THE PENNSYLVANIA STATE UNIVERSITY

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EFFECTS OF INDUCED HYPOTHYROIDISM DURING NEONATAL DEVELOPMENT ON SPERM PRODUCTION IN ADULT RAMS

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Abstract

Daily sperm production (DSP) in livestock species is important because the majority of genetic gain is made through the male germline. In rodents the use of 6-propyl-2-thiouracil (PTU) to induce a hypothyroid state during the neonatal period has been shown to increase DSP by 136%. Oral PTU administration to ram lambs during the neonatal period was studied to measure its effect on sperm production in adult rams. An experimental group of 3 lambs and control group of 4 lambs were formed. From 10 to 65 days of age lambs received PTU orally to suppress normal thyroid function or vehicle control. The dose of PTU from 10-20 days was 5 mg/kg body weight (BW), from 20-30 days was 10 mg/kg BW, from 30-40 days was 20 mg/kg BW, and from 40-65 days was 30 mg/kg BW. Body weight and scrotal circumference (SC) were recorded once weekly during treatment and every other week following treatment. Blood was collected every 10 days during treatment, and at 5, 6.5, 8 and 12 months of age following treatment and samples were run for triiodothyronine (T_3) concentration. Semen collection was performed by electro-ejaculation at 9 months of age to determine sperm motility, morphology and concentration. Another semen collection by electro-ejaculation was made at 11 months. Five samples were taken, each 2 days apart, and the last three were used to calculate DSP. Rams were sacrificed at about 1 year of age and the testicles were weighed and parenchymal samples taken for examining morphology of the seminiferous epithelium.

A hypothyroid state was achieved in the PTU-treated rams between 30-65 days of age. The BWs of treated rams were lower ($p < 0.05$) than controls during treatment, paralleled controls following treatment in the pre-pubertal period, and were not different ($p > 0.1$) from controls at 1 year of age. SC was not different between PTU-treated and control animals during the study. Diameter of the seminiferous tubules in treated lambs was larger ($p < 0.05$) than controls, but the number of Sertoli cells per tubule was not different. DSP was decreased ($p <$

0.01) by PTU treatment. Thus, neonatal hypothyroidism had a negative impact on the sperm production in adult rams, but no lasting impact on BW or SC.

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Introduction

Increasing sperm production in livestock species would have major benefit because use of sperm from superior sires greatly enhances the genetic improvement of a species. Genetically superior animals reduce the total number of animals necessary to generate animal products and therefore decrease waste associated with raising additional animals. Sheep are a good model animal for reproduction studies in large livestock species because they reach puberty at a relatively young age of 6 to 9 months in comparison to bulls which do not reach puberty until 9 to l8 months of age (Senger, 2003). In addition, scrotal circumference (SC) is easily measured in rams and is an acceptable indicator of testis size and sperm production while this measurement is not as easily obtainable in some other species such as the pig. Thus, to determine whether induction of hypothyroidism during neonatal development is a means to increase sperm production in a livestock species, we used rams as a model.

Administering the reversible goitrogen 6-proply-2-thiouracil (PTU) during the early postnatal period was shown to increase testicular size and daily sperm production (DSP) at adulthood in several species including rats, mice, chickens, and fish (Cooke and Meisami, 1991; Joyce et al., 1993; Kirby et al., 1996; Matta et al., 2009). In rats, testis weight and DSP were increased by 80% and 136%, respectively, at 160 days of age when given a dose of 0.006% PTU (the concentration in the water) through the mother's drinking water from birth to 25 days of age; this was found to be the maximally effective treatment period, dose, and length (Cooke et al., 1991; 1992; 1993). Maximal sperm production for PTU-treated rats was reached at 160 days versus 100 days for untreated (Cooke et al., 1991). Rats returned to a euthyroid state by 90 days of age (Cooke and Meisami, 1991). During exposure, body weight (BW) of PTU- treated animals was decreased compared to controls, but one week after treatment compensatory growth

that paralleled that of control animals occurred, although total BW of PTU-treated animals never reached the size of controls (Cooke and Meisami, 1991; Joyce et al., 1993).

Administration of PTU during the early postnatal period, when Sertoli cells were still proliferating, was shown to delay and extend this proliferation period (Cooke and Hess, 1992; van Haaster et al., 1992). Sertoli cell division was extended until postnatal day 30 in PTU-treated rats compared to day 15 in untreated rats (Bortolussi et al., 1990; van Haaster et al., 1992). Sertoli cells are necessary to support appropriate germ cell growth and form proper tight-junctions in the seminiferous tubules, making them central to testis function (de Franca et al., 1995). The number of Sertoli cells is positively correlated with the number of germ cells that can be produced, and because the early postnatal period is the only time when they proliferate, establishment of a Sertoli cell population during this time can affect adult fertility (Orth et al., 1988). Additionally, DSP is highly positively correlated with Sertoli cell number, accounting for 85-94% of the variation of DSP (Berndtson and Thompson, 1990). The increase in Sertoli cell number in PTU-treated rats led to secondary increases in germ cells, Leydig cells, and other testicular cells which resulted in an overall increase of testis size and DSP in adulthood (Hardy et al., 1993; Hess et al., 1993). During the period of treatment, testis weight in PTU-treated rats was actually 77% lower than controls at day 30, which was due to germ cell degeneration likely because Sertoli cells were not mature and did not support proper germ cell growth (van Haaster et al., 1992; Simorangkir et al., 1997).

The effects of PTU-treatment on Sertoli cell proliferation was due to decreased thyroid hormones which are normally produced in the thyroid gland are partially responsible for regulation of metabolism. The thyroid hormone triiodothyronine (T_3) is of particular importance in testis development because it has receptors in the testes during gestation and the perinatal

period. Jannini et al. (1990) showed that T_3 receptors were in highest concentration on Sertoli cells in the testis and decreased starting at day 19 of gestation, becoming almost undectable in adult rats. Treatment with PTU only increased testis size and DSP when administered in early postnatal life, showing that thyroid hormones effected testicular development only when Sertoli cells were still proliferating (Cooke et al., 1994a). Triiodothyronine normally induces a transition of Sertoli cells from the mitotic to non-mitotic state, so hypothyroidism led to delayed Sertoli cell differentiation and extended the period of mitogenesis, resulting in increased Sertoli cell numbers in PTU-treated rats (Cooke et al., 1994b). Treatment of rats with T_3 to generate a hyperthyroidic condition during the postnatal period agreed with these results because it shortened the period of Sertoli cell proliferation and resulted in decreased Sertoli cell numbers and testicular weight by 50% and 48%, respectively (van Haaster et al., 1993).

Sertoli cell and round spermatid numbers increased by 157% and 93%, respectively in PTU-treated rats, decreasing the round spermatid to Sertoli cell ratio by 30%, possibly due to decreased nucleolus size of Sertoli cells (Hess et al., 1993). There was no change in the serum testosterone (T) levels between control and PTU-treated rats; however, there was an increased Leydig cell number of 69% at day 180 in PTU-treated rats (Cooke and Meisami, 1991; Hardy et al., 1993). Leydig cell volumes of PTU-treated rats were 20% less than controls and PTU-treatment was shown to directly decrease T production per cell by 73% (Hardy et al., 1993). However, despite changes to various cells regarded as important to spermatogenesis, the motility, concentrations, and fertility of sperm taken from PTU-treated rats was normal (Cooke et al., 1991).

Due to the large increase in sperm production in rodents, the question of whether this treatment can be used in production animals to increase testes size and sperm production arises.

Production animals are valuable because of their ability to provide a product that is of use to humans such as milk, meat, or fiber. Increased genetic potential to efficiently produce these products could be obtained if sperm production were increased in males with desirable traits. This is especially true with today's extensive use of artificial insemination which extends the use of a single ejaculate to breed large numbers of females. Thus, genetic advancement of livestock species could proceed at an even greater rate if DSP were increased.

Previous studies increasing the testis size in rams involved hemicastration. Rams hemicastrated at 4 months of age showed increased testicular weight of 48% by 7 months of age, but their DSP/gram of testis was not affected (Brown et al., 1987). Additionally, Waites et al. (1983) reported that lambs hemicastrated within 1 week of birth showed compensatory growth in the remaining testis of 67% and 114% of controls at 8 and 12 weeks, respectively. Hyperplasia of Sertoli cells, at least in the neonatal hemicastrate, is believed to be the source of this compensatory growth because there were increased Sertoli cells per testis in hemicastrates compared to controls. The increase in Sertoli cell number was likely a result of the proliferative capabilities of Sertoli cells before puberty (de Reviers et al, 1980). However, Orth et al. (1988) demonstrated that once Sertoli cells had matured and stopped dividing, their capability to return to a proliferative state was lost. Hemicastration did not extend the proliferative period for the Sertoli cells, instead it increased the rate of proliferation (Bardin et al., 1994). Orth et al. (1984) demonstrated that rat hemicastrates had increased uptake of thymidine (used as an indicator of cell division) by Sertoli cells for 4 days after surgery with a concomitant 2-fold increase in serum follicle stimulating hormone (FSH) at day 4. However, hemicastrates administered testosterone (T) during the 4 days following surgery had no compensatory growth, so an increased concentration of T could reduce the rate of Sertoli cell proliferation. The increase in testis size in

the rats was not a practical means by which to increase sperm production because these animals have smaller total testicular volume and no increased DSP per gram testis. Increasing the rate of Sertoli cell proliferation at the expense of total testicular parenchyma did not improve the overall DSP. Testicular size is a reliable indicator of sperm production, so increasing size is likely to have a positive correlation with sperm production (Senger, 2003). Therefore, it would be of interest to increase DSP by increasing testis size in the ram. Genetic improvements in production species could be made at an even faster rate if sperm production in superior males could be increased. For this reason our objective was to determine if the results obtained in the rat studies from neonatal administration of PTU could be translated to sheep. In order to test this we treated neonatal cross-bred rams with PTU and examined the effects on testicular size and DSP in adulthood.

Monet-Kuntz et al. (1984) reported that the period of Sertoli cell differentiation in Romanov-Ile-de-France crossbred rams lasted until 40 days of age; with Sertoli cell numbers doubling between 25 and 40 days of age. After this period of proliferation, Sertoli cells continued to increase in size 3-fold until 100 days of age, but did not proliferate during this period. In addition, the receptors for FSH and androgens in the testis were also increased by 10 and 12-fold, respectively (Barenton et al., 1983; Monet-Kuntz et al., 1984). In previous studies involving PTU administration to sheep various doses were used to induce a hypothyroid response. However, studies using PTU to induced hypothyroidism in the ram have not been reported during the period of Sertoli cell proliferation. Wells et al. (2003) treated 6 month old Rambouillet ewes with orally administered gelatin capsules containing 20 mg PTU/kg BW and 40 mg PTU/kg BW and saw similar results for both. Thyroxin (T_4) levels were below 1 ng/mL after 2 weeks of treatment and T_3 levels fell below 1 ng/mL after 1 week of treatment. These

levels were then maintained throughout treatment, even following a 50% decrease in treatment dose. Another study by Achmadi and Terashima (1995) found that an oral dose of 4 mg PTU/kg BW had no affect on thyroid hormone concentrations in 30 month old Suffolk ewes, but 8 mg PTU/kg BW was sufficient to reduce both T_4 and T_3 concentrations by about 35% in treated animals. Fallah-Rad et al. (2001) used doses of 15 mg PTU/kg BW, administered by oral drench twice daily, to suppress thyroid function in 6-12 week old Suffolk rams.

Considering that PTU-treatment was able to induce a hypothyroid state in rams in previous studies we attempted to mimic the effects of PTU-treatment demonstrated in rats in our livestock model, rams. We hypothesized that an increasing dose from 5 mg PTU/kg BW to 30 mg PTU/kg BW between 10 and 65 days of age, when the Sertoli cells were known to be proliferating, would induce a hypothyroid state that would prolong the Sertoli cell proliferation period and increase sperm production in adult rams. If this response was achieved further studies in more economically important production animals would be warranted.

Materials and Methods

Animals

All animal use and experimental procedures were approved by the institutional animal care and use committee (IACUC). Eight Suffolk/Hampshire/Dorset crossbred lambs, born between March and April, were housed with natural lighting and ambient temperature. Lambs were allotted randomly to 2 groups and administered either PTU or a control treatment. Lambs were housed in the same pen and left with their mothers until weaning at about 70 days of age. Rams had *ad libitum* access to a ration of chopped hay and fresh water. One of the PTU-treated animals was removed from the study at about 4 months of age because of an inguinal hernia that could not be surgically repaired. Two other rams, one PTU-treated and one control, each

underwent surgical repair of inguinal hernias during the study at about 6 months of age. Inguinal hernias in rams are believed to be inherited as a recessive characteristic (Roberts, 1988).

Treatment

PTU-treatment began at 10 days of age and was administered once daily at 1500 h. PTU (Sigma-Aldrich; St. Louis, MO) was dissolved in 70% alcohol for the first 6 days and dissolved in water for the next 4 days and administered via esophageal tube. PTU was easily dissolved in alcohol, but treatment was changed when a method of stirring was able to dissolve PTU in water. Controls received equivalent administration of alcohol during the first 6 days of treatment, but were given no further treatment. Gelatin capsules (Torpac; Fairfield, NJ) were used to administer the PTU from 20-65 days of age. PTU-treated animals received 5 mg PTU/kg BW from 10-20 days of age, 10 mg/kg BW from 20-30 days of age, 20 mg/kg BW from 30-40 days of age, and 30 mg/kg BW from 40-65 days of age.

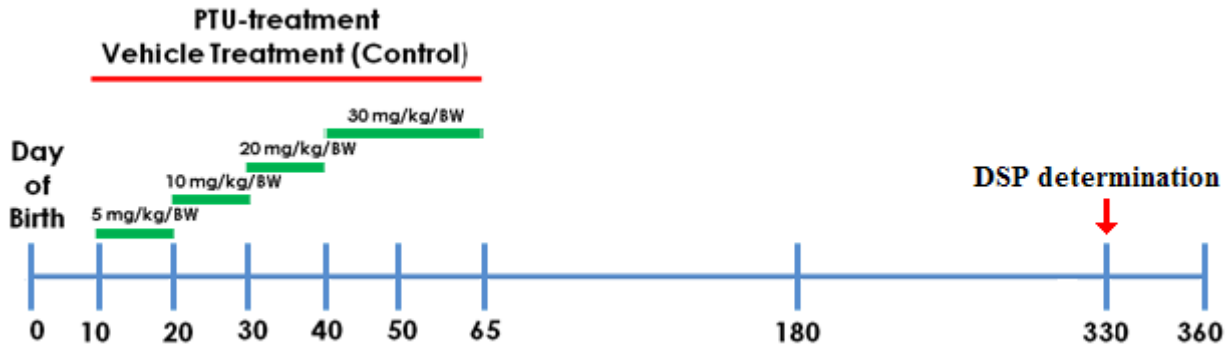

Figure 1 Timeline of PTU-treatment and DSP determination. Rams were given 5 mg PTU/kg BW from 10-20 days of age, 10 mg/kg BW from 20-30 days of age, 20 mg/kg BW from 30-40 days of age, and 30 mg/kg BW from 40-65 days of age. DSP was determined at approximately 330 days of age.

Growth measurements

Body weights were measured daily during treatment and SC (the distance around the testicles, contained within the scrotum, at the widest point) measurements were taken every 10 days with scrotal tape while lambs were restrained in a sitting position. Following treatment, body weights and SC were taken once weekly until rams were 6.5 months of age. At this time BW measurements were taken every two weeks and testicular diameter was measured once a month with a Pie Medical Ultrasound using a linear, 18 cm, 3.5 MHz transducer, as per the technique described by Cartee et al. (1990). Testicular diameter provided the size of the testes, excluding the scrotal tissue, however, this data was not converted to a testicular weight.

Blood sampling and triiodothyronine assays

Blood was collected from the jugular vein at 20, 30, 40, 50, 80, and 150 days of age. Additional blood collections occurred at approximately 6.5, 8 and 12 months of age. Samples were stored up to 16 hours at 4ºC to allow clotting. Samples were then transferred to 15 mL conical tubes and centrifuged for 20 minutes at 3000x g. Serum was collected in 5 mL tubes and stored at -20ºC until assayed for T_3. A T_3 enzyme-linked immunosorbent assay (ELISA) (Calbiotech; Spring Valley, CA) was conducted on the serum samples to determine blood concentrations of T_3 during PTU-treatment and the subsequent time of recovery to a euthyroid state.

Semen collection and determination of daily sperm production

Semen was collected by electro-ejaculation at 9 months of age. Semen was analyzed immediately for motility by the same experienced person through approximation of percent motility. Morphology was determined by counting 200 sperm on hematoxylin and eosin stained

smears of the semen. Concentration of semen was determined by using a dilution of 1:6000 in water and counting using a hemocytometer.

The DSP was determined at 11 months of age using electo-ejaculation. Five electro-ejaculates were taken every other day. The first two were used to clear epididymal reserves and the last three were counted for sperm concentration and averaged to estimate DSP. Semen volume was not used to assess DSP because of the variation in semen output produced by the electro-ejaculation collection technique.

Testicular histological analysis

Rams were sacrificed at approximately 1 year of age and the testicles were removed. Testes were weighed and random testicular parenchymal samples from each testis were taken and fixed in Bouin's solution for 4 h at 4ºC, followed by dehydration in ethanol and xylene, and embedded in paraffin. Histological sections were prepared using a microtome by cutting 5 µm thick cross-sections which were affixed to glass slides and deparaffinized. Staining for Sertoli cells was done with a primary antibody made in goat to GATA 4, and a secondary antibody of horse anti-goat IgG. Non-specific binding was blocked by incubation with horse serum. Reagents from Vector laboratories ABC kit (Vector laboratories; Burlingame, CA) were used and counter-staining was done with hematoxylin. Sertoli cells were counted per seminiferous tubule at 20X magnification, counting the number in 10 round seminiferous tubule cross-sections per animal. Digital images were captured with an Olympus DP71 microscope camera (Olympus America Inc.; Center Valley, PA). Each ram also had 10 round seminiferous tubules measured for diameter using the DP Controller software.

Statistical analyses

Growth measurements, semen analysis, and histological data were analyzed using one-tailed Student's T-tests. Effects with probability values (p values) of 0.05 or lower were considered significant. All data are shown as the mean ± SEM. For measurements taken over time data were analyzed in SPSS v.16 software (SPSS, Chicago, IL) using a repeated measures analysis of variance. Statistical Analysis Software v.9 (SAS Institute; Cary, NC) was used with mixed model analysis to determine the effects of age on BW and SC throughout the study.

Results

Effects of propylthiouracil-treatment on triiodothyronine concentrations

Analysis of T_3 concentrations during the study revealed a difference ($p < 0.01$) between PTU-treated and control rams during PTU administration. Administration of 5 mg PTU/kg BW did not completely remove thyroid function; PTU-treated rams had serum T_3 concentrations of 3.9 ± 0.20 ng/mL while controls had concentrations of 5.06 ± 0.08 ng/mL. Treatment with 10 mg PTU/kg BW induced a hypothyroid state with serum T_3 concentrations of 0.89 ± 0.50 ng/mL, while controls had T_3 serum concentrations of 4.57 ± 0.24 ng/mL following 10 days of PTU administration at this dosage. Serum concentrations of T_3 remained below 1.0 ng/mL from 30-65 days of age, but by 15 days after PTU administration PTU-treated rams returned to a euthyroid state ($p > 0.1$).

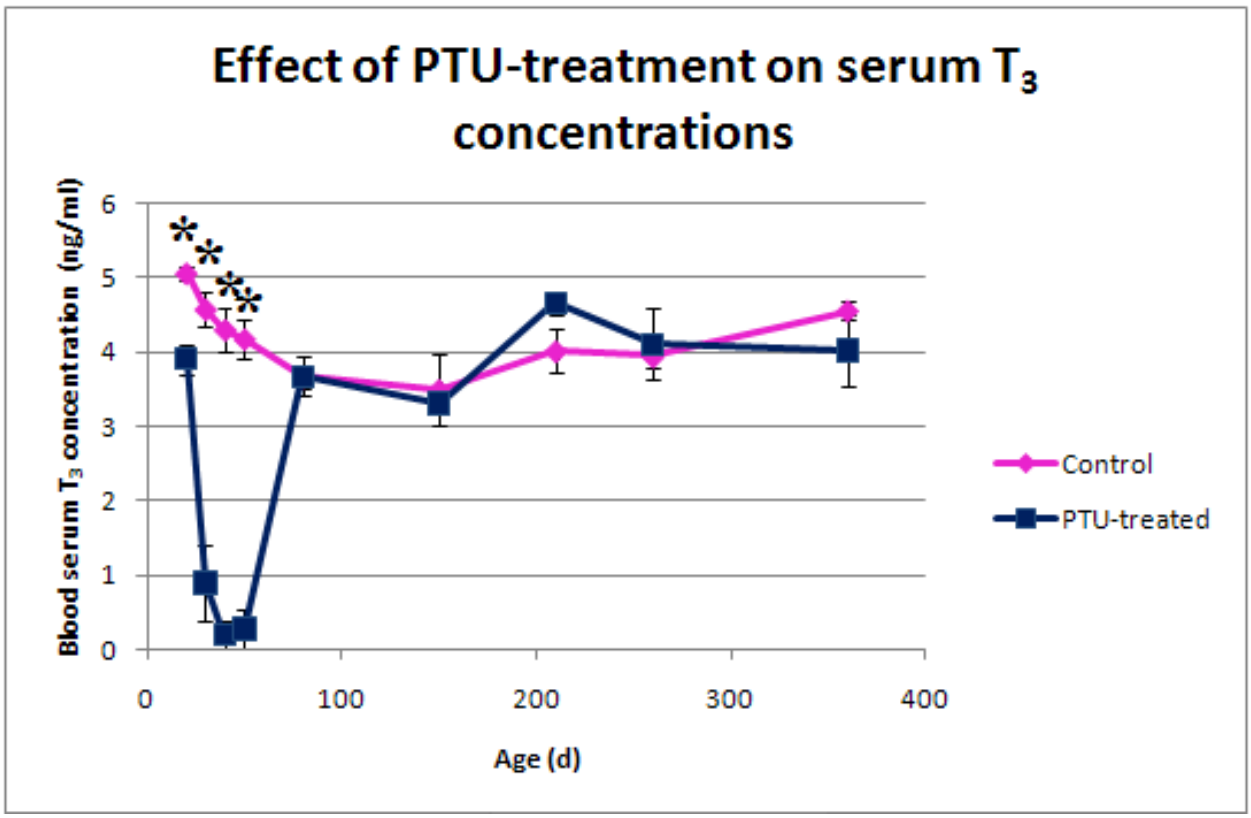

Figure 2 The effect of PTU-treatment on serum concentrations of T_3. PTU administration from 10-20 days of age reduced thyroid function, but did not induce a hypothyroid state (average T_3 serum concentrations of 3.9 ± 0.20 ng/mL in PTU-treated after 10 days of 5 mg PTU/kg BW). However, further administration from 30-65 days of age caused hypothyroidism with T_3 blood serum concentrations below 1.0 ng/mL in PTU-treated rams ($p < 0.05$). Within 15 days following PTU-treatment PTU-treated rams returned to a euthyroid state ($p > 0.1$). (* denotes significant).

Effects of propylthiouracil-treatment on growth during pre-pubertal development

BW in PTU-treated animals was decreased ($p < 0.05$) during the pre-pubertal period. At 150 days of age, PTU-treated lambs weighed an average of 59.5 ± 1.28 kg while controls weighed an average of 70.68 ± 1.8 kg. The difference was most pronounced during treatment as body weights of PTU-treated animals were lighter beginning at 30 days of age. BW of PTU-treated lambs began to parallel control growth immediately following the end of PTU administration.

Effects of propylthiouracil-treatment on body weight following puberty

At puberty (240 days of age) the BW of PTU-treated animals remained smaller than controls ($p < 0.01$). PTU-treated animals had an average BW of 79.53 ± 0.84 kg while controls averaged 88.8 ± 2.49 kg. However, at 330 days of age there was no difference ($p > 0.1$) in BW between PTU-treated and controls; PTU-treated rams averaged 106.13 ± 1.62 kg and controls averaged 112.7 ± 4.46 kg.

Effects of propylthiouracil-treatment on scrotal circumference during the pre-pubertal period

SC was not different ($p > 0.05$) between PTU-treated and control rams at any time throughout the study period. The variation in SC for the controls was high and likely impacted our ability to detect treatment effects during the study. At 150 days of age the PTU-treated rams had an average SC of 323.67 ± 6.89 mm while controls averaged 363.25 ± 16.14 mm. The difference between PTU-treated and control rams which began at 60 days of age was not significant.

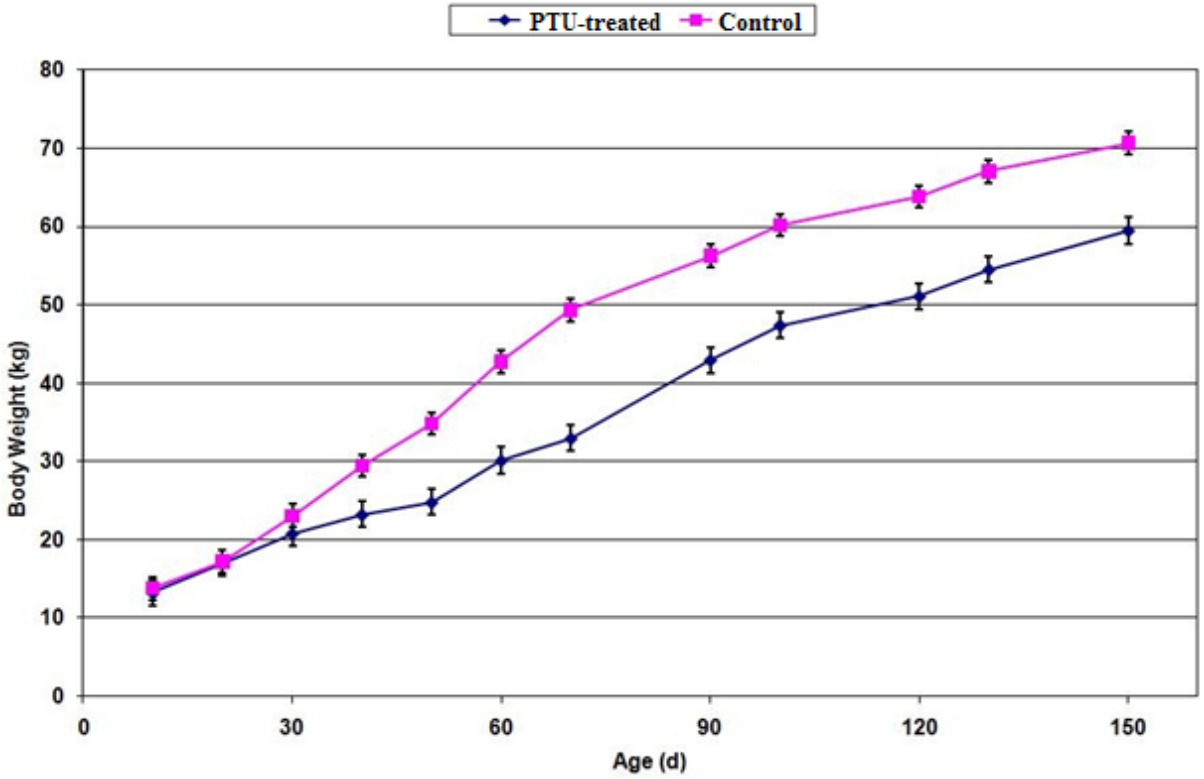

Figure 3 Effect of PTU treatment on BW during prepubertal development (10 to 150 days of age). BW was significantly ($p < 0.05$) reduced in treated animals beginning at 30 days of age and remained below controls during the entire pre-pubertal period. Once treatment was stopped compensatory growth occurred from 65-90 days. Then growth paralleled controls to 150 days of age.

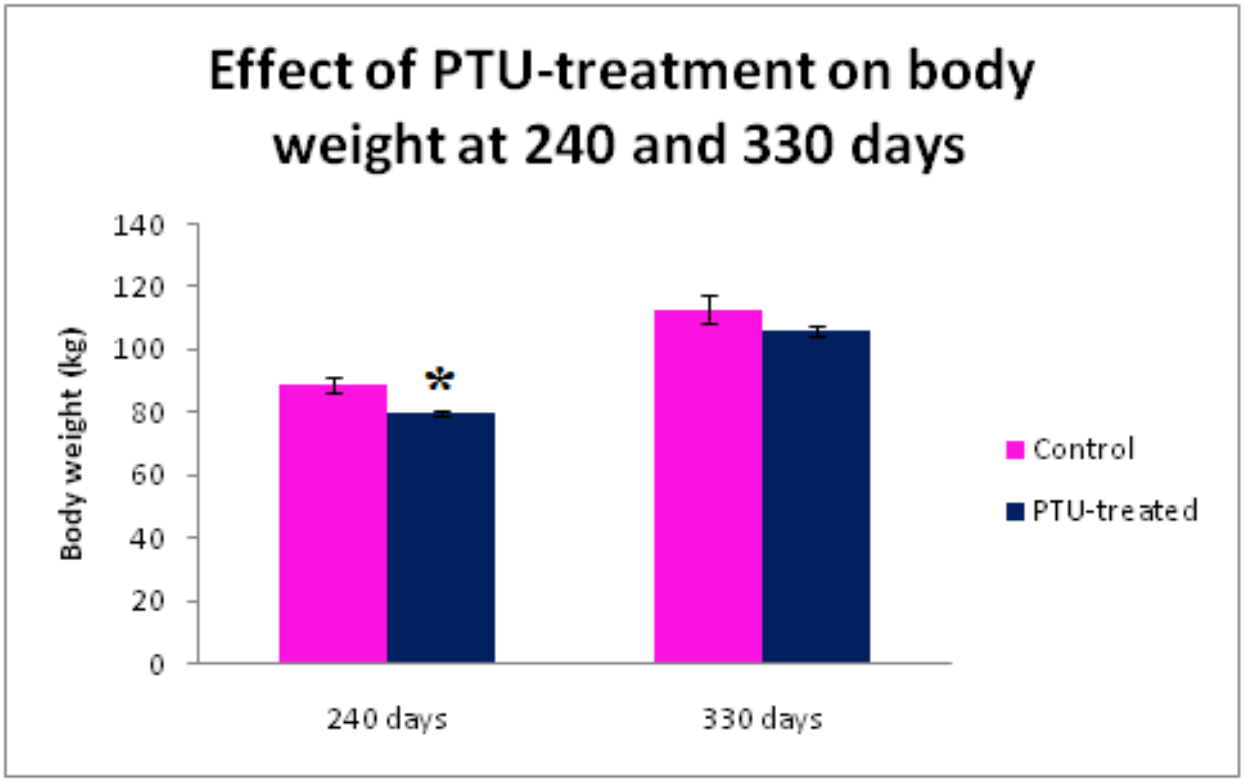

Figure 4 The effects of PTU-treatment on BW at puberty (240 days of age) and 330 days of age. The average BW of PTU-treated rams was significantly ($p < 0.05$) less than controls at puberty, but there was no significant ($p > 0.05$) difference in BW at 330 days of age. (* denotes significant).

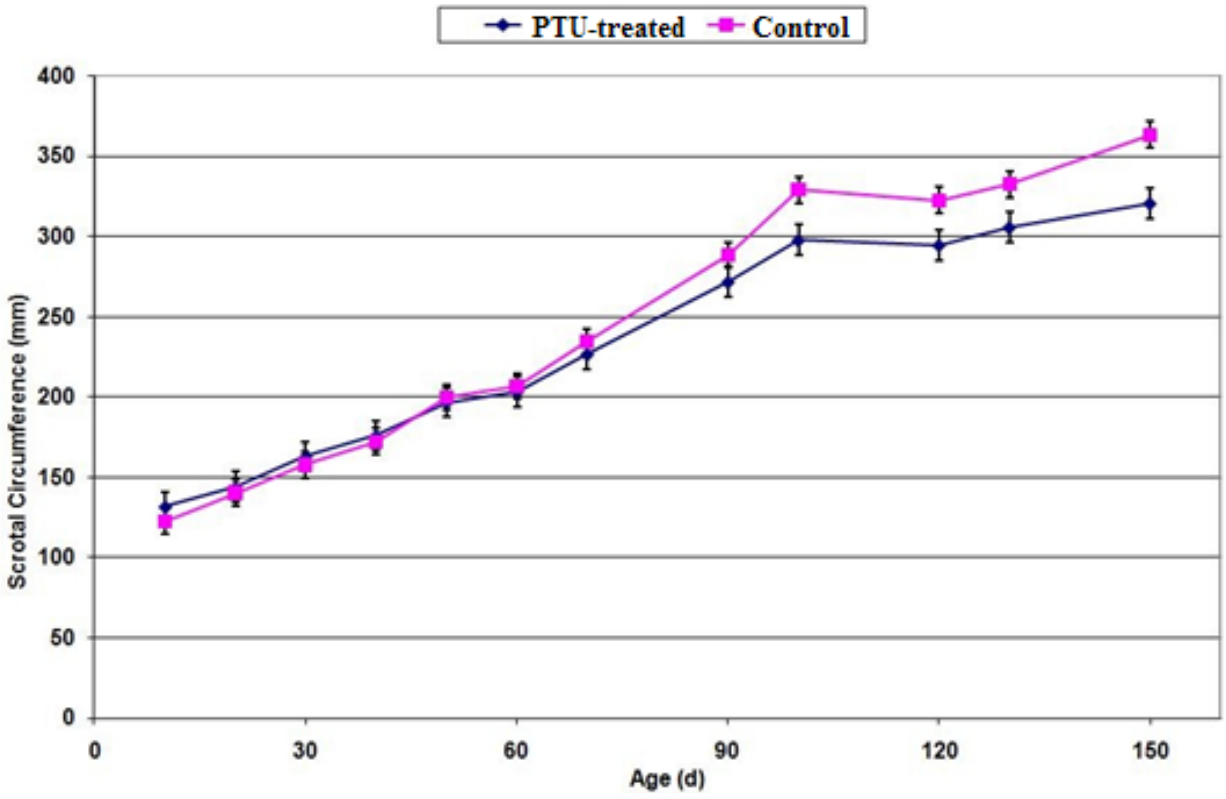

Figure 5 The effects of PTU treatment on SC during pre-pubertal growth (10 to 150 days of age). SC was not different ($p > 0.05$) between PTU-treated and control rams during the pre-pubertal period.

Effects of propylthiouracil-treatment on testicular diameter following puberty

Measurements taken after puberty were taken using ultrasound causing an apparent decrease in testis size between 150 and 240 days of age, which was likely due to the different measurement techniques utilized. At puberty (240 days of age) there was no difference ($p > 0.1$) in testis diameter between PTU-treated rams which had an average testis diameter of 290 ± 13.58 mm, and controls which averaged 304.25 ± 5.27 mm. There was no difference ($p > 0.1$) in testis diameter at 330 days of age either. At 330 days PTU-treated rams had an average testicular diameter of 397 ± 5.57 mm and controls averaged 382 ± 33.3 mm.

Effects of propylthiouracil-treatment on the scrotal circumference:body weight ratio

The effects of treatment on the SC:BW ratio was significant ($p < 0.01$). Using a SAS mixed model we determined that, during PTU-treatment and most of the prepubertal period, PTU-treated animals maintained a higher SC:BW ratio. However, beginning at 100 days, PTU-treated and control rams SC:BW ratio was not different ($p > 0.05$). At the end of PTU-treatment (65 days) SC:BW averaged 6.8 ± 0.44 mm/kg for PTU-treated and 4.85 ± 0.14 mm/kg for controls. However, SC:BW at 150 days of age averaged 5.4 ± 0.26 mm/kg for PTU-treated and 5.13 ± 0.2 mm/kg for controls.

Effects of propylthiouracil-treatment on morphology of the seminiferous epithelium

Diameter of seminiferous tubules was increased ($p < 0.001$) in PTU-treated rams versus controls, with an average size of 209.43 ± 4.11 μm while the diameter in control's was 181.2 ± 3.49 μm. The number of Sertoli cells per seminiferous tubule was not different ($p > 0.05$) between PTU-treated and control rams. The average Sertoli cell number per seminiferous tubule cross-section was calculated to be 3.5 ± 0.27 cells and 2.98 ± 0.22 cells in PTU-treated and control rams, respectively.

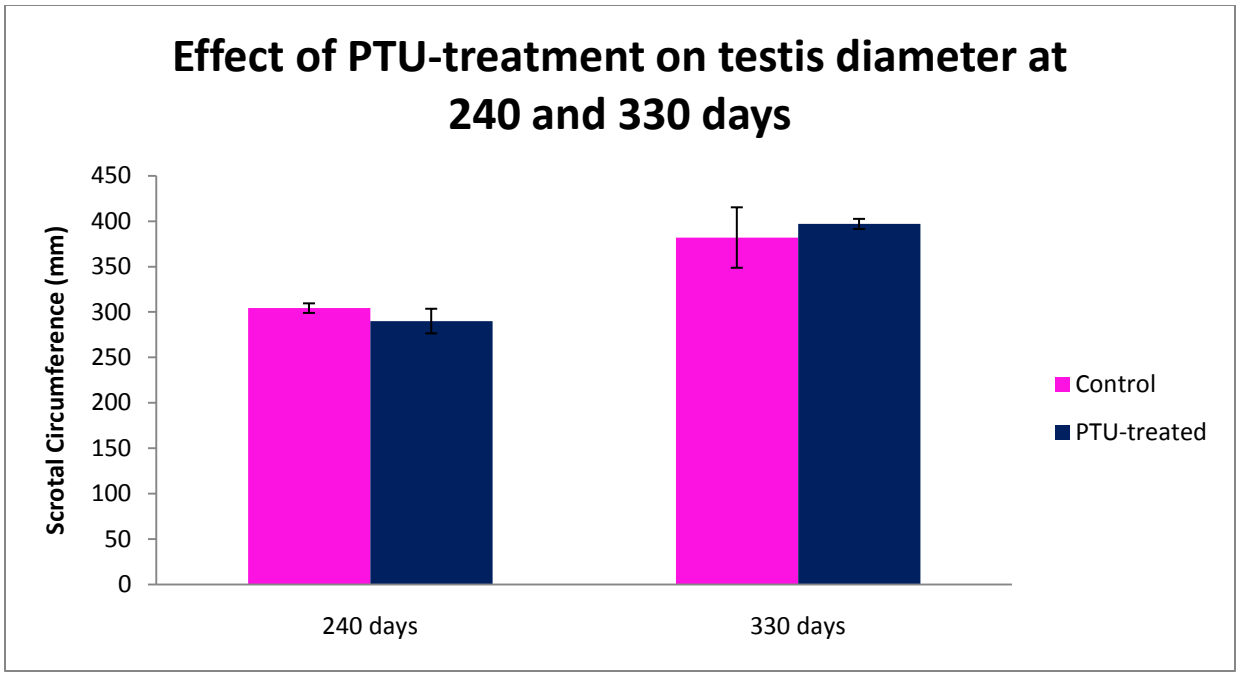

Figure 6 The effect of PTU-treatment on testis diameter at puberty (240 days) and 330 days of age. There was no difference ($p > 0.1$) in the testis diameter at puberty or 330 days of age.

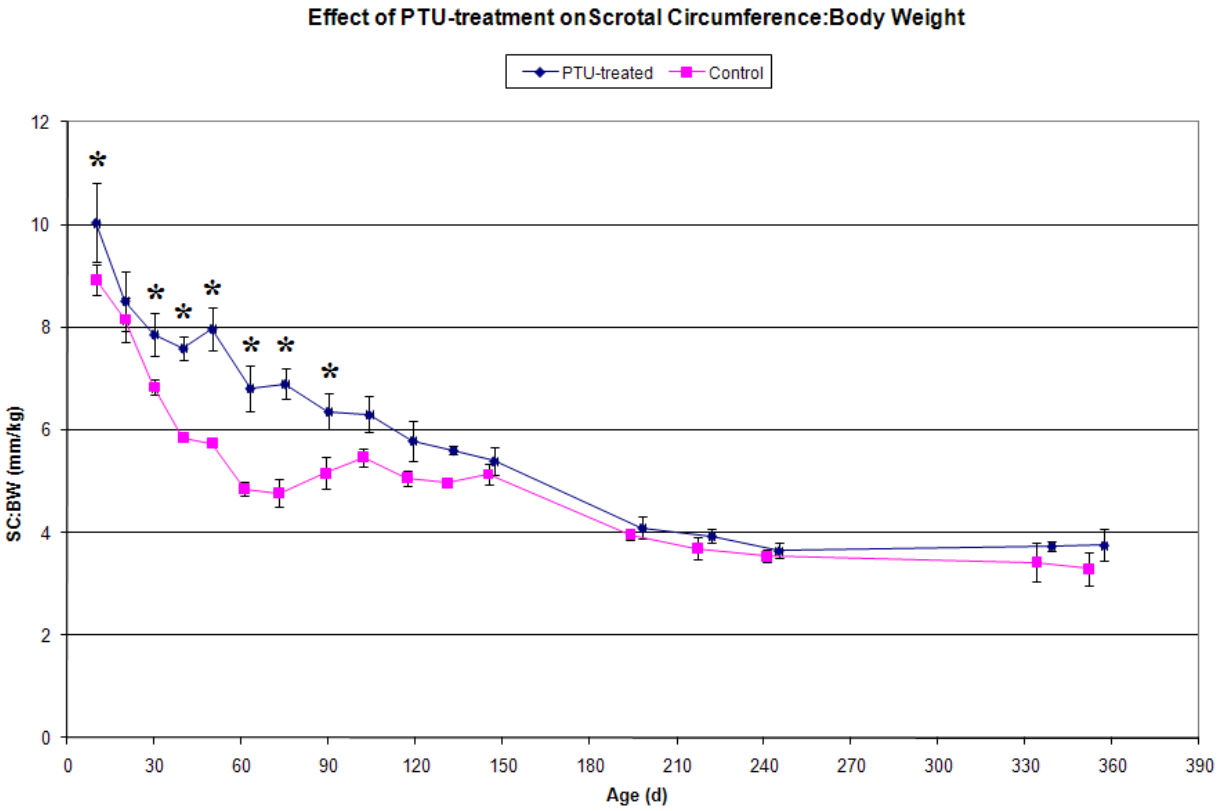

Figure 7 Ratio of SC:BW from the start of PTU-treatment until 360 days. There was an increase ($p < 0.01$) in the SC:BW ratio in PTU-treated lambs during treatment, starting at 30 days of age and lasting until 90 days of age (*denotes significantly different). However, no difference ($p > 0.1$) was found from 100-360 days of age.

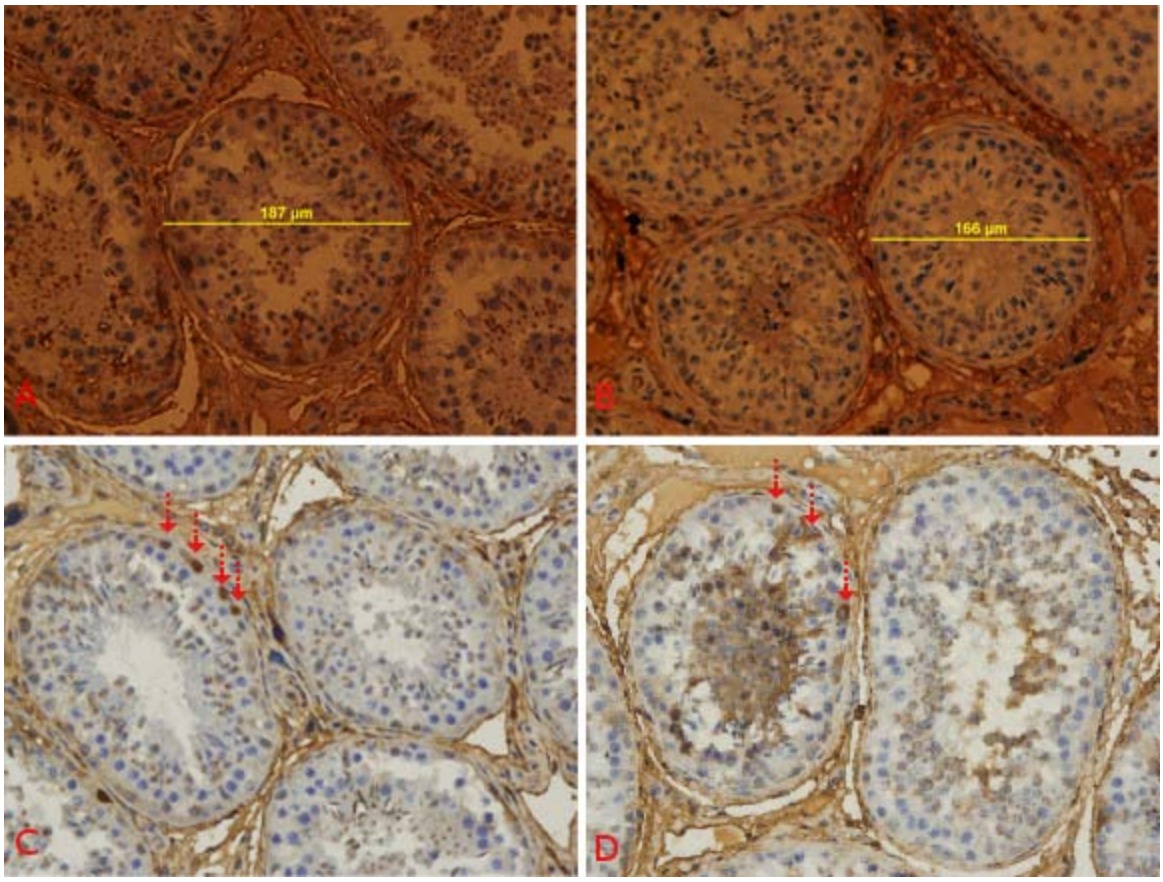

Figure 8 Photomicrographs of measurements taken of seminiferous tubules diameters and Sertoli cell counts/seminiferous tubule cross-section captured with an Olympus DP71 microscope camera. A) Measurement of the diameter of a seminiferous tubule in a PTU-treated ram. B) Measurement of the seminiferous tubule diameter in a control ram. C) Red arrows point to the Sertoli cells counted in the seminiferous tubule of a PTU-treated animal. D) Red arrows point to the Sertoli cells counted in the seminiferous tubule of a control animal.

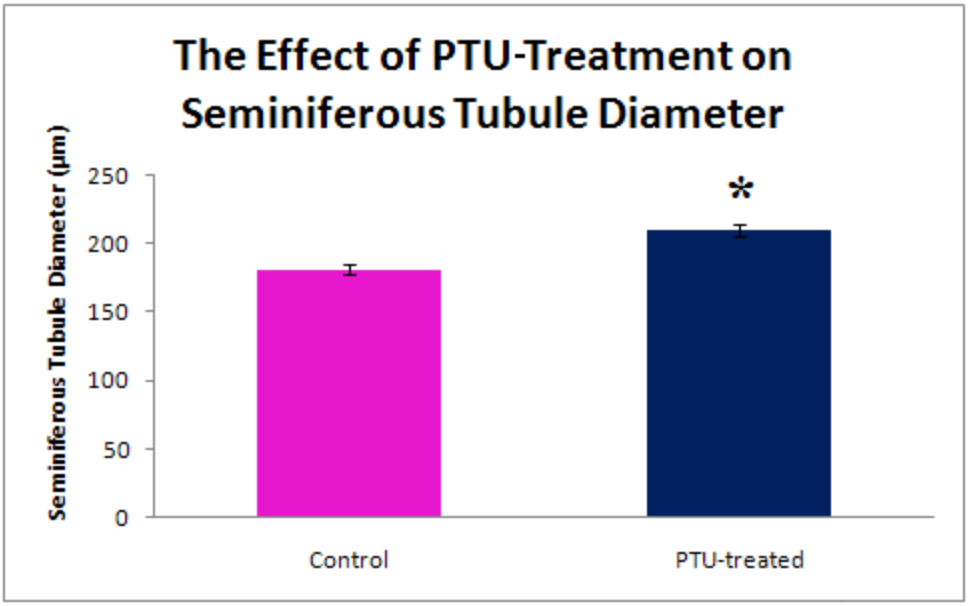

Figure 9 Effect of PTU-treatment on seminiferous tubule diameter. PTU-treated rams had larger ($p < 0.001$) seminiferous tubule diameters than controls (*denotes significantly different).

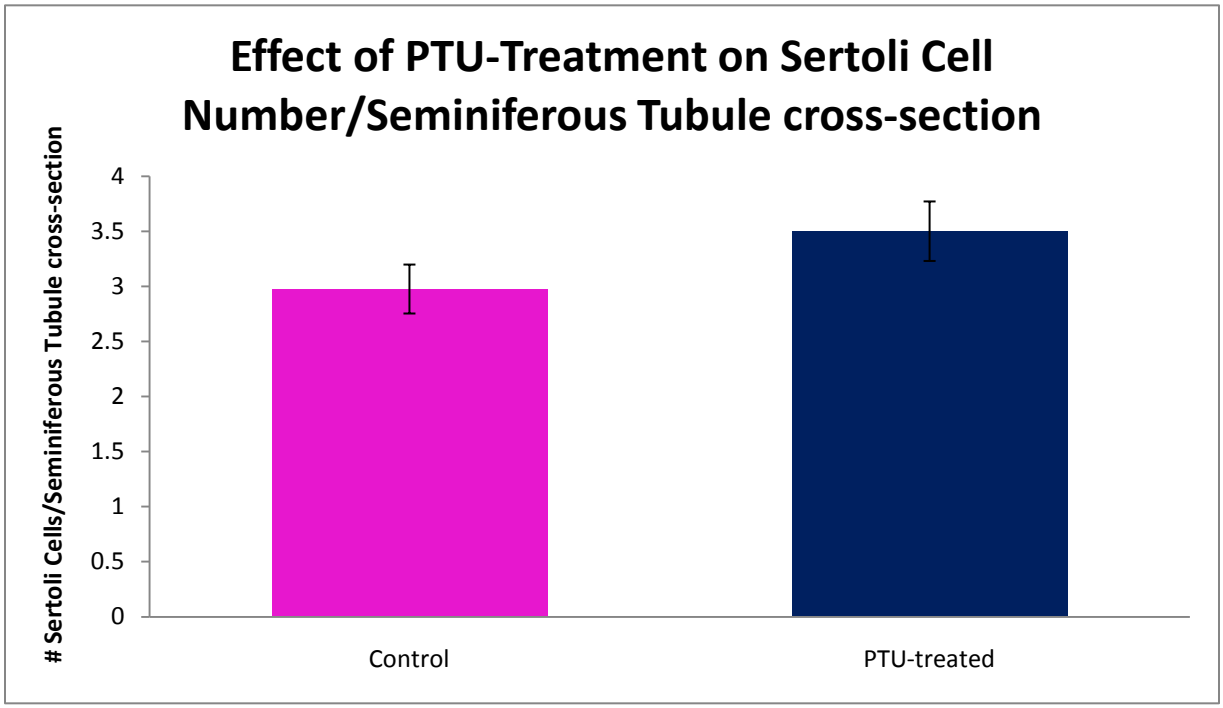

Figure 10 Effect of PTU treatment on Sertoli cell number per seminiferous tubule cross-section. No difference ($p > 0.05$) in the number of Sertoli cell cells per seminiferous tubule cross-section between PTU-treated and control rams was found.

Effects of propylthiouracil-treatment on semen quality

From the electro-ejaculation conducted at 9 months there was no difference ($p > 0.1$) in sperm motility between PTU-treated and control animals. Sperm from PTU-treated rams had an average motility of 26.67 ± 8.82 % while controls had an average motility of 17.50 ± 2.50 %. No difference ($p > 0.1$) in sperm morphology between PTU-treated and control rams was found. PTU-treated rams averaged 77.33 ± 10.58 % normal sperm and controls averaged 82 ± 3.97 % normal sperm. At 9 months, sperm concentration was not different ($p > 0.1$) between treatments. The average sperm count for PTU-treated rams was 5.80 X 10^9 ± 2.03 spermatozoa mL^{-1} while controls averaged 3.48 X 10^9 ± 1.05 spermatozoa mL^{-1}.

Effects of propylthiouracil-treatment on daily sperm production

There was a difference ($p < 0.01$) in average sperm concentration per mL semen in PTU-treated and control rams at 11 months of age. PTU-treated rams had a reduction in sperm production compared to controls, although the standard error was high for the PTU-treated group. PTU-treated rams had an average sperm concentration of 7.10 X 10^9 ± 2.84 spermatozoa mL^{-1} while controls averaged of 1.12 X10^{10} ± 0.58 spermatozoa mL^{-1}. DSP was recorded as a measure of ejaculate concentration and not on a total volume level because semen was collected by electro-ejaculation which does not give a good representation of true ejaculate volumes and consequently underestimates DSP.

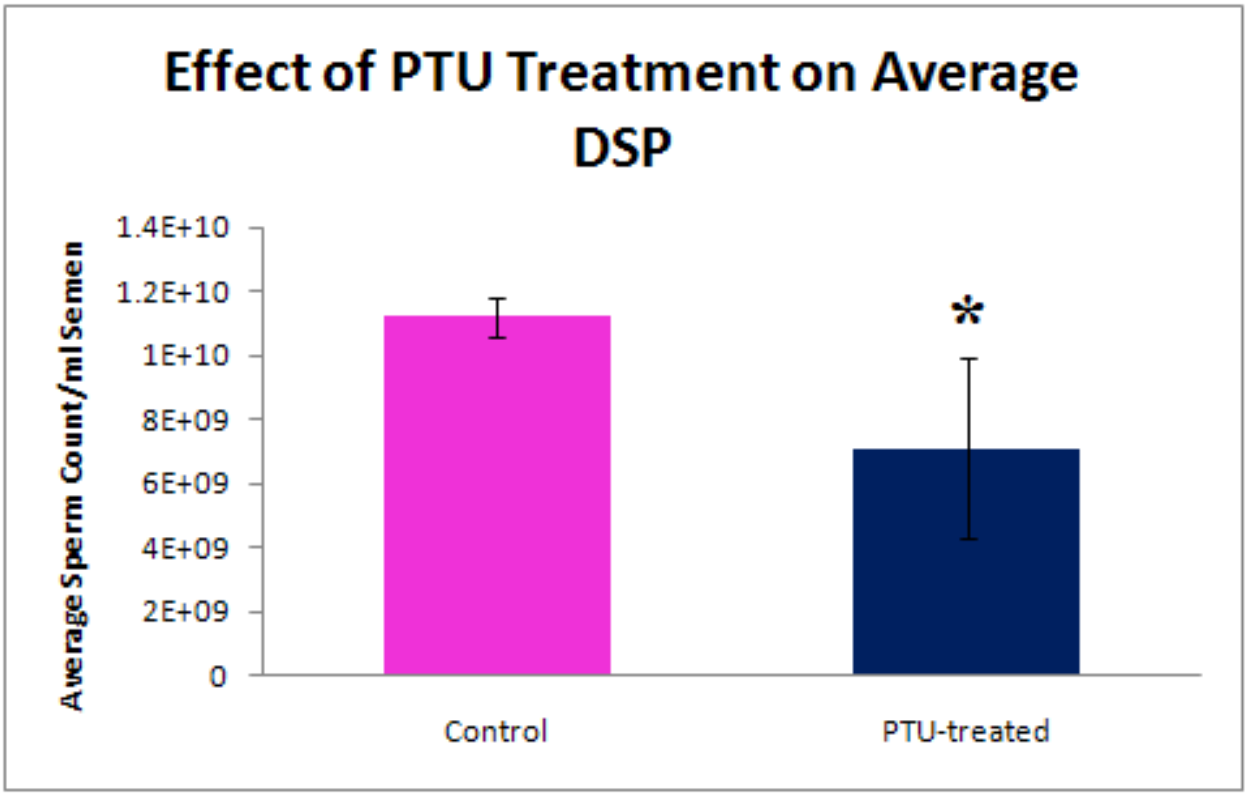

Figure 11 Sperm concentration from repeated electro-ejaculation at 11 months of age. There was decreased ($p < 0.01$) sperm concentration for PTU-treated compared to control rams (*denotes significantly different).

Discussion

Neonatal PTU-induced hypothyroidism in the ram decreased DSP in adult rams. Essential to the evaluation of the PTU-induced hypothyroid state was its ability to decrease T_3 blood serum concentrations. Although administration of 5 mg PTU/kg BW was unable to produce significant hypothyroidism, the 10 mg PTU/kg BW did induce a hypothyroid state during the period of Sertoli cell proliferation in the ram lamb. The establishment and maintenance of this state between 30-65 days of age should have prevented thyroid hormones from exerting their effects on the Sertoli cells during most of their proliferative period. Thus, evaluation of the DSP and testicular histology of these rams was a good representation of what effects neonatal hypothyroidism causes in the ram. However, it is still possible that the critical period to begin PTU-treatment in the ram occurs before the hypothyroid state was established in our study, which would affect results.

Assuming that Sertoli cells in the ram are not more sensitive to thyroid hormones during a different time period, the effects of neonatal PTU-treatment in the rat and the ram are different. The question remains whether we have missed the period of time in which the same effects can be achieved, or if there is a fundamental difference in Sertoli cell development which would not allow for a prolonged period of proliferation in the ram (Cooke et al., 2004). As was seen in the rat and mouse models, there was marked reduction in BW of rams during PTU-treatment and rapid compensatory growth following treatment, but testes size leveled off at similar levels to controls and sperm production in PTU-treated animals was decreased. A factor in the different response seen between rats and rams may be the period of time in which the Sertoli cells proliferate. In the ram, this period of proliferation is maximal between postnatal days 20 and 40 (Monet-Kuntz et al., 1984). In rats the proliferation period of Sertoli cells has been clearly

identified as beginning at day 16 of gestation, reaching a maximum at 20 days of gestation, and slowly declining after birth until proliferation stops at day 21 (Orth, 1982). Our results were similar to results achieved from treatment of neonatal boars with PTU. Klobucar et al. (2003) demonstrated that hypothyroidism produced no lasting effects on testis size or sperm production in boars, although there was a slight decrease in testis size before puberty. They also showed that Sertoli cells expressed proliferating cell markers beyond the typical proliferation period. This suggested that the decrease in thyroid hormones may have allowed Sertoli cells to proliferate for a longer period of time, but still did not explain why the increased number of Sertoli cells in PTU-treated rodents did not transfer into larger animal species. Hypothyroidism in livestock species may have resulted in prolonged Sertoli cell proliferation, however, because there was not a permanent increase in Sertoli cell numbers they would have to have undergone increased rates of necrosis or apoptosis. This could result from failure of Sertoli cells to mature due to lack of a growth factor which was no longer present at the later period of differentiation. This was difficult to assess without testis samples from animals during the proliferative period.

On the other hand, our experiment may not have extended the period of Sertoli cell development. Because the study utilized only 7 animals we could not sacrifice the animals at different stages of development in order to see how long the Sertoli cell proliferation lasted. In future studies it would be helpful to measure the duration of proliferation after neonatal PTU-treatment. This would allow for an understanding of how the proliferating Sertoli cells respond to lack of thyroid hormones and might reveal differences in localized growth factors that were present during the normal proliferation period, but lacking during prolonged proliferation or vice versa. Gondos and Berndtson (1993) suggested that factors important to proliferation may include testosterone (T), follicle stimulating hormone (FSH), Müllerian inhibiting hormone,

insulin-like growth factors, transforming growth factor-beta, fibroblast growth factor, nerve growth factor, interleukin-1, inhibin and activin. Sharpe et al. (2003) suggested that receptor numbers may be a major factor in the proliferative abilities of the Sertoli cell. It may be that despite prolonged proliferative ability, the Sertoli cells lack the normal number of receptors for factors that are needed for mitosis. Since T_3 interacts with androgens and possibly FSH to cause maturation of the Sertoli cell through increased androgen receptor expression, an increase in the sensitivity of the Sertoli cell to T_3 could greatly impair the rate of proliferation and differentiation.

A fundamental component to assessing whether the experiment could actually have an effect on Sertoli cells is the presence of thyroid hormone receptors on the cells during the neonatal period. Fallah-Rad et al. (2001) suggest that there are T_3 and T_4 receptors on ram lamb Sertoli cells during the neonatal period. They found that inducing a hyperthyroid state in neonatal rams led to decreased age at puberty and hypertrophy of Sertoli cells. This was similar to the effect in rats. Cooke et al. (1994b) reported that T_3 directly inhibited mitogenesis and increased the rate of differentiation in cultured neonatal rat Sertoli cells, thereby decreasing the age at puberty. A major difference between hyperthyroidism in rats and rams was its effect on testis size; whereas rats which had undergone neonatal hyperthyroidism had decreased testicular size, rams had increased testicular size (van Haaster et al., 1993; Fallah-Rad et al., 2001). This suggested a fundamental difference in the way rat and ram Sertoli cells respond to thyroid hormones.

Although there was no effect of PTU treatment on rats *in utero*, this may be a time when some effect could be attained through administration of PTU to pregnant ewes (Cooke et al., 1992). Cooke et al. (1992) demonstrated that the age of rats at the start of PTU administration

had a large effect on changes in testicular size and DSP. In rats the critical time to begin treatment was within the first week of birth. This was likely because, at birth the rat Sertoli cell is undifferentiated; it then undergoes structural maturation and changes in receptor expression during early postnatal life (Gondos and Berndtson, 1993). Coincident with Sertoli cell differentiation is the establishment of the hypothalmic-pituitary-thyroid axis which begins thyroid function (Jannini et al., 1990). In the ram the Sertoli cell development is maximal in postnatal life, but thyroid activity begins *in utero* (Gondos and Berndtson, 1993). If the release of thyroid hormones in prenatal life was inhibited in sheep there may be some impact on Sertoli cell proliferation that cannot be seen with postnatal treatment. PTU-treatment of rats blocked the effect of thyroid hormones entirely because it was begun coincident with onset of thyroid function, but ram Sertoli cells in our study were exposed to some thyroid hormones before treatment was begun. Since the treatment period in rats was so time-sensitive, there is still a possibility that we have not yet discovered the critical period for PTU-treatment of rams. Chandrasekhar et al. (1985) found similar results as those in the current study when they studied a methyl thiouracil-induced hypothyroid state in 16 week old rams. They found it had no effects on pubertal maturation though it caused decreased T and steroidogenic function. However, the euthyroid state was not necessary for the reproductive endocrine axis to function normally. Since this study was done after the period of Sertoli cell proliferation in the ram, these results would be expected because the effect of hypothyroidism in rats was on Sertoli cells undergoing mitosis (Cooke et al., 1994a). Therefore, although it is known that disruption of the hypothalmic-pituitary-thyroid axis postnatally did not affect reproductive development, it has not yet been shown whether disturbance of the hypothalmic-pituitary-thyroid axis *in utero* would impact ram DSP.

Our current study showed a decrease in the DSP of PTU-treated rams. However, there was a high standard error of the mean in PTU-treated rams. There was a possibility that the genetic variation in individual rams could have influenced the number of Sertoli cells, and thus, results of DSP may be skewed due to the small number of subjects. Rams had similar birth weights and dates of birth; however, Matos et al. (1992) suggested that testicular size could be greatly affected by genetics because heritability for testis size in various sheep breeds is between 0.22 and 0.60. Sertoli cells are the major determinant of testis size and sperm production; therefore, if there was a predisposition for more or less Sertoli cells this could skew the results (Sharpe et al., 2003). As the DSP for PTU-treated animals was much lower than controls, it was concluded that neonatal PTU-treatment in rams led to decreased sperm production in adults, though overall testis size was not affected.

An interesting event in the testis growth occurred following treatment, but before puberty (Fig. 4). During PTU-treatment there was no difference in SC between PTU-treated and control rams. However, shortly after PTU-treatment was discontinued PTU-treated rams began to have decreased testis growth. This difference was not maintained because at puberty there was no difference in testicular diameter between PTU-treated and control rams. During PTU-treatment, lack of thyroid hormones may have prevented the action of T on the Sertoli cells, but following cessation it would have regained its normal action. Monet-Kuntz et al. (1984) reported that androgen receptors on Sertoli cells in the ram increased continuously from 25 to 100 days of age. Therefore, in the PTU-treated rams, if the effect of T was reduced, there would have been a sudden surge in the sensitivity of Sertoli cells to T following PTU-treatment. This increased sensitivity to T may have caused the Sertoli cells to stop proliferating, as was seen in hemicastrated rats administered T (Orth et al., 1984). It might also limit their ability to maintain

germ cell development because of an attempt to support more germ cells than is physiologically possible, resulting in increased germ cell apoptosis. This could explain the decreased SC growth following PTU-treatment. Chandrasekhar et al. (1985) demonstrated that hyperthyroidism in 16 week old rams caused retardation of testis growth. This may be similar to the current results because, after prolonged hypothyroidism, normal levels of thyroid hormones may have mimicked a hyperthyroid state within the testis. Considering that in normal development there is a gradual increase in T receptors on Sertoli cells, a sudden change due to recovered T_3 action may have been physiologically incompatible with Sertoli cell function. However, if this were the case, most Sertoli cells were able to recover from this insult because the SC decrease in PTU-treated animals was not permanent. Because Sertoli cells do not undergo mitosis once initial proliferation has ended, only the cells left after an insult would provide further testis growth into puberty and adulthood. Adult testis size thus indicates that the number of Sertoli cells was unchanged by PTU-treatment. However, other data suggest that Sertoli cell numbers were decreased due to lowered DSP.

A good indicator for DSP is the number of Sertoli cells because they directly impact the number of germ cells that can be produced (Orth et al., 1988). There was no difference between Sertoli cell counts per tubule cross section in PTU-treated and control rams, but there was a significant increase in seminiferous tubule diameter of PTU-treated animals. Because DSP was decreased in PTU-treated rams, it is reasonable to suggest that the number of Sertoli cells was also decreased. However, we did not calculate the number of Sertoli cells or seminiferous tubules per testis, so it remains unclear what ratio of Sertoli cells to germ cells was present in PTU-treated rams. However, it has been shown repeatedly that Sertoli cells can only support a limited number of germ cells, accounting for about 85 to 94% of the variation in DSP (Orth et al., 1988;

Berndtson and Thompson, 1990). Therefore, it seems unlikely that individual Sertoli cells were supporting a decreased number of germ cells, and thus Sertoli cell numbers in PTU-treated animals were presumably decreased. It appears that the normal testis size seen in adult PTU-treated rams was due to increased lumen size or number of interstitial cells rather normal numbers of Sertoli cells.

Despite lacking a positive effect on reproductive function in the ram, PTU-induced hypothyroidism in the neonatal period did not have dramatic or long-lasting effects on ram growth. Though BW of PTU-treated animals was reduced during the pre-pubertal period, it was not permanently affected. This was similar to results in rats where BW was decreased during PTU-treatment, but paralleled controls beginning 1 week following PTU-treatment (Cooke and Meisami, 1991). Additionally, scrotal size was not affected in adult animals. Based on this, hypothyroidism can be induced transiently during the neonatal period in rams without affecting final size of the animals. However, it seems that should hypothyroid treatment be necessary during prepubertal development in rams, it would likely result in decreased DSP which would not be desirable in animals of high reproductive value. Therefore, we must reject our hypothesis that neonatal hypothyroidism in rams would cause increased testes size and DSP, because PTU-treatment had no effect on testes size and decreased DSP.

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Dr. Oatley's Research Laboratory **PSU**
Undergraduate Researcher *Feb. 2008-Present*

- Administered PTU treatments to lambs
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- Created scientific protocol
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- Recorded statistical data
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Hopkins' Dairy Farm **Spartansburg, PA**
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- Milked 30-40 cows twice daily
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- Observed cows and heifers for estrus
- Treated foot-rot and foot abscesses
- Operated heavy farm equipment
- Treated mastitis infected quarters
- Operated milk system washing and sanitation
- Dehorned and castrated calves
- Implemented rotational grazing
- Assisted cows with dystocia

Lynch Creek Animal Clinic **Plains, MT**
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- Developed x-ray films
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- Utilized autoclave
- Prepared vaccine injections
- Scheduled appointments
- Prepped for surgery using aseptic technique
- Assembled surgery packs
- Restrained pets
- Monitored anesthesia during surgeries
- Systematized client records