

## Section 3 Bioactive Compounds

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## **Executive Summary- Bioactive Compounds Soy/Swine Nutrition Program Year 1**

- Bioactive compounds in soybean meal were examined for effects on reproductive and growth characteristics. Ovulation propensity, embryo survival, and lactogenesis were done with in vitro work. Isoflavone effects on estrous and growth and muscle development were done using pigs and rats.
- Cultured porcine granulosa cells were treated with varying concentrations of genistein, daidzein, diethylstilbestrol, or estradiol to measure the effects on cell apoptosis (the natural programmed cell death).
- Daidzein and genistein were shown to decrease apoptosis, with daidzein ten times more potent than genistein. Both were more potent than the animal's natural estrogen, estradiol. These compounds can potentially increase follicle survival, leading to improved litter size.
- Synchronized breeding age gilts were fed diets prepared that lacked isoflavones, saponins, and phytosterols, or had standard soybean meal with these compounds present. Blood samples were taken for baseline measures and then at 15-minute intervals over 24 hours. Data have been collected to date, but analysis is still ongoing.
- Embryos from the 2-cell to hatching stage were collected from gilts and cultured with or without increasing doses of daidzein. There was no significant difference in the formation of blastocysts for all but one treatment dose of daidzein, compared to the controls. At 3  $\mu$ M of daidzein, there was a trend for improved blastocyst development.
- A bioassay to determine the tissue-level effects of isoflavones on mammary cell development and lactation was emphasized. Problems encountered in developing a tissue culture system to measure mammary tissue growth meant work moved to characterizing the effects of genistein as a potential tyrosine kinase inhibitor on lactogenesis. Genistein present at levels consistent with those typically found in swine diets had no negative effect on indicators of lactogenesis.
- Littermate gilts were fed diets with varying levels of isoflavones to measure their effects on carcass muscle content and body growth rate. While isoflavones increased daily body weight, daily feed intake and gain:feed were not affected. Isoflavones increased the percentage carcass muscle and estimated muscle gain/day, but did not affect percentage carcass fat. Isoflavones increased the weight of predominantly red-fibered muscles.
- Pregnant rats were used as a model to determine the effects dietary isoflavones have on growth and muscle development in offspring. The isoflavones were shown to be bioavailable to the pregnant rats, and were detectable in fetal tissues. The effect of the isoflavones fed to the pregnant rat were mixed. Female offspring were more efficient in feed utilization and had improved growth rate. In males, the hind limb muscle content increased. For the mother rat, body weight decreased with increasing isoflavone content up to the fourth day of lactation.

## **The Effect of Dietary Soybean Isoflavones on Carcass Muscle Content and Body Growth in Pigs Fed from 6 to 30 Kg Bw**

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**Institution:** Iowa State University

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### **Summary**

Fifteen pairs of littermate gilts were used to evaluate the effect of dietary soybean isoflavones on carcass muscle content and body growth rate. Within a litter, pigs were allotted to a basal diet supplemented with 0 or 1585 mg isoflavones (normalized concentration)/kg supplied by an extract of soybeans. The extract contributed 670 mg genistein, 705 mg daidzein and 210 mg glycitein/kg diet, primarily in glycosidic forms, equivalent to 410, 420 and 133 mg, respectively, of the free forms. Pigs were from a high lean genetic strain, weaned at 8 to 12 days of age, reared via SEW procedures and self-fed a milk-based diet until initiation of the study. The basal diet (1.8% lysine) consisted of a corn, soy protein concentrate, lactose, crystalline amino acids and vitamin-mineral mix formulated to exceed nutrient needs of high lean pigs experiencing minimal antigen exposure. Isoflavones were added at the expense of cornstarch. Pigs were penned individually and given ad libitum access to water and diets. Pig weights and feed consumption were recorded every 4 days and pigs were slaughtered at  $30.7 \pm 1.2$  kg BW. Isoflavones increased daily BW gain (579 vs 595 g,  $P < .06$ ) but did not affect daily feed intake (911 vs 912 g,  $P > .1$ ) nor gain:feed (.639 vs .656 g/g,  $P > .1$ ). Isoflavones increased percentage carcass muscle (59.1 vs 60.1,  $P < .07$ ) and estimated muscle gain/day (234 vs 245 g,  $P < .01$ ), but did not affect percentage carcass fat (14.1 vs 13.3,  $P > .1$ ) or carcass percentages of bone and skin nor visceral organ weights. Isoflavones increased the weight of predominantly red-fibered muscles (triceps brachii, rectus femoris, infraspinatus and psoas major) but not the weight of predominantly white-fibered muscles (vastus lateralis, semimembranosus, gluteus medius and longissimus dorsi). Based on these data, dietary isoflavones increased body growth rate, carcass muscle content, and growth of red-fibered muscles.

## Problem Addressed

Isoflavones are compounds found in soybeans (Reinli and Block, 1996) at concentrations ranging from 100 to 5000 ppm (normalized concentration) depending on growing location, season and soybean variety (Kudou et al., 1991; Barnes et al., 1994; Wang and Murphy, 1994b; Ding et al., 1995). When consumed by mammals, isoflavones are absorbed and subsequently can be quantitated in blood and urine (Xu et al., 1994; Xu et al., 1995; King et al., 1996). Because soybean processing to produce soybean meal does not remove isoflavones, and pig diets may contain as much as 40 % soybean meal, the potential effects of isoflavones on pig growth are important.

The biological activities of isoflavones are numerous and include estrogenic properties (Medlock et al., 1995; Santell et al., 1997; Zava and Duwe, 1997), antioxidant effects (Esaki et al., 1994; Wei et al., 1995; Hodgson et al., 1996), immunomodulation (Trevillyan et al., 1990; Wang et al., 1997; Zhang et al., 1997), inhibition of DNA synthesis (Hamlin and Soares, 1995), and suppression of myoblast proliferation and differentiation, which are critical processes for postnatal muscle growth (Hashimoto et al., 1995; Ji et al., 1997). Thus isoflavones have the potential to impact a wide variety of biological processes. Although numerous effects of individual isoflavones *in vitro* have been documented, the impact of soy isoflavones on pig growth has not been quantified. Therefore, the objective of this study was to determine the effect of feeding supplemental soybean isoflavones to pigs from 6 to 30 kg BW on the animals' rate and efficiency of growth and carcass muscle content.

## Approach Used

Pigs were fed a basal diet supplemented with 0 or 1585 ppm soy isoflavones from 6 to 30 kg BW. The basal diet (17 ppm isoflavones) consisted of a corn, lactose, and soy protein concentrate mix fortified with minerals and vitamins (Table 1). Diets were formulated to meet or exceed nutrient recommendations of high lean pigs experiencing minimal antigen exposure. The supplemental isoflavones were supplied by a soy extract, which was produced at Archer Daniels Midland Co., and analyzed as 31.8 % isoflavones (glycosidic and aglycone forms Table 2) with the remainder consisting of saponins (Personal communication, Eric Gugger, Archer Daniels Midland Co., Decatur, IL). The isoflavones consisted of glycosidic and aglycone forms of daidzein (13.4%), genistein (14.0%), and glycitein (4.4%).

Fifteen pairs of littermate gilts with a high capacity for lean growth (370 g/d) were reared via a segregated early weaning scheme and randomly allotted within litter to the basal diet supplemented with either 0 or 1585 ppm soy isoflavones (normalized concentration). Pigs were penned individually and were given *ad libitum* access to a commercial milk pellet diet for 7 d postweaning, then to experimental diets from 6.1 to 30.7 kg BW.

Pig BW and feed consumption were recorded every 4 d. At 6.1 and 30.7 kg BW, pigs were bled via the orbital sinus. Serum was analyzed for alpha-1 acid glycoprotein (AGP) concentrations. AGP was determined via radial immuno-diffusion assay kit (Developmental Technologies, Frederick, MD).

As pigs reached a BW of  $30 \pm 1.5$  kg they were given a lethal dose of sodium pentobarbital and immediately exsanguinated. The right triceps brachii, vastus lateralis, rectus femoris, and semimembranosus muscles were immediately excised, trimmed of visible fat, and weighed.

Carcasses were eviscerated and the offal components were isolated and weighed. The right and left carcass sides and offal components were then stored at  $-20^{\circ}\text{C}$  for subsequent analysis. The right carcass side was thawed and then dissected into muscle, fat, skin, and bone. During dissection, the longissimus dorsi, gluteus medius, infraspinatus, and psoas major muscles were excised intact, trimmed of visible fat, and weighed. Each individual muscle isolated was classified according to its fiber type composition (Beecher et al., 1965; Kiessling et al., 1982; Ono et al., 1995). Muscles with a high percentage of type-I and type-IIA fibers were considered red muscles; whereas, muscles with a high percentage of type-IIB fibers were considered white muscles. The frozen left carcass side and viscera were each cut into pieces, ground, and then subsampled (Williams et al., 1997). Samples were analyzed for nitrogen, ether extractable lipid, and dry matter according to (AOAC, 1995) procedures. Dissected muscle tissue was prepared and analyzed via the same procedures as outline above.

Carcass muscle and fat tissue accretion rates were estimated by subtracting estimated tissue weights at initiation of the trial from their dissected carcass tissue quantities and dividing by number of days on test.

The soy extract and experimental diets were analyzed for isoflavone concentration courtesy of Pat Murphy, Iowa State University according to the methods of (Wang and Murphy, 1994a; Wang and Murphy, 1994b).

Data were analyzed as a randomized complete block design with variance techniques using the GLM procedure of SAS (1997) with litter serving as the block and pig as the experimental unit. Carcass weight was used as a covariate in the analysis of carcass tissues and individual muscle weights. Daily BW gain, daily feed intake, and gain:feed ratios were analyzed at three stages of growth via a repeated measures model.

## Results

Supplemental isoflavones did not affect ( $P > .1$ ) daily feed intake (911 vs 911 g), or gain:feed ratios (639 vs 656 g/kg) but increased ( $P < .06$ ) daily BW gain from 579 g to 595 g (Table 3). The concentration of the acute phase protein, alpha-1 acid glycoprotein was assayed in serum at the initiation and termination of the trial and was not altered ( $P > .1$ ) by isoflavone regimen (Table 3). Daily BW gain during three phases of development (10.0, 18.0, and 26.0 kg) was independent ( $P > .1$ ) of dietary isoflavone concentration.

Supplemental isoflavones increased ( $P < .07$ ) the percentage of carcass muscle (Table 4) from 59.1 to 61.0, but did not ( $P > .1$ ) affect carcass percentages of fat (14.1 vs 13.3), bone (17.7 vs 17.1), and skin (9.0 vs 8.6). Estimated carcass muscle accretion rate was increased ( $P < .02$ ) by isoflavones (251 vs 266 g/d). Estimated fat accretion was not altered ( $P > .1$ ) by isoflavones (63 vs 61 g/d). Although carcass muscle was increased by isoflavones, the chemically-determined percentages of carcass protein (19.8 vs 20.5) and fat content (14.0 vs 13.4) were not altered ( $P > .1$ ) by feeding isoflavones. The chemical composition of dissected muscle did not differ ( $P > .1$ ) between treatments.

Eight individual muscles were dissected intact and trimmed free of external fat prior to carcass dissection (Table 5). The combined weight of four red muscles (psoas major, infraspinatus, triceps brachii, and rectus femoris) was increased ( $P < .01$ ) by dietary isoflavone inclusion (380 vs 409 g), but the combined weight of four white muscles (longissimus dorsi, semimembranosus, gluteus

medius, vastus lateralis) was not altered ( $P > .1$ ) by isoflavones (1153 vs 1142 g). Thus the muscle growth response to dietary isoflavones was dependent on muscle fiber type.

Visceral component weights of head, liver, kidneys, heart/lungs, and gastrointestinal tract were not altered ( $P > .1$ ) by isoflavones nor was chemical composition of the combined offal components ( $P > .1$ ).

## Discussion and Implications

The composition of supplemental isoflavones in the current study was similar to the isoflavone composition of soybeans, although the genistein concentration was slightly lower and daidzein slightly higher than soybeans. The total concentration of supplemental isoflavones used (1585 ppm) is equivalent to a diet containing 35% soybean meal, which in turn contains 4285 ppm isoflavones (normalized concentration). The soy extract was reported to be 70% saponins which do have some biological activities (reviewed by Hostettmann and Marston, 1995). But soybean saponins failed to alter animal growth (i.e. body weight gain) when fed at concentrations five times that found in soybeans (Ishaaya et al., 1986). Thus diet-induced differences in this study are discussed in the context of the biological activities of isoflavones.

Although measures of isoflavone absorption were not undertaken in the present study, we quantified urinary recoveries of genistein and daidzein in gravid rats fed the same soy extract as was used in this study (Objective 3.3.2). Based on these data, both daidzein and genistein in the soy extract are bioavailable. Further studies at our station showed that both genistein and daidzein from a different dietary source were absorbed in a dose-dependent fashion based on serum concentrations in pigs (Cook, 1999). Thus, we have established that isoflavones are bioavailable to pigs and that isoflavones from the source used in the current study also are bioavailable.

Daily BW gain and carcass muscle percentage were increased by dietary isoflavones in the current study suggesting that the negative effects of genistein observed in vitro on myoblast proliferation (Hashimoto et al., 1995; Jiet et al., 1997) and glucose transport (Smith et al., 1993; Vera et al., 1996) did not impact pig growth in vivo at the isoflavone concentration tested. The increased muscle growth occurred predominantly in muscles classified as red. Recently, daidzein has been shown to increase secretion of the cytokine, interleukin-3 (Wang et al., 1997), which has been shown to promote glucose transport by regulating affinity of the glucose transporter for glucose (Berridge and Tan, 1995; McCoy et al., 1997). Muscles with a predominance of type-I fibers (red) possess greater concentrations of glucose transporters relative to muscles with a predominance of type-II (white) fibers (Goodyear et al., 1991). If muscle tissue also is responsive to interleukin-3, daidzein-induced interleukin-3 production could increase glucose transport, and thus energy supply and growth, to a greater extent in red vs white muscles. The biological effects of interleukin-3 have not been characterized in pigs.

Other cytokines, such as interleukin-1, and tumor necrosis factor alpha are classified as "proinflammatory" and have negative effects on muscle growth when produced in response to immune stimulation (Klasing, 1988; Williams et al., 1997). Conversely, minimizing chronic immune activation, and thus proinflammatory cytokine level, and increases muscle growth. Daidzein increases production of the non-proinflammatory cytokine, interleukin-2 (Zhang et al., 1997). Thus, daidzein-induced IL-2 secretion could result in enhanced immune function, and reduced chronic immune activation and lower levels of pro-inflammatory cytokines. But serum

concentrations of AGP, an acute-phase protein produced in response to proinflammatory cytokines, were not altered by isoflavones in this study suggesting that pro-inflammatory cytokine levels were not altered.

Daidzein and genistein are referred to as "phytoestrogens" because of their estrogenic activity in several different experimental models (Medlock et al., 1995; Santellet al., 1997; Zava and Duwe, 1997). Estrogens stimulate growth hormone release (Tannenbaum et al., 1992; De Leo et al., 1993; Marin et al., 1994; Metzger and Kerrigan, 1994; Moe et al., 1998), growth hormone receptor expression, growth hormone action in bone cells (Slootweg et al., 1997), and IGF-I in tissue and serum (Murphy et al., 1987; Norstedt et al., 1989; Pollak et al., 1990; Pollak et al., 1992; Sahlin, 1995). Moreover, tyrosine kinase inhibitors, including genistein, enhance growth hormone releasing hormone-stimulated growth hormone release in rat anterior pituitary cells (Ogiwara et al., 1997). However, administration of porcine somatotropin to pigs from 30 to 60 kg BW increases the cross sectional areas of all fiber types (Solomon et al., 1990; Solomon et al., 1991) whereas our data suggest a muscle growth response specific to red-fibered muscles.

Based on our data, dietary soy isoflavones increase muscle growth in young pigs and this response is greatest in muscles, which have a high proportion of red fibers. The activity of soy isoflavones can serve as a value-added trait that will enhance the market value for soybeans and soy products. Therefore, the bioactive nature of soy isoflavones have important biological and economical impacts on muscle growth and meat quality.

### **Future Directions**

With the determination that soy isoflavones are bioactive compounds that enhance postnatal muscle accretion, the next steps should include: determining the optimum dietary concentration of the soy isoflavones (the biological effects of the isoflavones during the various physiological stages of the pigs body development (growth, sexual maturation)) and the identification of the ideal composition of the isoflavone mixture. Another possibility would be to analyze the enhancement of protein accretion when different ratios of the soy isoflavones are fed in swine diets. Both of these areas would provide valuable information when selecting the variety of soybean used for swine diets.

**Table 1. Basal diet composition, %<sup>a</sup>**

<b>Item</b>	<b>Amount, %</b>
Corn	37.59
Soy protein concentrate	38.28
Lactose	15.00
Corn oil	2.00
Corn Starch	.50
Soy extract <sup>c</sup>	---
Mono-dicalcium phosphate	3.90
Limestone	.41
Salt	.40
Crystalline amino acids	1.02
Trace mineral-vitamin mix <sup>b</sup>	.90

<sup>a</sup>Basal diet contained 17ppm isoflavones.

<sup>b</sup>Provided the following per kg of diet: Biotin, .30 mg; choline, 1800 mg; folic acid, 1.80 mg; niacin, 90.0 mg; riboflavin, 21.0 mg; pantothenic acid, 60.0 mg; pyridoxine, 9.0 mg; thiamine, 6.0 mg; Vit E, 96 IU; Vit A, 13,200 IU; Vit D<sub>3</sub>, 1323 IU; menadione, 3.0 mg; Vit B<sub>12</sub>, .105 mg; Cu, 17.5 mg; Fe, 175 mg; Mn, 60 mg; Se, .30 mg; Zn, 226 mg;

<sup>c</sup>Isoflavone composition of soy extract described in table 2.

**Table 2. Isoflavone composition of soy extract.**

Isoflavone form	Isoflavone content, mg/g <sup>a</sup>	
	Total	Normalized
Glycosidic forms		
Daidzin	107.83	68.32
6"-O-Acetyldaidzin	16.40	9.41
6"-O-Malonyldaidzin	15.55	8.11
Genistin	105.91	68.60
6"-O-acetylgenistin	14.93	8.78
6"-O-Malonylgenistin	12.37	6.64
Glycitin	33.93	23.62
6"-O-acetylglycitin	1.96	1.24
6"-O-Malonylglycitin	5.03	2.92
Total glycosidic forms	313.91	197.64
Aglycone forms		
Daidzein	1.41	1.41
Genistein	1.13	1.13
Glycitein	1.07	1.07
Total aglycone forms	3.61	3.61
Total isoflavones	317.51	201.25

<sup>a</sup>Normalized amount = (molecular weight of aglycone form/molecular weight of glycosidic form)x mg of glycosidic form in soy extract.

**Table 3. Effect of dietary soy isoflavones on pig growth and feed utilization and on serum alpha-1 acid glycoprotein (AGP) concentrations<sup>a</sup>.**

Item	Supplemental isoflavones, ppm		CV, %	Probability
	0	1585		
No. of pens	13	15		
Pig weight, kg				
Initial	6.17	6.18	2.6	.17
Final	30.90	30.57	1.8	.71
Pig growth and feed utilization <sup>b</sup>				
Body gain, g/d	579	595	3.0	.06
Feed intake, g/d	911	911	5.9	.98
Gain:feed, g/kg	639	656	5.1	.26
Serum AGP, $\mu$ g/ml				
Initial	754	750	21.8	.95
Final	435	409	11.0	.48

<sup>a</sup>Pigs penned individually and self-fed a basal diet supplemented with 0 or 1585 ppm soy isoflavones.

<sup>b</sup>Final pig weight used as covariate for analysis of pig growth and feed utilization data.

**Table 4. Effect of supplemental soy isoflavones on carcass tissue percentages, accretion rates, and chemical composition.**

Item	Supplemental isoflavones, ppm		CV, %	Probability
	0	1585		
Carcass wt., kg	21.2	21.0	1.8	.15
Carcass tissue, kg <sup>a</sup>				
Muscle	12.48	12.87	3.5	.08
Fat	3.02	2.80	12.9	.21
Bone	3.77	3.58	10.0	.27
Skin	1.92	1.79	9.6	.11
Carcass tissue gains, g/d <sup>a</sup>				
Muscle	234	248	5.0	.02
Fat	53	50	12.8	.41
<b>Dissected muscle chemical content, %</b>				
Protein	21.4	21.3	3.7	.62
Fat	5.1	5.0	5.1	.22
Carcass chemical content, %				
Protein	19.8	20.5	6.1	.26
Fat	14.0	13.4	14.9	.53

<sup>a</sup>Carcass weight used as a covariate.

**Table 5. Effect of dietary soy isoflavones on weights of red and white-fibered muscles.<sup>a</sup>**

Item	Supplemental isoflavones, ppm		CV, %	Probability
	0	1585		
Red-fibered muscles, g				
Psoas major	100.1	107.4	12.6	.22
Infraspinatus	51.3	59.6	14.3	.03
Triceps brachii	102.0	107.9	6.6	.07
Rectus femoris	126.5	133.9	3.0	.01
Total	379.8	408.8	5.4	.01
White-fibered muscles, g				
Longissimus dorsi	621.5	618.7	8.1	.90
Gluteus medius	84.8	83.6	9.8	.75
Semimembranosus	341.9	328.1	5.8	.14
Vastus lateralis	104.5	112.0	7.6	.06
Total	1152.6	1142.3	5.2	.70

<sup>a</sup>Carcass weight used as a covariate.

## **Effects of Dietary Soybean Isoflavone Concentrations in Gravid Rats on Rate and Efficiency of Growth and Muscle Content of the Offspring**

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**Institution:** Iowa State University

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### **Summary**

Gravid rats were given ad libitum access to a basal, corn-soy concentrate diet supplemented with 0, 431, 862, 1724 ppm (normalized concentration) soy isoflavones (42.3% genistein, 44.5% daidzein, 13.2% glycitein) from mating through parturition. In experiment one, urinary daidzein and genistein recoveries in dams during d 8-20 of gestation averaged 45% and 17%, respectively, indicating that a portion of soy isoflavones were digested and absorbed. Both isoflavones were detected in fetal tissues indicating placental transfer had occurred. In experiment two, the dietary isoflavone concentrations consumed by the dam during pregnancy altered the body weight gain, efficiency of feed utilization, and muscle development of the dam's offspring fed a basal diet from weaning to 215 g BW. In male offspring, gain:feed ratios improved linearly and hind limb muscle and testicle weights at 215 g BW increased quadratically ( $P < .03$ ), as maternal dietary isoflavone intake increased but hind limb bone weights were not altered ( $P > .15$ ). In female offspring, gain:feed ratios improved quadratically ( $P < .04$ ) and body growth rate and feed intake increased linearly ( $P < .01$ ) as maternal dietary isoflavone concentration increased. Hind limb muscle and bone weights were not altered ( $P > .1$ ) but the carcass protein content in hind limb muscle increased quadratically ( $P < .08$ ). Based on these data, soy isoflavones fed to gravid rats are bioactive compounds that are digested and absorbed in the gastrointestinal tract, cross the placenta during gestation, and elicit biological responses on offspring by increasing muscle and testicular growth in male offspring and the rate and efficiency of growth in female offspring. These results are consistent with our data showing increased body and muscle growth rates in young pigs fed supplemental dietary isoflavones.

### **Problem Addressed**

In a previous paper (Objective 3.3.1), we reported that dietary isoflavones fed to pigs from 6 to 30 kg BW increased muscle growth and the rate of BW gain. Muscle growth occurs through a process of myoblast proliferation and subsequent fusion to form muscle fibers. Altering certain

factors during gestation, such as nutrient supply, (Dwyer et al., 1993; Dwyer and Stickland, 1994; Dwyer et al., 1994; Dwyer et al., 1995) hormone levels (Ogata et al., 1988; Bell et al., 1989), and the presence of nonnutritive compounds (i.e. genistein) (Maltin et al., 1990; Kim et al., 1994), are reported to affect postnatal muscle growth potential. Some of isoflavones' biologic activities that could influence prenatal muscle development and thus postnatal muscle growth include estrogenic effects (Medlock et al., 1995b; Santell et al., 1997; Zava and Duwe, 1997), immunomodulation (Trevillyan et al., 1990; Wang et al., 1997; Zhang et al., 1997), inhibition of tyrosine kinase activity (Akiyama et al., 1987), inhibition of DNA synthesis in placental trophoblasts (Hamlin and Soares, 1995), and suppression of myoblast proliferation and differentiation (Hashimoto et al., 1995; Ji et al., 1997). Based on these data, isoflavones may impact a wide variety of biological processes including muscle growth and development. Therefore, our objective was to determine the effect of feeding soy isoflavones to gravid animals on postnatal rate and efficiency of growth and muscle content of the offspring.

### **Approach Used**

Sprague-Dawley female rats were mated with Sprague-Dawley males. Prior to mating, animals were allowed to consume a basal diet (Table 1) ad libitum. The morning pregnancy was detected, female rats were randomly allotted to the basal diet supplemented with 0, 431, 862, or 1724 ppm isoflavones (normalized concentrations). The basal diet was formulated to meet or exceed the nutrient requirements of rats during gestation and lactation (NRC, 1995). The supplemental isoflavones were supplied by a soy extract (Archer Daniels Midland Co., Decatur, IL) that replaced cornstarch on an equal weight basis. The composition of the soy extract is described in Objective 3.3.1. The gravid females were allowed to consume feed and water ad libitum and were maintained on a 12 h light-dark cycle in a room sustained at 27 ° C.

#### ***Experiment one.***

Twenty, first-parity females (215 g BW) were penned individually in metabolism cages from d 6 to 20 of pregnancy. From d 8 to 20 post-mating, dam's feed intake and BW were measured every 2 d. Each dam's total 48 hr urine output also was collected every 2 d and stored at -20 ° C for subsequent analysis of genistein and daidzein concentrations. At d 20 postmating, rats were rendered unconscious via CO<sub>2</sub> inhalation and immediately decapitated. The conceptus tissues (uterine, placental, and fetuses) were separated and weighed. Fetuses were cervically dislocated, frozen in liquid nitrogen, and stored at -20 ° C for analysis of tissue isoflavone content.

#### ***Experiment two.***

Eighty-five first and second parity females were penned individually in cages. Immediately after whelping, dams were allowed ad libitum access to the basal diet and water for lactation. Litter size was standardized to not more than seven pups on the day of whelping and to not more than six pups on day four of lactation by removing the largest and smallest pups from each litter. Pups were not cross fostered. All litters were weaned at 20-23 days of age, and one male and one female pup (close to the mean weight of the litter) per litter were individually penned for a growth trial. Pups were given ad libitum access to a corn-soy protein concentrate diet (Table 1) formulated to contain 1.4 % lysine and not less than 110 % of the ideal ratio other essential amino acids relative to lysine. The diet was formulated to meet or exceed nutrient requirements for growing rats (NRC, 1995).

Dam BWs were measured at breeding and immediately following parturition, and dam feed intakes were quantified from d 1 of gestation through parturition. Litter weights were recorded within 3 hours after parturition and every four days throughout lactation. Following weaning, pup weights and feed disappearance were measured at four-day intervals. All diets (gestation, lactation, and postweaning) were fed in meal form.

At a BW of  $215 \pm 3$  g, pups were anesthetized via CO<sub>2</sub> inhalation and immediately decapitated. Hind limb muscle tissue was dissected free from the bone and weighed to the nearest mg, external fat was removed and placed with the carcass, and muscle was frozen at  $-20^{\circ}$  C for subsequent protein analysis. Bone was weighed to the nearest mg and placed with the carcass. Internal organs of heart-lungs, liver, kidneys, gastrointestinal tract, reproductive tract were weighed to the nearest mg and frozen for subsequent protein analysis. Hind limb feet and skin and the head all were stored with the carcass at  $-20^{\circ}$  C for subsequent nitrogen analysis (AOAC, 1995). All animal procedures were approved by the Iowa State University Committee on Animal Care.

Urinary and fetal tissue isoflavone concentrations were analyzed using modifications of the procedures reported by King et al. (1996) and Franke et al. (1995). Standards consisting of 12.5, 25, 50, 100, 200, 300, 400, and 500  $\mu$ M daidzein and genistein in 80% aqueous methanol were used to calculate a standard curve using peak area. Using these procedures, the sensitivity was approximately .4  $\mu$ g/ml, and percent recoveries for daidzein and genistein were 94.5 and 104.1 with intra-assay CVs of 7.4 and 7.8 %, respectively.

Data were analyzed by variance techniques using the GLM procedure of SAS (1997). Data were analyzed as a completely randomized design and dam was considered the experimental unit. The orthogonal linear, quadratic, and cubic contrasts were used to evaluate the response to dietary isoflavone concentrations. Carcass weight was used as a covariate for the analysis of offspring muscle and bone weights, and muscle and carcass protein. Pup BW gain/d, feed intake/d, and gain:feed ratios over four stages of growth postweaning were analyzed via a repeated measures analysis. Separate analyses were performed for male and female pups postweaning as a result of large differences in growth rate and efficiency of feed utilization. All data presented are least square means.

## Results

### *Diets.*

The basal diet contained 149 ppm total isoflavones and the diets supplemented with 431, 862 and 1724 ppm diets contained 582, 1023, and 1941 ppm total isoflavones, respectively.

### *Experiment one.*

#### *Growth and Tissue.*

Increasing dietary dam isoflavone levels (Table 2) decreased dam feed intake and BW linearly ( $P < .01$ ). As expected, feed intake and BW increased with increasing days of gestation ( $P < .01$ ). Total conceptus weights, litter weights, placental weights, and uterine weights were not altered ( $P > .1$ ) by the dam's isoflavone level. Fetal number, and placenta weight/fetus were also not altered by isoflavone regimen ( $P > .1$ ).

***Urinary and tissue isoflavones.*** Percent urinary recovery of daidzein (Table 3) increased quadratically ( $P < .01$ ) with increasing isoflavone level and percent urinary recovery of genistein

(Table 5) responded in a cubic fashion ( $P < .02$ ). Daidzein recovery did not change ( $P > .1$ ) over time but genistein recovery decreased ( $P < .01$ ) with increasing day of gestation. Fetal tissue isoflavone levels were below the detection limit of our assay (.4 nM) with the exception of the highest isoflavone level. Daidzein and genistein were detected in fetal tissue from all rat dams consuming the highest isoflavone level and averaged .78 and .80  $\mu\text{M}$ , respectively.

### ***Experiment two.***

#### ***Gestation.***

The initial BW of rat dams at breeding averaged 270 g. During gestation, isoflavones (Table 4) decreased feed intake, and thus post partum BW, linearly ( $P < .01$ ). However, total pups born and pup birth weight were not altered ( $P > .1$ ) in response to isoflavones

#### ***Lactation.***

Dam feed intake and pup BW gain from parturition to weaning were not altered by maternal isoflavone intake ( $P > .1$ ) (Tables 5). Although post-partum BW of dams decreased linearly in response to isoflavones, BW change was not altered ( $P > .1$ ) in response to dietary treatments at any stage of lactation. Dam feed intake from d 4-8 of lactation was decreased at the lowest level of supplemental isoflavones, then increased in response to higher levels of isoflavones resulting in a quadratic response ( $P < .05$ ).

After adjustment on d 4, litter size was not significantly different between treatments ( $P > .1$ ) indicating litter size was successfully standardized. Average pup BW gain during lactation (Table 6) was decreased ( $P < .09$ ) at 4-8 and 8-12 d of lactation with increasing dam isoflavone intake.

#### ***Postweaning.***

Male pups daily BW gains and feed intakes (Table 7) were not altered ( $P > .1$ ) by maternal isoflavone intake but gain:feed ratios increased linearly as isoflavone level increased ( $P < .08$ ). The impact of maternal isoflavone intake on daily BW gain, daily feed intake, and gain:feed ratios was not dependent ( $P > .1$ ) on the male offspring's stage of growth (Table 8).

Female daily BW gains and feed intakes (Table 7) were increased linearly ( $P < .01$ ) and gain:feed ratios increased quadratically ( $P < .04$ ) as the dam's isoflavone level increased. Similar to responses in male pups, effects of maternal isoflavone intakes on female offspring's growth, feed intakes, and efficiencies of feed utilization were not dependent ( $P > .1$ ) on the female pups' stage of growth (Table 8).

#### ***Body tissues.***

At slaughter, carcass weights (Table 9) averaged 142 and 148 g in male and female offspring and were not altered by isoflavone level ( $P > .1$ ). As the dam's isoflavone intake increased, muscle weights increased quadratically ( $P < .05$ ) in male, but not in female pups. Bone weights were not altered ( $P > .1$ ) in either gender by the dam's isoflavone intake.

Offal component weights of liver, kidney, and heart/lungs in male and female pups (Table 10) were not altered ( $P > .1$ ) by isoflavone level. Weights of reproductive organs increased quadratically ( $P < .01$ ) in male but not in female pups in response to increasing maternal isoflavone intake.

Total hind limb muscle protein and total body protein content increased quadratically ( $P < .04$ ,  $P < .09$ , respectively), in male pups, but were not altered ( $P > .1$ ) in female pups as maternal isoflavone intake increased (Table 11). Visceral protein content responded in a cubic fashion ( $P < .01$ ) in male pups and increased linearly ( $P < .06$ ) in female pups with increasing maternal

isoflavone level. Carcass protein was not altered ( $P > .1$ ) in male pups but increased quadratically ( $P < .05$ ) in female pups with increasing maternal isoflavone intake.

## Discussion and Implications

Our data indicate that both daidzein and genistein (based on urinary recoveries), from the isoflavone source in this study, were bioavailable to gravid rats. In addition, fetal tissues with detectable levels of genistein and daidzein suggest that both isoflavones crossed the placenta. Thus, fetuses were directly exposed to isoflavones, which establishes a basis for isoflavone-induced changes in postnatal muscle growth as a result of maternal isoflavone intake during pregnancy.

Critical processes occur during fetal development that affect postnatal muscle growth potential of the offspring. Specifically, that rate of fetal myoblast proliferation and subsequent extent of muscle fiber formation determine muscularity in mature animals. Thus, manipulation of factors which influence fetal muscle development, such as nutrient supply (Dwyer et al., 1993; Dwyer and Stickland, 1994; Dwyer et al., 1994; Dwyer et al., 1995), hormone levels (Ogata et al., 1988; Bellet et al., 1989), and the presence of nonnutritive compounds (i.e. genistein) (Maltin et al., 1990; Kimet et al., 1994), likely will affect postnatal muscle growth potential.

In the current study, hind limb muscle weights of male rats increased in response to increasing maternal dietary isoflavone level during gestation, but bone weights were not different. In a previous study at our station, isoflavones from the same soy extract increased muscle growth when fed to young pigs. The muscle growth response in the current study was paralleled by an increase in testicle weight suggesting that changes in muscle may have been related to altered androgen levels and the anabolic effects of androgens. Isoflavones have recently been shown to inhibit thyroid hormone production (Divi et al., 1997). Neonatal hypothyroidism in male rats increases both Leydig and Sertoli cell proliferation (Hardy et al., 1996) resulting in greater numbers of these cells in adults and thus, greater number of cells capable of producing testosterone. The direct impact of this alteration on testicular development in a fetus is not clear. However, it is clear that genistein has the ability to alter steroid hormone metabolism, and the response of hind limb muscle weights in males was paralleled by testicular weights suggesting that isoflavones may have altered muscle weights in male rats by impacting steroid hormone metabolism and testicular development.

In female rats, hind limb muscle weights and total body protein was not altered by maternal dietary isoflavones, but carcass protein content increased slightly suggesting a generalized anabolic effect. Increased hind limb muscle in male but not female offspring suggests isoflavones' mode of action may not be a direct effect on developing muscle, but rather, an indirect effect via a gender-dependent mechanism (i.e. altered androgen levels). Thus, the impact of fetal exposure to isoflavones on muscle growth postnatally in castrated animals would be valuable information.

In females, increasing maternal isoflavone intake improved growth rate and efficiency of feed utilization. The majority of the response seemed to be in pups from dams fed the highest isoflavone level which gained BW 15% faster than control pups and had gain:feed ratios 10% greater than control pups. The response was consistent during four stages of growth. When administered from d 1-5 postnatally, the estrogenic compound coumesterol has been reported to decrease estrogen receptor levels in growing female rats (Medlock et al., 1995a). As mentioned

earlier, exogenous estrogen decreases feed intake in rats. Exposure to isoflavones during fetal development might have lowered estrogen receptor expression postnatally. Decreased responsiveness to endogenous estrogens postnatally might then allow increased feed intake in female offspring. We did not, however, quantify estrogen receptor expression in response to prenatal administration of isoflavones. Based on these data, the level of dietary isoflavones consumed by dams during pregnancy has a permanent effect of the offspring's postweaning rate of feed intake and body and muscle growth.

Maternal BW at parturition decreased linearly with increasing dietary isoflavones. But by d 4 of lactation, no differences in maternal BW were detected, and feed intake from parturition to d 4 of lactation did not differ among treatments. This discrepancy may be related to hydration level of dams during isoflavone consumption. In experiment one, urine volume increased with increasing dietary isoflavone levels (data not shown). In fact, urine volume of rats on the highest level of isoflavones was twice that of control rats. Genistein has a salidiuretic action in isolated perfused rat kidneys, which results in diuresis when genistein is administered in vivo (Gimenez et al., 1998). Thus rats consuming large amounts of genistein may have been dehydrated until shortly after parturition at which time, all rats received the basal diet with a low level of genistein. Removal of genistein would then allow rehydration and a rapid increase in BW. However, the reduction in maternal food intake cannot be ignored. Although not statistically different, the mean weight of dams fed the highest isoflavone level was 8 g less than control dams at d 4 of lactation. Thus maternal body energy stores and milk output potential may have been decreased.

Litter size was standardized to not more than six pups early in lactation in an attempt to minimize potential confounding effects isoflavones could have on mammary development, maternal body energy stores, and thus milk output. Even so, pup BW gain from d 4-12 was decreased with increasing isoflavone level. But after d 12 of lactation, pup BW gain was not altered. Rat pups begin to open their eyes around 10-12 days of age and start consuming maternal food shortly thereafter. Thus decreased pup BW gain early in lactation, followed by a lack of difference in late lactation may reflect reduced maternal body energy stores and decreased milk output as a result of decreased gestational maternal feed intake. Upon consumption of maternal food around d 12 of lactation, rat pups could alleviate their dietary restriction by consuming maternal feed. Rat dams restricted to 75% of ad libitum intake during gestation had pups at d 14 of lactation that were 20% lighter than controls (Young and Rasmussen, 1985). Our data are consistent with this observation because a 10% reduction in maternal food intake during gestation was associated with a 10% reduction in the rate of pup BW gain from d 8-12 of lactation.

Urinary daidzein and genistein recoveries averaged 45 and 17% respectively pooled across time. King et al. (1996) reported 17% urinary recovery of genistein in rats that received a bolus dose of either aglycone genistein or soy extract. Initial plasma concentrations of genistein were almost three-fold greater in rats that received the aglycone form of genistein vs the glycosidic form in the soy extract. However, plasma concentrations from 4-24 h post administration did not differ suggesting glycosidic genistein has a similar bioavailability as aglycone genistein. Greater bioavailability of daidzein relative to genistein based on urinary excretion has been reported previously in humans (Xu et al., 1994; Xu et al., 1995) and suggested to be partly a result of shorter intestinal half live for genistein relative to daidzein. However, plasma concentrations of daidzein and genistein in these studies did not differ and thus do not support greater bioavailabilities of daidzein. Our data indicate that daidzein and genistein do cross the placenta

and are found in fetal tissue in similar proportions relative to their dietary concentrations. Unfortunately, only fetuses from dams fed the highest dietary concentration of isoflavones had detectable tissue isoflavone levels and thus, the dose-dependency of isoflavone placental transfer is not apparent from our data. Daidzein recoveries did not change with increasing day of gestation while genistein recoveries decreased.

In conclusion, dietary isoflavones consumed by gravid rats are absorbed, cross the placenta, and impact fetal development resulting in altered rates of postnatal body and muscle growth in the offspring. These changes seem to be consistent with documented effects of isoflavones and estrogenic compounds on perinatal development. Our data on relative urinary excretion of daidzein vs genistein are consistent with other studies in that daidzein excretion through the urine seems to be greater than genistein when similar doses are consumed. Further, similar concentrations of daidzein and genistein in fetal tissue suggest similar bioavailabilities of genistein and daidzein.

### **Future Directions**

Dietary soy isoflavones, when fed to gravid animals (rats), can impact postnatal growth and muscle content of the offspring. The observations that isoflavones have a plethora of biological activities, cross the placenta, and impact postnatal muscle growth responses and meat quality (increase the weight of predominantly red-fiber muscles), suggest that high isoflavone intakes during gestation in other livestock species (i.e. swine) may alter other responses which merit investigation. Elucidation and quantification of these responses in swine will allow manipulation of dietary isoflavone levels via novel soy processing techniques or genetic manipulation of soybeans, to optimize performance criteria in livestock.

**Table 1. Composition of basal diets, %.**

Item	Stage of growth	
	Dam, gestation/lactation	Offspring, postnatal growth
Corn, ground	67.67	60.96
Soy protein concentrate	23.20	31.19
Corn oil	4.00	4.00
Mono-dicalcium phosphate	1.81	.46
Limestone	.91	.88
<b>Salt</b>	.20	.20
D,L-methionine	.55	.80
Premix <sup>b</sup>	.91	.76
Corn Starch	.75	.75
Soy extract <sup>a</sup>	---	---
<b>Analyzed composition</b>		
Protein, %	21.35	26.06
Fat, %	6.36	6.11

<sup>a</sup>Soy extract described in table 2.

<sup>b</sup>Contributed the following per kg of maternal diet: Biotin, .60 mg; choline, 2250 mg; folic acid, 3.0 mg; niacin, 45.5 mg; riboflavin, 12.0 mg; pantothenic acid, 30.0 mg; pyridoxine, 18.0 mg; thiamine, 12.0 mg; Vit E, 81 IU; Vit A, 6900 IU; Vit D<sub>3</sub>, 3000 IU; menadione, 3.0 mg; Vit B<sub>12</sub>, .15 mg; Cu, 17.5 mg; Fe, 175 mg; Mn, 60 mg; Se, .36 mg; Zn, 150 mg

**Table 2. (Exp. 1) Effect of dam's isoflavone regimen on feed intake and BW gain of gravid rats at six, 2-d periods during gestation.**

Item	Days of Gestation	Dam's isoflavone regimen, ppm				CV, %	Probability		
		0	431	862	1724		Isoflavone <sup>a</sup>	Day	Iso X Day
Feed intake, g	8-10	34.3	34.8	32.8	32.1	11.5	.01L	.01	.78
	10-12	36.1	37.6	34.3	31.9				
	12-14	40.2	36.5	34.3	33.1				
	14-16	36.3	38.2	37.3	35.0				
	16-18	42.3	43.9	39.8	39.8				
	18-20	40.2	42.2	37.9	32.4				
BW gain, g	8-10	11.5	11.8	11.3	10.0	5.1	.01C	.01	.99
	10-12	2.0	9.4	6.5	4.4				
	12-14	6.0	11.6	10.0	10.5				
	14-16	17.6	19.4	19.8	17.9				
	16-18	27.2	25.8	21.2	25.2				
	18-20	24.7	22.8	23.0	15.6				

<sup>a</sup>L=linear; C=cubic.

**Table 3. (Exp. 1) Effect of dam's isoflavone regimen on percentage urinary daidzein and genistein recovery of gravid rats at six, 2-d periods during gestation.**

Item	Days of Gestation	Supplemental isoflavone regimen, ppm				CV, %	Probability		
		0	431	862	1724		Isoflavone <sup>a</sup>	Day	Iso X Day
Daidzein, %	8-10	25.9	45.1	50.3	67.9	37.0	.01Q	.22	.23
	10-12	29.3	51.6	66.4	54.4				
	12-14	23.9	58.7	57.6	57.7				
	14-16	26.9	55.1	51.4	44.0				
	16-18	24.5	34.5	43.3	54.8				
	18-20	14.6	41.2	37.5	68.9				
Genistein, %	8-10	8.1	25.9	22.3	34.2	46.7	.02C	.01	.70
	10-12	8.2	25.0	25.5	26.0				
	12-14	7.0	21.1	22.1	24.0				
	14-16	7.7	19.5	19.3	17.0				
	16-18	6.4	13.0	15.3	19.4				
	18-20	3.6	10.3	13.5	19.4				

<sup>a</sup>Q=quadratic; C=cubic.

**Table 4. (Exp. 2) Effect of dam's isoflavone regimen on body weights<sup>a</sup> and feed intakes<sup>a</sup> of gravid dams, litter size,<sup>a</sup> and pup body weights at parturition<sup>b</sup> and weaning.<sup>a</sup>**

Item	Dam's isoflavone regimen, ppm				CV, %	Probability
	0	431	862	1724		Isoflavone <sup>c</sup>
Dam's data, mating to parturition						
No. of dams	20	25	24	20		
BW						
Breeding, g	267.0	275.6	270.5	269.8	5.2	.64
Parturition, g	304.2	312.9	300.3	292.3	5.9	.01L
Feed, g	405.6	407.3	398.9	363.5	6.7	.01L
Litter data at birth						
Total born	12.6	12.3	12.9	11.4	24.7	.48
Litter wt., g	73.8	71.5	75.5	64.3	21.9	.46
Pup wt., g	5.89	5.88	5.90	5.75	8.9	.27
Litter size, d 0 <sup>a</sup>	6.63	5.70	6.63	6.47	19.4	.01C
Litter data after litter size standardized <sup>b</sup>						
No. of pens	20	25	24	20		
Litter size, d 4	5.88	5.08	5.83	5.77	34.3	.13
Litter size, wean	5.28	4.54	5.04	5.01	32.2	.32
Pup wean wt., g	57.41	56.87	56.37	56.47	11.9	.17
Daily BW gain, g	2.73	2.66	2.59	2.64	10.5	.20

<sup>a</sup>Litter size standardized to not more than 7 pups.

<sup>b</sup>Litter size standardized to not more than 6 pups.

<sup>c</sup>L=linear; C=cubic.

**Table 5. (Exp. 2) Effect of dam's isoflavone regimen on dam feed intake and BW gain at 6 stages of lactation.**

Item	Day of Lactation	Dam's isoflavone regimen, ppm				CV, %	Probability
		0	431	862	1724		Isoflavone <sup>a</sup>
Dam feed intake, g							
	0-4	92.3	81.1	92.1	93.3	25.8	.61
	4-8	149.3	136.1	138.5	151.9	20.9	.06Q
	8-12	186.4	160.5	168.2	166.0	22.8	.17
	12-16	190.9	186.3	189.3	187.7	25.8	.61
	16-20	203.9	196.8	205.7	212.1	28.2	.54
	0-wean	984.4	956.3	1004.3	985.2	24.9	.49
BW change, g							
	0-4	9.2	3.3	7.4	13.5	6.1	.20
	4-8	13.5	15.3	17.0	17.2	5.5	.48
	8-12	6.5	7.5	7.5	10.5	5.4	.68
	12-16	1.4	-.6	.4	.4	5.3	.56
	16-20	-11.1	-9.1	-8.8	-9.4	5.6	.79
	0-wean	14.3	9.4	14.2	24.4	6.0	.46

<sup>a</sup>L=linear; Q=quadratic.

**Table 6. (Exp. 2) Effect of dam's isoflavone regimen on pup BW gain at 5 stages of lactation.**

Item	Day of Lactation	Supplemental isoflavone regimen, ppm				CV, %	Probability
		0	431	862	1724		Isoflavone <sup>a</sup>
BW gain, g	0-4	1.25	1.15	1.17	1.22	26.0	.27
	4-8	2.49	2.36	2.22	2.37	19.3	.09Q
	8-12	3.23	3.13	3.05	2.92	16.0	.05L
	12-16	3.07	3.10	3.02	3.17	12.0	.40
	16-20	3.16	3.08	3.00	3.10	10.6	.15

<sup>a</sup>L=linear; Q=quadratic.

**Table 7. (Exp. 2) Effect of dam's isoflavone regimen on feed intake and rate and efficiency of growth in male and female offspring.**

Item	Dam's isoflavone regimen, ppm				CV, %	Probability
	0	431	862	1724		Isoflavone <sup>a</sup>
Males						
Pup BW, g						
Initial	66.0	66.1	65.7	67.2	11.8	.43
Final	215.2	215.3	218.3	215.7	2.3	.08Q
Pup growth and feed utilization						
Feed, g/d	14.17	14.03	13.83	13.77	5.9	.13
BW gain, g/d	5.87	5.84	5.79	5.89	7.8	.53
Gain:feed, g/kg	414	416	419	428	6.8	.08L
Females						
Pup BW, g						
Initial	63.2	62.3	63.7	62.5	12.1	.61
Final	214.1	214.0	212.6	216.2	2.5	.15
Pup growth and feed utilization						
Feed, g/d	12.71	12.88	12.91	13.28	4.6	.01L
BW gain, g/d	2.69	2.76	2.63	3.12	16.3	.01L
Gain:feed, g/kg	211	214	203	234	13.2	.04Q

<sup>a</sup>L=linear; Q=quadratic.

**Table 8. (Exp. 2) Effect of dam's isoflavone regimen on BW gain of male and female offspring at 4 stages of postweaning growth.**

Item	Pup BW, g	Dam's isoflavone regimen, ppm				CV, %	Probability
		0	431	862	1724		Isoflavone <sup>ab</sup>
Males							
BW gain, g/d	wean-98	4.26	4.28	4.33	4.25	17.2	.43
	98-131	6.28	6.07	6.28	5.90		
	131-163	6.86	7.12	6.70	7.00		
	163-195	7.82	7.08	6.65	7.46		
Females							
BW gain, g/d	wean-98	3.58	3.67	3.68	3.81	21.0	.01Q
	98-131	4.47	4.48	4.32	5.03		
	131-163	3.20	3.30	3.06	3.64		
	163-195	2.50	2.20	2.28	2.75		

<sup>a</sup>No isoflavone by BW interaction detected ( $P > .10$ ) for male or female pups.

<sup>b</sup>Q=quadratic.

**Table 9. (Exp. 2) Effect of dam's isoflavone regimen on carcass, head, and hindlimb bone and muscle weights in male and female offspring<sup>a</sup>.**

Item	Dam's isoflavone regimen, ppm				CV, %	Probability
	0	431	862	1724		Isoflavone <sup>c</sup>
<b>Males</b>						
Carcass wt., g	142.62	142.62	142.72	142.03	2.5	.25
Head wt., g	17.01	17.27	16.77	17.11	4.6	.06C
Bone wt., g	3.21	3.27	3.22	3.23	7.5	.17
Muscle wt., g	22.65	22.96	23.24	22.74	6.1	.03Q
<b>Females</b>						
Carcass wt., g	148.54	149.04	147.42	149.18	2.4	.17
Head wt., g	18.43	18.54	18.61	18.44	4.2	.42
Bone wt., g	3.37	3.39	3.41	3.34	4.3	.27
Muscle wt., g	26.47	26.41	26.59	26.30	3.7	.56

<sup>a</sup>Carcass weight used as a covariate.

<sup>c</sup>Q=quadratic; C=cubic.

**Table 10. (Exp. 2) Effect of dam's isoflavone regimen on offal component and reproductive tract weights in male and female offspring.**

Item	Dam's isoflavone regimen, ppm				CV, %	Probability
	0	431	862	1724		Isoflavone <sup>a</sup>
<b>Males</b>						
Liver wt., g	9.64	9.59	9.76	9.57	7.0	.61
Kidney wt., g	1.91	1.95	1.92	1.92	5.4	.36
Heart/lung wt., g	2.56	2.45	2.53	2.50	8.8	.13
GI tract wt., g	20.99	20.72	22.25	21.71	9.8	.08C
Testicle wt., g	4.50	4.76	4.84	4.72	7.6	.01Q
<b>Females</b>						
Liver wt., g	7.15	7.12	7.16	7.19	8.1	.81
Kidney wt., g	1.67	1.66	1.66	1.63	5.7	.12
Heart/lung wt., g	2.33	2.36	2.33	2.36	7.5	.63
GI tract wt., g	17.63	17.25	17.53	18.44	6.1	.03Q
Uterine/ovary wt., g	.85	.90	.82	.95	36.1	.33

<sup>a</sup>Q=quadratic; C=cubic.

**Table 11. (Exp. 2) Effect of dam's isoflavone regimen on protein percentage and content of carcass, muscle and viscera in male and female offspring.**

Item	Dam's isoflavone regimen, ppm				CV, %	Probability
	0	431	862	1724		Isoflavone <sup>d</sup>
<b>Males</b>						
Carcass protein, g <sup>ac</sup>	37.42	37.76	37.94	37.82	3.0	.24
Muscle protein, g <sup>c</sup>	5.02	5.13	5.16	5.05	4.6	.04Q
Visceral protein, g	5.86	5.79	6.15	5.93	5.4	.01C
Total protein, g <sup>b</sup>	43.28	43.56	44.09	43.75	2.7	.09Q
<b>Female</b>						
Carcass protein, g <sup>ac</sup>	38.47	38.92	39.02	38.65	2.7	.07Q
Muscle protein, g <sup>c</sup>	6.15	6.24	6.25	6.12	4.7	.11
Visceral protein, g	4.53	4.51	4.49	4.67	5.4	.06L
Total protein, g <sup>b</sup>	43.00	43.43	43.51	43.32	2.4	.14

<sup>a</sup>Includes muscle protein.

<sup>b</sup>Sum of carcass and viscera.

<sup>c</sup>Carcass weight used as a covariate.

<sup>d</sup>L=linear; Q=quadratic; C=cubic.

## **Soy Phytoestrogens: Effects on Ovulation Propensity in Swine**

**Investigators:** Todd A. Winters, Gary A. Apgar, William J. Banz,

**Institution:** Southern Illinois University

**Report Date:** February 7, 2000

**Project Start Date:** January 1, 1999

**Project End Date:** December 31, 1999

**Funding Level:** \$32,000

### **Summary**

Soybeans and soy feed ingredients contain bioactive compounds that effect the physiology and metabolism of animals and humans that ingest these products. These compounds have also been called nutraceuticals or functional foods/feeds. The most studied of these compounds are the isoflavones (commonly called phytoestrogens). These compounds have been shown to effect, positively or negatively, reproductive function in animals. In addition to the isoflavones, other compounds found in soybeans may be estrogenic. These include the phytosterols, steroidal saponins, and other phenolic acids. Soybean meal makes up a significant percentage (~20%) of most swine diets. If soybean meal is to attain its full potential as a swine feed, a greater understanding of specific attributes of the meal, including bioactive compounds, must be developed. Whether beneficial or detrimental, a better physiological understanding of these bioactive components in swine is needed. Once understood, these components can be manipulated in the soybean meal by plant breeding, biotechnology, and/or processing, and feeding regimes at certain phases of the reproductive cycle can be developed to optimize production. Specific aims of this proposal were to determine the effects of soy phytoestrogens on the endocrinology of the swine estrous cycle and ovulatory capacity of the porcine ovary. Studies of cultured granulosa cells have found that the soy isoflavones genistein and daidzein inhibit natural programmed cell death (apoptosis) of these ovarian cells in a dose dependent manner, and that this action is at least in part through the estrogen receptor. Daidzein is the most potent of the two phytoestrogens tested with stronger effects than the natural female hormone, estradiol-17 $\beta$  or the synthetic estrogen, DES. Such results could be construed to mean that soy isoflavone may increase ovulation rate. Animal feeding studies are underway to determine the effects of these compounds on the hormonal milieu of the estrous cycle and how they are metabolized in gilts.

### **Problem Addressed**

Our overall objectives were to determine if soy phytoestrogens affect factors that would lead to an increase in swine reproductive prolificacy. Specifically we plan to examine biological

mechanisms that soy phytoestrogens may exert on the swine estrous cycle as it relates to ovarian function, especially ovulation propensity.

We hypothesize that soy phytoestrogens might have positive influences on ovulation rate thus increasing average litter size and number of pigs weaned per litter. Therefore, this research has the potential to benefit both the soybean and the swine industries.

### **Approach Used**

#### ***Ovarian Cell Culture Experiments.***

Cultured primary porcine granulosa cells were plated on 8-well polylysine-coated microscope slides (Nunc, Naperville, IL; n=5 independent populations of granulosa cells per slide). In the first set of experiment, cells were treated for 48 h with genistein, daidzein, diethylstilbestrol (DES) or estradiol at  $10^{-9}$ ,  $10^{-7}$ , and  $10^{-5}$  M, or EtOH vehicle control in serum-free media. In the second set of experiments, cells were treated as before except at the concentrations from  $10^{-17}$  M to  $10^{-5}$  M. In a third set of experiments cells were treated with phytoestrogens alone and in combination with 4-hydroxytamoxifen or ICI 182,780 (antagonistic anti-estrogens used to inhibit estrogen activity). After treatment, cells were processed using an *in situ* apoptosis assay kit (Oncor, Gaithersburg, MD). Degree of apoptosis was quantified microscopically (9 measurements/treatment/slide from a preplanned grid) using an image analysis system (Optimas 5.23, Edmonds, WA). Data were subjected to an analysis of variance and means separated using contrast analysis. The apoptosis experiments give us information about how phytoestrogens affect the cellular mechanism responsible for follicular atresia. Important knowledge about ovulation propensity.

#### ***Studies examining Endocrinology of the Estrous Cycle and Metabolism.***

Eight breeding age gilts (4/trial) had estrous cycles synchronized by moving animals to another barn and exposure to a boar. The gilts will be randomly assigned (blocked by litter) to one of two diets: (1) Ethanol-washed soybean concentrate (essentially void of isoflavones, saponins, and phytosterols; donated by ADM); (2) standard soybean meal (relatively high in isoflavones, and contains saponins and phytosterols) and fed in metabolism crates. The gilts were surgically fitted with vena cava catheters. Blood samples were taken daily for baseline measurement of serum reproductive steroids (progesterone, estrogen, and testosterone), gonadotropins (LH, FSH), and phytoestrogens. In addition, 15-minute blood samplings were collected over a 24-hour window during diestrus (day 9 after estrus) and proestrus (day 19 after estrus) to determine the effects that phytoestrogens may have on gonadotropin pulsatility. Urine and fecal samples were also collected for later analysis of phytoestrogen metabolites. Data will be subjected to analysis of variance and multiple regression analysis, and means separated using Tukey's HSD test. Hormone pulsatility data will be analyzed via deconvolution analysis. These endocrine studies will provide very important and novel information on the effects that soy bioactive factors exert on the hypothalamic-pituitary-ovarian axis, the key modulation system of reproductive function in all mammals.

## Results

### *Ovarian Cell Culture Experiments.*

The soy phytoestrogens, daidzein and genistein decreased granulosa cell apoptosis in a dose responsive manner. In experiment 1, daidzein treatment decreased ( $P < 0.05$ ) mean apoptosis (% area) from 62.9% in the controls to 27.3% in the daidzein-treated cells at  $10^{-9}$  M and higher concentrations ( $10^{-7}$  M=31.2%;  $10^{-5}$  M=23.2%)(Figure 1). Genistein decreased ( $P < 0.05$ ) apoptosis at  $10^{-7}$  M (34.7%) and  $10^{-5}$  M (36.1%), but not at  $10^{-9}$  M. DES decreased ( $P < 0.05$ ) apoptosis at  $10^{-7}$  and  $10^{-5}$  M and estradiol decreased ( $P < 0.05$ ) apoptosis at  $10^{-5}$  M only. In experiment 2, control levels of apoptosis were 80.5%. Daidzein decreased ( $P < 0.05$ ) apoptosis at  $10^{-11}$  M (39.5%) to  $10^{-5}$  M (26.6%), where genistein decreased apoptosis from  $10^{-9}$  M (49.5%) to  $10^{-5}$  M (49.9%). Estradiol decreased apoptosis at  $10^{-7}$  (39.9%) and  $10^{-5}$  M (40.5%). In experiment 3, a 10-fold excess ( $10^{-5}$  M) of the antiestrogens 4-hydroxytamoxifen or ICI 182,780 inhibited the survival effects of genistein and daidzein at  $10^{-7}$  M (Qualitative, quantitative image analysis underway). The Daidzein results of Experiment 1 were reported at the Society for the Study of Reproduction Meetings in College Station, TX (July, 1998), and the remainder of Experiments 1, 2, and 3 were presented at the Global Soy Forum in Chicago, IL (August, 1999). A manuscript to be submitted to the Journal of Animal Science is being prepared from these ovarian cell culture studies.

### *Studies examining Endocrinology of the Estrous Cycle and Metabolism.*

Two trials of 4 gilts each have been completed during the Fall, 1999. These studies were delayed because of problems with the surgical technique and longevity of the cannulas. No data has been analyzed. Additional trials are underway or planned (3-5 during the spring/summer 2000). An internet website has been developed at <http://www.siu.edu/~tw3a/cfarsoy.htm> to update the public on the progress of our soy isoflavone studies.

## Discussion

These results indicate that both genistein and daidzein have the ability to decrease follicular cell atresia by inhibiting apoptosis. Daidzein was approximately 10X more potent than genistein. Both phytoestrogens were more potent than the animals natural estrogen, estradiol. This observation suggests that the phytoestrogens may, at least in part, be working through a pathway different from the classical estrogen receptor mechanism; however, in Experiment 3 antagonist studies have shown that the soy isoflavones do elicit at least part of their effect through an estrogen receptor. Additional studies are underway: 1) to better understand the mechanism(s) of action for these soy phytoestrogens on granulosa cell apoptosis; and 2) to determine if phytoestrogens in the diet effect the estrous cycle and specifically ovulation in the whole animal. In summary, these results suggest that increased use of soy phytoestrogens in the swine diet could lead to more follicle recruitment and ovulations, thus potentially increasing litter size. Such a phenomena would have a great economic impact on the swine industry and thus increase the demand for high isoflavone soybeans.

## Future Directions

### Ovarian Cell Culture Experiments.

In the second year of this research, we will expand on our cell studies by examining how the phytoestrogens interact with other regulators of ovarian function. These will include: follicle stimulating hormone (FSH), a classical regulator of ovarian function working through a cyclic AMP second messenger pathway; insulin-like growth factor I (IGF-I), a regulator of ovarian function working through a tyrosine kinase pathway; and testosterone, a known inhibitor of ovarian function including induction of apoptosis. In addition, we have started examining the third isoflavone in soy, glycitein, and plan on examining other soy bioactive factors including phytosterols, flavones, and steroidal saponins in this ovarian cell culture system. In related studies funded by the Illinois Council on Food and Agricultural Research (C-FAR) we have also be looking at how soy isoflavones affect gene expression in estrogen sensitive cells. These studies have found unique regulation patterns, which are different than the classical estrogens. These studies continue; examining how the transcriptional machinery (molecular regulators of gene expression) is affected by soy isoflavones. All of this research together will help determine how soy isoflavone exert a follicle survival effect on the ovary.

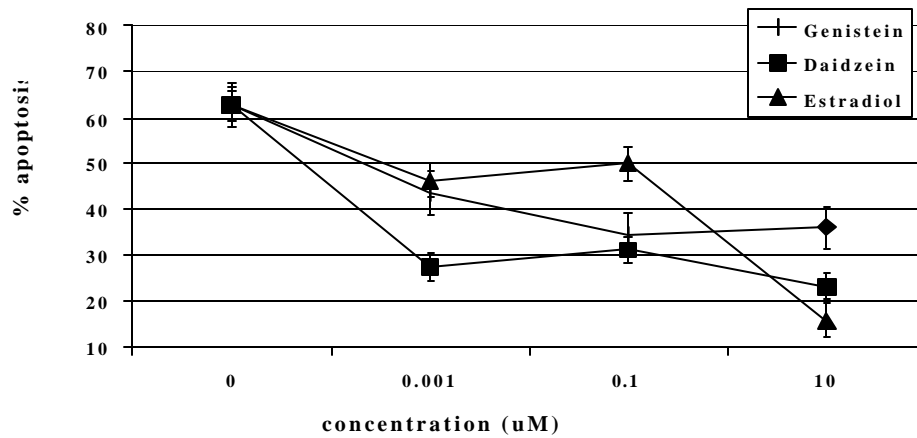
### Studies examining Endocrinology of the Estrous Cycle and Metabolism

In the second year these feeding/metabolism studies will continue. Since daidzein appears to be the strongest regulator of follicle survival (which could lead to an increase in ovulation rate), we are in the process of obtaining a third treatment diet which is high in daidzein from Archer Daniels Midland Co. This treatment will be added to our metabolism trials. In addition, we are in the third year of a long term feeding trial (funded by C-FAR) examining the effects of diets high and low in soy isoflavones on swine reproduction. Preliminary results from the studies have found a trend for an increase in litter size of about one pig. Additional trials are underway to confirm and potentially strengthen this observation. Additional funding is being solicited from C-FAR to continue these studies at least two more years. This would add to and leverage additional funding for a third year from ISPOB.

### Publications and Presentations:

- Winters, T.A., W.J. Banz, T.L. Rosenthal, and R.D. Arthur. 1998. Soy Phytoestrogens and Swine Reproduction. Annual Report to the Illinois Council on Food and Agricultural Research (T.M. Prow, Ed.), p. 34. Information Technology and Communication Service, CACES, University of Illinois, Urbana, IL.
- Suttner, A., N.A. Danilovich, W.J. Banz, and T.A. Winters. 1998. The effects of the phytoestrogen daidzein on *in situ* apoptosis in primary porcine granulosa cells. Biol. Reprod. 58 (Suppl.1): 74.
- Winters, T.A., D.A. Lightfoot, and W.J. Banz. 1999. Soy Phytoestrogens: Genetics, Health, and Reproduction. Poster presentation at the C-FAR Research Day, Springfield, IL, Mar 3, Mimeos, 3 pp.
- Raymer, A.M. 1999. Effects of soy isoflavones and doisynolic-type acids on apoptosis in porcine granulosa cells. M.S. Thesis, Southern Illinois University, Carbondale, IL
- Winters, T.A., W.J. Banz, M.A. Mallon, and S. Adler. 1999. Estrogenic gene activation and estrogenic gene repression by phytoestrogens define two functional

- classes. Proceedings of the Sixth World Soybean Research Conference, Global Soy Forum 99, Chicago, IL, Aug. 7, pg. 710.
- Raymer, A.M., N.A. Hoefling, W.J. Banz, and T.A. Winters. 1999. Soy isoflavones: Effects on *in situ* apoptosis in swine ovarian granulosa cells. Proceedings of the Sixth World Soybean Research Conference, Global Soy Forum 99, Chicago, IL, Aug.7, pg. 686.
- Winters, T.A. 1999. Soy Phytoestrogens in Swine Production. Invited Lecture at Nonruminant Nutrition Symposium entitled, ANutraceuticals: Nutrition or Nirvana?@ 32<sup>nd</sup> Midwestern Sectional Meeting of the America Association of Animal Science, March 15.
- Winters, T.A. 1999. Soy Phytoestrogens & Animal Reproduction. Wise and Helen Borroughs Lectureship. The Nutritional Sciences Council, Iowa State University. Lecture # 148, Feb. 3.



**Figure 1:** Effects of Soy Isoflavones and Estradiol on Porcine Granulosa Cell Apoptosis. Cells were treated for 48 hr and subjected to an in situ apoptosis assay. Percent apoptosis was determined using an image analysis system. Error bars = +/- Standard Error of the Mean.

## **Influence of Isoflavones on Embryonic Development and Embryonic Gene Expression**

**Investigators:** Matthew B. Wheeler

**Institution:** University of Illinois at Urbana-Champaign

**Report Date:** January 15, 2000

**Project Start Date:** January 1, 1999

**Project End Date:** December 31, 1999

**Funding Level:** \$26,183

### **Summary**

Embryonic development in swine is one of the most critical factors in reproduction in the pig. It is directly related to litter size and therefore, reproductive performance. It has been shown that the first embryos which proceed from the spherical stage to the filamentous stage are the embryos that survive to produce piglets. The number of embryos which reach this filamentous stage is directly related to litter size in the sow. The factors involved in embryo development in the pig are largely unstudied. They are likely however, genetic, environmental, nutritional and hormonal influences that affect embryo development and litter size. Estrogens are known to play an important in this early embryonic development period through their influence on maintenance of pregnancy.

The maternal recognition of pregnancy in the pig is a reproductive process about which little is known. It is known that embryos must be present in the uterus between days 12 through 15 after fertilization to maintain pregnancy. In the pig, enough embryos must be present between days 12-15 to maintain the corpus luteum. It has also been shown that a minimum of four embryos are required to maintain pregnancy. One important factor that is known for maternal recognition is estrogen production which is required by the embryo between days 12-15 to prevent the uterus from secreting prostaglandin F<sub>2</sub> alpha into the bloodstream. This prevention of prostaglandin F<sub>2</sub> alpha allows the embryo to survive within the uterus. The mechanism of estrogen production is unknown in the pig, whether the embryo produces estrogen or converts another substance into estrogen. There may be other factors such as growth factors that are involved in estrogen production.

Presently, little research has been done to determine the role of isoflavones in pig reproduction. Isoflavones may play a role in embryo development and affect the gene expression of important growth factors needed for embryo survival. With an increase in the number of embryos surviving, the litter size of the pig is also increased. The present research is necessary to evaluate the role of isoflavones in embryo development and ultimately its role in reproduction of swine. This research may help increase the consumption of soy products by the domestic pigs in the U.S.

## **Problem Addressed**

To determine the effects of isoflavones on development *in vitro* of swine embryos.

## **Approached Used**

### ***Embryo Collection***

Prepubertal Duroc, cross-bred(Duroc Yorkshire) and Yorkshire gilts will be injected with PG6007 at 200 days of age to induce an estrous cycle 3-5 days later. All injections are made subcutaneously. Yorkshires will be mated to Durocs and Durocs mated to Yorkshires to yield a genetically similar fertilized embryos for culture. The donors will be bred naturally by a boar at estrus.

On the day of collection anesthetized donors will then be wheeled into the surgery, the cart placed on the operating table, and a heart monitor and the anesthesia apparatus (supplying, oxygen and halothane) connected. A midline, lower abdominal incision will be made through the body wall, the uterus exteriorized, and embryos either flushed from or introduced into the uterus, depending on whether the sow is a donor or recipient. All incisions will be repaired with absorbable sutures, the wound covered with tape and the sow returned to a clean, dry pen to recover.

### ***In Vitro Embryo Development***

First, we will collect and culture embryos from 2-cell to hatching with or without increasing doses (0, 10nM, 30nM, 100nM, 300nM, 1FM, 3FM) of isoflavones to determine their effects on the rate of development of pig embryos. The base culture medium used will be North Carolina State University-23 (NCSU-23) medium + a protein supplement. Embryos will be cultured and compared in medium containing no steroids (charcoal-stripped serum) with and without the isoflavone doses and in medium with bovine serum (BSA) as a control. At least 100 embryos will be examined per sub-class. The embryos will be cultured for 96 hours. The stage of development will be evaluated and recorded every 24 hours (White et al., 1998). When an embryo ceases development to the next morphological stage, it will be fixed in aceto-orcein and the individual nuclei counted and recorded. This will give us an index of cell division and embryo growth that can be compared across treatments. Embryos reaching the blastocyst stage will be stained with propidium iodide and Hoescht 33342 to allow quantification of the number of inner cell mass cells as well as total cell number.

## **Data Analysis**

Four replicates for each isoflavone concentration, embryonic stage, embryo development were performed. The number of cells from each experimental condition will be analyzed by one-way analysis of variance (ANOVA). Differences between mean cell number for each appropriate comparison including interactions will be compared using treatment (isoflavone concentration, embryonic stage, and embryo development) as the main effect. If non-homogeneity of variance is detected by Bartlett's test, data will be subjected to logarithmic or other appropriate

transformation prior to analysis. For this and subsequent experiments, to permit multiple comparisons among different treatment groups, the Newman-Keuls procedure will be employed. To compare growth factor dose-response curves more precisely, the 4-parameter logistic fit model of David Rodbard, NIH adapted to IBM-PC will be used. This program permits simultaneous analysis of families of sigmoidal curves with statistical comparisons of maximal and ED<sub>50</sub>'s doses (concentrations effectively stimulating half-maximal response). The ED<sub>50</sub>'s will be compared using Student's T-test. Such comparisons are essential to a proper understanding of dose-dependent interactions between effector systems.

## Results

Embryo development data was observed and recorded every 24 h. Developmental data was analyzed by chi-square analysis with the aid of the computer program Statview 5.0 (SAS Institute, Inc., Cary, N.C.). There was no significant difference ( $p < 0.05$ ) in the formation of blastocysts between embryos treated with 1 FM (20/41 49%), 10 nM (24/40 60%), 100 nM (18/39 46%) compared to controls (25/43 58%). Similar results were obtained with embryos treated with 3 FM (30/42 71%), 30 nM (21/43 49%), 300 nM (22/42 50%). Our data suggest that there is trend for improved blastocysts development when cultured in the presence of 3 FM daidzein compared to controls. This data was presented at the 26th annual International Embryo Transfer Society Meeting in Maastricht, Netherlands. Currently, we are beginning to collect two-cell embryos to determine the effect of genistein on embryonic development. The genistein dose curves and analysis are currently underway.

## Discussion and Implications

An increase from 8 to 11 piglets weaned per litter would decrease the cost of producing a weaned piglet by \$9. Therefore, it is very clear that an increase in litter size of domestic pigs, by even 1 pig, would have a dramatic impact on the pork industry. Further, I could increase the consumption of soy products by increasing piglet numbers. By determining the factors that influence prolificacy, including embryo development, we can select or transform pigs from domestic breeds, based on these factors, which will allow us to keep the carcass quality that pork producers have strived so long to achieve. Then, producers will not lose money on the finished product but gain from the improved efficiency of their swine operation.

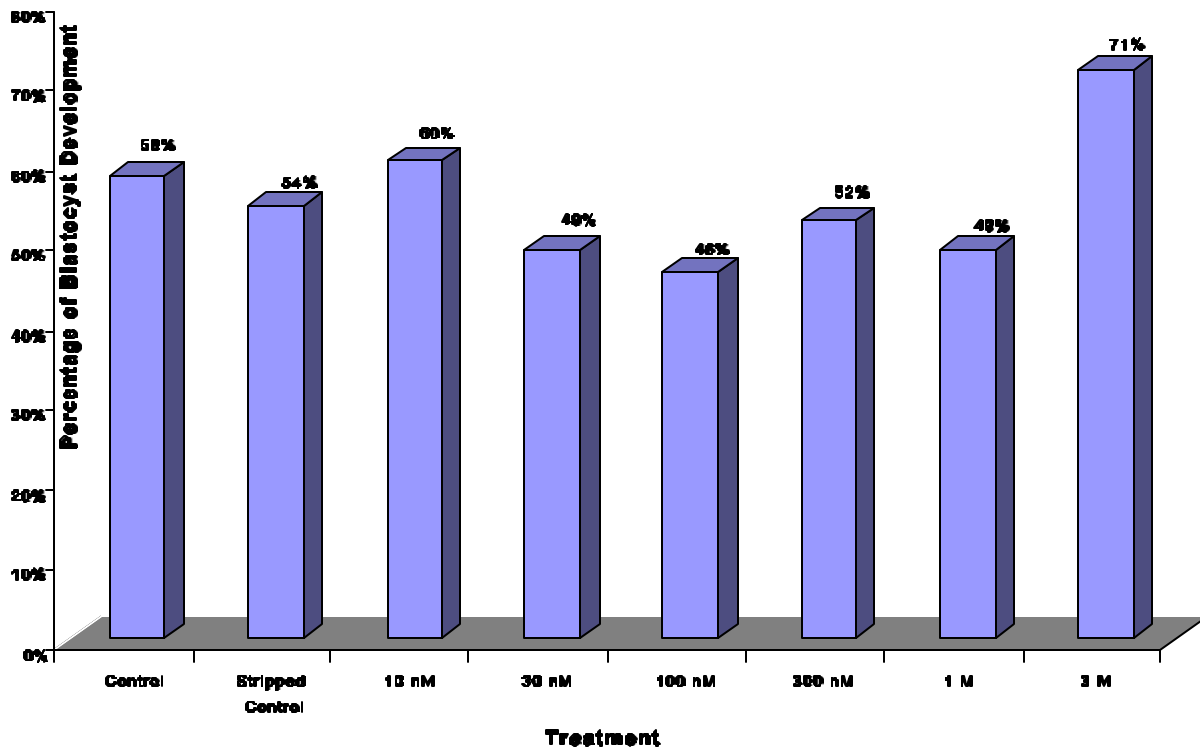
The outlined experiments are providing direct evidence on the role of isoflavones on embryo development and will begin to provide some information on the role of isoflavones on growth factor/cytokine expression in the developing embryo. *The deliverable would be a protocol for using isoflavones and thus soy beans to improve embryo development in the pig.* This could have a profound effect on production efficiency and soy consumption. It is also a potential value-added product for use in the animal biotechnology industries which use swine or swine embryos to produce pharmaceuticals.

The current results indicate that an increased concentrations of isoflavones in swine diets does not adversely impact embryo development and may in fact improve embryo development with certain concentrations. Further, studies are underway to confirm these observations.

### Future Directions (Year 2-3)

1. To systematically investigate growth factor, growth factor receptor and cytokine levels present in embryos in response to increasing levels of isoflavones.
2. To determine the expression levels of messenger RNA=s for growth factor genes, growth factor receptor genes and cytokine genes, through the use of the Polymerase Chain Reaction (PCR), and their effects on in vivo embryo development in response to increasing levels of isoflavones.

### Daidzein



## **Local Tissue Effects of Soybean Phytoestrogens on the Porcine Mammary Gland**

**Investigators:** Walter Hurley

**Institution:** University of Illinois at Urbana-Champaign

**Report Date:** February 11, 2000

**Project Start Date:**

**Project End Date:** December 31, 1999

**Funding Level:** \$26,183

### **Summary**

The long-range goal is to optimize sow productivity by making the most effective use of soybeans and soybean phytoestrogens for enhancing porcine mammary gland growth and lactation. This proposal directly addresses the ISPOB Soy/Swine Nutrition Program objective area # 3, "Investigate the effects of the bioactive molecules in soybean meal on reproduction and carcass composition".

The lactating sow is at the heart of the swine industry, consuming considerable soy protein herself and producing the tremendous numbers of pigs that also consume soy protein. Soy phytoestrogens are of particular interest in the estrogen-sensitive reproductive tract and mammary gland. Physiological effects of soy phytoestrogens on sow mammary gland growth and development have not been described. Actions of soy phytoestrogens on the mammary gland are probably mediated through their estrogenic effects. To determine the nature of phytoestrogen actions on mammary growth in swine, experimental protocols must be developed which provide measurable endpoints for phytoestrogen actions.

The specific objective of the first year of this project was to develop bioassays which may be used to determine the direct tissue-level effects of soy phytoestrogens on mammary gland growth and lactogenesis in swine. Developmental efforts focused upon two bioassays aimed at determining A) the local tissue effects of soy phytoestrogens on growth of porcine mammary tissue *in vitro*, and B) the local tissue effects of soy phytoestrogens on induction of lactation (lactogenesis) in porcine mammary tissue *in vitro*.

During the first year of this project the potential effects of phytoestrogens on porcine mammary tissue in tissue culture systems have been examined. In spite of its dramatic effects *in vivo*, it is difficult to demonstrate effects of estrogen on mammary tissue *in vitro*. Efforts to demonstrate *in vitro* estrogenic or antiestrogenic effects of genistein on mammary tissue *in vitro* have led to equivocal results. Supraphysiological concentrations of genistein can inhibit *in vitro* hormone-induced lactogenic properties of mammary tissue. This effect apparently happens via genistein's properties as a tyrosine kinase inhibitor. For the second year of this project, we are turning our attentions to evaluation of the *in vivo* effects of genistein on mammary growth in pigs.

## **Problem Addressed**

In addition to the nutrient contributions of soybeans to growth and development of pigs, levels of phytoestrogens contained in the soybeans may have significant effects on the physiology of the animal, especially on tissues where estrogens play a central role. Such a tissue is the sow mammary gland where estrogens have multiple roles in driving mammary growth and function. A primary impact of soy phytoestrogens on the mammary gland is probably mediated through the estrogenic effects of the phytoestrogens, although other nonestrogenic effects may also occur.

Phytoestrogens, particularly the soy phytoestrogen genistein, can affect reproductive function in mammals. However, effects of phytoestrogens on the mammary gland are considerably less well documented and poorly characterized. Studies in rats point to effects of genistein or other phytoestrogens both before puberty and after puberty. Exposure of adult nonpregnant ewes to dietary phytoestrogen by grazing subterranean clover results in significant estrogenic-like changes in the reproductive tract and in the mammary glands. The effect on mammary glands includes production of a small amount of a milky substance from the gland, suggesting an estrogenic effect on mammary development. In contrast, genistein inhibits *in vitro* prolactin stimulation of milk component synthesis in cultured mouse mammary tissue, probably via the highly specific tyrosine kinase inhibiting activity of genistein.

Genistein consumption during induction of lactation could have an inhibitory effect on subsequent milk yield in sows. However, it is difficult to extrapolate the apparent positive or negative effects of genistein on the mammary gland of rodents and other mammals to mammary function in the pig. The effects of soy phytoestrogens like genistein on mammary gland development or induction of lactation in the pig are unknown and need to be directly tested in pigs. The first year of this project has been aimed at determining if genistein has effects on mammary tissue in an *in vitro* culture system.

The specific objective of the first year of this project was to develop bioassays which may be used to determine the direct tissue-level effects of soy phytoestrogens on mammary gland growth and lactogenesis in swine. Developmental efforts focused upon two bioassays aimed at determining A) the local tissue effects of soy phytoestrogens on growth of porcine mammary tissue *in vitro*, and B) the local tissue effects of soy phytoestrogens on induction of lactation (lactogenesis) in porcine mammary tissue *in vitro*.

## **Approach Used**

Several *in vivo* animal models were tested in an effort to obtain appropriate tissues for *in vitro* evaluation of phytoestrogen effects on mammary tissue growth. These included inducing mammary development with estrogen and progesterone injections, with or without prolactin injections, in barrows, in virgin gilts, and in a non-lactating sow.

Mammary tissue explant culture was used to assess the *in vitro* effects of phytoestrogen (genistein) on *in vitro* induced lactogenesis. Tissue was obtained by surgical biopsy from late pregnant sows. Explant cultures of mammary tissue were incubated in the presence of lactogenic hormones (insulin, cortisol, and prolactin) with various concentrations of phytoestrogen. Effects of phytoestrogen on protein synthesis, glucose oxidation, and lysine uptake were evaluated.

## Results

Mammary development was induced in four post-pubertal gilts, four pre-pubertal barrows, and a sow by treatment with estradiol and progesterone. Mammary tissues from gilts treated with prolactin displayed the greatest degree of lobuloalveolar development. Many alveoli with a monolayer of epithelial cells and a well- rounded lumen were present. Mammary tissue still contained stromal tissue between alveoli (similar to that seen during involution), as well as between lobules. Mammary tissue from the gilts that did not receive prolactin and the sow displayed lobuloalveolar development as well, but most of the alveoli contained more than a monolayer of epithelial cells and the lumen were collapsed (similar to early involution). All barrows displayed ductal development, large amounts of stromal tissue (collagen), but no lobuloalveolar development in their mammary glands. Mammary tissues from barrows treated with prolactin seemed to have more ducts present, especially more smaller ducts that were fairly homogeneously spread throughout tissue sections. Mammary tissue from barrows that did not receive prolactin was mostly stromal tissue with a few large ducts present. None of these models of in vivo-induced mammogenesis resulted in sufficient tissue to be useful for in vitro assays of phytoestrogenic effects in mammary tissue.

In view of the problems with developing a suitable source of tissue which might provide a model for demonstrating consistent in vitro phytoestrogenic effects on mammary tissue growth, we turned our attention to development of an in vitro assay to characterize the effects of genistein as a potential tyrosine kinase inhibitor on lactogenesis. Tissue was obtained by surgical biopsy from late pregnant sows. Results of these studies suggested that genistein at high concentrations (200  $\mu$ M) inhibits the increases in glucose oxidation, lysine uptake and total protein synthesis induced by lactogenic hormones. These results suggest that genistein at high concentrations may be inhibitory to lactogenesis and that this effect may occur via tyrosine kinase inhibition. Culture of mammary tissue in lower concentrations did not have the inhibitory effect on protein synthesis.

## Discussion and Implications

Although high concentrations of genistein inhibited indicators of lactogenesis in vitro, it should be noted that the inhibitory effects of genistein on tyrosine kinase activity are typically apparent at supraphysiological concentrations of genistein and occur in vitro. Lower concentrations of genistein did not inhibit protein synthesis by explant cultures. If concentrations of genistein typically found in pigs eating normal diets are not inhibitory in vitro, then genistein's tyrosine kinase inhibitor activity may not be relevant to lactogenesis in vivo. The results of this first year of the project have helped to better frame the issue of the potential for tyrosine kinase inhibition of mammary function by genistein, but the in vivo effect of genistein on the mammary gland remains an open issue. Similarly, estrogen effects on mammary tissue have always been difficult to determine in vitro. Most studies of estrogenic effects on mammary growth or other functions are conducted in the whole animal, whether done with local-release implants or by systemic administration.

Consumed at the proper time, soy phytoestrogens may be used to enhance mammary development. Conversely, if soy phytoestrogens are found to have antiestrogenic or negative

effects on mammary gland growth or lactogenesis, then diets can be altered at critical times in mammary development to minimize such effects. In either case, careful management of dietary soy protein by pork producers may result in enhanced mammary gland growth, enhanced milk production, and consequently in enhanced pig survival and growth. This increased efficiency of sow productivity ultimately results in increased demand for soy protein.

### **Future Directions**

The experimental protocol for the second year of this project will evaluate the in vivo effects of genistein on mammary growth in pigs. The effort will take advantage of the strong physiological interaction between estrogenic compounds and the hormone relaxin to develop an in vivo model for study of phytoestrogen effects on porcine mammary growth. Relaxin has dramatic mammogenic effects during late pregnancy. Estrogen and relaxin have synergistic effects on mammary growth in ovariectomized nonpregnant gilts. This synergy between an estrogenic compound and relaxin also provides a sensitive assay of the mammogenic role of the estrogenic compound. This experimental protocol provides an excellent model for determining the mammogenic effects of phytoestrogens in the presence and absence of relaxin.