

Influence of season and low-level oestradiol immunoneutralization on episodic LH and testosterone secretion and testicular steroidogenic enzymes and steroidogenic acute regulatory protein in the adult ram

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The regulation of LH-dependent and -independent increases in testosterone secretion by key proteins in the testes of adult rams was investigated. Serial blood samples were collected from groups of four control and passively immunized (oestradiol antiserum for 3 weeks) rams and the animals were gonadectomized in either the non-breeding season (April) or the breeding season (September). LH pulse frequency and basal (interpulse) concentrations were several times greater ($P < 0.01$) in the breeding season than in the non-breeding season. Neither of these parameters nor LH pulse amplitude were affected by oestradiol immunization. Parameters of testosterone episodic secretion and response to an injection (i.v.) of 15 µg NIH-LH-S25 were also greater ($P < 0.05$) in the breeding season and, with the exception of pulse frequency, in immunized rams versus controls. Substrate utilization established that testosterone biosynthesis was predominantly via the 5-ene pathway. Increases in blood testosterone concentration in the breeding season were associated with a fivefold higher ($P < 0.01$) activity of cytochrome P450 17 α -hydroxylase/C-17,20 lyase (P450_{17 α}) and a 65% higher ($P < 0.05$) relative amount of mRNA for cytochrome P450 cholesterol side-chain cleavage enzyme complex (P450_{scc}) in the testis. Of the steroidogenic enzyme activities examined, only that for 17 β -hydroxysteroid dehydrogenase (17 β -HSD) tended to be increased by oestradiol immunization. Blood concentrations of cholesterol lipoproteins and expression of the testicular low density lipoprotein receptor were not affected by season or immunization. The amount of steroidogenic acute regulatory protein (StAR) mRNA was 65% higher ($P < 0.01$) in the breeding season and 20% higher ($P < 0.01$) in immunized rams versus controls. These results indicate that greater LH stimulation may increase testosterone biosynthesis in the breeding season by increasing StAR mRNA (and presumably delivery of cholesterol to P450_{scc}) and the activity of P450_{17 α} and possibly that of P450_{scc} (activity not measured). More moderate increases in StAR mRNA and 17 β -HSD activity may explain, in part, the increases in testosterone secretion with oestradiol immunization.

Introduction

Mammalian species inhabiting temperate regions usually show seasonal variation in testicular activity which, if pronounced enough, is associated with androgen-dependent changes in secondary sex characteristics and sexual and

aggressive behaviour (Lincoln, 1989). Testosterone production by the testes of some species such as sheep and reindeer is estimated to vary between five- and ten-fold during the year (Whitehead and West, 1977; Darbeida and Brudieux, 1980). Higher production rates in the breeding season are made possible mainly by hypertrophy of Leydig cells (Lincoln, 1989; Hochereau-de Reviers *et al.*, 1992). Greater steroidogenic capacity of Leydig cells in the ram testis corresponds to increased development of cellular organelles, including the mitochondria and smooth

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endoplasmic reticulum responsible for steroid biosynthesis (Lunstra and Schanbacher, 1988; Lincoln, 1989). Maintenance of the structural and functional integrity of Leydig cells involves primarily LH (Saez, 1994). In rams, LH pulse frequency (Sanford *et al.*, 1984; Sanford and Baker, 1990) and basal (interpulse) LH concentration (Chase *et al.*, 1988) are the most important release parameters regulating seasonal changes in testosterone secretion.

Although testosterone secretion in the ram is highly regulated by LH, there is probably a moderating paracrine and autocrine control by a number of factors produced within the testis (Saez, 1994). One factor that may suppress testosterone secretion is oestradiol (Moger, 1980). In mature rams, oestradiol is thought to be produced mainly by Leydig cells and is present in the testes in extremely high concentrations (Bilinska *et al.*, 1997; Hötzel *et al.*, 1998). Oestradiol exerts a negative effect on gene expression and activity of cytochrome P450 17 α -hydroxylase/C-17,20 lyase (P450_{17 α}) in rats (Wang *et al.*, 1980; Majdic *et al.*, 1996) by an oestrogen-receptor mediated action (Melner and Abney, 1980; Nozu *et al.*, 1981) and is a competitive inhibitor of the activity of 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β -HSD) in mice (MA-10 Leydig tumour cells) (Freeman, 1985). Other studies indicate that oestrogen exerts similar actions in rams; low-level oestradiol immunoneutralization can result in three- to sixfold higher blood testosterone concentrations independently of increases in LH release and the number of LH receptors in the testis (Sanford, 1989; Sanford *et al.*, 1991), although Leydig cells clearly become more responsive to LH (Sanford, 1985).

As is found in ruminants generally (Conley and Bird, 1997), testosterone synthesis in the ram testis involves the 5-ene pathway (Fields *et al.*, 1980). In this pathway, pregnenolone formed from cholesterol by cytochrome P450 cholesterol side chain cleavage enzyme complex (P450_{scc}) is metabolized to 17 α -hydroxypregnenolone and then to dehydroepiandrosterone (DHEA) by P450_{17 α} . DHEA in turn is metabolized to androstenedione and then to testosterone by 3 β -HSD and 17 β -hydroxysteroid dehydrogenase (17 β -HSD), respectively. Investigations have not been undertaken in rams to determine how increases in testosterone secretion in the breeding season (mainly LH dependent) and with low-level oestradiol immunoneutralization (mainly LH independent) may relate to changes in the gene expression and activity of these four steroidogenic enzymes. Histochemical localization studies with deer have shown more intense staining for 3 β -HSD in Leydig cells when blood testosterone concentrations are increased in the rut (Markwald *et al.*, 1971; Lincoln, 1989).

The aim of the present study was to examine the effects of season and passive immunization against oestradiol on steady-state mRNA concentrations and activity of key proteins involved in: (i) testosterone biosynthesis (P450_{scc}, P450_{17 α} , 3 β -HSD and 17 β -HSD); and (ii) cholesterol substrate availability to the steroidogenic pathway (low density lipoprotein receptor, LDL-R; and steroidogenic acute regulatory protein, StAR). Testicular protein mRNA concentrations and activity, and blood lipoprotein concentrations were determined for adult control and immunized rams in relation to episodic LH and testosterone secretion in the non-breeding and breeding seasons.

Materials and Methods

Animals

Sixteen adult (6 years of age) DLS rams were used in this investigation. The DLS is a relatively new breed of sheep developed by crossbreeding Dorset, Leicester and Suffolk. Rams were entrained to natural photoperiod change (latitude 45°48'N) during the experimental period, which began in mid-August and ended in early April. For DLS rams kept at this latitude, August and April represent months when testicular size and testosterone secretion are near, or at, yearly high and yearly low values, respectively (Sanford and Robaire, 1990). DLS ewes become seasonally anoestrus by the end of April (Fahmy and Dufour, 1986). Therefore, in the present study, experimental observations on rams were made in both the non-breeding and breeding seasons. The experiments were carried out in accordance with guidelines of the Canadian Council on Animal Care and the Animal Care Committee of McGill University.

Blood and testicular tissue collection

Beginning on August 17 (day 1) and again on March 15 (day 1), separate groups of four rams were passively immunized against oestradiol for 3 weeks. Other groups of four rams served as controls. On day 21 of immunization, a 7 ml sample of blood was taken from a jugular vein of each ram every 20 min for 10 h (beginning at 09:30 h). After collection of the 25th sample, a single i.v. injection of 15 μ g NIH-LH-S25 was given to assess the testosterone response to a standardized physiological LH challenge. Melnyk *et al.* (1992) reported that injections of 10 μ g NIH-LH-S25 given to adult rams of similar body weight produced 'LH pulses' of an amplitude that was indistinguishable from those of endogenous LH pulses.

On day 23 of immunization, rams were gonadectomized 30 min after receiving an i.v. injection of the tranquillizer Ketamine[®] (1 ml per 50 kg body weight, 100 mg ketamine hydrochloride ml⁻¹) (Rogar/STB, London, ON) and a series of s.c. injections of the local anesthetic Xylocaine[®] (15 ml, 20 mg lidocaine hydrochloride plus 0.01 mg adrenaline ml⁻¹) (Astra Pharmaceuticals, Mississauga, ON) in the scrotal neck area. As the testes were removed, a number of samples of testicular tissue (1–2 g) were taken for each ram and frozen on dry ice. Tissue for RNA extraction was placed immediately into 4 mol guanidinium isothiocyanate l⁻¹ containing 0.12 mol β -mercaptoethanol l⁻¹ before it was frozen. All samples of testicular tissue were stored at -70°C. Testicular mass and body weight were recorded at the time of gonadectomy.

Oestradiol antiserum development and passive immunization

The oestradiol antibody used in passive immunization had been developed in gonadectomized adult DLS rams. Three rams were actively immunized against oestradiol-6-(O-carboxymethyl)oxime-BSA conjugate as described by Sanford (1987a) and several litres of antisera were harvested and stored frozen (-20°C). In the present study, individual collections of antisera were thawed, pooled and treated with

charcoal–dextran (10:1 (w/w); 550 mg per 200 ml serum) to remove endogenous steroids. After 6 h of incubation at 4°C, charcoal–dextran was removed from the pooled antiserum by centrifugation (twice at 600 g for 20 min) and passage through progressively smaller millipore filters (0.45 and 0.22 µm). The titre of oestradiol antibody in the pool, defined as the dilution of serum that bound 50% of added [2,4,6,7,16,17-³H]oestradiol (10 700 c.p.m. or 18 pg) (New England Nuclear, Mississauga, ON) in an overnight incubation at 4°C, was 1:4500. The antiserum was specific for oestradiol, crossreacting only slightly with oestrone (15%).

Passively immunized rams were injected with sufficient oestradiol antiserum to develop an antibody titre of about 1:200 within 1 week, as described by Sanford (1987a) and Sanford *et al.* (1991). Antiserum (60 ml) was injected into the jugular vein on day 1 of immunization, and then again every 3–4 days for 3 weeks (total of seven immunizations). The amount of antiserum injected into rams in the breeding season was increased to 120 ml per injection in week 2 to take into account the 50–100% higher oestradiol concentration in peripheral blood at this time of the year (Sanford *et al.*, 1993).

Testosterone binding by oestradiol antiserum

The ability of oestradiol antibody to bind testosterone under *in vivo* conditions (for example, in serum at the low titre established in the passively immunized rams) was evaluated to examine the possibility that any increase in testosterone concentration with immunization was the result of greater retention of testosterone within the blood circulation (Webb *et al.*, 1984) as well as increased secretion (Schanbacher *et al.*, 1987). Negligible crossreaction of the oestradiol antiserum with testosterone (< 2%) under *in vitro* conditions (for example at high antibody dilution in the absence of other steroids) may not hold true *in vivo*. This was evaluated by determining: (i) the percentage binding of labelled testosterone in serum pools representing the first 8 h of blood sampling from immunized and control rams in the breeding season; and (ii) the ability of oestradiol to displace labelled testosterone in these same samples. Binding of [1,2,6,7-³H]testosterone (16 000 c.p.m. or 48 pg) (New England Nuclear, Mississauga, ON) by 100 µl undiluted serum was determined in an overnight incubation at 4°C. Radioactivity in the serum-bound fraction (supernatant) was counted after free and bound fractions were separated by charcoal–dextran. The displacement of testosterone binding by oestradiol was assessed by first incubating 200 µl serum with labelled testosterone overnight at 4°C and removing the free steroid with charcoal–dextran. The supernatant (100 µl) was then incubated overnight at 4°C with 100 µl PBS (1% (w/v) gelatin) containing 3.2, 32.0 or 320.0 ng oestradiol. Free steroid was removed by charcoal–dextran and radioactivity remaining in the supernatant was counted.

Blood hormone assays and pulse analysis

Blood sera obtained during the 10 h collection periods were assayed for LH (Niswender *et al.*, 1969) and

testosterone (Sanford, 1985) using double-antibody radioimmunoassay. The primary antiserum in the LH assay, provided by N. C. Rawlings (University of Saskatchewan, Saskatoon, SK; NCR no. 7) and validated for this assay system (Sanford, 1987a), was used at the initial dilution of 1:80 000. The reference preparation was NIH-LH-S24. The minimum detectable concentrations of LH and testosterone with assay at the serum volumes indicated were 0.08 ng ml⁻¹ (200 µl) and 0.45 ng ml⁻¹ (20 µl), respectively. Intra- and interassay coefficients of variation were all less than 12%.

Patterns of episodic LH and testosterone secretion were characterized from hormone concentrations obtained over the first 8 h of serial blood collection (Sanford *et al.*, 1993). Briefly, increases in concentration were identified as pulses when: (i) peak values occurred within 40 (LH) or 60 (testosterone) min of the preceding basal value; (ii) the increase exceeded twice the minimum detectable concentration of the assay; and (iii) the increase was greater than two standard deviations associated with duplicate estimates of the preceding basal value. Pulse frequency is the total number of pulses occurring over the 8 h period, and pulse amplitude is the mean of the differences between peak and preceding basal concentrations. Basal (interpulse) concentration is the mean of all values not associated with a pulse or pulses. The total testosterone response to LH injection was determined by measuring the area under the 2 h response curve above concentration at time 0.

Testicular steroidogenic enzyme activities

The radiolabelled steroids [7-³H]pregnenolone (25.0 Ci mmol⁻¹), [1,2,6,7-³H]progesterone (109.5 Ci mmol⁻¹), [1,2,6,7-³H]-dehydroepiandrosterone (DHEA) (89.2 Ci mmol⁻¹) and [7-³H]androstenedione (24.5 Ci mmol⁻¹) were obtained from New England Nuclear (Mississauga, ON). The unlabelled carrier steroids pregnenolone, progesterone, 17α-hydroxy-pregnenolone, 17α-hydroxyprogesterone, DHEA, 4-androstenedione, 5-androstene-3β,17β-diol and testosterone were obtained from Steraloids Inc. (Wilton, NH). Nicotinamide cofactors came from Sigma Chemical Co. (St Louis, MO). Organic solvents were obtained from BDH (Montreal, QC) and plastic coated silica gel thin-layer chromatography plates from Merck (Darmstadt). All other chemicals were of reagent grade.

At the time of assay, the testes were thawed and homogenized in a motor-driven Potter-Elvehjem homogenizer in Tris–HCl (50 mmol l⁻¹) buffer, pH 7.5, containing sucrose (0.25 mol l⁻¹), KCl (25 mmol l⁻¹), MgCl₂ (5 mmol l⁻¹) and mercaptoethanol (7 mmol l⁻¹). The homogenate was centrifuged at 10 000 g for 10 min at 4°C (Beckman L7 Ultracentrifuge, Beckman Instruments, Montreal, QC) and the supernatant re-centrifuged under the same conditions to yield a post-mitochondrial supernatant. The microsomal fraction was obtained by centrifuging the post-mitochondrial supernatant at 176 000 g for 1 h at 4°C. Microsomes were resuspended in Tris–HCl buffer, pH 7.5, and used immediately for assessment of steroidogenic enzyme activities. Protein was estimated using the method of Lowry *et al.* (1951).

Radiometric enzyme assays were conducted according to

a modified method of Cooke (1991) and Cooke *et al.* (1998). For 17-hydroxylase activity, aliquants of microsomal fraction were added to Tris-HCl buffer, pH 7.5 (3 ml), containing pregnenolone ($1 \mu\text{mol l}^{-1}$, 40 000 c.p.m. ^3H) ($1 \text{ c.p.m.} = 0.0167 \text{ Bq}$) and NADPH and NAD^+ (both at 0.25 mmol l^{-1}) at 37°C in a reciprocating water bath. After 30, 60 and 90 min, 1.0 ml was withdrawn and transferred to a glass screw-cap test tube containing hexane (5 ml) and carrier steroids (pregnenolone, progesterone, 17α -hydroxypregnenolone, 17α -hydroxyprogesterone, DHEA, 4-androstenedione, 5-androstene- $3\beta,17\beta$ -diol and testosterone; $30 \mu\text{g}$ each). The tubes were vortexed vigorously to terminate the reactions and extract the steroids, and were centrifuged (800 g for 10 min) to achieve phase separation. The organic phase was transferred to conical tubes and evaporated (Savant, Speedvac Plus, Fisher Scientific, Ottawa, ON). The residue was applied to plastic-coated thin-layer plates (Whatman PE SIL G) which were developed in a two-dimensional system to separate the steroids. The first dimension was chloroform-acetone (9:1, v/v) and the second dimension was hexane-ethyl acetate (5.0:3.5, v/v) (Hurden *et al.*, 1984). Carrier steroids were visualized by ultraviolet illumination and exposure to iodine vapour. The regions corresponding to the carrier steroids were excised and quantified by scintillation counting. The combined quantities of radiolabelled 17α -hydroxypregnenolone, 17α -hydroxyprogesterone, DHEA and 4-androstenedione were used to determine $\text{P450}_{17\alpha}$ activity (testosterone and other steroids were produced in negligible quantities). The same methodology was used to determine $\text{P450}_{17\alpha}$ activity using progesterone as the steroid substrate ($1 \mu\text{mol l}^{-1}$, 40 000 c.p.m. ^3H) and NADPH (0.25 mmol l^{-1}) as cofactor (Cooke *et al.*, 1998). 3β -HSD activity was determined by combining the amounts of 4-ene-3-oxosteroids in pregnenolone incubations and was verified in assays using DHEA as the steroid substrate ($1 \mu\text{mol l}^{-1}$, 40 000 c.p.m. ^3H) and NAD^+ (0.25 mmol l^{-1}) as the cofactor, and pH of the incubation was 8.4. The carrier steroids were DHEA, 5-androstene- $3\beta,17\beta$ -diol, 4-androstenedione and testosterone, and steroid separation was achieved using unidimensional thin-layer chromatography (chloroform-acetone, 9:1 (v/v), run once). The only product was 4-androstenedione. Identical methods were used to determine 17β -HSD activity except that 4-androstenedione was the steroid substrate ($1 \mu\text{mol l}^{-1}$, 40 000 c.p.m. ^3H), NADPH (0.25 mmol l^{-1}) was the cofactor, and the pH of the reaction was 7.5. The quantity of testosterone produced was used to determine activity.

Optimal conditions for all enzymes were determined with respect to steroid substrate, cofactor and protein concentrations and linearity of reaction with time. Incubation conditions were chosen that maintained the conversion of a saturating concentration of steroid substrate to less than 15% in accordance with the criteria for enzyme kinetic analysis (Cleland, 1967). Reaction rates were obtained by linear regression analysis ($r > 0.95$).

Testicular steroidogenic enzyme mRNA concentration

Testicular tissue was thawed and homogenized in $4 \text{ mol guanidine isothiocyanate l}^{-1}$ containing 0.12 mol

β -mercaptoethanol l^{-1} . Total RNA was isolated by centrifugation for 21 h at $174\,000 \text{ g}$ on a cushion of $5.7 \text{ mol cesium chloride l}^{-1}$. The pellet was resuspended in $3 \text{ mol sodium acetate l}^{-1}$, precipitated twice with ethanol and quantified by measuring absorbance at 260 nm.

For northern blot analysis, total RNA ($20 \mu\text{g}$) was size fractionated on a 1% (v/v) formaldehyde-agarose gel. The RNA was transferred overnight by capillary action onto a nylon membrane (Hybond-N Nylon, $0.45 \mu\text{m}$, cat. RPN.1510N; Amersham, Oakville, ON) which was subsequently cross-linked in a commercial UV chamber (Bio Rad, Mississauga, ON). Membranes were incubated for 2 h in pre-hybridization solution containing 10% (w/v) dextran sulfate, $5 \times \text{saline-sodium phosphate-EDTA (SPPE)}$, $5 \times \text{Denhardt's solution}$, 0.5% (w/v) SDS and herring sperm DNA (200 mg ml^{-1}). Hybridization with labelled cDNA probes was performed overnight at 65°C . After hybridization, membranes were washed in $2 \times \text{SSPE-0.1\% (w/v) SDS}$ twice at room temperature and twice at 65°C . The labelled membranes were exposed to Kodak X-Omat film (Kodak Canada Inc., Toronto, ON) at -70°C in the presence of an intensifying screen for 1–5 days. Membranes were stripped and rehybridized to a labelled 28S ribosomal cDNA probe as internal control. The relative abundance of signal was quantified by densitometry (Collage Analysis; Fotodyne Inc., Hartland, WI). The density of hybridization signals was corrected for loading using hybridization to 28S, and these corrected data were expressed relative to a control ram sample that was included in the two blots required.

mRNA concentrations were determined for P450_{scc} (activity not measured) and $\text{P450}_{17\alpha}$ (activity differed with season). The bovine cDNA probes for P450_{scc} (1.7 kb insert) and $\text{P450}_{17\alpha}$ (2 kb insert) were a gift from Dr M. R. Waterman (Vanderbilt University School of Medicine, Nashville, TN). These cDNA contain the complete coding sequences for each enzyme (John *et al.*, 1984; Zuber *et al.*, 1986). The probes were labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ by random primer extension (Boehringer Mannheim, Laval, QC) to a specific activity of $1.5\text{--}3.0 \times 10^9 \text{ d.p.m. } \mu\text{g}^{-1}$. Labelled probes were purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification (Promega, Ottawa, ON).

Blood cholesterol and testicular LDL-R and StAR mRNA

Total cholesterol concentration in peripheral blood (8 h serum pools) of rams was measured with the commercially prepared A-Gent[®] cholesterol test kit (Abbott Laboratories, Montreal, QC). Combined very low and low density lipoprotein (VLDL and LDL) and high density lipoprotein (HDL) fractions were quantified specifically by measuring cholesterol content of serum HDL after the VLDL and LDL fraction was precipitated with A-Gent[®] HDL reagent. The difference between total and HDL cholesterol concentrations was taken as VLDL and LDL concentration.

Testicular mRNA concentrations for the LDL-R and StAR protein were assessed by slot blot analysis, loading approximately $6 \mu\text{g}$ sample per well. The bovine LDL-R cDNA was generated in this laboratory (Soumano and Price, 1997) and corresponds to +643 to +792 (from the start codon)

Table 1. Parameters of episodic LH and testosterone secretion in control and oestradiol-immunized rams on day 21

Hormonal parameter	Non-breeding season		Breeding season	
	Control rams	Immunized rams	Control rams	Immunized rams
LH				
Mean concentration (ng ml ⁻¹)	0.29 ± 0.14 ^a	0.17 ± 0.04 ^a	0.83 ± 0.10 ^b	0.88 ± 0.13 ^b
Basal concentration (ng ml ⁻¹)	0.23 ± 0.10 ^a	0.15 ± 0.02 ^a	0.51 ± 0.05 ^b	0.56 ± 0.12 ^b
Pulse frequency (number per 8 h)	0.7 ± 0.7 ^a	0.3 ± 0.3 ^a	3.3 ± 0.3 ^b	3.3 ± 0.3 ^b
Pulse amplitude (ng ml ⁻¹)	0.62 (1)	0.70 (1)	1.04 ± 0.24 ^a	1.08 ± 0.05 ^a
Testosterone				
Mean concentration (ng ml ⁻¹)	1.9 ± 0.6 ^a	2.8 ± 1.1 ^a	11.5 ± 0.9 ^b	20.1 ± 2.1 ^c
Basal concentration (ng ml ⁻¹)	0.9 ± 0.2 ^a	1.9 ± 0.3 ^b	5.7 ± 0.9 ^c	10.3 ± 1.1 ^d
Pulse frequency (number per 8 h)	0.7 ± 0.7 ^a	0.3 ± 0.3 ^a	3.8 ± 0.5 ^b	3.5 ± 0.5 ^b
Pulse amplitude (ng ml ⁻¹)	5.3 (1)	14.7 (1)	14.2 ± 1.9 ^a	24.6 ± 3.4 ^b

Blood samples were taken from the jugular vein every 20 min for 8 h.

Data represent the mean ± SEM of three (non-breeding, control) or four rams, unless indicated otherwise in parentheses.

^{abcd}Within rows, means with different superscripts are significantly different ($P < 0.05$).

of the bovine sequence reported by Russell *et al.* (1984). The full-length cDNA encoding mouse StAR (Clark *et al.*, 1994) was provided by D. Stocco (Texas Tech University, Health Sciences Center, Lubbock, TX).

Statistical analyses

The statistical significance of season and immunization differences was determined by two-way ANOVA (SAS, 1989). When main effects or the season × immunization interaction were significant ($P < 0.05$), group means were compared using the predicted difference option of least-squares means. When appropriate, a logarithmic transformation was applied to the data for analysis to normalize variances between treatment groups. Data from one of the control rams (non-breeding season) were excluded from these analyses because of abnormally high LH release. Mean and basal LH concentrations and LH pulse frequency in this ram were more than six- and twofold greater than in other rams in the non-breeding and breeding seasons, respectively.

Blood parameters of episodic LH and testosterone secretion were correlated with selected testicular mRNA and enzyme activity measurements by linear regression analysis. Pearson or Spearman (pulse frequency) correlation coefficients were calculated on data combined for all four groups of rams.

Results

Body weight and testicular mass

Body weight and paired testes mass of rams were greater (season, $P < 0.05$) in the breeding season (90.9 ± 2.4 kg, 753 ± 38 g) than in the non-breeding season (75.7 ± 3.5 kg, 586 ± 43 g). However, testes mass as a percentage of body weight was the same in both seasons (breeding season,

0.83 ± 0.04%; non-breeding season, 0.78 ± 0.06%). None of these measurements was affected by oestradiol immunization.

Blood parameters of episodic LH and testosterone secretion

Mean and basal LH concentrations and LH pulse frequency in rams were three- to sixfold higher (season, $P < 0.01$) in the breeding season than in the non-breeding season. Oestradiol immunization did not influence any of the parameters of LH release in either season (Table 1). Parameters of testosterone secretion were also greater (season, $P < 0.05$) in the breeding season and, with the exception of pulse frequency, were increased (immunization, $P < 0.05$) by oestradiol immunization. However, increases in mean testosterone concentration in immunized versus control rams were apparent only in the breeding season (season × immunization, $P < 0.05$) (Table 1).

Total testosterone response to LH injection was more than 2.5-fold greater (season, $P < 0.01$) in the breeding season than in the non-breeding season. Total response in immunized rams was twice that (immunization, $P < 0.01$) in control rams in both seasons (Fig. 1c), mainly due to greater increments in testosterone concentration at 40, 60 and 80 min (Fig. 1a,b).

Testosterone binding by oestradiol antiserum

A near doubling ($P < 0.01$) in mean testosterone concentration with oestradiol immunization in the breeding season (control rams, 11.5 ± 0.9 ng ml⁻¹; immunized rams, 20.1 ± 2.1 ng ml⁻¹) was not associated with greater binding of testosterone tracer in serum of immunized rams (67.8 ± 0.6% versus 62.0 ± 2.3% for controls). Tritiated testosterone was not displaced from serum by the addition of 3.2 ng oestradiol, but 32 ng oestradiol displaced 35.6 ± 1.9% and 25.3 ± 8.2% of the tracer bound to control and immune sera, respectively. Addition of 320 ng oestradiol displaced

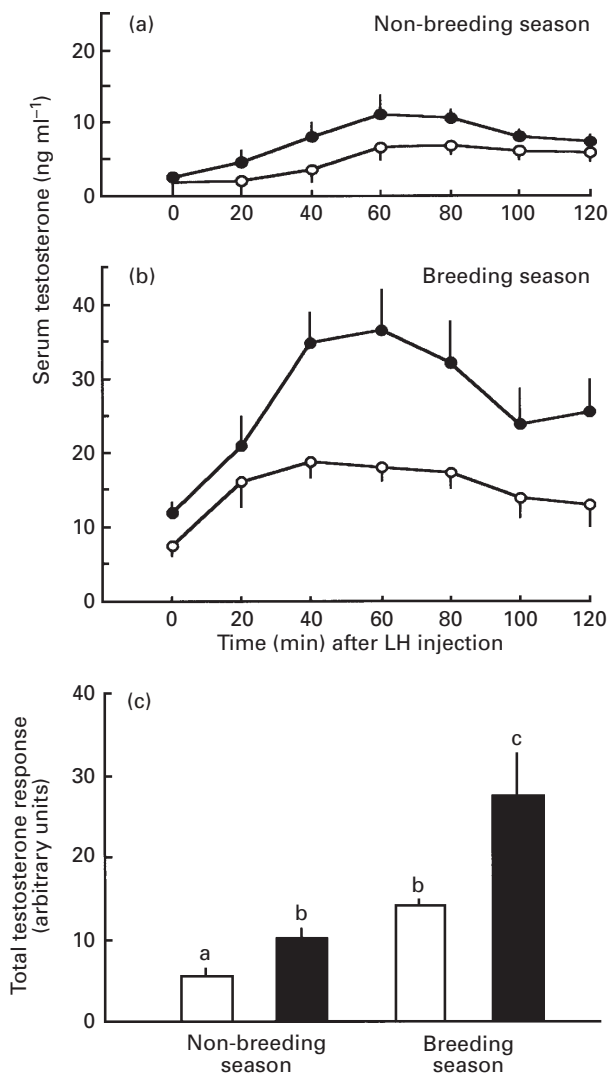


Fig. 1. Serum testosterone concentrations at 20 min intervals (a,b) and total testosterone response (area under curve above time 0 concentration) (c) for control (○, □) and oestradiol-immunized (●, ■) rams given an i.v. injection of 15 µg NIH-LH-S25 (day 21) in the non-breeding and breeding seasons. Means (\pm SEM) represent three (controls, non-breeding season) or four rams. Different letters indicate significant ($P < 0.05$) differences in total testosterone response between groups.

$68.7 \pm 5.3\%$ and $77.0 \pm 0.8\%$ of tracer bound to control and immune sera, respectively. Differences between treatment groups were not statistically significant (Student's *t* test).

Activity of testicular steroidogenic enzymes

Substrate utilization established that pregnenolone metabolism by ram testis microsomes proceeded predominantly through the 5-ene route; 40–50% of the pregnenolone appeared as 17α -hydroxypregnenolone and DHEA. Less than 5% of the pregnenolone was metabolized through the 4-ene route to progesterone and 17α -

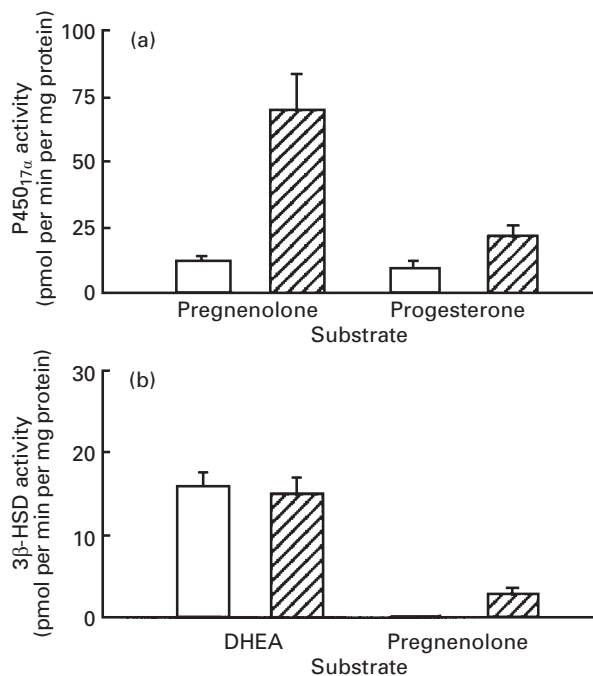


Fig. 2. Activity of P450_{17α} with pregnenolone or progesterone as substrate (a) and of 3β-hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3β-HSD) with dehydroepiandrosterone (DHEA) or pregnenolone as substrate (b) in testicular tissue taken from control and oestradiol-immunized rams (day 23). Means (\pm SEM) represent seven (□, non-breeding season) or eight (▨, breeding season) rams.

hydroxyprogesterone. When progesterone rather than pregnenolone was supplied as substrate, the activity of P450_{17α} was reduced by 30% and 70% in the non-breeding and breeding seasons, respectively (Fig. 2a). Activity of 3β-HSD with pregnenolone rather than DHEA as substrate was undetectable in the non-breeding season and was only 20% of the activity with DHEA in the breeding season (Fig. 2b).

Activity of P450_{17α} (pregnenolone as substrate) was more than fivefold greater (season, $P < 0.01$) in the breeding season than in the non-breeding season (Fig. 3a). When activity was expressed as pmol per min per g tissue or per testis rather than mg microsomal protein, P450_{17α} activity was still fourfold greater (season, $P < 0.01$) in the breeding season. In contrast, the activity of 3β-HSD (DHEA as substrate) expressed either on a protein or testis basis was unchanged with season (Fig. 3b). When expressed as per g tissue, 3β-HSD activity was about 35% lower (season, $P < 0.05$) in the breeding season (102 ± 14 pmol min⁻¹) than in the non-breeding season (164 ± 21 pmol min⁻¹), as was the concentration of microsomal protein. The activity of 17β-HSD did not differ between seasons (Fig. 3c). Immunization against oestradiol did not significantly affect the activity of any of the steroidogenic enzymes examined, although 17β-HSD activity expressed on a protein basis tended to be higher in immunized rams (57 ± 7 pmol min⁻¹) than in controls (39 ± 6 pmol min⁻¹). A similar trend was observed when 17β-HSD activity was expressed on a per g tissue or testis basis.

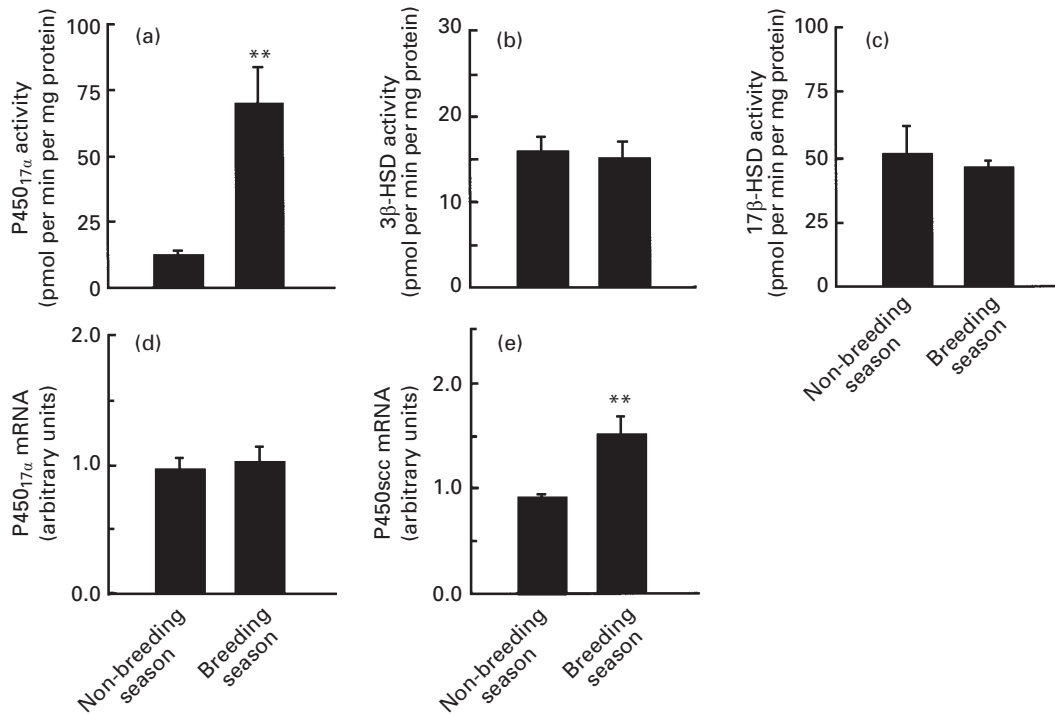


Fig. 3. Activity of P450_{17α} with pregnenolone as substrate (a), 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴ isomerase (3β-HSD) with dehydroepiandrosterone (DHEA) as substrate (b) and 17β-hydroxysteroid dehydrogenase (17β-HSD) with androstenedione as substrate (c), and relative amount of mRNA of P450_{17α} (d) and P450_{sc} (e) in testicular tissue taken from control and oestradiol-immunized rams (day 23). Estimates of mRNA abundance were corrected for loading (pixel density of target probe divided by pixel density of 28S rRNA probe) and are expressed relative to a control sample included in all blots. Means (± SEM) represent seven (non-breeding season) or eight (breeding season) rams. Asterisks indicate significant (***P* < 0.01) differences between seasons.

mRNA abundance for testicular steroidogenic enzymes

The relative amount of P450_{sc} mRNA (Fig. 3e) in ram testes was 65% higher (season, *P* < 0.05) in the breeding season than in the non-breeding season. The relative abundance of P450_{17α} (Fig. 3d) was not different between seasons. Oestradiol immunization did not affect the amount of mRNA for either of these enzymes.

Blood cholesterol concentrations and testicular mRNA abundance for LDL-R and StAR

Serum concentrations of total cholesterol in rams averaged 52.3 ± 3.0 mg dl⁻¹, of which slightly more (Student's *t*-test, *P* < 0.05) was in the form of VLDL and LDL (28.7 ± 2.1 mg dl⁻¹, 54%) than HDL (23.5 ± 1.2 mg dl⁻¹, 46%). Neither combined VLDL and LDL nor HDL were affected by season (Fig. 4a,b) or immunization. The same was true for relative amount of mRNA for the LDL-R in the testis (Fig. 4c). However, the abundance of StAR mRNA was affected by both season and immunization; it was 65% higher (season, *P* < 0.01) in the breeding season compared with the non-breeding season (Fig. 4d), and was 20% greater (immunization, *P* < 0.01) in immunized rams than in controls in both seasons (for example breeding season, 1.05 ± 0.04 arbitrary units versus 0.87 ± 0.06 arbitrary units for controls).

Correlation of blood hormone with testicular protein measurements

Three parameters of episodic LH release (mean and basal concentrations, and pulse frequency) were consistently correlated with testicular mRNA or activity of StAR, P450_{sc} and P450_{17α} (Table 2). The correlations of LH parameters with P450_{17α} activity were all highly significant (*r* = 0.81–0.90, *P* < 0.001). StAR mRNA and P450_{17α} activity were, in turn, correlated with parameters of episodic testosterone secretion (mean, basal and stimulated concentrations) (Table 3). Most of these correlations were highly significant (*r* = 0.82–0.86, *P* < 0.001).

Discussion

This is the first study to demonstrate that increases in testosterone secretion in adult rams in the breeding season are associated with increases in key proteins in the testes which are known to be rate-limiting or highly regulated by LH. Substantial increases in StAR and P450_{sc} mRNA and in P450_{17α} activity may all contribute towards maintaining increased testosterone biosynthesis. Although these results provide some explanation for the seasonal rise in testosterone concentration (for example mean increase of 9.6 ng ml⁻¹ in control rams) the reasons for the comparable LH-

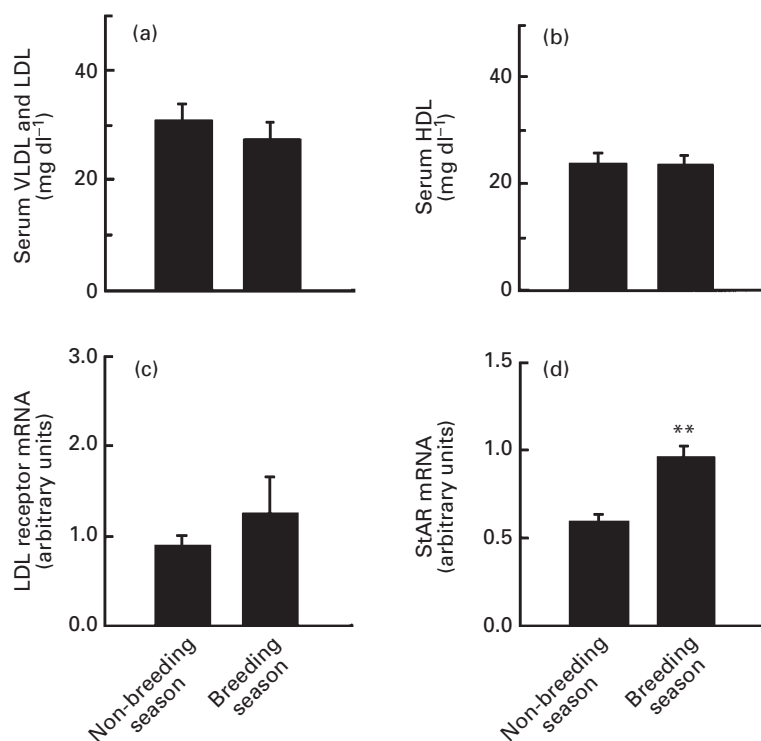


Fig. 4. Serum concentrations of combined very low and low density lipoprotein (VLDL and LDL) (a) and high density lipoprotein (HDL) (b) (day 21), and relative abundance of mRNA of the LDL receptor (c) and steroidogenic acute regulatory protein (StAR) (d) in testicular tissue (day 23) for control and oestradiol-immunized rams. Estimates of mRNA abundance were corrected for loading (pixel density of target probe divided by pixel density of 28S rRNA probe) and are expressed relative to a control sample included in all blots. Means (\pm SEM) represent six or seven (non-breeding season) or eight (breeding season) rams. Asterisks indicate significant (** $P < 0.01$) differences between seasons.

Table 2. Correlation (r) between parameters of episodic LH release and testicular steroidogenic acute regulatory protein (StAR) and steroidogenic enzyme mRNA concentration and enzyme activity in rams^a

LH parameter	StAR mRNA	Enzyme RNA		Enzyme activity		
		P450 _{scc}	P450 _{17α}	P450 _{17α}	3 β -HSD	17 β -HSD
Mean ^b	0.73**	0.70**	0.07	0.82***	0.08	0.24
Basal ^c	0.71**	0.70**	0.08	0.81***	0.11	0.24
Frequency ^d	0.76**	0.81***	0.01	0.90***	-0.04	0.02
Amplitude ^e	0.44	0.33	0.05	0.43	0.12	-0.33

^aPearson or Spearman (pulse frequency) correlation coefficients representing the four groups of rams; asterisks indicate levels of significance (** $P < 0.01$, *** $P < 0.001$).

^bMean LH concentration over the 8 h sampling period.

^cInterpulse LH concentration with blood sampling every 20 min.

^dNumber of LH pulses per 8 h.

^ePeak LH concentration minus preceding nadir.

3 β -HSD: 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase; 17 β -HSD: 17 β -hydroxysteroid dehydrogenase.

independent increases in testosterone with oestradiol immunization (for example mean increase of 8.6 ng ml⁻¹ in the breeding season) remain obscure. Increases in StAR mRNA and 17 β -HSD activity were small and probably do not explain fully how low-level oestradiol immunization led to increased testosterone secretion in rams. It is concluded that other as yet unidentified oestradiol actions within the testis may also be involved.

As the testes of adult rams undergo recrudescence in the summer, the volume and possibly number of Leydig cells increase (Garnier *et al.*, 1981; Hochereau-de Reviers *et al.*, 1985, 1992). Increased blood testosterone concentration in the breeding season relates to a greater total volume of Leydig cells per testis and cross-sectional area of Leydig cells (Hochereau-de Reviers *et al.*, 1985). In seasonally breeding males, increased steroidogenic capacity of Leydig cells in

Table 3. Correlation (*r*) between parameters of testosterone synthesis and testicular steroidogenic acute regulatory protein (StAR) mRNA concentration and steroidogenic enzyme activities in rams

Testosterone parameter	StAR mRNA	Enzyme activity		
		P450 _{17α}	3β-HSD	17β-HSD
Mean ^b	0.86***	0.82***	0.22	0.16
Basal ^c	0.85***	0.82***	0.16	0.40
Stimulated ^d	0.84***	0.74**	0.15	0.12

^aPearson correlation coefficients representing the four groups of rams; asterisks indicate levels of significance (***P* < 0.01, ****P* < 0.001).

^bMean testosterone concentration over the 8 h sampling period.

^cInterpulse testosterone concentration with blood sampling every 20 min.

^dArea under the 2 h testosterone response curve (above time 0 concentration) to a single physiological injection of NIH-LH-S25. 3β-HSD: 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴ isomerase; 17β-HSD: 17β-hydroxysteroid dehydrogenase.

reactivated testes is associated with more highly developed mitochondria and smooth endoplasmic reticulum (Lincoln, 1989), which in rams can be as much as three times more abundant (Mortimer and Lincoln, 1982). In the present study, testosterone mean concentration and total response to LH injection were more than six- and 2.5-fold greater in the breeding season than in the non-breeding season. It was confirmed that, as in males of closely related ruminants such as deer (Bilinska, 1985) and goats (Mori *et al.*, 1980), testosterone biosynthesis involves the 5-ene pathway (Fields *et al.*, 1980), which predominates irrespective of season.

Episodic testosterone secretion in rams is tightly coupled to episodic LH release (Pelletier *et al.*, 1982; Lincoln, 1989). Evidence indicates that increases in LH pulse frequency are largely responsible for the seasonal increases in testosterone secretion. Monthly changes in LH pulse frequency and mean testosterone concentration are highly correlated (Sanford *et al.*, 1977), and experimentally increasing LH pulse frequency during testicular recrudescence increases blood testosterone concentration by 3.5-fold within days (Sanford and Baker, 1990; Melnyk *et al.*, 1992). Increases in basal (interpulse) LH concentration (Chase *et al.*, 1988) and in the number of testicular LH receptors (Barenton and Pelletier, 1983) likely combine with increases in LH pulse frequency to increase the steroidogenic capacity and responsiveness of Leydig cells to LH in the breeding season. In rams in the present study, seasonal increases in LH release (pulse frequency, mean and basal concentrations) were strongly related to corresponding increases in P450_{17α} activity and to a lesser extent in the amount of P450_{17α} mRNA in the testis. Although LH is necessary for expression of all four enzymes involved in testosterone biosynthesis, only P450_{17α} expression in mice (Payne and Youngblood, 1995) and P450_{scc} and P450_{17α} expression in rats (Saez, 1994) are highly dependent upon LH. Whether there is a causal relationship between specific parameters of episodic LH release and the expression of these enzymes in rams, as implied by the results of the present study, remains to be determined.

Blood parameters of episodic testosterone secretion (mean and basal concentrations) and the testosterone response to an LH challenge were highly correlated with two key regulatory proteins in the testis, StAR (mRNA concentration) and P450_{17α} (activity). In most biological systems investigated, StAR facilitates the delivery of cholesterol from

the outer to the inner mitochondrial membrane and the P450_{scc} enzyme, enabling greater conversion of cholesterol to pregnenolone (Stocco, 1997, 1998). Continuously high trophic stimulation of the testes of rams in the breeding season was associated with a 65% higher steady-state concentration of StAR mRNA, which presumably led to an increase in the synthesis of StAR protein since transcriptional and translational activity are normally tightly coupled (Stocco, 1997, 1998). In the present study, the several-fold increases in testicular steroidogenesis with season would not have been possible without greater *de novo* synthesis of StAR, known to be the rate-limiting step in LH-induced testosterone production (Stocco, 1997, 1998). However, it should be noted that StAR transcription and translation occasionally can be dissociated (Clark and Stocco, 1997) and that 10–20% of the steroidogenic potential of Leydig cells may be StAR independent (Clark *et al.*, 1997). Testicular proteins other than StAR may also be involved in regulating cholesterol transport (Stocco, 1998). Thus, precise relationships among StAR mRNA and protein expression and testosterone production need to be established in rams, as does the significance of the 65% seasonal increase in P450_{scc} mRNA; the activity of P450_{scc} was not measured.

The second testicular protein that was highly correlated with blood testosterone parameters was P450_{17α}. The activity of this enzyme was more than fivefold higher in the breeding season than in the non-breeding season. This enzyme plays a key regulatory role in species in which the 5-ene pathway predominates by competing with 3β-HSD and directing pregnenolone metabolism towards DHEA (Conley and Bird, 1997). P450_{17α} is highly responsive to LH, at least in rodent species (Saez, 1994; Payne and Youngblood, 1995) and thus might also be expected to play an important role in regulating the seasonal changes in testosterone biosynthesis as the trophic stimulation to Leydig cells changes. The results of the present study indicate that this may be true for rams since the activity of P450_{17α} increased in proportion to mean testosterone concentration in the breeding season whereas the activities of 3β-HSD and 17β-HSD remained the same. These observations also indicate that the last two enzymes in the biosynthetic pathway were not rate limiting. Alterations in testosterone metabolism within the testes were probably not responsible for differences in blood testosterone concentrations between groups of rams since concentrations of

testosterone 5 α -reductase and cytochrome P450 aromatase were very low (both were undetectable, data not presented). Finally, greater activity of P450_{17 α} in the breeding season was not associated with a simultaneous increase in mRNA transcript for this enzyme, perhaps because of differences in the half-life of enzyme protein and mRNA or because other mechanisms were directly regulating enzyme activity *per se*.

Low-level oestradiol immunoneutralization for 3 weeks produced increases in various parameters of episodic testosterone secretion in rams which, in the breeding season, were equivalent in value to seasonal increases in controls. Differences in blood testosterone concentration between immunized and control rams most likely reflect differences in testosterone secretion. Schanbacher *et al.* (1987) showed that when rams are actively immunized against oestradiol, testosterone metabolic clearance rate does not change and tenfold increases in testosterone mean concentration are in proportion to the estimated daily production rate. The results of the present study demonstrate that twofold increases in testosterone at much lower titres of oestradiol antibody are not an artefact of antiserum administration, since neither the capacity of serum to bind labelled testosterone nor the displacement of serum-bound testosterone by oestradiol increased with immunization.

Increases in testosterone secretion in immunized rams occurred independently of increases in any of the parameters of episodic LH release, as has been observed with low titres (approximately 1:100–200) of oestradiol antibody developed and maintained over 2–4 weeks (Sanford, 1985, 1987b, 1989; Sanford *et al.*, 1991). In the absence of a change in trophic stimulation, a reasonable explanation for the testosterone increases with immunization is that there was a reduction in oestradiol negative feedback action within the testes. Oestradiol antibody could easily have penetrated post-capillary venules and entered interstitial fluid (Bergh and Damber, 1993) and subsequently bound a portion of the 'free' available oestradiol diffusing in either from Leydig cells (Bilinska *et al.*, 1997) or Sertoli cells (Bardin *et al.*, 1994). The end result may have been a reduction in effective oestradiol concentration (Hillier *et al.*, 1975; Wickings and Nieschlag, 1978) in the vicinity of Leydig cells.

Oestradiol immunization was associated with a 20% increase in the abundance of StAR mRNA. Northern blot analysis of StAR transcripts in mouse Leydig tumour cells stimulated with hCG showed that relatively small increases in StAR transcription can be meaningful physiologically (Manna *et al.*, 1999). However, it is difficult to see how StAR requirement for the near doubling of testosterone secretion in immunized rams in the breeding season could have been met by such a small increase in transcription, unless reducing oestradiol action in the testis resulted in more complete StAR translation, which is proposed to be differentially regulated (Clark and Stocco, 1997). It has been reported that oestradiol stimulates rather than depresses StAR expression in the rabbit corpus luteum (Townson *et al.*, 1996) and in pig granulosa cells (Chedrese *et al.*, 1996). The only other protein change in the testes of immunized rams was a relatively small increase in 17 β -HSD activity, which may have facilitated testosterone biosynthesis by increasing androstenedione metabolism. It is possible that oestrogen

affected P450_{17 α} and 3 β -HSD, but that these effects were negated by induction of a testosterone autoregulation mechanism which, as in mice (Payne and Youngblood, 1995), limits the synthesis or activities of these enzymes at high testosterone concentrations.

Season and oestradiol immunization did not affect blood cholesterol lipoprotein concentrations or testicular LDL-R mRNA. In the present study, cholesterol was fairly evenly distributed between combined VLDL and LDL (54%) and HDL (46%) fractions. This is in contrast to previous reports showing that much more of the plasma cholesterol in sheep is in either the LDL or the HDL fraction (Vitic and Stevanovic, 1993; Meyer *et al.*, 1996). Both LDL and HDL support steroidogenesis in ovine adrenal and luteal cells *in vitro* (Durand *et al.*, 1987; Rainey *et al.*, 1988; Wiltbank *et al.*, 1990) and in Leydig cells in rodent species (Saez, 1994). However, steroidogenic activity is not correlated with LDL-R or HDL binding protein in corpora lutea in ewes (Tandeski *et al.*, 1996) or with blood lipoprotein concentrations or testicular LDL-R mRNA in rams (present study). These observations indicate that changes in lipoprotein receptor dynamics were not part of the mechanism or mechanisms regulating gonadal steroidogenesis in the present study.

The correlative results of the present study strongly imply that in the testes of adult rams: (i) StAR and P450_{17 α} may have central roles in regulating the LH-driven seasonal increases in testosterone biosynthesis; and (ii) that StAR and 17 β -HSD may help facilitate LH-independent increases in testosterone secretion with oestradiol immunization. Other intratesticular regulatory factors affected by oestradiol, possibly from Sertoli cells or germ cells, which in rats contain oestrogen receptor β (Saunders *et al.*, 1998; van Pelt *et al.*, 1999), may also be involved. These hypotheses remain to be tested.

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