DOES CONSUMPTION OF BEEF FROM CATTLE ADMINISTERED GROWTH-ENHANCING TECHNOLOGY TRIGGER EARLY ESTRUS IN PRE-PUBERTAL GILTS?

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE – NON-THESIS OPTION

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ABSTRACT

The objective was to determine if pre-pubertal gilts supplemented ground beef obtained from steers implanted with growth enhancing technology caused precocious puberty. Twenty-four gilts were selected for the same birth date from common parentage. Upon reaching 61 days of age, daily delivery of the low-estrogenicity base diet was supplemented with: 114 g beef natural patty (NAT), 114 g beef patty from steers that had received growth promoting implants (100 mg trenbolone acetate and 14 mg estradiol benzoate; IMP), 198 g tofu patty (TOFU), or the negative control (base diet only; CON). The estradiol equivalents (ng/kg) of the TOFU were approximately 500 fold times the NAT and 350 fold the IMP supplement. No differences ($P = 0.55$) were observed in number of days to reach estrus, feed efficiency ($P > 0.19$), live weight gain ($P = 0.89$), loin muscle development ($P = 0.45$), or subcutaneous fat deposition ($P = 0.71$).

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INTRODUCTION

A review of literature by Nebesio and Pescovitz (2005) reported that young American girls are reaching the onset of puberty at an earlier age. These authors acknowledged that the trigger for precocious puberty can be a complex interaction between genetics, hormones, and environmental factors including contact with industrial chemicals, pesticides, estrogen-containing cosmetics, and phytoestrogens. An earlier review by Anderson and Skakkebeak (1999) stated that hormone residues in meat obtained from animals implanted with sex-steroid hormones (to promote growth) had been largely ignored as a possible cause of precocious puberty, and suggested that they were "very likely the cause". More recently, Cuffman et al. (2011) suggested that a mother's consumption of beef obtained from estrogenically implanted cattle may have an influence on the development of the unborn fetus which could lead to the early onset of puberty of her female offspring.

The anatomy and physiology of pigs are very similar to humans. A pig's gastrointestinal system, body composition, and nutrient requirements make the pig an ideal model for evaluation of how diet influences physiological responses in growth and development (Tumbleson, 1986; Tumbleson and Schook, 1996). Hughes (1982) reported that factors such as genotype, social environment, season of the year, boar exposure, growth rate, body composition, and age influence the development and onset of first estrus in gilts, similar to corresponding factors described above for humans. The use of swine as a biomedical model for young girls is advantageous because many intrinsic and extrinsic factors can be controlled by the researchers to allow for an accurate assessment of the influence of the dietary treatments.

Using pre-pubertal gilts as an animal model, our hypothesis is that beef obtained from cattle receiving growth promoting technology that was implanted post-weaning does not alter the

timing of puberty, or the body composition post-puberty compared to females fed non-implanted "natural" beef, or a common meat alternative, tofu.

MATERIALS AND METHODS

Animals

All methods and procedures involving animals were reviewed and approved by the North Dakota State University Animal Care and Use Committee (IACUC #A0958). Thirty-three crossbred gilts from a common sire (Danbred 610) and dam line (Danbred 241) were selected at weaning (18 days of age) on the same birth day from Progressive Swine Technologies (St. Edward, NE) for inclusion in the experiment. On the day of selection, piglets were transported by pick-up truck (livestock box 1.22 meters x 1.83 meters) to the Animal Nutrition and Physiology Center at North Dakota State University (725 km, Fargo, ND). At approximately16 kg of BW, animals were individually penned in the nursery room. Gilts were fed a pelleted commercial nursery diet (Table 1) *ad libitum.*

Gilts were vaccinated with Ingelvac Circo Flex (product # 30902-04, Boehringer Ingelheim, Ridgefield, CT) and Ingelvac MycoFlex (product # 27302-01, Boehringer Ingelheim, Ridgefield, CT), and treated with 1 cc Baytril 100 (Bayer Pharmaceuticals, Shawnee Mission, KS) to promote health and aid the transition process. Twenty four pigs were selected for the project based on uniformity of littermates and weight (24.5 ± 3.2 kg) in order to provide balance across treatments. Piglets were selected from litters birthed to eight sows. A minimum of two and a maximum of 4 piglets per sow were included in the project and were stratified over the four dietary treatments based on BW rank within litter. Animal health was continuously monitored and one gilt was removed early in the project for health issues not related to treatment.

Table 1. Stage 1, Stage 2, Stage 3, Stage 4, and Stage 5 diets expressed on an as-fed basis.

	Stage 1[1]	Stage 2	Stage 3	Stage 4	Stage 5
Days of age per feeding stage	0 – 50	51 – 82	83 – 117	118 – 154	155 – 170
Ingredient, %					
Duration of feeding in kg BW	10-20	18-30	30-60	60-100	100-152
Corn	62.30	56.28	66.04	69.49	74.73
Casein	10.00	—	—	—	—
Whey Powder	10.00	10.00	—	—	—
Canola Meal	12.00	28.00	28.00	25.00	20.00
Soy Oil	2.00	2.00	2.00	2.00	2.00
Cornstarch	1.00	1.00	1.00	1.00	1.00
Limestone	1.05	0.90	1.00	0.80	0.80
MCP	0.85	0.55	0.80	0.65	0.45
Lysine-HCL	0.10	0.45	0.40	0.33	0.30
DL-Methionine	—	0.02	—	—	—
L-Threonine	—	0.06	0.03	—	—
L-Tryptophan	—	0.04	0.03	0.03	0.02
Salt	0.40	0.40	0.40	0.40	0.40
Vitamin Pre-mix[2]	0.30	0.30	0.30	0.30	0.30
Calculated composition, %					
CP	19.52	15.85	15.45	14.67	13.32
Lys	1.32	1.17	1.07	0.96	0.84
Met	0.48	0.34	0.32	0.30	0.28
Thr	0.84	0.74	0.67	0.60	0.54
Trp	0.22	0.22	0.20	0.18	0.15
Ca	0.77	0.70	0.71	0.59	0.53
Total P	0.63	0.63	0.64	0.58	0.51
ME, Mcal/kg	3.40	3.21	3.21	3.24	3.29
Actual composition, %					
Dry matter	94.32	92.57	91.42	91.52	93.25
Crude protein	23.16	16.46	17.17	16.16	14.73
Crude fat	3.41	4.76	4.38	4.70	4.74
Calcium	0.97	1.11	1.17	0.79	0.74
Phosphorus	0.84	0.73	0.75	0.73	0.59
Ash	7.65	6.17	5.74	5.47	4.34
Gross energy, Mcal/kg	4.10	4.16	4.21	4.33	4.29
Estradiol activity (ng/kg; as fed basis)	—[3]	884	254	NA[4]	354

[1]Acclimation diet where no supplemental treatments were fed. Each phase diet was formulated to meet the requirements of the lightest pig on test.

[2]Vitamin Pre-mix supplied 10,000,000 IU of vitamin A, 1,500,000 IU of vitamin D_3, 50,000 IU of vitamin E, 40 mg of vitamin B_{12}, 4,000 mg of menadione, 155 mg of biotin, 1,000 mg of folic acid, 50,000 mg of niacin, 30,000 mg of d-pantothenic acid, 3,000 mg of vitamin B_6, 9,000 mg of riboflavin, and 3,000 mg of thiamine per pound of feed (Trouw Nutrition USA, LLC, Highland, IL).

[3] Supplements were not started until feeding of Stage 2

[4] Not assayed

Upon reaching approximately 62 kg BW, gilts were ultra- sounded every 14 days.

Longissimus muscle area (LMA) and subcutaneous fat depth (FD) was obtained via ultrasonography at each weigh period at an anatomical location adjacent the 10^{th} and 11^{th} thoracic rib, as a means to monitor muscle and adipose development over time on test.

Diet and Treatment

A corn and canola-base diet was formulated to meet or exceed *National Research Council* nutrient requirements for maximizing lean growth for all phases of production (Table 1). Canola was selected as a replacement for soybean meal in an effort to reduce the overall estrogenicity of the base diet. Gilts were phase-fed the base diets as outlined in Table 1. The base diet was fed as a percentage of BW and adjusted weekly (based on weekly obtained BW) to equal the percentage consumed by the gilt consuming the least feed. In this way energy intake was restricted, ensured that all treatment supplements were consumed, and allowed consistency across animals and treatments as intake was equal on a percentage of BW basis (approximately 3.8% BW).

Four treatments were administered beginning at 61 days of age: 1) negative control- (CON; low estrogenicity base diet only), 2) positive control- (TOFU; base diet + 198g [pre-cooked wt.] tofu patty), 3) Natural (NAT: base diet + 114g [pre-cooked wt.] beef patty obtained from feedlot steers raised without the use of growth promotant technology (GPT), and 4) Implanted- (IMP; base diet + 114g [pre-cooked wt.] beef patty obtained from feedlot steers provided GPT (Synovex Choice implants, Fort Dodge Animal Health, Fort Dodge, IA) containing 100mg of trenbolone acetate and 114 mg of 17β-estradiol that was administered to the live steers at weaning (approximately 230 kg BW) and again at approximately 410 kg BW. Tofu and beef patties were cooked to internal temperature of 76˚C, cooled, and fed daily at 1600h before feeding of the base diet. Beef patty size was chosen to equate to that offered at fast food restaurants as a quarter-pound burger reflecting the uncooked weight of the ground beef. The

required weight of the tofu burger was then calculated to provide equivalent caloric and nitrogenous intake. Table 2 contains the compositional analysis of the supplemental treatments.

Table 2. Compositional analysis (%) of supplemental dietary treatments as fed.

	Treatments[1]		
	NAT	**IMP**	**TOFU**
Raw patty weight, g	113.00	113.00	198.00
Cooked patty weight[2], g	64.00	70.00	144.00
DM (as-fed), %	44.61	39.19	17.85
Estradiol equivalents, ng/kg supplement (as fed)[3]	10 ± 3.4	14 ± 3.7	5133 ± 643
Nutritional composition, %			
Crude protein	75.00	85.68	57.99
Crude fat	19.81	9.74	24.24
Calcium	0.08	0.07	0.82
Phosphorus	0.58	0.63	0.90
Gross energy, Mcal/kg	6.20	5.68	6.03

[1]**NAT** = cooked beef patty obtained from a feedlot steers raised without the use of growth promoting technology; **IMP** = cooked beef patty obtained from feedlot steers provided Synovex Choice (100 mg of trenbolone acetate and 14 mg of 17β-estradiol) at approximately 230 (weaning) and 410 kg BW; **TOFU** = cooked tofu.

[2]All supplemental treatments were cooked to 76˚C and cooled to room temperature prior to feeding.

[3]E-Screen analysis of estradiol equivalents, mean ± S.D. of three extractions.

Estrous Detection

Gilts were physically isolated from boars to prevent precocious puberty (Estienne and Harper, 2009). Upon reaching 90 kg BW, gilts were allowed access to a community pen for 30 minutes a day, where visual signs of estrus were monitored (swelling and redness of the vulva, lordosis, and mucus discharge). A functional estrus was confirmed chemically based on presence of circulating progesterone production by a functioning corpus luteum, defined as serum progesterone greater than 1ng/mL. For this assessment, blood was collected twice a week after gilts reached 68 kg of BW. Two gilts (one CON, and one TOFU) failed to exhibit heat or present serum progesterone levels greater than 1 ng/ml progesterone before termination of the experiment. All gilts were slaughtered at the NDSU Meat Science abattoir a minimum of three days after the first visible sign of estrus.

Analysis of Progesterone

Serum. Blood samples were collected weekly on Monday and Thursday. Upon confirmation of estrus, gilts were slaughtered on the following Tuesday. There was no blood sample collected on the day of slaughter. Blood samples were collected (1-inch 18 gauge needle with 12cc sterile syringe), via jugular venipuncture. Blood samples were refrigerated for 2 hours, centrifuged, serum transferred to 2 ml vials, and frozen at -20^{o}C until analysis. Progesterone concentration was analyzed using the Immulite 1000 (# LKPG1, Siemens Medical Solutions Diagnostics, Los Angeles, CA).

Analysis of Estrogenicity

Glassware and Reagents. All glassware used for extraction or analysis of estrogenicity of diets was solvent washed and baked. The process was: wash in Liquinox detergent (MG Scientific, Pleasant Prairie, WI), rinse with nanopure water (npH_2O), dry, rinse with series of solvent (acetone, methanol, acetonitrile [ACN], ethyl acetate, and methylene chloride), and bake at 450°C for 4 h. All solvents used were high performance liquid chromatography grade (99.8% purity). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Extraction of Rations. The base diet for each phase (Stage 2, 3, 4 and 5) were extracted as previously described for sugar beet by-product feed samples (Shappell et al., 2012) with exception of an additional hexane extraction step. Briefly, triplicate subsamples of feed (1g as fed) were extracted using sonication and 25 mL of 60% acetonitrile at 37˚C for 1h (47kHZ), shaking samples every 10 min. Particulates were removed by centrifugation (900 X g, 10 min). Supernatants were filtered through solvent-washed glass wool to remove remaining fines and volumes recorded. Acetonitrile was volatilized off under N_2 at 37˚C. Hexane was added to the supernatants (1:1, v:v), samples vortexed for 1 min and then held at -20˚C for ≥ 1h. Dietary

phases were then separated by centrifugation as described above. The upper hexane layer was discarded. The lower aqueous layer was diluted to approximately 90 mL with npH_2O (after removing any pelleted material), concentrated by solid phase extraction using OASIS HLB cartridges, and taken to dryness exactly as described by Shappell et al. (2012).

Dry eluates were stored at -20^{o}C for later analysis. While rations were not expected to contain known phytoestrogens, extraction efficiency was assessed using genistein as a surrogate (400μg genistein/g of ration, using a stock solution of 2.5 mg/mL in EtOH).

Extraction of Tofu Burger. Cooked tofu patties were extracted by the method described Murphy et al. (1999) for isoflavone analysis of foods modified with an additional hexane extraction step. Crumbled patty (3g as fed) was added to 19 ml 0.01M HCl 53% ACN and rotated for 2 h at room temperature (60 RPM, RKVSD rotator, Appropriate Technical Resources, Laurel, MD). Solids were removed by filtration through solvent-washed glass wool and ACN was volatilized off as described above, followed by hexane extraction and processing through SPE as described above for the base diet rations. Extraction efficiency was assessed with genistein, adding 800 μg of genistein/3 g tofu burger to ensure estrogenic activity greater than what was due to the endogenous genistein contained in the tofu samples.

Extraction of Ground Beef. Cooked ground beef burgers were extracted in a modification of a method for extraction of estrogens from fish (Al-Ansari et al., 2011). The crumbled patty (5g as fed) was mixed with approximately 2 g of diatomaceous earth (ASE Prep DE, Dionex Corp., Salt Lake City, UT) that had been previously processed through the solvent extraction method to be used on the Accelerated Solvent Extractor (ASE 200, Dionex). Samples were loaded on 2 solvent-washed glass filters (19.8mm diameter, Dionex) into an 11 mL ASE cell topped with more DE to fill the cell, and a final third filter on top. Samples were extracted using

100% acetone under 1500 psi, at 60˚C, with 5 min preheat time, two cycles of 5 min static time followed by a cell flush of 20% cell volume, and completed with a 2 min purge with N_2. Extracts were transferred to conical glass centrifuge tubes, measured, and formic acid added to a final concentration of 1%. Samples were vortexed (1 min) and held at -20˚C for 1 h, followed by centrifugation at 900 X g, 10 min, -4˚C. The liquid phase was removed from the pellet to a second conical glass tube and extracted with an equal volume of hexane, vortexing for 1 min, and repeating the centrifugation step. The upper lipid layer was removed and acetone in the lower layer was volatilized, followed by SPE of the remaining aqueous material diluted to 100mL with npH_2O, and processed as described above. Extraction efficiency was assessed by fortifying ground beef with 17ß-estradiol (17β-E_2, 600 pg/5 g of beef).

E-Screen. Estrogenic activity was determined by E-Screen, an *in vitro* assay that assesses estrogen-dependent proliferation of human mammary epithelial cell line (MCF7-BOS, from the laboratory of Drs. Anna Soto and Carlos Sonnenschein, Tufts University School of Medicine, Boston, MA). The assay (as described Shappell, 2006) was performed with the only change being an increase in cell plating density to 4 X 10^3 cells/well in the 96 well plate. Briefly, cells were plated in complete medium including 5% fetal bovine serum (FBS). After 24 h, media was replaced with medium containing no phenol red (due to estrogenicity) and 10% FBS that had steroids removed. After 5 days of exposure, cells were fixed with trichloroacetic acid, stained for protein with sulforhodamine blue, solubilized, and absorbance read (A 490 nm). Estradiol equivalents were calculated based on dilutions falling within the linear range of the standard curve (typically 1 x 10^{-12} to 1 X 10^{-11}M 17β-E_2). Dilutions that fell within that range were as follows: Grower Rations 1 and 2, 1:500-700; Stage 4 and 2, 1:250-500; tofu burgers, 1:25,000-50,000; and ground beef, 1:100-200. Specificity of estrogenicity of samples (reflected by

proliferation of cells) was confirmed by E_2-receptor antagonist ICI 182,780 (Tocris, Ellisville, MO) as described by Rassmussen and Nielsen (2002).

Statistical Analysis

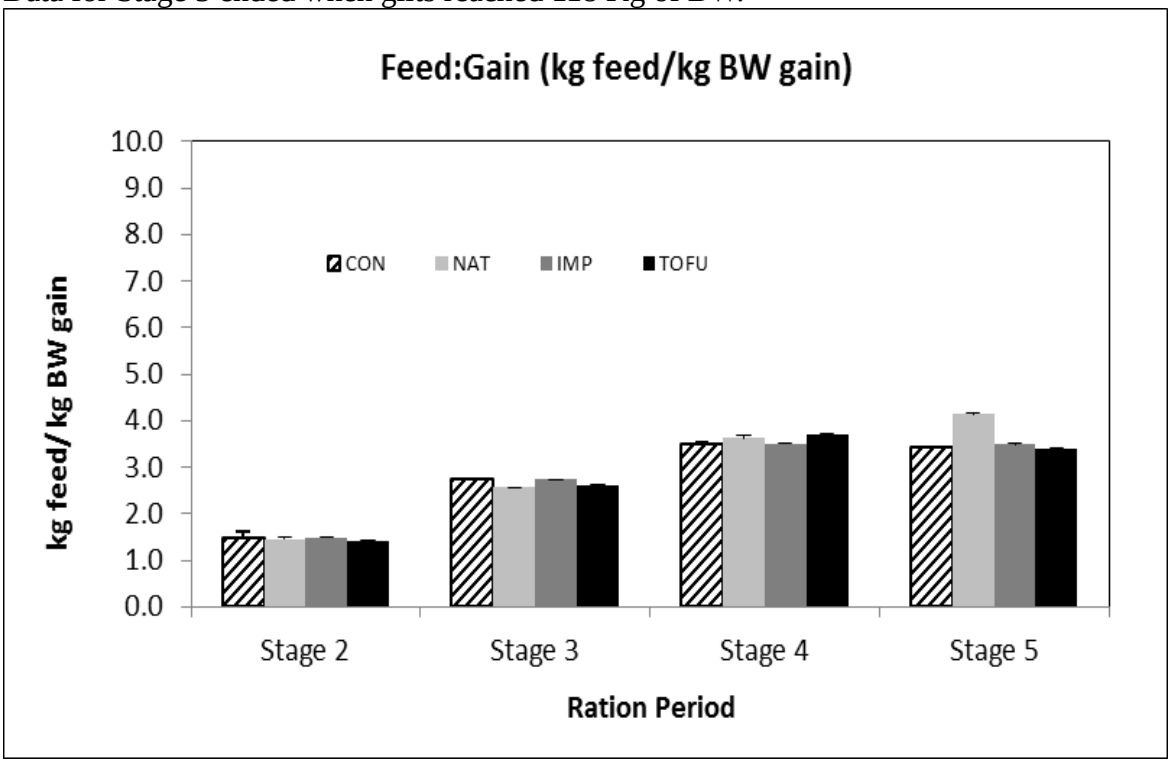
Differences in kg BW, LMA, and FD for each weigh period, and number of days to reach estrus were analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC; version 9.2), with treatment as a fixed effect, litter as a random variable, and gilt as the experimental unit. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

The composition (percentage of ingredients) of all phases of diets is presented in Table 1. Stage 1, Stage 2, Stage 3, Stage 4, and Stage 5 diets are expressed on an as-fed basis. These were the "base diets' administered to all gilts and the supplementary treatments (TOFU, NAT, and IMP) were administered daily in addition to the base diet. The CON diet received no supplement. The estrogenic activity of each phase diet was evaluated for Stage 2, Stage 3, and Stage 5 with Stage 2 possessing the highest estrogenic activity. The authors speculate that the greater level of estrogenicity in the Stage 2 base diet may be attributed to the presence of whey protein added to this phase ration; however the estrogenicity of the whey protein alone was not assayed. The Stage 3 and Stage 5 diets possessed very low levels of estrogenicity (254 and 354 ng/kg of feed, as fed; respectively).

The major protein source in a traditional swine diet is obtained from the addition of soybean meal. Soy and soy-products contain a high degree of estrogenicity as a result of naturally occurring phytoestrogens (Brown and Setchell, 2001). This was evident in our current study which showed that the estradiol equivalents (ng/kg, as fed) of the TOFU (a soy-based product) treatment were approximately 500 fold the NAT supplement and approximately 350 fold the IMP supplement (Table 2). In order to reduce the overall estrogenicity of the base diets, canola meal was used as a replacement for soybean meal. It is also important to note that each of the dietary supplements were similar in caloric content (Table 2). No differences were observed across treatment for feed efficiency (kg of feed/kg BW gain; Figure 1) indicating that the supplemental treatment had no influence on the conversion of diet to kg of BW gain (P values > 0.19).

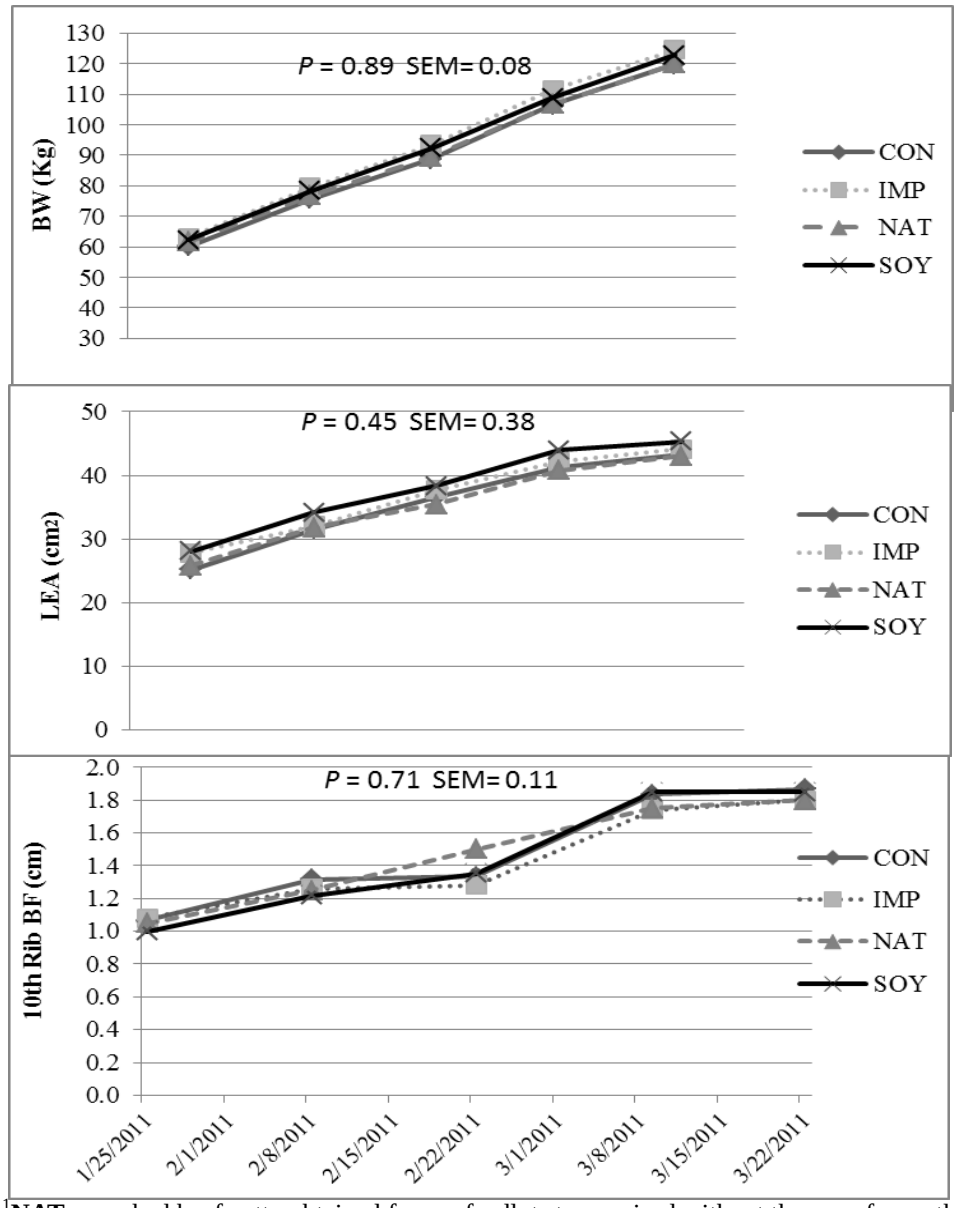
Figure 1. Average feed efficiency for each ration (kg feed/kg BW gain, mean ± standard error). Data for Stage 5 ended when gilts reached 118 Kg of BW.



Stage 2- *P* = 0.37; Stage 3- *P* = 0.19; Stage 4- *P* = 0.85; Stage 5- *P* = 0.97

This is further exemplified in Figure 2 showing no treatment effect on BW (P = 0.89), loin muscle area developed at the 10th and 11th rib interface (P = 0.45), and subcutaneous fat deposition (P = 0.71) at the same location over time on test. In other words, diet did not influence muscle or fat accumulation.

Figure 2. Average body weight (kg), *Longissimus thoracis* cross-sectional muscle area (LMA; cm^2) at the $10^{th}/11^{th}$ rib interface, and subcutaneous fat depth (BF) located at the $10^{th}/11^{th}$ rib interface (cm) for gilts over time across supplemental treatment.



[1]**NAT** = cooked beef patty obtained from a feedlot steers raised without the use of growth promoting technology; **IMP** = cooked beef patty obtained from feedlot steers provided Synovex Choice (100 mg of trenbolone acetate and 14 mg of estradiol 17β) at approximately 230 (weaning) and 410 kg BW; **TOFU** = cooked tofu.

[2]All supplemental treatments were cooked to 76C and cooled to room temperature prior to feeding.

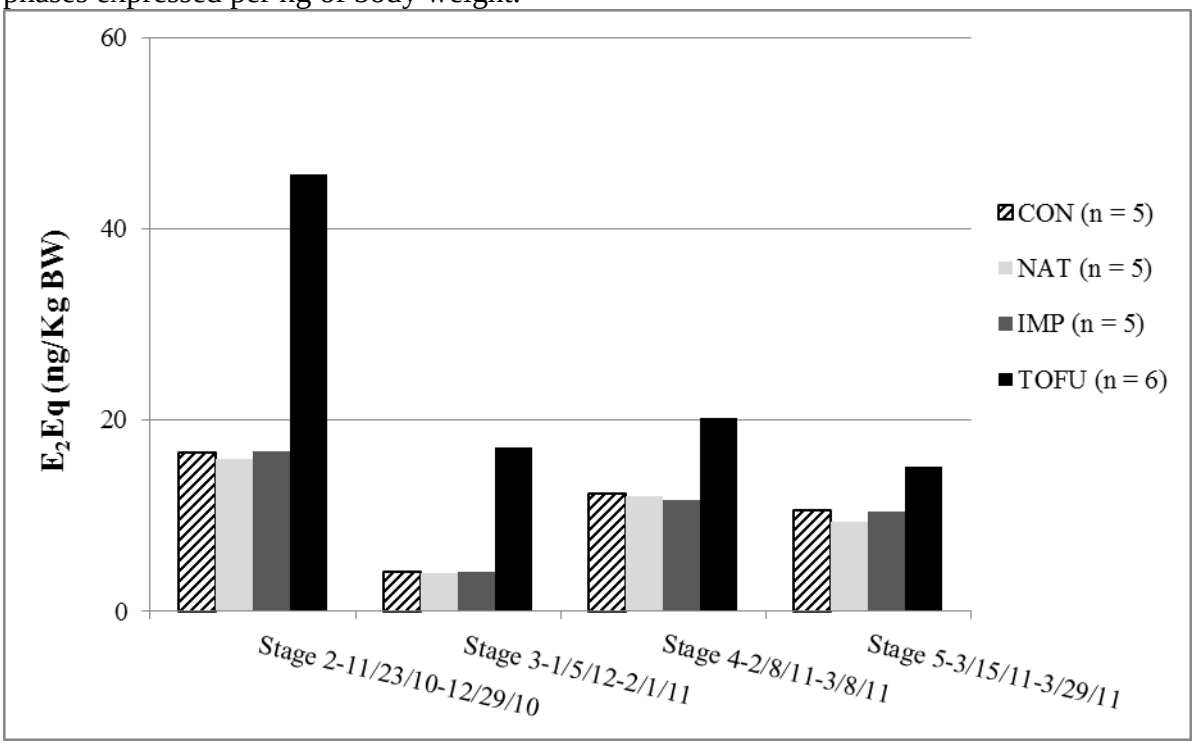
The level of estrogenic activity between NAT and IMP beef are similar and both are extremely low compared to the estrogenic activity of TOFU. According to Food and Drug

Administration regulations, the amount of extra hormone in meat must not exceed one percentage of the average amount of that hormone produced by a person in the most sensitive segment of the human population (Webster, 1989). Hoffman and Evers (1986) reported that pre-pubertal girls (avg. weight = 27 kg) produce an average of 54,000 ng of E_2 each day; approximately 2000 ng/kg BW. Therefore 1% would equate to a dietary allowance of 20 ng E_2/kg BW. Using the supplemental treatments from the current study, this would equate to 88% of the 20 ng E_2/kg BW/day for 60 kg gilts (17ng/kg BW) and 42% for 120 kg gilts (8.5ng/kg BW) on TOFU treatment, while those receiving NAT or IMP were approximately 0.15 and 0.05% for 60 and 120 kg BW gilts, respectively. This data is represented graphically in Figure 3, where TOFU is seen to consistently provide higher estrogenic activity than the other supplements. The impact of ground beef, either NAT or IMP, is insignificant by comparison.

When assessing the contribution of dietary "hormones", food preparation must be considered. Tittlemier et al. (2011) reported the cooking process reduces the amounts of E_1, E_2, and catechol estrogens in ground beef, apparently in relationship to fat content. While uncooked regular ground beef contained approximately 25% fat, the composition of the juices collected after cooking, (known to contain liquefied fat), was approximately 75% fat, which resulted in a concomitant reduction in estrogen content of the cooked beef (Tittlemier et al. (2011). As seen in Table 2, the crude fat composition of NAT was actually higher (approximately 20%) than the IMP beef (approximately 10%), an expected consequence of the steroid treatment (a combination of estrogen and androgen agonist). Yet on a cooked, as-fed basis the E_2Eq of the two patties were nearly identical. The estrogen content of beef reported in the literature frequently reports levels for uncooked tissues. For example, Anderson and Skakkebeak (2005) provided no indication that the meat samples evaluated in their study were cooked, thus the values reported

may be uncharacteristically elevated relative to levels that would be consumed as part of a normal human diet.

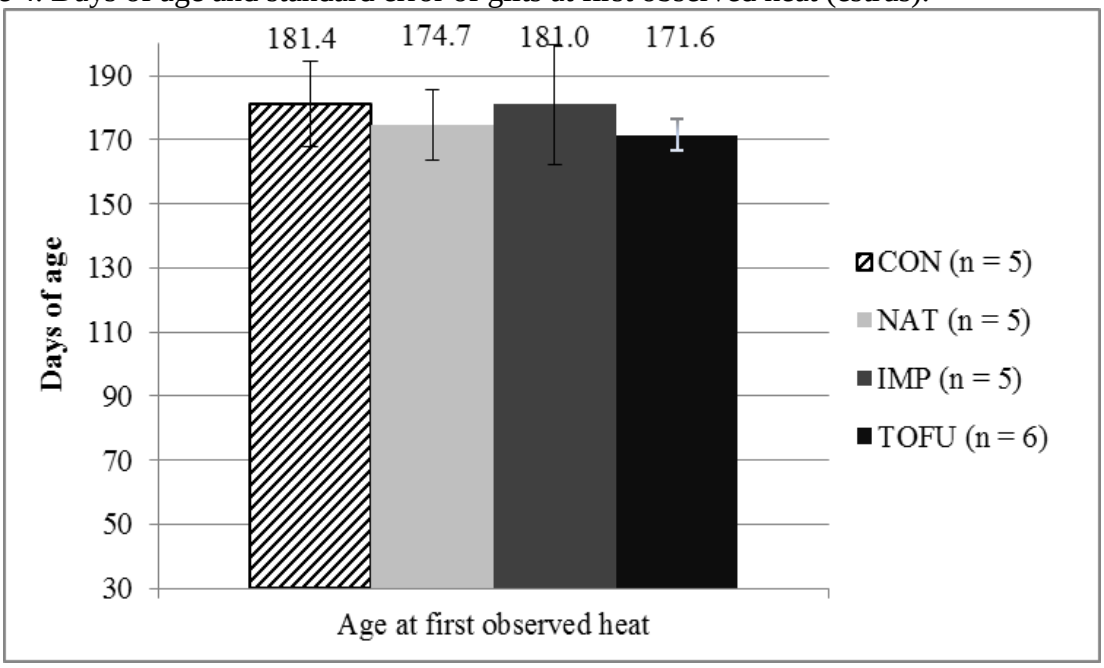
Figure 3. Estradiol equivalence (ng) for each supplemental treatment across the four dietary phases expressed per kg of body weight.



In a study with men, women, pre-pubertal girls, and boys conducted by Hartmann et al. (1998), the average daily intake of steroid hormones was estimated. Their study concluded that meat, meat products, eggs, vegetables, and fish did not play a dominant role in the daily intake of hormones, but rather milk products were the main source of estrogen and progesterone. This finding support our speculation that whey protein contributes to the estrogenicity of the Stage 2 diet in the present study. Hartmann et al. (1998) estimated that the first-pass-effect of the liver (glucuronidation, sulfation, or other metabolism) drastically reduces the biological activity of 90% of the hormones that are ingested, and hypothesized exposure to phytoestrogens from plants or environmental chemicals have a greater influence on human beings than the exposure to naturally occurring hormones from food. Consistent with this premise was the present study's

failure to demonstrate feeding beef from hormonally treated animals would result in early onset of puberty in gilts. We observed no difference (P = 0.55) in the number of days to reach visual detection of estrus across supplemental treatment (Figure 4).

Figure 4. Days of age and standard error of gilts at first observed heat (estrus).



NS; P = 0.55, SEM= 5.71

In contrast, a case study conducted by Jihye, et al. (2011) evaluated 108 girls with central precocious puberty (8.6 ± 0.8 years of age) with 91 age-matched controls (8.5 ± 0.8 year of age) that did not exhibit precocious puberty revealing that high serum isoflavone concentrations were associated with the risk of precocious puberty. In the present study, genetics and environmental factors were completely controlled, while control of these aspects is difficult if not impossible to manage in human studies evaluating the same objectives.

The mean 17β-estradiol (E_2) residues determined for Synovex Choice when tested by FDA (2002) were below the allowable incremental increases for all of the sampled tissues, permitted under 21 CFR 556.240 (120 ppt for muscle, 240 ppt for liver, 360 ppt for kidney, and 480 ppt for fat). Food and Drug Administration documentation (2002) reported that the

concentrations of E_2 in fat and muscle were determined by the method involving solid-phase extraction, high-performance liquid chromatographic separation, and quantification by radioimmunoassay.

In the present study, we found that beef patties obtained from implanted steers had 14ng of E_2Eq/Kg, or 0.014ng of E_2Eq/g of cooked patty. By comparison, Stephany (2004) reported median dietary intake of E_2 in 250g of "hormone-free cattle" steak was less than 2.5 ng, and 250 g of steak from "hormone- treated cattle" was 5ng, or 0.02ng E_2/g. In our study, the E_2Eq of the IMP and NAT patties were similar (0.014 and 0.010 ng/g, respectively) and were less than those reported by Stephany (2004) for beef from hormone treated cattle, and between 10 and 20 fold less than the limits imposed by the FDA for muscle (0.120 ng/g) and fat (0.240ng/g).

IMPLICATIONS

Consumption of beef obtained from cattle that had received Synovex Choice as part of a growth promoting implant strategy from weaning through feedlot finishing did not alter the timing of puberty or body composition post-puberty of gilts used in this study. Likewise, daily consumption of beef from non-implanted (i.e. natural) cattle or a common meat alternative food (tofu) did not influence days to estrus despite the high level of estrogenicity of the tofu supplement. Accepting gilts as a viable bio-medical animal model for human girls, we conclude that daily consumption of one serving (common equivalent to one quarter pound beef hamburger) very early in physiological development does not lead to precocious puberty.

LITERATURE CITED

Anderson, A.M. and N.E. Skakkebeak. 1999. Exposure to exogenous estrogens in food: possible impact on human development and health. Eur. J. Endocrinol. 140:477-485.

Brown, N. M. and Setchell, K. D. R. 2001. Animal Models Impacted by Phytoestrogens in Commercial Chow: Implications for Pathways Influenced by Hormones. Clinical Mass Spectrometry, Children's Hospital Medical Center, Cincinnati, Ohio. 81:735–747

Cuffman, A.L., S.M. Rejanovic, K.M. McNeils, T.T. Dong, S.A. Lewis, L.W. Jackson, and B.A. Cromer. 2011. Premature thelarche in infants and toddlers: Prevalence, natural history and environmental determinants. J. Ped Adol Gyn. 24:338-341.

Estienne, M.J. and A.F. Harper. 2009. Type of accommodation during gestation affects growth performance and reproductive characteristics of gilt offspring. J. Anim. Sci. 88:400-407.

Food and Drug Administration (FDA). 2002. Freedom of Information Summary. Supplemental New Animal Drug Application. Synovex® Choice (Trenbolone Acetate and Estradiol Benzoate) Implants. NADA 141-043 .

Hoffman, B. and P. Evers. 1986. Anabolic agents with sex hormone-like activities: Problems of residues. Drug Residues in Animals. (p 111-146). Edit. A. Rico. Academic Press. London.

Hughes, P. E. 1982. Factors affecting the natural attainment of puberty in the gilt. In *Control of Pig Reproduction*, Cole, D.J.A., and G.R. Foxcroft, Eds., Butterworth Scientific, Boston.

Hartmann, Sonja, Lacorn, Markus and Steinhart, Hanz. 1997. Natural occurrence of steroid hormones in food. Department of Biochemistry and Food Chemistry, University of Hamburg, Grindelallee 117, D-20146, Hamburg, Germny. Food Chemistry. Volume 62, Issue 1, May 1998, Pages 7-20.

Jihye Kim, et al. 2011. High serum isoflavone concentrations are associated with the risk of precocious puberty in Korean girls. Clinical Endocrinology. Volume 75, Issue 6, pages 831–835.

Oregon Physicians for Social Responsibilities (PSR). 2009. U.S. Meat Production. Viewed at: http://www.psr.org/chapters/oregon/safe-food/industrial-meat-system.html.

Nebesio, T.D. and O. Hirsch Pescovitz. 2005. Historical perspectives: Endocrine disruptors and the timing of puberty. Endocrinologist. 15:44-48.

Pimentel, D and Pimentel, M (2003). Sustainability of meat-based and plant-based diets and the environment. American Journal of Clinical Nutrition, Vol. 78, No. 3, 660S-663S.

Rasmussen TH, Nielsen JB: Critical parameters in the MCF-7 cell proliferation bioassay (E-Screen). Biomarkers 2002, 7:322-336.

Shappel, N. 2011. Estrogenicity of sugar beet by-products. USDA Agricultural Research Service - Publications from USDA-ARS. "Accepted for Publication".

Shappell, N. 2006. Estrogenic Activity in the Environment: Municipal Wastewater Effluent, River, Ponds, and Wetlands. USDA Agricultural Research Service - Publications from USDA-ARS / UNL Faculty. P 122.

Soto, A.M., et al. 1995. The E-SCREEN Assay as a Tool to Identify Estrogens: An Update on Estrogenic Environmental Pollutants. University School of Medicine, Department of Anatomy and Cellular Biology, Boston, Massachusetts. Page 114.

Stephany R.W., et al. 2004. Hormonal Growth Promoting Agents in Food Producing Animals. Handbook of Experimental Pharmacology 195. Page 364.

Tittlemier et al. 2011. Effect of cooking on concentrations of B-Estradiol and Metabolites in Model Matrices and Beef. J. Agric. Food Chem. 59, 915.

Tumbleson, M. E. 1986. *Swine in Biomedical Research*, Vols. 1, 2, and 3, Plenum Press, New York.

Tumbleson, M. E. and L. C. Schook. 1996. *Advances in Swine in Biomedical Research*, Vols. I and II, Plenum Press, New York.