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# Effects of immunization against luteinizing hormone-releasing hormone and treatment with trenbolone acetate on reproductive function of beef bulls and steers<sup>1</sup>

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**ABSTRACT:** The objectives of this study were 1) to evaluate the ability of trenbolone acetate (TBA) administered in tandem with LHRH immunization to suppress reproductive function in bulls and 2) to examine the effects of LHRH and androgen (TBA) signaling on pituitary gland function. Forty-four Angus × Hereford crossbred calves (BW = 225 ± 2 kg; age = 187 ± 6 d) received castration, LHRH immunization, or TBA administration in a 2 × 2 × 2 factorial design. Treatment groups receiving LHRH immunization contained 6 animals, whereas other treatment groups contained 5 animals. Animals immunized against LHRH received a primary injection and 2 booster injections of ovalbumin-LHRH-7 fusion protein on d 0, 42, and 196, respectively. Animals treated with TBA were implanted on d 224. Serum LHRH antibodies increased ( $P < 0.05$ ) after each booster for immunized animals, but were negligible in nonimmunized animals throughout the experiment. Serum testosterone concentration ( $P < 0.001$ ) and scrotal circumference ( $P < 0.05$ ) were depressed in LHRH-immunized bulls compared with nonimmunized bulls by d 84 and 168 of the experiment, respectively. Treatment with TBA tended ( $P = 0.08$ ) to decrease serum testosterone concentrations of nonimmunized bulls. Weights of testes at slaughter

were decreased ( $P < 0.001$ ) for LHRH-immunized (232 ± 41 g) compared with nonimmunized (752 ± 45 g) bulls, but did not differ ( $P = 0.80$ ) between TBA-implanted (500 ± 49 g) and nonimplanted bulls (484 ± 36 g). Both LHRH immunization and castration decreased pituitary gland stores of LH and FSH ( $P < 0.001$ ). There was no effect ( $P > 0.10$ ) of TBA on pituitary gland FSH content and only a tendency ( $P = 0.09$ ) to increase pituitary gland LH content. Immunization against LHRH decreased expression of LH β-subunit and common α-subunit genes ( $P < 0.001$ ). Castration increased expression of LH β-subunit and common α-subunit genes ( $P = 0.02$ ). Treatment with TBA further suppressed ( $P = 0.04$ ) α-subunit mRNA expression in LHRH-immunized steers. In summary, LHRH immunization decreased synthesis and storage of LH and decreased storage, but not synthesis of FSH in bulls. The increased synthesis of LH and FSH in nonimmunized, but not LHRH-immunized steers suggests that castration removes the negative feedback on gonadotropin synthesis but that LHRH is still needed for release of these hormones. Androgen replacement with TBA did not restore the negative feedback control of gonadotropin synthesis.

**Key words:** androgen, bull, cattle, gonadotropin, gonadotropin-releasing hormone immunosterilization, luteinizing hormone-releasing hormone

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## INTRODUCTION

The idea of active immunization against LHRH, or immunocastration, began when Arimura et al. (1973) demonstrated the ability of anti-LHRH antibodies to neutralize the reproductive axis. Recent studies with a recombinant ovalbumin protein containing multiple LHRH inserts (Zhang et al., 1999) resulted in excellent immune and biological responses in beef heifers and bulls (Hernandez et al., 2005; Stevens et al., 2005; Geary et al., 2006).

Castration decreased aggressive behavior, rate of growth, and feed efficiency in beef bulls (Field, 1971; Huxsoll et al., 1998). To attenuate the negative effects of castration, it became common practice in the beef industry to treat steers with growth-promoting implants (Preston, 1999). It was suggested that immunocastration may be a viable alternative to surgical castration (Reeves et al., 1989). Aïssat et al. (2002) reported similar growth and muscling characteristics between immunocastrated bulls and steers treated with trenbolone acetate (**TBA**) implants.

Trenbolone acetate is a 17-carbon steroid whose metabolite, trenbolone-17 $\beta$  hydroxide (**TBOH**), binds the androgen receptor with a large degree of specificity (Delettré et al., 1980). Administered alone, TBA decreased circulating concentrations of LH and FSH in steers (Gettys et al., 1984; Aïssat et al., 2002). Therefore, the primary objective of this study was to test the hypothesis that LHRH immunization along with TBA administration results in a more pronounced suppression of reproductive characteristics than LHRH immunization alone. The secondary objective was to examine anterior pituitary gland changes in gonadotropin hormones and gene expression in response to the aforementioned treatments.

## MATERIALS AND METHODS

All treatments and procedures involving animals were approved by the Fort Keogh Animal Care and Use Committee.

### *Antigen Preparation*

The antigenic fusion protein, ovalbumin-LHRH-7 (**ova-LHRH-7**), was prepared as described previously (Zhang et al., 1999; Quesnell et al., 2000). Briefly, ova-LHRH-7 is an ovalbumin protein with a total of 7 LHRH sequences inserted at 4 separate immunogenic sites and a 6-histidine sequence (His-tag, Novagen EMD Biosciences, Madison, WI) at the carboxyl terminus. The ova-LHRH-7 was overexpressed in *Escherichia coli* cultures. Solubilized cell lysates were purified over a Ni<sup>2+</sup> affinity column, which is facilitated by the His-tag. Soluble proteins were then dialyzed against 6 M urea and stored at  $-20^{\circ}\text{C}$  until use.

### *Animals and Treatments*

Angus  $\times$  Hereford calves utilized in this study were maintained at the USDA-ARS Fort Keogh Livestock and Range Research Laboratory in Miles City, MT. Twenty-two bull and 22 steer calves (BW =  $225 \pm 2$  kg),  $187 \pm 6$  d of age (bulls =  $219 \pm 8$  d, steers =  $154 \pm 5$  d), were randomly assigned to 1 of the following 8 treatments in a  $2 \times 2 \times 2$  factorial arrangement: control bulls, no treatment; bulls treated with a 200-mg TBA implant; bulls immunized against LHRH; bulls immunized against LHRH and treated with a 200-mg TBA implant; control steers; steers treated with a 200-mg TBA implant; steers immunized against LHRH; and steers immunized against LHRH and treated with a 200-mg TBA implant. Thus, castration (bull vs. steer), LHRH immunization, and TBA implant were the main factors. Five animals were used in each treatment group not receiving LHRH immunization, and 6 animals were used in each treatment group receiving LHRH immunization. Cattle immunized against LHRH received a primary injection containing 2 mg of ova-LHRH-7 emulsified in modified complete Freund's adjuvant on d 0. Booster injections of 2 mg of ova-LHRH-7 emulsified in incomplete Freund's adjuvant were administered on d 42 and 196. The TBA was administered as a subcutaneous, slow-release implant placed in the center of the ear on d 224 of the experiment. Implants contained 200 mg of TBA and had an expected delivery life of 100 d. The timing of the implant was chosen based on our goal of slaughtering animals while androgen concentrations were still increased rather than optimizing effects on bull testicular function. Implants were purchased commercially as Finaplix-H (Intervet Inc., Millsboro, DE). Steers used in this study were surgically castrated at approximately 30 d of age.

Scrotal circumference (**SC**) and BW were measured at d 0, then once every 28 d throughout the trial. Average daily gain was calculated over 2 distinct periods; d 0 to 224 (pre-TBA implant), and d 224 to slaughter (post-TBA implant). Blood (9 mL) was collected on d 0, 7, and 14, then every 14 d until d 56, after which samples were taken once every 28 d and at slaughter. Blood samples were collected via coccygeal venipuncture. All blood samples were stored at  $4^{\circ}\text{C}$  overnight then centrifuged at  $1,200 \times g$  for 30 min at  $4^{\circ}\text{C}$ . Serum was collected and stored at  $-20^{\circ}\text{C}$  until RIA.

### *Diet and Housing*

Animals were housed in a single pen from initiation of the experiment until placed on a finishing diet. Calves were placed on an initial growing diet consisting of 46.5% corn silage, 26.3% ground corn, 18.1% alfalfa hay, and 9.1% supplement from d 0 to 196 for bulls or d 0 to 201 for steers. At this time, calves were placed on a finishing diet of 67.4% ground corn, 16.4% corn silage, 8.0% alfalfa hay, and 8.2% supplement. All diets

are expressed on a DM basis. Growing and finishing diets were calculated to contain 16.8 and 16.3% CP, 1.69 and 1.96 Mcal/kg of NE<sub>m</sub>, and 1.10 and 1.34 Mcal/kg of NE<sub>g</sub>, respectively. Cattle consumed the finishing diet for a minimum of 72 d before slaughter.

### *Semen Evaluation*

Semen samples were collected from all bulls on d 191 and 266 (approximately 410 and 485 d of age) using electroejaculation. Collections were made and evaluated via a standard breeding soundness exam as outlined by Lunstra (2002). Sperm concentration was calculated for all bulls from which an ejaculate was obtained. Microscope slides of semen stained with eosin/nigrosin morphology stain were prepared for later analysis of percentage live/dead sperm and sperm morphology. One hundred sperm were randomly counted to calculate the percentage of live sperm in the ejaculate, whereas 200 sperm were counted to evaluate the percent normal and abnormal sperm. Abnormalities were classified as one of the following based on definitions of Barth and Oko (1977): tapered/pyriform head, microcephalic (small)/macrocephalic (large) heads, distal midpiece reflex, bent/coiled tail, proximal cytoplasmic droplet, detached head, and other. Included in the other category were unspecified midpiece defects, misshapen heads, and various other abnormalities occurring at low frequency.

### *Slaughter*

All animals were slaughtered at a local abattoir. Because of storage space and time constraints, not all animals could be slaughtered at a single slaughter date. Animals from each treatment were stratified across slaughter dates. Animals determined to be the most market ready based on BW and external fat deposition were slaughtered first. Once slaughtered, testis with epididymis weights were collected from all bulls as an indication of biological response to treatment. Anterior pituitary glands were collected, cleaned of external blood, and outer connective tissue was removed. Anterior pituitary glands were then hemisected mid-sagittally by removal of an approximately 2 mm slice of tissue. The tissue slice was placed in Bouin's fixative solution. The 2 halves were snap-frozen in liquid nitrogen for later analysis of hormone content and gene expression.

### *Pituitary Extractions*

One-half of each pituitary gland was lyophilized and weighed. Tissue was then ground by mortar and pestle and homogenized in distilled water. Tissue homogenates were centrifuged at  $800 \times g$  for 15 min at 4°C.

The resulting supernatant was assayed for LH and FSH concentrations.

Total cellular RNA was isolated from pituitary gland tissue after the Trizol (Invitrogen, Carlsbad, CA) directions and treated with DNase to eliminate genomic DNA from samples. Total RNA was resuspended in RNase-free water (Ambion, Austin, TX). Integrity of RNA samples was determined by agarose gel electrophoresis. An aliquot of RNA from each animal was reverse transcribed into cDNA using oligo(dT) priming and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Synthesis of cDNA was confirmed using reverse transcriptase-PCR (**RT-PCR**) with primers for  $\beta$ -actin [5'-TCCTGTGGCATCCAC-GAAACT-3' (forward) and 5'-GAAGCATTGCG-GTGGACGAT-3' (reverse)].

### *Antibody and Hormone Assays*

Serum concentrations of testosterone and LHRH-antibody activity were evaluated as indications of biological and immune responses to treatment. Serum antibodies against LHRH were quantified as the percentage of <sup>125</sup>I-LHRH bound in a serum dilution of 1:1,000 as defined by Johnson et al. (1988). Intra- and interassay CV for antibody quantification were 1.7 and 12.0%, respectively. Serum testosterone concentrations were evaluated using solid-phase RIA (Diagnostic Products Corporation, Los Angeles, CA) using procedures previously outlined (Richards et al., 1999) with the exception that tubes were incubated overnight. All samples were analyzed in 2 assays and intra- and interassay CV for testosterone assays were 2.7 and 4.0%, respectively, and sensitivity of the assay was 10.0 pg/mL. Testosterone concentrations were measured in all serum samples from bulls. Testosterone concentration was measured at d 0 and 252 in samples obtained from steers, to confirm negligible testosterone production and that TBOH did not cross-react with our assay, because TBA is quickly hydrolyzed into TBOH upon administration (Henricks et al., 1997). Serum TBOH concentrations were measured in samples obtained from animals that received TBA implants. Concentrations of TBOH were measured after HPLC extraction as described by Henricks et al. (1997) in 2 assays. Intra- and interassay CV were 2.4 and 1.7%, respectively, and TBOH recovery was 90.2%. Concentrations of LH in anterior pituitary gland extractions were determined using the procedure validated by Golter et al. (1973), with NIH-LH-B5 as the standard. The intra-assay CV for the single LH assay was 2.6%. Concentrations of FSH in anterior pituitary gland extractions were determined using the assay validated by Acosta et al. (1983), with NIH-FSH-B1 as the standard. The intra-assay CV for the FSH assay was 14.0%. Concentrations of gonadotropins in anterior pituitary gland extractions are expressed as nanograms of gonadotropin per milligram of lyophilized anterior pituitary gland tissue.

**Real-Time PCR**

All cDNA samples were evaluated for the expression of bovine LH  $\beta$ -subunit (**LH $\beta$** ), FSH  $\beta$ -subunit (**FSH $\beta$** ), common  $\alpha$ -subunit, androgen receptor (**AR**), estrogen receptor- $\alpha$  (**ER $\alpha$** ), estrogen receptor- $\beta$  (**ER $\beta$** ), activin receptor II (**ACVR2**), activin receptor II B (**ACVR2B**), LHRH-receptor (**LHRH-R**), and ribosomal protein S2 (**RPS2**) mRNA. Primers for genes of interest were designed using real-time PCR primer design (GeneScript Co., Piscataway, NJ) and obtained from Invitrogen. Primer sequences are shown in Table 1. All samples were analyzed in duplicate, and all 10 genes were detected on the same 96-well plate for each sample. Four samples were randomly assigned to each plate. The reaction mixture contained 200 ng of cDNA, 20 pmol of each primer, 12.5  $\mu$ L of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in a 25- $\mu$ L reaction volume. Reactions were performed in an iCycler iQ Real-Time PCR Detection System (Bio-Rad). The amplification reaction consisted of 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Visualization of amplification products with agarose gel electrophoresis confirmed amplification products were of the expected size compared with a DNA ladder and verified the absence of other PCR amplification products for each primer set. Melt curves following real-time PCR were evaluated to confirm that a single PCR product was generated and used for subsequent calculations. Dilution curves were run for all primer sets, and the amplification efficiencies were determined with the use of the qGene program (Muller et al., 2002). Primers were designed to span exon boundaries. Primer efficiency analysis was conducted with qGene for each real-time run. Amplification efficiencies were 97 to 100% for all primer pairs. Negative control reactions were performed including reactions with no reverse transcriptase, reactions with no primers, and reactions containing no template. Mean normalized gene expression was calculated based on the equation of Muller et al. (2002), relative to *RPS2* expression. Cycle threshold values for *RPS2* were evaluated on each real-time run and were always within 0.2 units for a CV of less than 1%.

**Statistical Analysis**

Paired testis weight, ejaculate characteristics, anterior pituitary gland weight, pituitary gonadotropin concentration, and gene expression data were all analyzed by ANOVA for a completely randomized design with the GLM procedure (SAS Inst. Inc., Cary, NC). Note that the pen effect could not be accounted for in performance data. The model included effects of castration (bull vs. steer), LHRH immunization, and TBA implant. Animals treated with LHRH immunization or TBA implant, but either not expressing a significant response to LHRH immunization or not having a detectable serum concentration of TBOH, were excluded

from analyses of all data listed above, except ejaculate characteristics. Evaluation of semen ejaculate data included TBOH concentration as a linear covariate. When a significant effect of treatment was observed ( $P \leq 0.05$ ), all pair-wise comparisons were performed using Fisher's LSD test.

Percentage of  $^{125}$ I-LHRH bound, serum testosterone concentration and SC were analyzed by the PROC MIXED repeated measures procedure in SAS using individual within treatment as the random variable. Because TBA was not administered until d 224, percentage of  $^{125}$ I-LHRH bound was analyzed as a 2  $\times$  2 factorial arrangement of treatments with castration (bull vs. steer), LHRH immunization, and day as fixed effects until d 224. Thereafter, percentage of  $^{125}$ I-LHRH bound was analyzed as a 2  $\times$  2  $\times$  2 factorial arrangement of treatments with castration, LHRH immunization, TBA, and day as fixed effects. Testosterone was analyzed similarly except that castration was removed as a fixed effect. Bull SC was analyzed as LHRH immunization vs. control until d 252. When a significant effect ( $P \leq 0.05$ ) of treatment was observed, comparisons were performed using Fisher's LSD test.

**RESULTS**

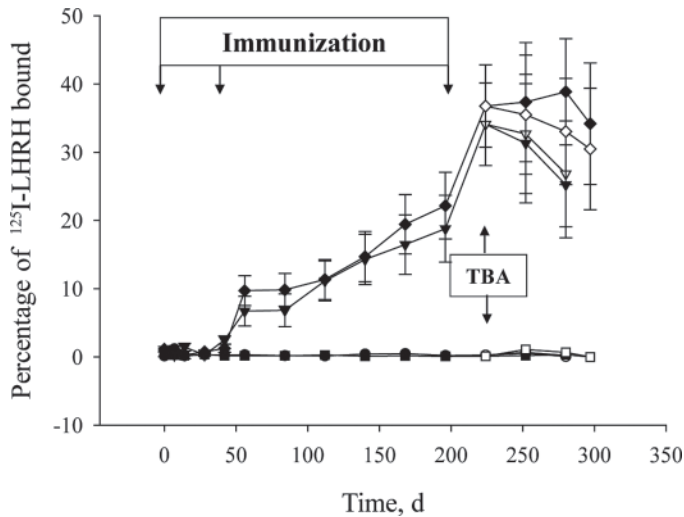
**Serum LHRH Antibody, Testosterone, and TBOH**

Antibody production against LHRH was quantified as the percentage of  $^{125}$ I-LHRH bound in a 1:1,000

**Table 1.** Nucleic acid sequences of primers used for real-time reverse-transcriptase PCR

Gene primer <sup>1</sup>	Nucleotide sequence (5' to 3')
<i>LH<math>\beta</math>-F</i>	TAA GGA TGC CCC ACT TCA AC
<i>LH<math>\beta</math>-R</i>	GGG AAG AGT GTC TGC TGG TT
<i>FSH<math>\beta</math>-F</i>	CGG TGG AGA AAG AGG AAT GT
<i>FSH<math>\beta</math>-R</i>	AGT CCC GGG TGT AGC AGT AG
<i><math>\alpha</math>-Subunit-F</i>	GCA GCT GTC ATT CTG ACC AT
<i><math>\alpha</math>-Subunit-R</i>	AGC ATC TGG CTT GGA GAA GT
<i>AR-F</i>	AGA GAG GGA AAA AAC AGG GTT GA
<i>AR-R</i>	AGA GGC CCA AGG ACA TGA AA
<i>ER<math>\alpha</math>-F</i>	ATG ATG AAA GGC GGA ATA CG
<i>ER<math>\alpha</math>-R</i>	ATC ATC TCT CTG GCG CTT GT
<i>ER<math>\beta</math>-F</i>	AAA GAG TCC TTG GCG TGA AA
<i>ER<math>\beta</math>-R</i>	GCT CCC ACT AGC CTT CCT TT
<i>ACVR2-F</i>	AAA GTT TGA GGC TGG CAA GT
<i>ACVR2-R</i>	GCA TCC CTT TGG AAG TTG AT
<i>ACVR2B-F</i>	TCA AGG GGA ACA TCA TCA CA
<i>ACVR2B-R</i>	GTG GGC AAT AGA TGG CTT GT
<i>LHRH-R-F</i>	TTG GGA TGA TCC ATT TAG CA
<i>LHRH-R-R</i>	ATG AAG AGG CAG CTG AAG GT
<i>RPS2-F</i>	GGA GCA TCC CTG AAG GAT CA
<i>RPS2-R</i>	TCC CCG ATA GCA ACA AAG G

<sup>1</sup>F indicates a forward primer, and R indicates a reverse primer. *LH $\beta$*  = LH  $\beta$ -subunit; *FSH $\beta$*  = FSH  $\beta$ -subunit; *AR* = androgen receptor; *ER $\alpha$*  = estrogen receptor- $\alpha$ ; *ER $\beta$*  = estrogen receptor- $\beta$ ; *ACVR2* = activin receptor II; *ACVR2B* = activin receptor II B; *LHRH-R* = LHRH-receptor; *RPS2* = ribosomal protein S2.



**Figure 1.** Least squares means  $\pm$  SEM of LHRH-immunized bulls (closed triangles) and steers (closed diamonds), and nonimmunized bulls (closed squares) and steers (closed circles) from d 0 until d 224. After d 224, LHRH-immunized bulls (open triangles) and steers (open diamonds) and nonimmunized bulls (open squares) and steers (open circles) receiving a trenbolone acetate (TBA) implant (Finaplix-H, Intervet Inc., Millsboro, DE) are also depicted until slaughter. Animals received a primary immunization on d 0, followed by booster injections on d 42 and 196. Percentage of  $^{125}\text{I}$ -LHRH bound was affected by LHRH immunization ( $P < 0.001$ ), day ( $P < 0.001$ ), and the interaction between immunization and day ( $P < 0.001$ ).

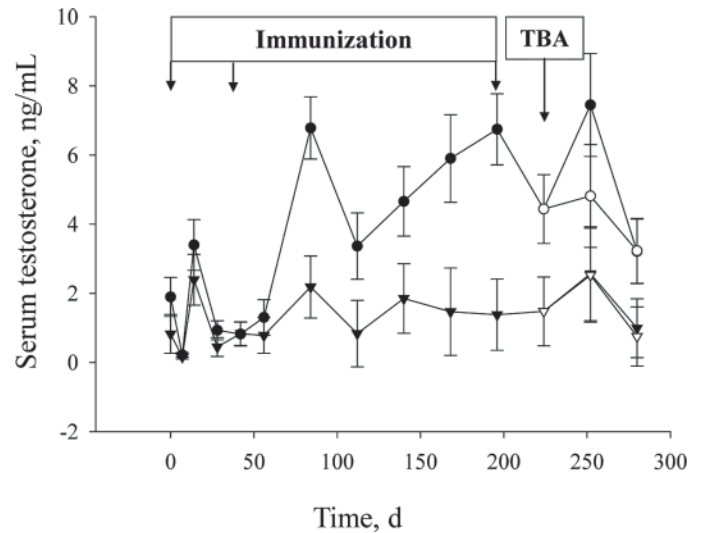
serum dilution. Only 75% of the bulls (9 of 12) and steers (9 of 12) developed an antibody response against LHRH, and 1 of the bulls did not develop an antibody response until after receiving the booster immunization. Percentage of  $^{125}\text{I}$ -LHRH bound was affected by LHRH immunization ( $P < 0.001$ ), day ( $P < 0.001$ ), and the interaction between immunization and day ( $P < 0.001$ ; Figure 1). Although percentage of  $^{125}\text{I}$ -LHRH bound in serum of immunized bulls and steers increased after the first booster on d 42, the difference between immunized and nonimmunized groups was not significant ( $P = 0.04$ ) until d 42.

Serum testosterone concentrations in bulls were affected by LHRH immunization ( $P < 0.001$ ), day ( $P < 0.001$ ), and immunization  $\times$  day interaction ( $P < 0.001$ ; Figure 2). Serum testosterone concentrations were greater ( $P = 0.001$ ) in nonimmunized than LHRH-immunized bulls from d 84 through slaughter. Implanting bulls with TBA tended ( $P = 0.08$ ) to decrease serum testosterone concentrations of nonimmunized bulls.

Serum TBOH was undetectable 28 d after receiving a TBA implant in 5 of 11 bulls. Only 1 of 11 steers implanted with TBA had undetectable concentrations of serum TBOH postimplantation. Bulls and steers receiving implants, but having undetectable concentrations of TBOH, were consequently removed from statistical analyses of response variables.

### SC and Paired Testis Weight

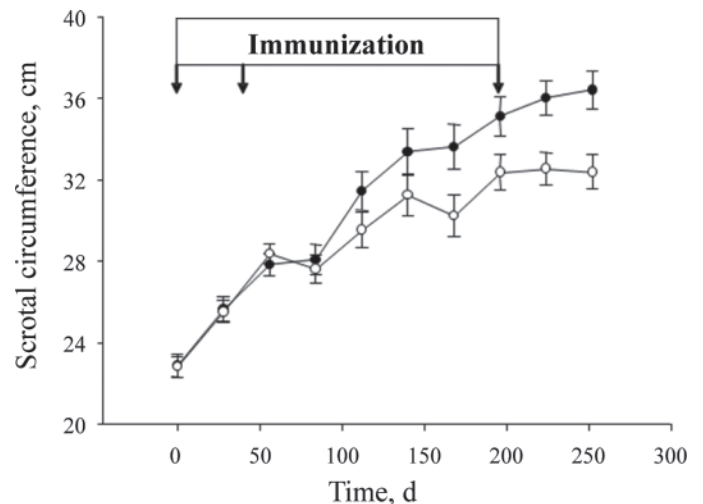
Scrotal circumference was greater ( $P < 0.05$ ) for nonimmunized bulls than LHRH-immunized bulls from d



**Figure 2.** Least squares means  $\pm$  SEM of serum testosterone concentrations of bulls (closed circles) compared with LHRH-immunized bulls (closed triangles) from d 0 until d 224. After d 224, bulls receiving a trenbolone acetate (TBA) implant (open circles) and LHRH-immunized bulls receiving TBA implant (open triangles) are also depicted until slaughter. Bulls received a primary immunization on d 0, followed by booster injections on d 42 and 196. Serum testosterone concentration differed by LHRH immunization ( $P < 0.001$ ) and day ( $P < 0.001$ ), and tended to differ ( $P = 0.08$ ) by the interaction of day  $\times$  TBA in nonimmunized bulls.

168 through d 252 of the experiment (Figure 3). The SC of 3 LHRH-immunized bulls was immeasurable on d 252 due to increased fat in the scrotum and small testis size; thus, SC was held constant from the previous measurement for these bulls. Because SC was measured just once (d 252) after TBA implantation, effect of TBA treatment on SC was not evaluated.

Paired testis weight (with epididymides attached) of bulls were greatly reduced ( $P < 0.001$ ) by LHRH immunization, but not TBA implant ( $P = 0.80$ ). Least



**Figure 3.** Least squares means  $\pm$  SEM scrotal circumferences of LHRH-immunized bulls (open circles) were less ( $P < 0.05$ ) than nonimmunized bulls (closed circles) from d 168 through d 252. Bulls received a primary immunization against LHRH on d 0, followed by booster injections on d 42 and 196.

squares means  $\pm$  SEM paired testis weights for LHRH-immunized and nonimmunized bulls were  $232 \pm 41$  and  $752 \pm 45$  g, respectively. Paired testis weights of TBA-implanted and nonimplanted bulls were  $500 \pm 49$  and  $484 \pm 36$  g, respectively.

### *Anterior Pituitary Gland Weight and Gonadotropin Content*

Bulls immunized against LHRH had lighter ( $P = 0.01$ ) anterior pituitary hemisections ( $97.7 \pm 6.5$  mg) compared with nonimmunized bulls ( $121.4 \pm 6.1$  mg). Anterior pituitary gland LH concentration was decreased by castration ( $P < 0.001$ ), LHRH immunization ( $P < 0.001$ ), and castration by immunization interaction ( $P < 0.001$ ; Figure 4). Anterior pituitary gland FSH concentration was decreased by castration ( $P < 0.001$ ) and LHRH immunization ( $P < 0.001$ ). Anterior pituitary weight and FSH concentrations were not affected ( $P = 0.16$ ) by TBA implants, but there was a tendency ( $P = 0.09$ ) for LH content to be greater in bulls and steers receiving TBA.

### *Anterior Pituitary Gene Expression*

All 9 candidate genes evaluated in anterior pituitary samples were expressed in each sample. Transcripts for only 3 genes, *LH $\beta$* , *FSH $\beta$* , and common  $\alpha$ -subunit, were affected by the treatments applied. Expression of *LH $\beta$*  mRNA was increased by castration ( $P = 0.02$ ) and decreased by LHRH immunization ( $P < 0.001$ ). Expression of *FSH $\beta$*  mRNA tended to be decreased ( $P = 0.08$ ) by LHRH immunization. Expression of common  $\alpha$ -subunit mRNA was decreased by LHRH immunization ( $P < 0.001$ ) and further decreased by TBA ( $P = 0.04$ ). Expressions of *LH $\beta$* , *FSH $\beta$* , and common  $\alpha$ -subunit mRNA are depicted in Figure 5 for each treatment group. Unfortunately, TBOH concentration data were not available until after real-time RT-PCR had been performed. Only 1 TBA-implanted bull chosen for gene expression analyses had detectable TBOH in serum. Consequently, values from this treatment were removed from analyses, but are shown in Figure 5 for reference.

The ADG of bulls and steers before d 224 (administration of TBA implant) was affected by both castration ( $P = 0.001$ ) and a castration  $\times$  LHRH immunization interaction ( $P = 0.04$ ; Table 2). After d 224, no effect ( $P > 0.10$ ) of castration, immunization, or TBA implant was evident.

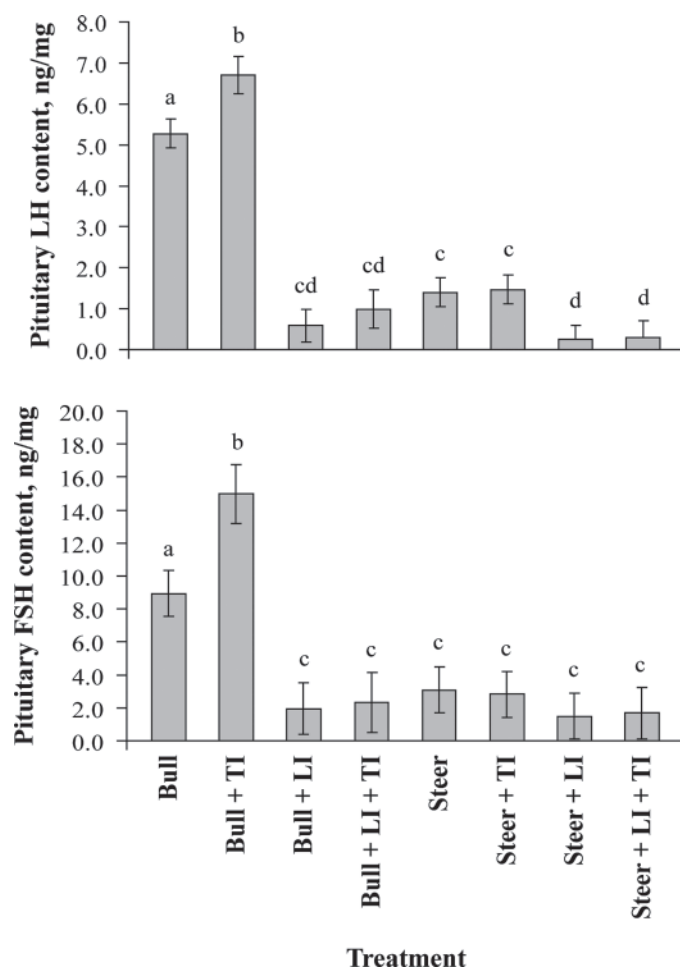
### *Sperm Morphology and Concentration*

On d 191, the percentage of abnormal sperm produced by bulls was increased ( $P = 0.03$ ) in LHRH-immunized (49.5%) compared with nonimmunized bulls (23.0%). Of the specific abnormalities, only distal midpiece reflex ( $P = 0.07$ ) and microcephalic/macrocephalic heads ( $P = 0.07$ ) tended to be increased by

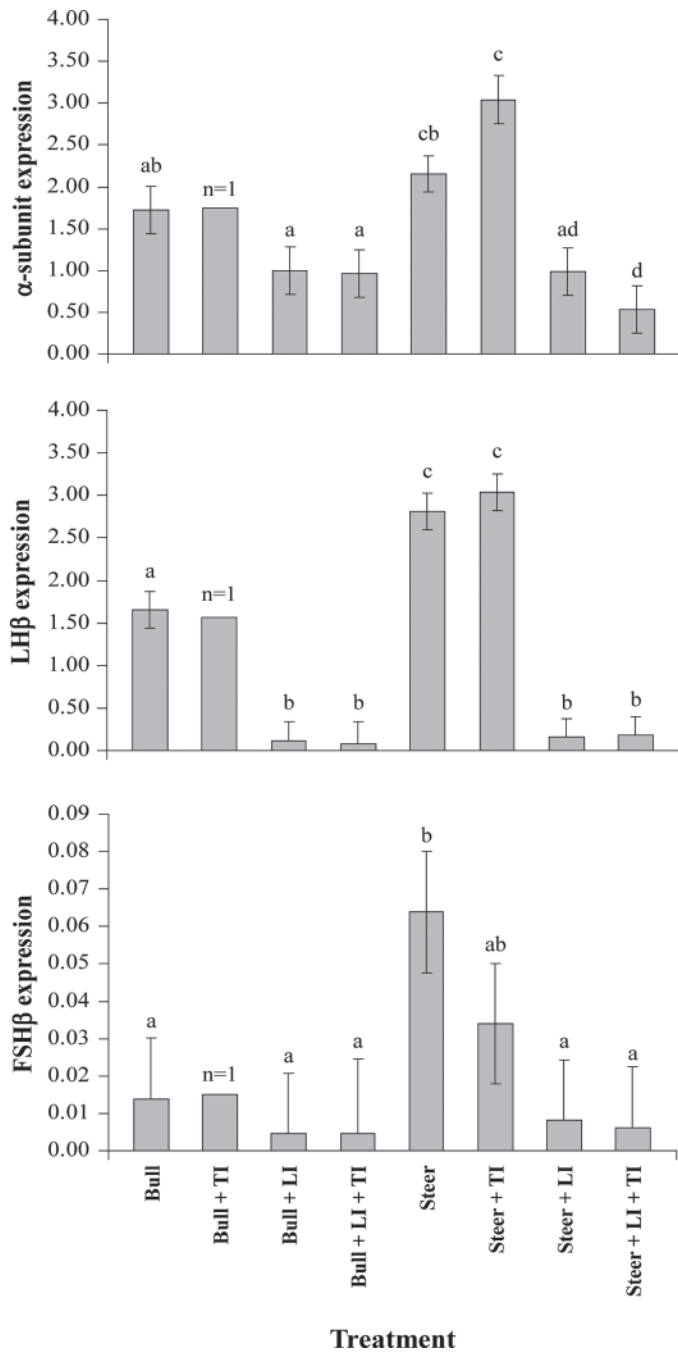
LHRH immunization. Immunization against LHRH continued to increase the percentage of abnormal sperm in bull ejaculates collected on d 266 (54.0 and 18.5% for immunized and nonimmunized bulls, respectively;  $P = 0.008$ ). The percentage of proximal droplets tended ( $P = 0.06$ ) to be increased, whereas detached heads ( $P = 0.007$ ) and other abnormalities ( $P = 0.05$ ) were increased by LHRH immunization at the second collection. Including TBOH concentration as a covariate revealed increased percentage of bent/coiled tails ( $P < 0.001$ ) for TBA-implanted bulls. Immunization did not affect ( $P > 0.10$ ) sperm concentration per ejaculate or the number of bulls providing an ejaculate at either collection time. Serum TBOH concentration had no effect ( $P = 0.48$ ) on sperm concentration at the second collection date.

## DISCUSSION

Immunization against LHRH with the ova-LHRH-7 antigen was effective in decreasing circulating concen-



**Figure 4.** Anterior pituitary gland content (least squares means  $\pm$  SEM) of LH and FSH from bulls and steers utilized in a  $2 \times 2 \times 2$  factorial arrangement of treatments with LHRH immunization and trenbolone acetate (TBA; Finaplix-H, Intervet Inc., Millsboro, DE) implant as treatment factors. On the treatment axis, LI = LHRH immunization, and TI = TBA implant. Within a graph, bars without a common letter (a–d) differ ( $P < 0.05$ ).



**Figure 5.** Relative mRNA expression (least squares means  $\pm$  SEM) of gonadotropin subunit genes [common  $\alpha$ -subunit, LH  $\beta$ -subunit (*LH $\beta$* ), and FSH  $\beta$ -subunit (*FSH $\beta$* )] to bovine ribosomal protein S2 in anterior pituitary glands of bulls and steers used in a  $2 \times 2 \times 2$  factorial arrangement of treatments with LHRH immunization and trenbolone acetate (TBA) implant as treatment factors. On the treatment axis, LI = LHRH immunization, and TI = TBA implant. Gene expression was analyzed for 3 animals in each treatment, except Bull + TI ( $n = 1$ ) and Bull + LI + TI ( $n = 2$ ). Treatment Bull + TI was not included in statistical analyses, but is shown for reference. Within a graph, bars without a common letter (a-d) differ ( $P < 0.05$ ).

treatments of testosterone, scrotal circumference, and testes weights. These results are consistent with the findings of Hernandez et al. (2005). Nonetheless, the percentage of animals responding to LHRH immunization with significant LHRH antibody production (75%) or other biological response was low compared with

**Table 2.** Least squares means of ADG (kg/d) and days on feed for bulls and steers treated with LHRH immunization and trenbolone acetate (TBA) implant in a  $2 \times 2 \times 2$  factorial treatment structure, both before and after TBA implant

Period	Treatment <sup>1</sup>							
	Bull	Bull + TBA	Bull + implant + TBA	Bull + implant	Steer	Steer + TBA	Steer + implant	Steer + implant + TBA
Pre-TBA implant (d 0 to 24)	1.20 $\pm$ 0.04 <sup>a</sup>	—	—	1.12 $\pm$ 0.04 <sup>ab</sup>	0.93 $\pm$ 0.04 <sup>c</sup>	—	1.03 $\pm$ 0.04 <sup>bc</sup>	—
Post-TBA implant (d 224 to slaughter)	1.56 $\pm$ 0.19	1.52 $\pm$ 0.15	1.71 $\pm$ 0.17	1.56 $\pm$ 0.14	1.18 $\pm$ 0.17	1.46 $\pm$ 0.19	1.40 $\pm$ 0.15	1.53 $\pm$ 0.14
Days on feed <sup>2</sup>	283.2 $\pm$ 4.2	283.2 $\pm$ 4.2	284.8 $\pm$ 3.8	282.5 $\pm$ 3.5	301.4 $\pm$ 5.1	301.4 $\pm$ 5.1	303.5 $\pm$ 4.7	303.5 $\pm$ 4.7

<sup>a-c</sup>Within a row, means without a common superscript differ ( $P < 0.01$ ).

<sup>1</sup>Implant = LHRH immunization; TBA = TBA implant (Finaplix-H, Intervet Inc., Millsboro, DE). The LHRH-immunized animals received a primary injection on d 0 and boosters on d 42 and 196. Animals receiving TBA were implanted on d 224.

<sup>2</sup>Days on feed were different ( $P < 0.05$ ) between bulls and steers, but not between treatments ( $P > 0.10$ ) within sex.



other studies utilizing the same antigen (Hernandez et al., 2005; Stevens et al., 2005; Geary et al., 2006). Immunization against LHRH also increased the percentage of abnormal sperm per ejaculate to 49.5 and 54.5% at 2 separate collections. Barth and Oko (1977) indicated that bulls with >17% abnormal sperm are not likely to be highly fertile. In addition, fewer LHRH-immunized bulls (75%) provided an ejaculate containing sperm at the second collection date, but the mean concentration of sperm for those bulls that provided an ejaculate was not affected by treatment.

As evidenced by the limited effect of TBA on reproductive characteristics, LHRH immunization in tandem with TBA implant did not result in a more pronounced suppression of reproductive function. Perhaps greater effects would have been observed if we had administered the TBA implant earlier in the study. Bulls that received Synovex-C (containing 100 mg of progesterone and 10 mg of estradiol benzoate) at 3.6 mo of age and Synovex-S (containing 200 mg of progesterone and 20 mg of estradiol benzoate) at weaning had decreased serum testosterone concentrations, decreased SC, and decreased testis concentrations of spermatozoa at slaughter compared with control bulls (Adams et al., 1993). Therefore, it seems reasonable to suggest that TBA could have had a more significant effect if administered earlier in reproductive development. Fabry et al. (1983) reported that bulls implanted with a combination of TBA and zeranol at 90 d of age had significantly reduced SC compared with nonimplanted bulls. Fabry et al. (1983) and Renaville et al. (1988) both reported treatment with TBA in combination with estrogen to decrease pituitary gland responsiveness to exogenous LHRH. Additionally, Keel et al. (1987) demonstrated that wether lambs implanted with dihydrotestosterone (DHT), estradiol, or DHT + estradiol at the time of normal puberty had decreased pituitary gland concentrations of LH. It is also possible that bulls and steers in the present study did not retain the TBA pellets in the implant site, or did not release TBA into the bloodstream because serum TBOH was not increased in some animals. No observations of TBA implant sites were conducted at slaughter. The limited number of animals coupled with immeasurable TBOH in serum of several animals was a severe limitation of this study.

The ability of TBA to decrease circulating concentrations of LH in steers (Gettys et al., 1984; Aïssat et al., 2002) and to decrease LH and testosterone release in response to exogenous administration of LHRH (Fabry et al., 1983; Renaville et al., 1988) is well established and was the basis for this experiment. Thus, we did not examine circulating concentrations of LH in the animals of this study. Both castration and LHRH immunization resulted in decreased pituitary gland concentrations of LH and FSH, which agrees with findings by Adams and Adams (1986) and Keel et al. (1987). Implanting control bulls with TBA increased pituitary gland LH and FSH concentrations, but exerted no ef-

fect on pituitary gland gonadotropin content of animals from other treatments. This finding differs from that of Keel et al. (1987), who reported that DHT administration decreased anterior pituitary LH content in wether lambs. Perhaps anterior pituitary gland LH and FSH expression and storage was too small in LHRH-immunized animals, and steers had become refractory to androgenic effects on LH and FSH secretion. It has been suggested that chronically castrated steers may become refractory to androgen replacement (D'Occhio et al., 1982). The suppressive effect of TBA treatment on serum concentration of LH was decreased in steers that received implants at an older age (and longer interval since castration; Aïssat et al., 2002). The aforementioned studies may account for the lack of an effect of TBA on steer pituitaries. Immunization against LHRH decreased anterior pituitary gland weight, which agrees with decreased concentrations of gonadotropin storage in the present study.

Unlike pituitary gonadotropin content, gonadotropin synthesis was increased in steers compared with bulls. Expression of *LH $\beta$*  mRNA was increased in steers. Immunization against LHRH decreased *LH $\beta$*  and common  $\alpha$ -subunit mRNA in both bulls and steers, and tended to decrease *FSH $\beta$*  mRNA expression. These data agree with Stumpf et al. (1992), who reported that pituitary concentrations of mRNA encoding *LH $\beta$*  and common  $\alpha$ -subunit were decreased by immunization of LHRH in ovariectomized cows.

The disparity between pituitary gland gonadotropin concentrations and mRNA expression is likely due to alternate profiles of synthesis and release in bulls and steers. The increased storage of both LH and FSH in the anterior pituitary glands is likely a reflection of less gonadotropin release in bulls. Greater circulating concentrations of LH, as well as greater frequency/amplitude of LH pulses in steers, has been reported previously (Schanbacher et al., 1983). Thus, it seems logical that steers would synthesize more gonadotropin hormone, resulting in the increased or the trend for increased pituitary gland gonadotropin expression in the present study. Decreased pituitary gland stores of gonadotropins in steers likely reflect a greater release of LH and FSH compared with bulls.

The increase in pituitary gland stores of LH and FSH in bulls treated with TBA may reflect an effect of TBA on hormone release because expression of LH and FSH subunits was not different. It has been proposed that androgen feedback may be imparted primarily at the anterior pituitary gland (Gettys et al., 1984). The lack of difference between nonimmunized steer groups may be a result of a refractory state of the chronically castrated steer pituitary gland to androgen replacement, or simply the greater concentration of basal hormone release in steers. Although an interaction between TBA and LHRH immunization affected  $\alpha$ -subunit mRNA expression, the results are challenging to interpret. Similar to pituitary gland gonadotropin content,  $\alpha$ -subunit

mRNA expression did not differ between immunized groups (bulls or steers).

Of the other genes evaluated, none were affected by castration, LHRH immunization, or TBA implant. The response of pituitary gland gonadotroph cells to LHRH is directly influenced by the number of LHRH-R expressed on the cell surface (Norwitz et al., 1999), which is partially mediated by *LHRH-R* mRNA expression (Turzillo and Nett, 1999). Due to the fact that LHRH is a positive regulator of its own receptor at the transcriptional level (Turzillo and Nett, 1999), we expected its mRNA expression to be affected by LHRH immunization. Although it is possible that LHRH-R number at the cell surface may have been affected, expression of *LHRH-R* mRNA was not affected by any of our treatments. Turzillo and Nett (1997) reported no change in *LHRH-R* mRNA expression in ovariectomized ewes passively immunized against GnRH.

In summary, LHRH immunization was effective in suppressing reproductive function. In addition, LHRH immunization decreased synthesis and storage of LH and decreased storage, but not synthesis of FSH in bulls. The increased synthesis of LH and FSH in non-immunized, but not LHRH immunized steers, suggests that castration removes the negative feedback on gonadotropin synthesis but that LHRH is still needed for release of these hormones. Androgen replacement with TBA did not restore the negative feedback control of gonadotropin synthesis. In the present study, TBA may not have been administered early enough to detect differences in testicular function.

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