Animal (2010), **4:3**, pp 454–465 © The Animal Consortium 2009
doi:10.1017/S1751731109991200

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Dietary genistein stimulates mammary hyperplasia in gilts

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(Received 10 June 2009; Accepted 15 September 2009; First published online 30 October 2009)

The possible role of the phytoestrogen genistein on prepubertal development of mammary glands, hormonal status and bone resorption was investigated in gilts. Forty-five gilts were fed a control diet containing soya (CTLS, n = *15), a control diet without soya (CTL0,* n = *15) or the CTLS diet supplemented with 2.3 g of genistein daily (GEN,* n = *15) from 90 days of age until slaughter (day 183* ± *1). Both basal diets were isonitrogenous and isocaloric. Jugular blood samples were obtained on days 89 and 176 to determine concentrations of isoflavone metabolites (on day 176 only), prolactin, estradiol, progesterone, insulin-like growth factor 1 (IGF1), and* N*-telopeptide of type I collagen (NTx; on day 176 only). At slaughter, mammary glands were excised, parenchymal and extraparenchymal tissues were dissected, and composition of parenchymal tissue (protein, fat, dry matter (DM), DNA) was determined. Histochemical analyses of mammary parenchyma were performed. Dietary genistein increased parenchymal protein (*$P < 0.05$*) while decreasing DM (*$P < 0.05$*) and tending to lower fat content compared with the CTLS, but not the CTL0, diet. There was more parenchymal DNA (1.26 v. 0.92 mg/g,* $P < 0.05$*) in GEN than CTLS gilts, likely reflecting an increase in the quantity of mammary epithelial cells. Circulating concentrations of genistein were increased in GEN gilts (*$P < 0.001$*) but concentrations of hormones or NTx (indicator of bone collagen resorption) were not affected by GEN (*$P > 0.1$*). Percentage of estradiol receptor alpha (ER*α*)-positive epithelial cells was lower (*$P < 0.05$*) in GEN than CTLS gilts, whereas 5-bromo-2′-deoxyuridine labeling index was unaltered (*$P > 0.1$*). Transcript levels for ER*α*, ER*β*, IGF1, epidermal growth factor (EGF), epidermal growth factor receptor and transforming growth factor alpha were not altered by treatments. Supplementation of the diet with genistein during the growing phase in gilts, therefore, led to hyperplasia of mammary parenchymal tissue after puberty; yet, even though circulating genistein was increased, this was not accompanied by changes in mammary expression of selected genes or circulating hormone levels.*

Keywords: genistein, mammary glands, phytoestrogen, pigs, puberty

Implications

Current findings indicate that it might be possible to feed phytoestrogens to gilts during critical stages of their development in order to enhance mammary development after puberty. Further work is needed to determine the optimal conditions of such a treatment (i.e. ideal dose, timing and duration), the potential effects on future lactation performance as well as the mechanisms of action. Feeding genistein to growing gilts, however, has no impact on bone resorption. Mammary development in gilts also does not appear to be stimulated solely by ingesting a standard diet containing soya compared with a soya-free diet.

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Introduction

Sow milk yield is a major determinant of piglet growth and is largely affected by the number of milk secretory cells present at the onset of lactation (Head *et al.*, 1991). Feeding management of gilts during the early phase of mammary cell accretion (from 90 days of age until puberty; Sorensen *et al.*, 2002) has an impact on mammary development (Farmer *et al.*, 2004; Sorensen *et al.*, 2006), and it is likely that specific feed ingredients may be useful in stimulating this process of mammogenesis. Phytoestrogens have gained increasing interest in recent years because of their potential health benefits in prevention of mammary cancer (review by Warri *et al.*, 2008) and other benefits such as alleviation of bone loss in postmenopausal women

(review by Lister *et al*., 2007). These naturally occurring plant compounds with estrogenic or anti-estrogenic activities (depending on the concentration of endogenous estrogens present; Sun Hwang *et al*., 2006) comprise three major classes. They are isoflavones (comprising genistein), lignans and coumestans, and isoflavones are present in large amounts in soya, which is a major component of swine feed.

In rodents, it was shown that neonatal exposure to genistein stimulates mammary development and differentiation (review by Jefferson *et al*., 2007) and there is one report in swine suggesting that postpubertal exposure to genistein also enhances mammogenesis (Ford, 2003). Because of its structural similarity to estradiol-17β, many of the actions of genistein are mediated through estradiol receptors alpha (ERα) and ER beta (ERβ; Nilsson *et al*., 2001). Indeed, genistein was shown to enhance mammary gland differentiation in prepubertal rats by increasing epidermal growth factor receptors (EGFRs) and transforming growth factor alpha (TGFα) in mammary terminal ductal structures (Brown *et al*., 1998). More recently, it was reported that neonatal exposure to genistein results in increased levels of ERβ mRNA in the mice mammary gland, whereas ERα protein and mRNA are decreased (Padilla-Banks *et al*., 2006). The insulin-like growth factor 1 (IGF1) is essential for mammogenesis (Ruan and Kleinberg, 1999) and was found to be a paracrine mediator of estrogen action in the bovine mammary gland (Li and Capuco, 2008). Interestingly, the IGF1 receptor is essential for mediating genistein effects in human breast cancer cells and the IGF1 receptor promoter activity, induced by genistein, requires the action ER (Chen *et al*., 2007), thus suggesting a cross-talk between the ER and IGF1 receptor signaling pathways in mammary cells. Mundy (2006) also suggested that genistein may prevent bone resorption by promoting bone formation, and this can be measured using specific markers, such as cross-linked *N*-telopeptide of type I collagen (NTx; Weiler *et al*., 2001), present in the circulation. This study was, therefore, undertaken to determine whether dietary supplementation with the phytoestrogen genistein during the prepubertal period affects mammogenesis and/or bone resorption in gilts and if feeding soya alone impacts these variables.

Material and methods

Animals and treatments

Forty-five crossbred gilts ((Yorkshire × Landrace) × Landrace) were fed a commercial diet until 89 days of age. The next day, they were equally divided into the following 3 nutritional regimens: (i) diet containing no soya (CTL0, $n = 15$), (ii) soya-based commercial diet (CTLS, $n = 15$) and (iii) CTLS with 2.3 g daily supplementation of genistein (GEN, $n = 15$). The genistein was offered once daily to gilts (at 1400) after 6 h of feed removal and was premixed in 15 g of corn flour. Gilts from all treatment groups received 15 g of corn flour in the same manner, and it was included in the compositional analyses of the diets. There was a 10-day adaptation period from 80 days of age, during which all gilts received 15 g of finely ground corn without genistein. All dietary regimens were isonitrogenous and isocaloric based on calculated values (Table 1), which were used in formulating diets that were fed *ad libitum* over two phases of growth, namely from 75 to 119 days of age (phase 1) and from 120 days to slaughter at 182 ± 1 days (phase 2). Compositions of all diets are shown in Table 1. Representative feed samples were taken twice weekly throughout the experiment for compositional analyses (shown in Table 1). Throughout the experiment, all gilts were housed in individual stalls (0.6 × 2.1 m) from 1500 to 0800 and were given fresh feed once daily (1500). Individual feed consumption was recorded daily from 90 days of age until slaughter. At 0730, gilts were roused and encouraged to stand up and eat any leftover feed, they were then housed in groups of 15 (5 from each dietary treatment group) from 0800 until 1400 (surface area of 29.7 m^2). As of 140 days of age, gilts were moved to a similar room with a boar present and estrous behavior was evaluated twice daily by allowing physical contact between gilts and the boar. Date and duration of first estrus were recorded, and all gilts had started cycling before slaughter. Gilts were weighed and their backfat thickness measured ultrasonically at the last rib (Scanmatic SM-1, Medimatic, Hellerup, Denmark) on days 89, 150, and 183, following an overnight fast. The experiment took place between September 2005 and February 2006. Animals were cared for according to a recommended code of practice (Agriculture and Agri-Food Canada, 1993).

Blood sampling and assays

Jugular blood samples were obtained at 0730, following an overnight fast, on 89 days of age and 10 ± 1 days following the last heat before slaughter (average age of 176.4 days at second blood sampling, ranging from 164 to 184 days, slaughter being at 183 ± 1 days) to determine concentrations of estradiol, progesterone, prolactin, IGF1, isoflavone metabolites (at last sampling before slaughter only) and NTx (at last sampling before slaughter only). Samples collected for isoflavones, progesterone and prolactin measurements were left at room temperature for 4 h, stored overnight at 4°C, centrifuged the following day and serum was harvested. Samples for estradiol and IGF1 assays were collected in EDTA-tubes (Becton Dickinson and Cie, Rutherford, NJ, USA) and those for NTx assays in heparinized tubes (Becton Dickinson and Cie); they were all put on ice and centrifuged within 20 min, and plasma was immediately recovered. Serum and plasma samples were frozen at −20°C until they were assayed.

The quantitative analysis of isoflavones and their metabolites in serum samples was completed with a method using liquid chromatography/mass spectrometry combined with photodiode array detection, as developed in Dr Gilani's laboratory (Sepehr *et al*., 2006).

Concentrations of prolactin were determined using a previously described radioimmunoassay (RIA; Robert *et al*., 1989). The radioinert prolactin was purchased from A.F. Parlow (US National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrence, CA, USA) and the first antibody to prolactin was purchased from Research Products International (Mount Prospect, IL, USA).

Table 1 *Actual ingredients, calculated and analyzed composition of the two basal diets on an as-fed basis, which were fed from days 75 to 119 (phase 1) or days 120 to slaughter at 183 ± 1 days (phase 2)*

	Phase 1		Phase 2	
	CTLS	CTL0	CTLS	CTL0
Ingredient, (kg) 1000/kg				
Ground corn	608.0	679.5	678.9	741.4
Soybean meal (48%)	234	–	159	–
Gluten feed (21%)	–	150	–	150
Wheat middlings	100	–	100	–
Fishmeal (60%)	–	77	–	67
Pork protein	–	52	–	27
Corn gluten meal (60%)	–	17	–	–
Canola meal	–	–	21	–
Animal fat (ruminant free)	16	10	5	–
Limestone	14.8	–	11.9	–
Calcium phosphate	13.1	–	10.3	–
NaCl	6.1	4.7	6.0	5.2
Trace mineral and vitamin premix[a]	2.0	2.0	2.0	2.0
Vitamin E premix (20 000 IU/kg)	1.5	1.5	1.5	1.5
Mold inhibitor (Mycocurb S)[b]	1.0	1.0	1.0	1.0
Biotin premix (400 PPM)	0.9	0.9	0.9	0.9
L-Lysine–HCl	1.7	3.0	1.9	2.8
L-Tryptophan	–	0.5	–	0.5
L-Threonine	–	0.5	–	0.3
Choline	0.2	0.2	0.2	0.2
Anti-oxidant (Ethoxyquin, 66%)	0.2	0.2	0.2	0.2
DL-Methione	0.5	–	0.2	–
	Chemical composition (calculated)			
DE (MJ/kg)	14.0	14.1	13.8	13.8
Crude fiber (%)	2.7	2.8	2.9	2.9
CP (%)	17.4	17.1	15.1	14.5
Fat (%)	4.6	5.3	3.7	4.1
Ca (%)	0.90	1.07	0.74	0.79
P (%)	0.66	0.83	0.59	0.69
Na (%)	0.25	0.25	0.25	0.25
	Chemical composition (analyzed)			
CP (%)	18.4	16.9	15.9	15.3
Fat (%)	4.4	5.0	3.8	3.9
Ca (%)	0.92	1.29	0.80	0.94
P (%)	0.72	0.90	0.67	0.79
Na (%)	0.29	0.28	0.29	0.30
Dry matter (%)	87.6	87.5	86.9	87.8
Phytoestrogens (mg/kg):				
Genistein	278.4	7.9	195.5	6.2
Daidzein	278.3	9.2	209.1	7.1
Glycitein	26.1	0.4	24.5	0.3
Total	582.8	17.5	429.1	13.6

Standard soya-based diet: CTLS, diet without soya: CTL0

[a]Provided the following per kilogram of diet: Cu, 25 mg; Zn, 145 mg; Se, 0.3 mg; Mn, 30 mg; Fe, 100 mg; I, 1.0 mg; Vitamin A, 3000 IU; Vitamin D, 900 IU; Vitamin E, 30 IU; Vitamin K, 1.5 mg; Vitamin B_{12}, 20 μg; thiamin, 2.0 mg; riboflavin, 3.5 mg; pantothenic acid, 15 mg; niacin, 20 mg; folate, 0.45 mg; biotin, 50 μg; pyridoxine, 2.0 mg.

[b]Kemin Industries, Des Moines, IA.

Parallelism of a pool of serum from sows was demonstrated. Average recovery, calculated by addition of various doses of radioinert hormone to 50 μl of a pooled sample, was 103.4%. Sensitivity of the assay was 1.5 ng/ml. Six samples of a representative pool of serum were carried in duplicates in all assays in order to calculate coefficients of variation (CV). The intra-assay CV, calculated from the mean values of the pools within each assay, was 6.1%. The interassay CV, calculated

from the mean values of the pools obtained for different assays, was 8.9%. Concentrations of IGF1 were determined with a previously described RIA (Abribat *et al*., 1993). The IGF1 was extracted using the formic acid-acetone method. The first antibody in the IGF1 assay was purchased from A.F. Parlow (US National Hormone and Pituitary Program). The radioinert IGF1 was purchased from GROPEP (Adelaide, SA, Australia). Parallelism of a plasma pool from sows was demonstrated. Average recovery was 101.6% and assay sensitivity was 62.5 pg/ml. Intra-assay CV was 5.4% and all tubes were analyzed in one assay. Estradiol (Diagnostic Systems Laboratories Inc., Webster, TX, USA) and progesterone (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) were quantified using commercial RIA kits. All samples were assayed in a single assay with an intra-assay CV of 1.2% for estradiol and 4.5% for progesterone. Concentrations of NTx were determined using a commercial ELISA kit (Osteomark NTx serum ELISA assay, Wampole Laboratories, Princeton, NJ, USA) validated for swine serum. The assay was performed as recommended by the suppliers except that samples were diluted 1 : 75 in assay buffer. The accuracy of the kit controls was 97% of expected values and agreement between duplicate samples ranged from 91% to 97%.

Mammary gland measurements and organ sampling

At slaughter, mammary glands were excised from the abdominal wall of all gilts. Parenchymal tissue from the fourth anterior mammary glands was used for mRNA measurements of ERα and ERβ, IGF1, epidermal growth factor (EGF), EGFR, and TGFα. These samples were stored at −80°C until analysis. The remaining mammary glands were then stored at −20°C until dissection and analyses for tissue composition. Frozen mammary glands were trimmed of skin and teats and subsequently stored at −20°C. They were then sawed into 2-cm slices and mammary parenchymal tissue from each slice was dissected from surrounding adipose (i.e. extraparenchymal tissue) at 4°C and both parenchymal and extraparenchymal tissue weights were recorded. Mammary parenchyma was defined as the region containing epithelial tissue, but also contained a large amount of adipose and connective tissues because mammary ducts and alveolar tissues are embedded within loose connective tissue of the mammary fat pad in gilts of this age. Parenchymal tissue from all dissected and sliced glands was homogenized and a representative sample was used for determination of composition by chemical analysis. The DNA content of parenchymal tissue was evaluated using a method based on fluorescence of a DNA stain (Labarca and Paigen, 1980). Dry matter (DM), protein, and lipid contents were also measured (AOAC, 2004). Six gilts per treatment group received an i.v. injection of 5 mg/kg (diluted in saline at a concentration of 20 mg/ml and pH of 8 to 8.5) of 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical, Saint Louis, MO, USA) in the marginal ear vein, 2 to 3 h before slaughter. Three cubes of parenchyma ($\sim 3 \times 3$ mm) from these gilts were obtained from two regions within the fourth anterior mammary gland and used to quantify the portion of epithelial cells that were labeled with BrdU, as an index of proliferation rate. Tissues were collected from the second anterior mammary gland and were fixed in 10% formalin in phosphate-buffered saline at 4°C for 24 h before being transferred to 70% ethanol until further processing. They were then dehydrated through a graded series of ethanol to 100% ethanol and embedded in paraffin according to standard techniques. Embedded tissue was later sectioned at 5 μm for immunohistochemical staining and evaluation. Ovaries were collected from all gilts and the number of corpora lutea counted.

Immunohistochemistry

Slides were prepared as described by Farmer *et al*. (2007) and bright field microscopic detection of BrdU-labeled cells was performed as described by Capuco *et al*. (2002). Immunohistochemical localization of ERα was performed on 3 randomly selected gilts per dietary treatment using a rabbit polyclonal antibody (sc-787 at 1 : 100 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Tissue sections were prepared for immunohistochemistry as described above. Following overnight incubation with primary antibody, sections were washed in phosphate-buffered saline (PBS; 3 times, 2 min at room temperature) and then incubated with second antibody-horseradish peroxidase conjugate (30 min at room temperature). Sections were washed in PBS and then were incubated with diaminobenzidine, counter stained with hematoxylin, and mounted with Permasilip.

To quantify the number of BrdU-labeled epithelial cells and ER-positive epithelial cells, photographs of stained tissue sections were captured as digital images. For quantifying the BrdU-labeled cells, a slide was prepared and processed for histochemistry from each of two parenchymal regions per gilt. For each slide, 10 tissue areas were photographed with a Spot® digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) on a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY, USA) with the ×40 objective. The number of BrdU-labeled epithelial cells and total epithelial cells were counted manually. At least 1100 epithelial cells were scored per gilt (average ± s.e. = 2753 ± 143). For quantifying ER-positive cells, slides were processed, images obtained and the number of ER-positive epithelial cells and total epithelial cells analogously determined. At least 1300 epithelial cells were scored per gilt (average ± s.e. = 2801 ± 141).

Real-time PCR analyses of selected genes

Total RNA was extracted from parenchymal tissue using Trizol™ reagent (Invitrogen, Grand Island, NY, USA) and according to the manufacturer's instructions. Extracted RNA was then treated with DNAse I (Amplification grade; Invitrogen) to remove contaminating DNA. First-strand complementary DNA was synthesized using oligo dT (13 to 18) primers (Invitrogen) and the Superscript II preamplification system (Invitrogen) following the manufacturer's instructions.

The relative mRNA abundance of selected genes was determined using real-time PCR analyses. Primers were designed based on available porcine sequence (Table 2)

Table 2 *Oligonucleotide primers used for real-time PCR amplifications of selected genes*

Genes	Primer sequences (5′-3′)	GenBank accession no.	Primer concentrations (nM)	Product size (bp)
EGF	Forward CAAATCAACAGGAAGGCACCAT	NM_214020	900	105
	Reverse CTGGATCAACTGCTACATTTGCA		900	
EGFR	Forward CCATAAATGCTACGAATATCAAGCA	NM_214007	300	96
	Reverse TGAAGGAGTCACCCCTAAATGC		150	
ERα	Forward TCCTACCAGACCCCTCAGTGA	NM_214220	150	97
	Reverse CACCCTCTTTGCCCAGTTGA		150	
ERβ	Forward GAGAGACATTGAAAAGGAAGGCTAGT	NM_001001533	300	102
	Reverse AATCACTGCAGACAGCACAGAAG		900	
IGF1	Forward ACATCACATCCTCTTCGCATCTC	NM_214256	300	95
	Reverse AGCCCCACAGAGGGTCTCA		300	
TGFA	Forward CGCGCTGGGTATCTTGTTG	NM_214251	300	121
	Reverse GTGGGAATCTGGGCAGTCAT		150	
GAPDH	Forward CAGCAATGCCTCCTGTACCA	AF017079	300	70
	Reverse GATGCCGAAGTTGTCATGGA		300	
PPIA	Forward GCACTGGTGGCAAGTCCAT	NM_214353	150	71
	Reverse AGGACCCGTATGCTTCAGGA		300	
ACTβ	Forward ATCACCATCGGCAACGA	AY550069	150	122
	Reverse TCGACGTCGCACTTCATGA		150	

EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; ERα = estradiol receptor alpha; ERβ = estradiol receptor beta; IGF1 = insulin-like growth factor 1; TGFA = transforming growth factor alpha; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; PPIA = peptidylprolyl isomerase A; ACTβ = beta actin.

and were selected using the Primer Express software 3.0 (PE Applied BioSystems, Foster City, CA, USA). Amplification of peptidylprolyl isomerase A, glyceraldehyde-3-phosphate dehydrogenase and beta actin (ACTβ) housekeeping genes (HKG) was also performed using porcine specific primers described in Table 2. The PCR amplifications were performed in a 10-μl reaction volume consisting of corresponding concentrations of each primer (Table 2), 5 μl of 2 × Power SYBRGreen Master Mix (PE Applied BioSystems), 3 μl of 15 × diluted cDNA, and 0.05 μl of UNG AmpErase (PE Applied BioSystems). Cycling conditions were 2 min at 50°C to activate the amperase, 10 min at 95°C, followed by 40 cycles of 3 s at 95°C and annealing and polymerization at 60°C for 30 s. Amplification, detection, and data analysis were performed with an ABI 7500 Fast Real-Time PCR System (PE Applied BioSystems). Specificity of amplified fragments was verified with the melting curve analysis. Amplifications were performed in triplicate and standard curves were established in duplicate for each gene. Selected genes and HKG amplifications were run in separate assays. A pool of parenchymal tissue cDNA was used to create a standard curve for quantification of transcripts using the relative standard curve method as described by Applied BioSystems (1997). For each experimental sample, the amount of target gene mRNA relative to HKG mRNA was determined from the corresponding standard curves. Relative quantity ratios were then obtained by dividing the relative quantity unit of selected genes by those of HKG. Mean values from triplicates were used to perform statistical analyses.

Statistical analyses

The MIXED procedure of Statistical Analysis Systems Institute (SAS, 1998) was used for statistical analyses. The ANOVA used for isoflavones, NTx, mammary gland and ovarian variables included the effect of nutritional treatments with the residual error being the error term used to test main effects of treatment. Analyses on weights of mammary extraparenchymal and parenchymal tissues were done using BW of gilts at slaughter as a covariate. Analyses were performed on weight gains and total feed intakes over each of the three growth periods studied (days 90 to 119, days 120 to 149, days 150 to 180). Repeated measures ANOVA with the factors treatment (the error term being gilt within treatment), day of age and the treatment × day of age interaction (the residual error being the error term for these last two factors) were carried out on backfat thickness and hormonal data. When the treatment × day interaction was significant, separate analyses were also performed for each day. Data were corrected with a square root or a logarithmic transformation (using natural logarithms) when appropriate and, in those cases, means presented in tables are back-transformed values. Real-time PCR data were analyzed using the same one-way ANOVA model. For all analyses, differences between means were evaluated through pair-wise multiple comparisons with a Tukey adjustment. The extent of histological development in mammary parenchymal tissue slices was scored using categorical data (visual scoring index from 1 to 5) and was, therefore, analyzed with the Cochran-Mantel–Haenszel test (Stokes *et al.*, 1995). Data in tables are presented as least squares means ± SEM unless indicated otherwise.

Results

Performance, hormonal, NTx and isoflavone data

Total feed intake of gilts was affected by treatments ($P < 0.001$) during the first growth period only, specifically from 90 to 119 days of age; values were lowest for CTL0

compared with the CTLS and GEN gilts ($P < 0.01$, Table 3). The same relationship held true for weight gains (Table 3), with values being altered by treatments from days 90 to 119 ($P < 0.01$), and those of CTL0 gilts being lower than those of CTLS and GEN gilts ($P < 0.01$). Feed conversion, however, was not affected by diets ($P > 0.1$, Table 3). There was a treatment × day interaction on backfat thickness of gilts ($P < 0.05$) and analyses for each day showed lower backfat in CTL0 than CTLS and GEN gilts on day 90 ($P < 0.05$) but only a tendency for values to be lower in these same animals on days 120 and 150 ($P < 0.1$), and no significant treatment effect on day 180 ($P > 0.1$, Table 3).

Circulating concentrations of isoflavone metabolites at the end of the experimental period are shown in Table 4. There was a significant effect of diets on all isoflavones measured ($P < 0.001$) with values being lower in gilts fed the CTL0 diet compared with either the CTLS ($P < 0.001$) or the GEN ($P < 0.001$) diets. Concentrations of genistein were also significantly increased ($P < 0.001$) in the GEN diet compared with the CTLS diet ($P < 0.001$). Diets had no impact ($P > 0.1$) on circulating concentrations of prolactin, IGF1, estradiol, progesterone or NTx in gilts at the end of the experimental period (Table 5), yet weight (but not age) of gilts at first estrus was lower ($P < 0.05$) for CTL0 than CTLS gilts, and duration of the first estrus also tended to be longer in CTL0 than in CTLS gilts ($P < 0.1$, Table 5).

Mammary gland data

Composition of mammary tissue at slaughter and BrdU incorporation are shown in Table 6. While amount of mammary parenchymal and extraparenchymal tissues were not altered by dietary treatments ($P > 0.1$), composition of parenchymal tissue was affected. More specifically, parenchymal tissue from GEN gilts contained less DM ($P < 0.05$), tended to have a lower percentage of fat ($P < 0.1$), had a greater percentage of protein ($P < 0.05$), greater concentrations of DNA ($P < 0.05$) and tended to have more total DNA ($P < 0.1$) than that of CTLS gilts. There was a significant reduction in

Table 3 *Growth and feed intake performances of gilts fed one of three diets from 90 to 183 days of age*

	Dietary treatments[a]				Contrasts, *P*-values		
Item	CTL0	CTLS	GEN	SEM[b]	CTL0 *v.* CTLS	CTL0 *v.* GEN	CTLS *v.* GEN
Weight gain (kg)							
Days 90 to 119	28.1	32.0	31.8	0.8	0.003	0.005	0.993
Days 120 to 149	28.8	30.0	25.6	1.1	0.670	0.987	0.528
Days 150 to 180	31.5	31.0	28.8	1.3	0.950	0.286	0.393
Total feed intake (kg)							
Days 90 to 119	65.6	75.3	73.5	1.7	0.0004	0.004	0.705
Days 120 to 149	85.3	93.9	88.3	3.2	0.117	0.763	0.338
Days 150 to 180	103.3	107.2	101.0	3.7	0.703	0.892	0.374
Feed/gain ratio							
Days 90 to 119	2.42	2.44	2.39	0.05	0.950	0.918	0.733
Days 120 to 149	2.97	3.15	3.11	0.08	0.247	0.409	0.932
Days 150 to 180	3.52	3.77	3.78	0.15	0.422	0.388	0.998
Backfat thickness (mm)							
Days 90	7.3	8.4	7.9	0.3	0.029	0.195	0.599
Days 120	10.3	11.5	10.9	0.4	0.085	0.580	0.418
Days 150	14.0	15.6	14.3	0.5	0.070	0.916	0.120
Days 180	19.9	20.5	18.6	0.8	0.832	0.466	0.151

Diets were: diet without soya, CTL0; standard corn-soya diet, CTLS; and standard corn-soya with 2.3 g of supplementary genistein daily, GEN.
[a]Significant overall treatment effect on weight gain and total feed intake for days 90 to 119 ($P < 0.01$) and on backfat on day 90 ($P < 0.05$). Tendency for an overall treatment effect on backfat on days 120 and 150 ($P < 0.1$).
[b]Maximum value of the standard error of the means.

Table 4 *Concentrations of isoflavone metabolites (μmol/l) at 176 days of age in serum of gilts that were fed one of three diets from 90 to 183 days of age*

	Dietary treatments[a] (means (lower and upper confidence interval))			Contrasts, *P*-values		
Item	CTL0	CTLS	GEN	CTL0 *v.* CTLS	CTL0 *v.* GEN	CTLS *v.* GEN
Daidzen	0.018 [0.003, 0.045]	0.256 [0.191, 0.330]	0.330 [0.254, 0.416]	0.0001	0.0001	0.237
Genistein	0.012 [0.001, 0.038]	0.144 [0.093, 0.205]	0.634 [0.518, 0.761]	0.0001	0.0001	0.0001
Glycitein	0.001 [0.000, 0.005]	0.048 [0.035, 0.064]	0.043 [0.030, 0.058]	0.0001	0.0001	0.633
Equol	0.000 [0.000, 0.000]	0.212 [0.166, 0.264]	0.192 [0.147, 0.243]	0.0001	0.0001	0.617

Diets were: diet without soya, CTL0; standard corn-soya diet, CTLS; and standard corn-soya with 2.3 g of supplementary genistein daily, GEN.
[a]Significant overall treatment effect on all variables ($P < 0.0001$).

Table 5 *Endocrine and first estrus data for gilts fed one of three diets from 90 to 183 days of age*

Item	Dietary treatments[a]			SEM[b]	Contrasts, *P*-values		
	CTL0	CTLS	GEN		CTL0 *v.* CTLS	CTL0 *v.* GEN	CTLS *v.* GEN
Prolactin (ng/ml)							
Days 89[c]	1.84	1.64	1.54	0.13	0.521	0.219	0.799
Days 176	2.09	1.78	2.38	0.34	0.772	0.799	0.337
IGF1 (ng/ml)							
Days 89	231.4	257.2	227.7	16.8	0.485	0.985	0.339
Days 176	236.4	234.1	220.8	9.1	0.980	0.408	0.469
Estradiol (pg/ml)							
Days 89[c]	12.0	11.8	11.7	0.4	0.929	0.846	0.978
Days 176	14.2	14.3	14.1	0.4	0.950	0.971	0.834
Progesterone[d] (ng/ml)							
Days 176	27.4	27.9	29.5	1.9	0.976	0.695	0.784
NTx, nM BCE[e]							
Days 89[c]	386.7	408.4	360.8	23.5	0.792	0.720	0.337
Days 176	272.2	251.5	205.8	26.6	0.847	0.197	0.454
First estrus data							
Weight (kg)	107.2	119.4	112.9	3.08	0.012	0.361	0.220
Age (days)	154.9	159.0	155.2	3.06	0.567	0.996	0.574
Duration (h)	50.3	37.8	47.6	4.2	0.076	0.884	0.162

IGF1 = insulin-like growth factor 1; NTx = *N*-telopeptide of type I collagen.
Diets were: diet without soya, CTL0; standard corn-soya diet, CTLS; and standard corn-soya with 2.3 g of supplementary genistein daily, GEN.
[a]Significant overall treatment effect on weight at first estrus ($P < 0.05$) and tendency for duration of first heat ($P < 0.1$).
[b]Maximum value of the standard error of the means.
[c]Significant effect of day on prolactin ($P < 0.05$), estradiol ($P < 0.001$) and NTx ($P < 0.001$).
[d]All values on day 89 were below assay sensibility (i.e. <0.15 ng/ml) so are not presented.
[e]Bone collagen equivalent.

Table 6 *Composition and BrdU incorporation of mammary glands at slaughter (day 183) in gilts fed one of three diets from 90 to 183 days of age*

Item	Dietary treatments[a]			SEM[b]	Contrasts, *P*-values		
	CTL0	CTLS	GEN		CTL0 *v.* CTLS	CTL0 *v.* GEN	CTLS *v.* GEN
Extraparenchymal tissue (g)	1391.5	1622.6	1412.3	87.5	0.127	0.983	0.139
Parenchymal tissue (g)	366.7	387.4	434.7	41.0	0.924	0.431	0.622
Dry matter (%)	72.1	73.9	69.2	1.4	0.556	0.264	0.020
Fat[c] (%)	93.0	93.7	92.7	0.4	0.299	0.840	0.078
Fat (g)	240.1	267.3	278.4	25.5	0.704	0.500	0.934
Protein[c] (%)	7.0	6.4	7.5	0.3	0.306	0.503	0.020
Protein (g)	18.3	18.0	22.1	1.9	0.992	0.299	0.199
DNA[c] (mg/g)	1.0	0.9	1.3	0.1	0.771	0.137	0.019
DNA (g total)	0.28	0.26	0.38	0.04	0.958	0.186	0.080
Protein/DNA	73.3	72.0	63.7	3.5	0.961	0.115	0.151
BrdU labeling index (%)	2.75	0.79	4.34	1.39	0.547	0.687	0.176
Estrogen receptors (%)[d]	21.4	27.5	15.9	3.0	0.338	0.411	0.031
Histological score[e]	2.6 ± 0.6[f]	1.8 ± 0.4	3.3 ± 0.4	NA	0.382	0.645	0.028

BrdU = 5-bromo-2′-deoxyuridine.
Diets were: diet without soya, CTL0; standard corn-soya diet, CTLS; and standard corn-soya with 2.3 g of supplementary genistein daily, GEN.
[a]Significant overall treatment effect on percentage of dry matter, percentage of protein, and DNA in mg/g ($P < 0.05$). Tendency for: gram of extraparenchymal tissue, % fat, total DNA and protein/DNA ($P < 0.1$).
[b]Maximum value of the standard error of the means.
[c]Expressed on a dry matter basis.
[d]Represents the percent of epithelial cells that are positive for estrogen receptor alpha.
[e]Histological score of mammary development utilizing a scale from 1 (simple ducts) to 5 (extensive lobular development). Data were analyzed using the non-parametric Mann–Whitney test.
[f]Mean ± s.e.

number of ER-positive cells ($P < 0.05$) in these same animals. Representative micrographs of tissue sections depicting mammary gland microanatomy and BrdU labeling at slaughter are shown in Figure 1. Micrographs depict increased mammary development evident by greater histological score of GEN *v.* CTLS gilts ($P < 0.05$, Table 6), and

lack of difference in histological score between CTL0 and GEN ($P > 0.1$, Table 6). Immunodetection of BrdU-labeled cells suggests reduced BrdU labeling of mammary epithelium in tissue from CTLS gilts, consistent with the numerical, but non-significant ($P > 0.1$), differences in means for BrdU labeling index (Table 6). Micrographs of BrdU-stained sections also clearly show heavy nuclear labeling with the BrdU. The relative abundance of transcripts for ERα, ERβ, IGF1, EGF, EGFR and TGFα genes in mammary parenchymal tissue at slaughter was not affected by diets (Table 7).

Discussion

The formulation of a diet without soya led to an unconventional diet that was not as appetizing as the other diets, as evidenced by the lower feed intake and decreased weight gain during the first growth period in CTL0 gilts compared with gilts from the other two groups. Yet, there seemed to be an adaptation to this diet because total feed intake and weight gain were not affected by dietary treatments in the second and third growth periods. The absence of treatment effects on growth and feed/gain ratio corroborates findings of Kuhn *et al.* (2004), who saw no effects of feeding diets differing in isoflavone content (soyabean meal *v.* soya protein concentrate) on the performance of weanling pigs. Furthermore, these authors reported no treatment differences in carcass composition, meat quality as well as expression of IGF1 receptor mRNA in the muscle of pigs. Current results also showed no effect of treatment on circulating IGF1 concentrations, yet circulating concentrations of isoflavones were increased in gilts fed a standard soya diet compared with a diet without soya. It therefore appears that these changes were not sufficient to elicit significant alterations in any of the growth or physiologic variables measured.

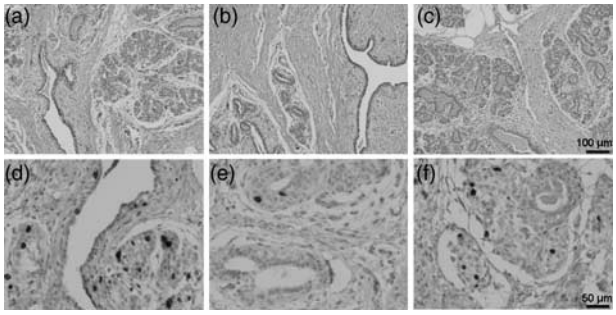

Figure 1 Mammary gland microanatomy and 5-bromo-2′-deoxyuridine (BrdU) labeling at slaughter (day 183) in gilts fed one of three diets from 90 to 183 days of age. Diets were: diet without soya, CTL0; standard corn-soya diet, CTLS; and standard corn-soya with 2.3 g of supplementary genistein daily, GEN. Representative micrographs of tissue sections from CTL0 (a), CTLS (b) and GEN (c) stained with hematoxylin and eosin depict increased mammary development in GEN *v.* CTLS gilts ($P < 0.03$), and lack of difference between CTL0 and GEN ($P > 0.1$). Immunolocalization of BrdU-labeled cells are depicted in micrographs from CTL0 (d), CTLS (e) and GEN (f) groups and suggest reduced BrdU labeling of mammary epithelium in tissue from CTLS gilts, consistent with numerical, but nonsignificant ($P > 0.1$), differences in means. BrdU immunohistochemistry also indicates heavy nuclear labeling with BrdU.

It is well documented that neonatal exposure of mice to genistein disrupts ovarian function during adulthood and reduces fertility (review by Jefferson *et al.*, 2007). To the best of our knowledge, only two studies looked at the effects of phytoestrogens on reproduction in swine. Drane *et al.* (1981) fed either a 20% soyameal diet or a soya-free diet to gilts from 10 to 24 weeks of age, at which time they slaughtered the animals and examined their reproductive tissues. Vulval enlargement was noticed in treated gilts, but there were no effects on the reproductive tracts. However, it is important to point out that these animals were prepubertal and it is unknown whether effects would have been apparent had they been evaluated after the onset of puberty. Indeed, in a more recent study, Ford *et al.* (2006) demonstrated that exogenous genistein does affect reproductive tissues of ovariectomized postpubertal gilts. More specifically, i.m. injections of increasing doses of genistein (varying from 50 to 400 mg/day) for 10 days stimulated uterine and cervical tissue growth in a dose-dependent manner. The highest dose of genistein (400 mg/day) also

Table 7 *Relative mRNA levels of selected genes in mammary parenchymal tissue at slaughter (day 183) in gilts fed one of three diets from 90 to 183 days of age*

Item	Dietary treatments[a]			SEM[b]	Contrasts, *P*-values		
	CTL0	CTLS	GEN		CTL0 *v.* CTLS	CTL0 *v.* GEN	CTLS *v.* GEN
Mammary gene expression[c]							
ERα	1.13	1.77	1.51	0.28	0.252	0.607	0.780
ERβ	0.43	0.59	0.67	0.19	0.821	0.646	0.952
IGF1	1.46	1.85	2.19	0.33	0.682	0.276	0.743
EGF	1.41	1.89	1.68	0.29	0.487	0.803	0.858
EGFR	1.45	1.54	1.79	0.23	0.960	0.552	0.718
TGFα	1.06	1.07	1.15	0.19	0.998	0.928	0.948

ERα = estradiol receptor alpha; ERβ = estradiol receptor beta; IGF1 = insulin-like growth factor 1; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; TGFα = transforming growth factor alpha.
Diets were: diet without soya, CTL0; standard corn-soya diet, CTLS; and standard corn-soya with 2.3 g of supplementary genistein daily, GEN.
[a]Overall treatment effect not significant for any of the measured genes ($P > 0.1$).
[b]Maximum value of the standard error of the means.
[c]mRNA levels. Values correspond to the relative abundance of studied genes mRNA.

increased the number of cells that stained positive for progesterone receptor and for proliferation in the uterus and cervix. Current findings showed a decrease in BW at puberty onset and a tendency for an increase in duration of first estrus in gilts fed the diet without soya compared with gilts fed the commercial soya diet, but there was no effect of added genistein. This is contrary to our expectation because mice treated neonatally with genistein generally show an increased length of the estrous cycle (review by Jefferson *et al*., 2007) and accelerated onset of puberty (Lewis *et al*., 2003). This discrepancy between our results and those reported in mice could be due to the difference in age at treatment (i.e. prepubertal *v.* neonatal) or to the fact that age at first estrus is very robust to dietary manipulation in gilts (Friend *et al*., 1981). It is also very likely that discrepancies between results in rat and swine studies are related to interspecies differences in isoflavone metabolism. Indeed, Gu *et al*. (2006) reported that the overall metabolism of isoflavones in pigs is closer to that of human than to that of rats or monkeys. Dosage could also be involved in differences between current results and those of Lewis *et al*. (2003), as these last authors saw an effect of treating rats with 40 mg/kg but not 4 mg/kg BW of genistein per day. It is obviously difficult to extrapolate the amount provided per day in the current trial to that provided in the rat trials as average weights of the gilts at the onset and end of treatments differed (i.e. 47.6 and 137.8 kg, respectively) but quantity of ingested genistein was not altered. Thus, 2 g of genistein per day in this study was equivalent to approximately 42.0 and 14.5 mg/kg BW, respectively, at the onset and conclusion of the experiment. The purpose in selecting this dose was to ensure that pigs obtained at least 40 mg/kg BW of genistein (as used by Lewis *et al*., 2003) at the onset of treatment. Nevertheless, it is obvious that more information on metabolism and tissue uptake of isoflavones in swine are needed to establish optimal dosage in that species. Circulating concentrations of isoflavone metabolites in this study showed that genistein, but not daidzen, glycitein or equol, increased in the blood of swine receiving an oral dose of genistein, but was unaffected solely by providing phytoestrogens via a standard soya diet. The undetectable levels of equol and greater concentrations of genistein and daidzen found in CTL0 gilts are in accordance with previous findings in piglets (Gu *et al*., 2006); however, the increase in concentrations of equol in CTLS gilts is in contradiction with findings from these last authors. Differences in diet or age of animals could be involved in this discrepancy because Gu *et al*. (2006) measured isoflavones in 28-day old piglets that had been fed a liquid diet based on soya protein isolate as sole protein source. Indeed, Adlercreutz *et al*. (1987) established that diet composition (i.e. fat and fiber intakes) can alter the metabolism of estrogens and phytoestrogens. Furthermore, the greater gastrointestinal tract development of pubertal gilts (in this trial) compared with that of suckling piglets could have come into play as intestinal bacteria are known to be involved in metabolism and absorption of phytoestrogens (Adlercreutz *et al*., 1987).

The lack of treatment effects on circulating concentrations of estradiol, progesterone and prolactin contradicts data in rats where concentrations of LH and prolactin were greater in adult male offspring from dams that were fed soya, compared with dams fed milk and eggs, as a protein source during two reproductive cycles (Pastuszewska *et al*., 2008). However, it is in agreement with another study where 3 s.c. injections of genistein to prepubertal rats had a biological effect on mammary tissue, but did not alter circulating concentrations of estradiol-17β or progesterone after puberty (Murrill *et al*., 1996). On the other hand, Rachon *et al*. (2008) noted increased concentrations of estradiol and prolactin in the circulation of ovariectomized rats receiving a high dose (i.e. 400 mg/kg feed) of the phytoestrogen, equol, for a 3-month period; but they did not look at the effect of equol on intact animals.

Neonatal exposure of mice to genistein affects the mammary glands. Indeed, advanced mammary gland development and differentiation were observed with exposure to genistein, moreover, the dose and timing of exposure has an impact on these effects (review by Jefferson *et al*., 2007). Mice injected s.c. with lower doses of genistein (0.5 and 5 mg/kg) showed advanced mammary gland morphogenesis at puberty, whereas stunted mammary development was observed at the highest dose (50 mg/kg; Padilla-Banks *et al*., 2006). The authors noted that morphological changes observed at the lower doses were due to expansion of the mammary gland through the fat pad at a faster rate. Rowell *et al*. (2005) looked at the mechanism of action of genistein on mammary tissue in rats and found that early prepubertal exposure to genistein enhanced cell proliferation by up-regulating the protein guanosine triphosphate-cyclohydrolase 1 and the EGF-signaling pathway. It is not known if that is the case in swine.

There is only one published report on the effect of genistein on mammogenesis in swine. Ford (2003) noted that i.m. injections of 400 mg/day of genistein to postpubertal ovariectomized gilts for 10 days enhanced mammary gland development. There was increased lobular development and alveolar cells, and less dense connective tissue and less fat in the stroma. This is consistent with our current findings of increased DNA in mammary parenchymal tissue and a tendency for lower fat content. Although there have been reports in the literature of genotoxic effects of genistein, these have been restricted to *in vitro* studies utilizing pharmacological concentrations of genistein that exceeded 5 μM (Klein and King, 2007). Evaluation of data from this study does not support a genotoxic effect of genistein. Tissue morphology and BrdU-labeling indices of mammary tissue from GEN gilts were not impaired compared with that of CTL0 or CTLS gilts and there was more extensive lobular development in GEN gilts. Finally, it should be noted that BrdU labeling was heavy and thus the labeling indices were not likely to have been compromised by labeling due to unscheduled DNA synthesis. Such repair processes result in incorporation of nucleotides and nucleotide analogs (BrdU) that are a minor fraction of the incorporation that

occurs during the semiconservative DNA synthesis necessary for cell proliferation (Valentin-Severin *et al.*, 2004).

The reduced number of ERα-positive mammary epithelial cells in GEN gilts from this study is in agreement with findings of Padilla-Banks *et al.* (2006) who observed lower ERα expression in mammary tissue of pubertal mice following neonatal exposure to three doses of genistein (s.c. injections of 0.5, 5 or 50 mg/kg BW per day). An increased expression of ERβ was also noted by Padilla-Banks *et al.* (2006) at the lowest dose of genistein, but this was not accompanied by increased protein expression of ERβ in the mammary ductal epithelium, as determined by histochemistry. The negative effect of genistein on ERα may be indicative of negative feedback, but the direct consequence of this lower expression remains unclear in light of the mammary hyperplasia observed. Even though not significant, numerically, there was a 60% increase in BrdU labeling index in GEN *v.* CTL0 gilts in this study. Conversely, the numerically lower value for CTLS gilts compared with CTL0 gilts was surprising. Consistent with our current observations of increased DNA in mammary parenchyma and reduced number of ERα-positive mammary epithelial cells in GEN gilts, Capuco and colleagues (Capuco *et al.*, 2002; Meyer *et al.*, 2006) noted that exogenous estrogen stimulates the *in vivo* proliferation of mammary cells in dairy heifers while decreasing expression of ERα.

Human epidemiological data and *in vitro* animal studies suggest that genistein may possess breast cancer preventive properties (review by Warri *et al.*, 2008) by altering expression of *BRACA1* and *PTEN* and perhaps by epigenetic mechanisms. Similarly, *in vitro* data support an effect on mammary tumorogenesis, as exposure of human mammary cells to genistein altered the expression of certain cancer-related genes (Moon *et al.*, 2007). The lack of effect of genistein on the mRNA expression of selected genes in this study could be due to an inadequate dose, yet positive effects on mammary development were observed. This suggests that the enhanced mammogenesis was not related to expression of the genes evaluated. It is of interest to note, however, that it was only at high concentrations (100 μM and not 1 or 10 μM) that genistein and daidzen affected (i.e. decreased) IGF- and EGF-associated gene expression in porcine skeletal muscle tissue *in vitro* (Kalbe *et al.*, 2008) and reduced protein synthesis and total DNA in semimembranosus muscle cell cultures from neonatal pigs (Mau *et al.*, 2008). Rehfeldt *et al.* (2009) also reported dose-dependent effects of genistein on protein metabolism in porcine skeletal muscle cells *in vitro*, with protein degradation being reduced with 0.1 nmol/l of genistein and protein synthesis being reduced with 100 μmol/l of genistein. *In vivo* and *in vitro* studies indicate that effects of phytoestrogens are highly dose dependent. Insufficient dose could therefore be related to the lack of effect on mammary gene expression in our study. Additionally, the timing of *in vivo* exposure could be important as Luijten *et al.* (2007) reported that the impact of phytoestrogen exposure on mammary tumorogenesis in mice appears to depend on the timing of exposure within the life cycle. The optimal time for phytoestrogen exposure to alter mammogenesis in swine is not known.

A recent review discussing the effects of phytochemicals on bone and joint health (Lister *et al.*, 2007) stated that there has been considerable investigation on the use of phytoestrogens to improve bone health and prevent osteoporosis. Studies in humans have shown promising, yet variable results. Consumption of isoflavones for up to a year increased expression of beneficial bone biomarkers, such as markers for bone mineral density (Williamson and Manach, 2005). On the other hand, feeding 1 mg of genistein to humans for a year did not alter bone density or bone resorption but increased markers of bone formation (Atkinson *et al.*, 2004). As hypothesized by Mundy (2006) genistein may prevent bone resorption by promoting bone formation. An impact on bone resorption was not apparent in this study as concentrations of NTx, which is a highly-specific parameter of bone collagen degradation and serves as an indicator of bone resorption (Weiler *et al.*, 2001), were not altered. This may be due to the shorter duration of treatment (i.e. 93 days) in our study compared with studies performed in humans. Indeed, Setchell and Lydeking-Olsen (2003) in their review stated that diets rich in phytoestrogens have bone sparing effects in the long-term but that the magnitude of the effects are still speculative. Kaludjerovic and Ward (2009) recently demonstrated that neonatal exposure of rats to genistein modulates bone development in adulthood. It is not known whether that would also be the case in swine.

Conclusions

In conclusion, it is apparent that exposure of growing gilts to the phytoestrogen genistein has a stimulatory effect on mammary development measured after puberty. However, this is not accompanied by changes in abundance of transcripts for selected genes in mammary tissue, or in circulating hormone levels. These results also show that mammary development in gilts was not stimulated solely by feeding a standard diet containing soya compared with a soya-free diet.

Acknowledgements

The authors thank L. Thibault, S. Horth, D. Beaudry and P. Robertson for their invaluable technical assistance, A. Hummel for assistance with immunohistochemistry, the staff of the Swine Complex, especially D. Morissette, J. Phaneuf, J. Marois and T. Marsh, for care and slaughter of the animals, S. Méthot for statistical analyses and the 'Centre d'Insémination Porcine du Québec' (CIPQ) for supplying the Landrace semen. Sherbrooke AAC contribution no. 1021.

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