover pastures in western Australia. Australian Veterinary Journal 22:2.

Boettger-Tong H, Murthy L, Chiappetta C, Kirkland JL, Goodwin B, Aldercreutz S, George M, Mäkela S. 1998. A case of a laboratory animal feed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. Environmental Health Perspectives. 106:369-373.

Brown NM, Setchell KDR. 2001. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. Laboratory Investigation. 81:735-747.

Cagen SZ, Waechter JM, Dimond Jr. SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, Harris LR. 1999. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicological Science. 50:36-44.

Harlan Teklad. 2003. Harlan Teklad Global Diets. Available: http://www.teklad.com [accessed 2003].

Jensen MN, Ritskes-Hoitinga M. 2007. How isoflavone levels in common rodent diets can interfere with the value of animal models and with experimental results. Laboratory Animals. 41:1-18.

Lee DH, Lee IK, Song K, Steffes M, Toscano W, Baker BA, Jacobs DR. 2006. A strong dose-response relation between serum concentrations of persistent organic pollutants and diabetes: results from the National Health and Examination Survey 1999–2002. Diabetes Care. 29:1638-1644.

Mäkelä S, DavisVL, Tally WC, Korkman,J, Salo L, Vihko R, Santti R, Korach KS. 1994. Dietary etrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environmental Health Perspectives. 102:572-578.

Mäkelä S, Santti R, Salo L, McLachlan JA. 1995. "Phytoestrogens are partial estrogen agonists in the adult male mouse. Environmental Health Perspectives. 103 (suppl 7) :123-127.

Newbold RR, Padilla-Banks E, Snyder RJ, Jefferson WN. 2005. Developmental exposure to estrogenic compounds and obesity. Birth Defects Research (Part A). 73:478–480.

Purina Mills Incorporated. 2003. Lab Diet. Available: http://www.labdiet.com [accessed 2003].

Ruhlen R. 2002. Unpublished data.

Setchell, KDR. 1998. "Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones." American Journal of Clinical Nutrition. 68 (suppl): 1333S-1146S.

Sasco, AJ. 2001. Epidemiology of breast cancer: an environmental disease? APMIS. 109:321-32.

Simonich, SL and RA Hites. 1995. Global distribution of persistent organochlorine compounds. Science. 269:1851-1854.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano, FO. 1995, The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environmental Health Perspectives. 103 (suppl 7):113 122.

Spearow JL, Doemeny P, Sera R, Leffler R, Barkley M. 1999. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science. 285:259 1261.

Swan SH, Brazil C, Brobnis EZ, Liu F, Kruse RL, Hatch M, Redmon JB, Wang C, Overstreet JW, and the Study for Future Families Research Group. 2003. Geographic differences in semen quality of fertile US males. Environmental Health Perspectives. 111:414-420.

Thigpen JE, Saunders HE, Haseman JK, Setchell KDR, Caviness G, Echerd L, et al. 2004. Variations in phytoestrogen content between different mill dates of the same diet produces significant differences in the time of vaginal opening in F344 rats.

Thigpen JE, Haseman JK, Saunders HE, Setchell KDR, Grant MG, Forsythe DB. 2003. Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. Comparative Medicine. 53:607-115.

Thigpen JE, Haseman JK, Saunders H, Locklear J, Caviness G, Grant M, Forsythe D. 2002. Dietary factors affecting uterine weights of immature CD-1 mice used in uterotrophic bioassays. Cancer Detection and Prevention. 26:381-393.

Thigpen JE, Setchell KDR, Goelz MF, Forsythe DB. 1999. The phytoestrogen content of rodent diets [letter]. Environmental Health Perspectives. 107:182-183.

Thigpen,JE, Setchell KD, Ahlmark,KB, Locklear J, Spahr T, Caviness GF, Goelz MF, Haseman JK, Newbold RR, Forsythe DB. 1999. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. Laboratory Animal Science. 49:530-536.

Wang MH and vom Saal FS. 2000. Maternal age and traits in offspring. Nature. 407:469-470.

Welshons WV, Thayer KS, Taylor JA, Judy B, vom Saal FS. 2003. Large effects from small exposures: I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environmental Health Perspectives. 111:904-106.

Welshons WV, Rottinghaus GE, Nonneman DJ, Dolan-Timpe M, Ross PF. 1990. A sensitive bioassay for detection of dietary estrogens in animal feeds. Jouranal of Veterinary Diagnostic Investigation. 2:268-273.

VITA

Maren Bell Jones was born in Omaha, Nebraska on October 13, 1982. She received Bachelors of Arts degrees in biological sciences and religious studies from the University of Missouri-Columbia in 2004 with departmental honors for research in biological sciences and an honors certificate from the Honors College. She began her graduate studies in the Division of Biological Sciences in 2004 under Dr. Frederick vom Saal at the University of Missouri-Columbia and graduated with a Masters of Arts in May 2007. She will be working towards a doctor of veterinary medicine degree at the University of Missouri College of Veterinary Medicine starting in August 2007.