Anti-Müllerian hormone (AMH) secretion in prepubertal and adult rams

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The aim of the present analysis was to determine whether anti-Müllerian hormone concentrations in prepubertal plasma or adult rete testis fluid are related to the number or function of Sertoli cells in rams or to the presence of the *Fec^B* Booroola gene. Twenty rams from two Booroola crosses, differing in their testicular masses were analysed; in each cross, half of the animals were heterozygous carriers of the *Fec^B* gene. The data from rams, during prepuberty and at adulthood during the non-sexual season, were analysed by two-way ANOVA and residual correlations. In 4-week-old intact male lambs, the mean anti-Müllerian hormone plasma concentration was 15 ng ml⁻¹, irrespective of cross, genotype or eCG stimulation; it was significantly negatively correlated with FSH ($r = -0.51$; $P = 0.02$; $n = 19$). In adults, anti-Müllerian hormone was not detectable in plasma and was 0.5 ng ml⁻¹ in rete testis fluid, irrespective of cross or genotype. The total number of Sertoli cells per testis was not related to anti-Müllerian hormone concentration in lamb prepubertal plasma or in adult rete testis fluid. The concentration of anti-Müllerian hormone in adult rete testis fluid was significantly and negatively correlated with the daily production of leptotene primary spermatocytes per testis ($r = -0.56$; $P = 0.02$; $n = 16$). The mean oestrogen concentration in the adult testicular vein was 2 pg ml⁻¹ and was correlated negatively with the rete testis fluid concentration of anti-Müllerian hormone ($r = -0.60$; $P = 0.02$; $n = 15$) and correlated positively with the daily production of leptotene primary spermatocytes per testis ($r = 0.53$; $P < 0.05$; $n = 19$). In conclusion, anti-Müllerian hormone secretion was not correlated with the total numbers of Sertoli cells per testis and cannot be used as a predictor of the number of Sertoli cells. Anti-Müllerian hormone secretions were not affected by the presence of *Fec^B* gene. However, anti-Müllerian hormone secretion could be considered to be inversely related to the daily production of primary spermatocytes by the testis.

Introduction

The fetal male gonad secretes a protein that inhibits the formation of Müllerian ducts in males (Jost, 1947). Anti-Müllerian hormone (AMH or Müllerian inhibiting substance or factor) is secreted by the fetal Sertoli cells surrounding the gonocytes (Tran *et al.*, 1981, 1987) but the hormonal and local control of its secretion has yet to be clarified. The mRNA encoding AMH is regulated by dibutyryl cAMP but not by FSH or hCG in cultured human fetal testicular cells (Voutilainen and Miller, 1987). Similarly, secretion of the AMH by bovine Sertoli cells in primary culture is not modified by FSH or testosterone (Vigier *et al.*, 1985). In contrast, in rat fetal or new-born testes *in vivo*, mRNA transcription of AMH is decreased by FSH treatment (Kuroda *et al.*, 1990) and cleavage

or dissociation of AMH is increased by testosterone (Kuroda *et al.*, 1991). Moreover, in boys presenting normal or precocious pubertal development, an inverse relationship between AMH and testosterone serum concentrations has been observed (Rey *et al.*, 1993). However, in a longitudinal comparative analysis of AMH, testis volume and hormonal plasma concentrations throughout puberty, no such relationship was observed (Hudson *et al.*, 1990). Furthermore, Baker and Hutson (1993) hypothesized a positive control of AMH on the transformation from gonocytes to spermatogonia.

In females, the *Fec^B* Booroola gene is known to affect litter size, ovulation rate, the pattern of follicular development and the onset of ovogenesis in the fetal ovary (McNatty *et al.*, 1990, 1995). The role of the *Fec^B* gene in the synthesis of oestrogen and on plasma concentrations of FSH in females is well documented. During the prepubertal period in males, the presence of a transient increase in LH and FSH plasma

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concentrations in heterozygous carriers of the *Fec^B* gene was observed by Seck *et al.* (1988) but remains controversial. In adult rams, no obvious differences in hormonal secretions or in somatic or germ cell compositions of the testis were recorded in carriers of the *Fec^B* gene (Hochereau-de Reviers and Seck, 1990). A role of an oestrogen-related mechanism, transiently expressed in males, could be suspected in the *Fec^B* gene; because of the control of AMH synthesis by oestrogen (Guerrier *et al.*, 1990), AMH secretion could be involved in *Fec^B* gene expression.

In adult males, AMH has been detected at low concentrations in the rete testis fluid in boars (Josso *et al.*, 1979) and bulls (Vigier *et al.*, 1983). However, the relationships between its secretion by the Sertoli cells and the number of Sertoli cells (Rey *et al.*, 1996) and spermatogenesis are unclear (Baarends *et al.*, 1995). Therefore, in the present study, the relationships between AMH, LH, FSH, oestrogen and testosterone secretions were explored before and after puberty in rams. Furthermore, the relationship between secretion of AMH and spermatogenesis was analysed in adult rams. This analysis was performed on males from two Booroola Merino crosses (Booroola $\times$ Mérimos d'Arles and Booroola $\times$ Romanov), which differed in the precocity of the onset of their testicular growth and in their mean adult testicular masses (Hochereau-de Reviers and Seck, 1990), to determine whether AMH secretion differed with the presence of *Fec^B* gene. In each cross, half of the males were carriers and half were non-carriers of the *Fec^B* gene but no difference in sperm production with the presence of the *Fec^B* gene was observed (Hochereau-de Reviers and Seck, 1990).

Materials and Methods

Materials

Purified ovine LH (YC 1086 or 1051: 1 mg of this preparation is equivalent to 2.1 mg of LH-NIH-S1) was kindly provided by Y. Combarous (URA 1291, PRMD, Nouzilly). FSH was donated by A. F. Parlow (FSH, NIH I₁, NIADDK NIH, Bethesda ML; assays in prepubertal lambs) and H. Grimek (FSH.HG.225; Madison, WI). 1,2,6,7-[³H]testosterone (88 Ci mmol⁻¹) and 2,4,6,7,16,17-[³H]oestrogen (155 Ci mmol⁻¹) were purchased from the Radiochemical Centre (Amersham, Bucks). Non-labelled steroids were obtained from Steraloids (Wilton, NH). The testosterone antibody was obtained by immunization of rabbits against testosterone coupled with 3-carboxyl-methyl-amine saturated by BSA, kindly provided by G. Picaper (CHR, Orléans). The antibody against oestrogen was provided by J. Saumande and was obtained by immunization of rabbits against 6-ketoestradiol-17 β 6-(O-carboxymethyl) oxime conjugated to BSA. Equine CG (Chronogest) was purchased from Intervet (Boxmeer). Na ¹²⁵I was purchased from Amersham International (Amersham, Bucks). The standard incubation medium of fetal calf testis was kindly provided by B. Vigier (Paris). Antibodies against bovine AMH (278 II and 61 A; Vigier *et al.*, 1985; Legeai *et al.*, 1986) were generous gifts of F. Legeai and B. Vigier. ELISAs were performed in polystyrene plates (Microtest Luxlon[®]; CEB, Angers) and read by a Titertek

multiskan MKII. The horse serum used for dilution of plasma or RTF samples originated from Gibco (Paisley).

Animals

Experiment 1. Blood samples were collected from six normal and four castrated Ile de France lambs on 3 successive days when they were 6 weeks old (three blood samples per animal) to validate the enzyme immunoassay for plasma concentrations of AMH in prepubertal lambs.

Experiment 2. Blood samples were collected from fourteen prepubertal Booroola $\times$ Romanov male lambs (4 weeks old with an average weight of 8 kg) at 14:00 h and 16:00 h on day 0 and at 9:00 h on day 1 to determine the daily variation in plasma concentrations of AMH. In the same lambs, the influence of an increase in gonadotrophin and testosterone plasma concentrations on plasma concentrations of AMH was tested by injection of eCG (80 iu kg⁻¹ of body weight, i.m.) at 10:00 h on day 1. The blood samples were collected at 6 h (day 1), 24 h and 30 h (day 2) after injection and the stimulation of testosterone secretion after these eCG injections was observed 24–48 h after injection (testosterone before injection: 0.5 ng ml⁻¹; 1–3 days after injection: 3 ng ml⁻¹; Hochereau-de Reviers *et al.*, 1990).

Experiment 3. Blood samples were collected from prepubertal male lambs from crosses between Booroola *Fec^B Fec⁺* or *Fec^B Fec^B* rams with ten Mérimos d'Arles ewes (BooMa in Domaine du Merle, ENSAM, 13300 Salon-de-Provence) or ten Romanov ewes (BooRo in Domaine de la Sapinière, INRA, 18390 Osmoy) once a week from 3 to 10 weeks of age. Each cross (BooMa and BooRo) contained five non-carriers *Fec⁺ Fec⁺* and five heterozygous carriers *Fec^B Fec⁺*, respectively. In BooRo lambs, the onset of testis growth was evaluated by comparative palpation and the lambs were weighed twice, at 4 and 6 weeks of age.

In the same BooRo and BooMa animals after puberty (at a similar mean body weight of 55–60 kg), a cannula was inserted under halothane anaesthesia in the rete testis of one testis per ram (Dacheux *et al.*, 1981) during the nonbreeding season, from the end of January to April. Rete testis fluid (RTF) was collected continuously in 16 of the 20 conscious animals; the hourly flow rate of RTF and the concentrations of spermatozoa in RTF were determined; concomitantly, blood samples were collected from animals at 9:00 h, 11:30 h, 14:30 h and 16:00 h. At castration, venous blood was sampled from the testicular vein just before the arrival of the vein in the pampiniform complex. The oestrogen secretion by the testis was estimated by analysis of its concentration in the testicular venous plasma.

Testis analysis

The testes of all BooRo and BooMa adult rams (Expt 3) were weighed and a piece (1–3 cm³) of parenchyma of one testis per ram was fixed in Bouin–Hollande solution. The histological analysis was performed as described by Hochereau-de Reviers *et al.* (1992). Briefly, the relative volume of intertubular tissue and of seminiferous tubules in the testicular parenchyma were

determined with a 25-point integrator on 20 fields per testis at a magnification of $\times 160$; the relative volume of Leydig cells in the intertubular tissue was determined in the same way at a magnification of $\times 800$. The total volumes of seminiferous tubules and of intertubular tissue were then calculated by multiplying their relative volumes in the parenchyma by the total mass of the testis. The mean cross-sectional area was measured on 20 round sections of seminiferous tubules per testis with a camera lucida attached to a microscope and the use of the Tablet Graphic planimeter program (Apple Computers, Cupertino, CA). The total length of the seminiferous tubules was then calculated by division of their total volume by the mean seminiferous cross-sectional area (Attal and Courot, 1963). The cross-sectional areas of 20 Leydig cells and of 20 Sertoli cell nuclei measured at stage 8 (classification of Roosen-Runge and Giesel, 1950) were determined using the same planimeter program at a magnification of $\times 800$. The mean numbers of Sertoli cell nuclei and of A_1 spermatogonia (stages 7–8), of leptotene primary spermatocytes (end of stage 1 and stage 2) and round spermatids (stages 7–8) were counted on ten cross-sections of seminiferous tubules. The mean corrected number of cells per unit length was calculated after correction for nuclear size and section thickness ($10\ \mu\text{m}$) according to the method of Abercrombie (1946). The total numbers of Sertoli cells and germ cells per testis were then obtained by multiplying their number per unit length by the total length of seminiferous tubules per testis. The daily production of germ cells (A_1 to round spermatids) was determined by division of their total number per testis by the mean duration of the seminiferous epithelium cycle in rams (Ortavant, 1959). The total volume of Leydig cells was obtained by multiplying their relative volume in the intertubular tissue by the total volume of intertubular tissue. Their total numbers were obtained by division of their total volume per testis by their mean individual volume, calculated from their mean cross-sectional area, and with the assumption that they were spherical. All values were corrected for shrinkage.

Hormone assays

Plasma concentrations of LH, FSH and testosterone were determined by radioimmunoassays in prepubertal and adult BooMa and BooRo, in two separate assays according to age. LH was assayed by displacement (Pelletier *et al.*, 1982); the detection level (B:Bo = 95%) was $6\ \text{pg ml}^{-1}$ of oLH 1051 or oLH 1086 and the intra-assay coefficient of variation was 10% for B:Bo = 50%. FSH rabbit polyclonal antibody (AS5; Pelletier *et al.*, 1982) was first incubated with oLH (37°C ; 90 min) to eliminate crossreactivity with LH (Blanc and Poirier, 1979). The crossreactivity of FSH antibody with LH after this incubation is 0.02%. The detection concentration of FSH (B:Bo = 95%) was $2\ \text{ng ml}^{-1}$ and the coefficient of variation for B:Bo = 50% was 7%. Plasma concentrations of testosterone were determined by a direct radioimmunoassay (Garnier *et al.*, 1978; modified by Hochereau-de Reviers *et al.*, 1990). The detection level for B:Bo = 95% was $60\ \text{pg ml}^{-1}$ and the coefficient of variation was 18% at $0.3\ \text{ng ml}^{-1}$ and decreased to $< 5\%$ at 3 and $10\ \text{ng ml}^{-1}$. Oestrogens were measured after extraction in 1–2 ml of testicular venous plasma (Saumande, 1981). The detection limit

of the assay was $0.5\ \text{pg ml}^{-1}$ and the coefficient of variation was 12% at $20\ \text{pg ml}^{-1}$ and decreased to 6% at $1\ \text{pg ml}^{-1}$.

Plasma concentrations of AMH were determined with a two-site enzyme immunoassay adapted for ovine AMH, using two different monoclonal IgG1 antibodies, raised against purified bovine AMH described by Vigier *et al.* (1983) and Legeai *et al.* (1986). The monoclonal antibodies, mAb 61 raised against purified bAMH ($K_a = 5.8 \times 10^9$) and mAb 278 raised against impure bAMH ($K_a = 17 \times 10^9$), were used since their respective target epitopes were close to the biological activity site but different from each other. Furthermore, they cross-reacted with AMH contained in fetal lamb sera (Legeai *et al.*, 1982). The coating of polystyrene plates was obtained with $100\ \mu\text{l}$ mAb 278 ($6.15\ \mu\text{g ml}^{-1}$ in Na_2CO_3 , NaHCO_3 $0.2\ \text{mol l}^{-1}$, pH 9.2, for 1 h at 37°C and overnight at 4°C). Saturation of residual binding sites was performed by an incubation in Tris buffer (Tris $0.1\ \text{mol l}^{-1}$, NaCl $0.15\ \text{mol l}^{-1}$, pH 7.5) containing BSA $1\ \text{mg ml}^{-1}$ for 2 h at 37°C . In addition, $100\ \mu\text{l}$ of plasma samples were incubated for 1 h at 37°C and overnight at 4°C ; samples were either diluted (1/100 or 1/50 in PBS buffer containing 5% heat-inactivated horse serum; equine AMH did not crossreact with these mAb; Legeai *et al.*, 1986), while RTF samples were not diluted because of their low protein content ($1\ \text{mg ml}^{-1}$; Dacheux *et al.*, 1981). After washing, the plates were exposed successively to the biotinylated (Josso *et al.*, 1990) mAb 61 ($5\ \mu\text{g ml}^{-1}$ in Tris buffer containing $1\ \text{mg BSA ml}^{-1}$) for 2 h at 37°C , and then to the streptavidin-alkaline phosphatase complex diluted to 1/500 in the same buffer. The colour reaction was obtained by addition of paranitrophenol-phosphate ($1\ \text{mg ml}^{-1}$) in diethanolamine $10\ \text{mmol l}^{-1}$, MgCl_2 $1\ \text{mmol l}^{-1}$, pH 9.5 for 30 min at 37°C : the 15 min absorbance was half that of 30 min of incubation. The reaction was stopped by addition of $50\ \mu\text{l}$ $0.1\ \text{mol glycine l}^{-1}$ to each well. Absorbance was read at 405 nm. Increasing doses of an incubation medium of fetal calf testis containing high concentrations of bAMH were used as standard of calibration for each plate. The regression curves were drawn and did not differ significantly from each other: the mean of 14 slopes was 0.99236 with a SD of 0.0466. All samples were assayed in duplicate. The minimal detection of the assay was $20\ \text{pg ml}^{-1}$ and the intra-assay coefficient of variation at B:Bo = 50% was $< 5\%$.

Statistical analyses

The effects of cross and of genotype intra-cross were analysed by a two-way analysis of variance (GLM procedure; type III SAS system). Residual Pearson correlation coefficients, corrected for the variation due to cross and to genotype intracross, were then calculated (SAS system). In addition, Spearman's rank correlation analysis was performed to analyse the relationships between AMH concentrations and total numbers of Sertoli cell per testis.

Results

The results presented (Tables 1 and 2) relate to cross differences (BooRo and BooMa) as no genotype (Fec^B or $++$) differences were observed, irrespective of age. However, the

Table 1. Mean $\pm$ SD plasma hormone concentrations (ng ml⁻¹) of prepubertal and adult male sheep

Age	Hormone	Crossbreed	
		BooRo	BooMa
1 month	Anti-Müllerian hormone in plasma	14.69 $\pm$ 4.13	12.64 $\pm$ 3.98
	LH	0.89 $\pm$ 0.41 ^a	0.35 $\pm$ 0.36 ^b
	FSH	4.28 $\pm$ 1.64	5.60 $\pm$ 1.33
	Testosterone	1.20 $\pm$ 0.50 ^a	0.67 $\pm$ 0.60 ^b
1.0–2.5 months	LH	0.82 $\pm$ 0.23	0.80 $\pm$ 0.72
	FSH	4.87 $\pm$ 1.18	5.15 $\pm$ 1.07
	Testosterone	1.08 $\pm$ 0.42 ^a	0.62 $\pm$ 0.27 ^b
	LH	1.41 $\pm$ 1.33 ^a	0.40 $\pm$ 0.38 ^b
Adult	FSH	3.12 $\pm$ 0.60	3.30 $\pm$ 1.08
	Testosterone	1.02 $\pm$ 1.04	0.58 $\pm$ 0.82

^{ab}Significantly different means.

genotype was always taken into account for variance analysis and the correlations were always residual ones to exclude any false interpretation. Only correlations related to AMH, oestrogen, gonadotrophins and testosterone, and spermatogenesis were presented.

Anti-Müllerian hormone in peripheral plasma and testis growth of male prepubertal lambs

Experiment 1. AMH was not detected in castrated lamb plasma while in normal prepubertal 4–6 week-old lambs, the mean plasma concentrations of AMH were 15 ng ml⁻¹.

Experiment 2. AMH concentration in the three serial blood samples taken at the same age did not vary significantly in the same animal. The mean coefficient of variation in the same lamb was 17%. There was no significant variation between morning (17.8 $\pm$ 4.5 ng ml⁻¹) and afternoon (17.2 $\pm$ 3.4 ng ml⁻¹) samples; the correlation coefficient between the two values per animal was 0.83 (n = 7).

Exogenous eCG injection, which increased testosterone plasma concentrations threefold, did not significantly modify the AMH concentration at 6–30 h after injection (before eCG: 15.9 $\pm$ 2.8 ng ml⁻¹, CV = 18%; and after eCG: 17.1 $\pm$ 3.7 ng ml⁻¹, CV = 22%).

Experiment 3. In young prepubertal lambs, the mean AMH concentration did not vary with breed or cross: Ile de France: 14.00 $\pm$ 2.6 ng ml⁻¹; BooRo: 14.7 $\pm$ 4.1 ng ml⁻¹ and BooMa: 12.7 $\pm$ 4.0 ng ml⁻¹. At 4 weeks of age, plasma concentrations of AMH did not differ significantly between cross and genotype; however, the mean coefficient of variation between animals, intracross and intragenotype, varied from 22 to 37%. Furthermore, in BooRo lambs, the mean plasma concentrations of AMH decreased by 30% between 4 (17.6 $\pm$ 3.9 ng ml⁻¹) and 5 (11.8 $\pm$ 2.7 ng ml⁻¹) weeks of age, irrespective of the genotype and the onset of testis growth.

Gonadotrophins and testosterone in prepubertal lambs

FSH did not differ significantly between BooRo and BooMa lambs but LH (+ 154%; P < 0.01%) and testosterone (+ 79%;

P = 0.05) were significantly higher in BooRo than in BooMa lambs (Table 1). During the entire period between 4 and 10 weeks of age, BooRo male lambs had higher plasma concentrations of testosterone (+ 74%; P = 0.01; n = 20) than did BooMa lambs. At 5 weeks of age, the onset of testis growth had begun in 5/10 BooRo lambs, irrespective of genotype (mean testis volume = 4.2 $\pm$ 1.1 ml), while it had not taken place in any BooMa lambs (testis volume $\leq$ 3 ml). At 4 weeks of age, AMH and FSH plasma concentrations were significantly negatively correlated (r = - 0.51; P = 0.02; n = 19). The testis volumes of BooRo lambs at 4 weeks of age were significantly related to the mean testosterone plasma values during the entire period between 4 and 10 weeks of age (r = 0.75; P < 0.01; n = 10) and to the body weight (r = 0.92; P < 0.01; n = 10) but not to the plasma concentrations of AMH. However, the variations in plasma concentrations of AMH of prepubertal lambs were not related to their testosterone plasma concentrations at either 4 weeks of age or during the whole period between 4 and 10 weeks of age (n = 19). When plasma concentrations of AMH were compared with total numbers of Sertoli cells per adult testis, no linear or rank correlations were observed.

Anti-Müllerian hormone in adult rete testis fluid and oestrogen in testicular venous plasma of adult rams

AMH was detected in RTF samples but no measurable AMH concentration was observed in the ovine testicular vein or testicular lymph. The mean AMH concentration, measured in three successive non-diluted samples of RTF of the same ram, was below 1 ng ml⁻¹, and the coefficient of variation between samples from the same animal was $\leq$ 20%. Mean AMH concentration did not differ with cross (BooRo: 0.52 $\pm$ 0.37; BooMa: 0.55 $\pm$ 0.38 ng ml⁻¹) or genotype; however, the mean coefficient of variation between rams, intracross and intragenotype, was very high (38–100%). Oestrogen concentrations in the testicular vein were 5 pg ml⁻¹ and did not differ with the cross (BooRo: 5.29 $\pm$ 5.06; BooMa: 5.77 $\pm$ 3.44 pg ml⁻¹) or the genotype; however, the mean coefficient of variation between rams, intracross and intragenotype, was very high (40–85%).

Table 2. Mean $\pm$ SD testicular parameters in adult rams from samples collected during the non-breeding season

Parameter	Crossbreed	
	BooRo	BooMa
Testis weight (g)	165 $\pm$ 31 ^a	211 $\pm$ 42 ^b
Leydig cell cross-sectional area (μm^2)	64 $\pm$ 5 ^a	58 $\pm$ 8 ^b
Number of Leydig cells per testis $\times 10^8$	40.3 $\pm$ 12.0 ^a	73.3 $\pm$ 26.0 ^b
Total length of seminiferous tubule per testis (m)	2199 $\pm$ 375 ^a	2750 $\pm$ 639 ^b
Seminiferous tubule diameter (μm)	226 $\pm$ 12	223 $\pm$ 18
Number of Sertoli cells per testis $\times 10^8$	23.6 $\pm$ 4.1	28.3 $\pm$ 6.7
Sertoli nuclear area (μm^2)	57 $\pm$ 5 ^a	72 $\pm$ 4 ^b
Number of A ₀ spermatogonia per testis $\times 10^7$	2.37 $\pm$ 0.67	2.63 $\pm$ 1.02
Daily production of A ₁ spermatogonia per testis $\times 10^7$	2.64 $\pm$ 0.87	3.08 $\pm$ 0.84
Daily production of leptotene primary spermatocytes $\times 10^8$	6.77 $\pm$ 2.00	8.10 $\pm$ 3.42
Daily production of round spermatids $\times 10^9$	2.03 $\pm$ 0.41	2.28 $\pm$ 0.57
Rete testis fluid flow rate (ml day ⁻¹)	1.17 $\pm$ 0.31 ^a	1.60 $\pm$ 0.36 ^b
Number of spermatozoa in rete testis fluid $\times 10^6$ ml ⁻¹	217 $\pm$ 196	182 $\pm$ 106
Rete testis fluid protein content (mg ml ⁻¹)	1.34 $\pm$ 0.82	1.51 $\pm$ 0.96

^{a,b}Significantly different means.

Gonadotrophins and testosterone in adult rams

In adulthood, during the non-breeding season, BooRo rams had significantly higher (+250%; $P = 0.04$; $n = 18$) plasma concentrations of LH than those of BooMa rams; however, their respective plasma concentrations of FSH and testosterone did not differ significantly (Table 1). The presence of the *Fec^B* gene did not significantly modify the hormonal plasma concentrations.

Testis parameters

BooRo rams exhibited lower mean testis weights (-22%; $P = 0.01$; $n = 20$), total number of Leydig cells (-45%; $P = 0.002$; $n = 10$), total length or total volume of seminiferous tubules (-20%; $P = 0.03$; $n = 20$), total number of Sertoli cells (-17%; $P = 0.07$; $n = 20$) and daily RTF flow rate per testis (-37%; $P = 0.025$; $n = 16$) than BooMa rams (Table 2), irrespective of genotype.

The daily production of germ cells and the concentrations of proteins and of spermatozoa in RTF did not differ significantly between the two crosses (Table 2). The mean cross-sectional areas of Sertoli cell nuclei were lower in BooRo than in BooMa rams (-20%; $P < 0.001$; $n = 20$), while the mean cross-sectional areas of Leydig cell were higher in BooRo than in BooMa rams (+11%; $P = 0.05$; $n = 20$).

Relationship between AMH secretions, prepubertal or adult hormonal and testicular parameters

The AMH concentration in RTF of adult rams were significantly negatively correlated with the oestrogen concentration in the testicular vein ($r = -0.60$; $P = 0.02$; $n = 15$). However, AMH concentration in the RTF of adult rams was not correlated with prepubertal plasma AMH concentrations ($n = 16$) or with adult FSH ($n = 15$), LH ($n = 15$) or testosterone

Fig. 1. Relationship between the concentrations of anti-Müllerian hormone (AMH) and FSH in jugular venous plasma at 4 weeks of age in 19 crossbred Booroola lambs ($r = -0.51$; $P < 0.05$).

($n = 14$) concentrations in plasma during the same period. However, the oestrogen concentration in the testicular vein was positively correlated with the mean FSH plasma concentrations ($r = 0.66$; $P = 0.004$; $n = 17$; Fig. 1) and with the mean testosterone plasma concentrations ($r = 0.58$; $P = 0.02$; $n = 16$).

The AMH concentrations in RTF or the oestrogen concentrations in the testicular vein were not related to any Sertoli or Leydig cell parameters. However, the daily productions of leptotene primary spermatocytes were significantly positively correlated with the oestrogen testicular venous plasma concentration ($r = 0.53$; $P = 0.02$; $n = 18$; Fig. 2a) and negatively

Fig. 2. Relationships between the daily production of leptotene primary spermatocytes expressed per Sertoli cell and: (a) oestrogen concentration (pg ml⁻¹ g⁻¹ of testis ($r = +0.53$; $P = 0.05$; $n = 19$); (b) anti-Müllerian hormone (AMH) concentration (ng ml⁻¹ expressed per 10⁷ Sertoli cells) in the rete testis fluid (RTF) ($r = -0.56$; $P < 0.05$; $n = 16$).

correlated with the AMH concentration in the RTF ($r = -0.56$; $P = 0.02$; $n = 16$; Fig. 2b).

Discussion

The immunoassay used in the present study did not crossreact with other non-gonadal proteins, as no activity was observed after castration. The AMH concentrations observed in prepubertal lambs corresponded to those obtained by Legeai *et al.*

(1986) in near-term male fetal ovine sera. The AMH is secreted by the testes with no evidence of sharp fluctuations among successive samples or with time of day, confirming previous data concerning the low amount of fluctuation (Lee *et al.*, 1994). Furthermore, injection of eCG, which possessed LH and FSH activities and stimulated testosterone production in the same lambs (before injection: 0.5 ng ml⁻¹; and 1–3 days after injection: 3 ng ml⁻¹; Hochereau-de Reviers *et al.*, 1990), did not modify the AMH plasma concentration within the period of sampling (6–30 h later). These results corroborate previous data obtained *in vitro* with Sertoli cell cultures, which show the absence of modulation of AMH secretion by FSH, LH or testosterone (Vigier *et al.*, 1985; Voutilainen and Miller, 1987). However, in 4-week-old lambs, a negative correlation between plasma FSH and AMH concentrations was observed; this negative relationship could be an indirect effect of differentiation of the Sertoli cells that modify the AMH degradation. In rat fetuses it has been suggested that FSH downregulates AMH transcription and that LH and sex steroids play a role in the control of AMH processing (Kuroda *et al.*, 1991). During the prepubertal period, BooRo lambs had higher plasma concentrations of testosterone than did BooMa lambs (Hochereau-de Reviers and Seck, 1990), but no relationship between AMH and testosterone plasma concentrations was observed in the present study. This could be due to the variability of plasma concentrations of testosterone as a consequence of the pulsatility of testosterone secretion; however, in weekly samples, which allow a mean estimation of the whole testosterone production, no correlation between AMH and testosterone was observed. The negative relationships observed in human males between testosterone, testicular growth and plasma concentrations of AMH remain controversial (Kuroda *et al.*, 1991; Baker and Hutson, 1993; Lee *et al.*, 1994). Most observations are based on comparisons between precocious or delayed and normal puberty at a given age. In a longitudinal analysis *in vivo* in the pubertal *Cynomolgus* monkey, it was observed that plasma concentrations of AMH were highly negatively correlated with testis volume, body weight and age, but not with testosterone plasma concentrations (Lee *et al.*, 1994). In boars, AMH secretion in the blood is maintained until pubertal testicular growth is achieved (Tran *et al.*, 1981). In the present study, BooRo lambs showed a 30% decrease in AMH concentration at the beginning of testis growth but without correlation between AMH plasma concentration and testis volume.

No correlation was observed between the prepubertal plasma AMH concentrations and the number of Sertoli cells in the same animals as adults; moreover, the BooRo lambs, which had the smallest number of Sertoli cells as adults, exhibited the same plasma concentrations of AMH as the BooMa, which had 20% more Sertoli cells (Hochereau-de Reviers and Seck, 1990; $P = 0.07$). In sheep, the Sertoli cells cease division around 1 month of age (Monet-Kuntz *et al.*, 1984) and the numbers of Sertoli cells per testis do not vary significantly later on (Hochereau-de Reviers and Courot, 1995). The concentration of AMH at 1 month of age in the present study could not be used as a predictor of the total numbers of Sertoli cells per adult testis.

In adult rams, AMH is present at a low but detectable concentration in the RTF (0.5 ng ml⁻¹) as has been observed in

pig RTF and bovine seminal plasma (Josso *et al.*, 1979; Vigier *et al.*, 1985). AMH was not detected in the lymph or in the venous testicular plasma and, if it is present in ovine plasma, its concentration is lower than the minimal detection limit (0.02 ng ml^{-1}). Hudson *et al.* (1990) observed an AMH concentration of 0.5 ng ml^{-1} in human adult serum which equalled the concentration observed in the present study in the RTF. The concentration of AMH in ovine RTF was much lower (20–25-fold) than that observed in peripheral plasma of the same animals before puberty. This difference is larger than that observed in humans (Hudson *et al.*, 1990; Rey *et al.*, 1993; Lee *et al.*, 1994).

The secretion of AMH by adult Sertoli cells towards the adluminal compartment and into the RTF did not differ significantly between the two crosses, although the mean testis mass differed between crosses. However, in terms of Sertoli cell function, the production of germ cells expressed per Sertoli cell did not differ between the crosses (Hochereau-de Reviers and Seck, 1990). The negative correlation observed between daily production of leptotene primary spermatocytes, expressed per testis or per Sertoli cell, and the AMH concentration in RTF, has not been observed previously. This finding could be compared with the absence of spermatogonial differentiation from gonocyte multiplications in the presence of high concentrations of AMH in the fetus (Jost, 1947). Moreover, at the onset of spermatogenesis, in both normal and intersex patients, AMH was present in premeiotic seminiferous tubules but was no longer detected in neighbouring tubules with meiotic development (Rey *et al.*, 1996). In the ovine testis, this could represent a seasonal imbalance of differentiated and undifferentiated secretions by the Sertoli cells, which varies among individuals, as has been observed in adult men (Rajpert-De Meyts and Skakkebaek, 1996). Moreover, in rats, the mRNA encoding AMH receptor II varies with stage of the seminiferous epithelium cycle and is maximal at stage VII when B type spermatogonia divisions occur (Baarends *et al.*, 1995), just before the formation of leptotene primary spermatocytes.

The concentrations of oestrogen observed in testicular venous plasma in the present study were comparable with those of Ile de France rams during the sexual season (Setchell *et al.*, 1991). Moreover, in hypophysectomized–hypophyseal extract-treated rams, the testicular venous concentration of oestrogen increased with plasma concentrations of FSH (Setchell *et al.*, 1991). The correlations observed between oestrogen concentrations in the testicular venous plasma, the AMH concentration in the RTF and the production of leptotene primary spermatocytes could depend on the same local control of the Sertoli cell function by the germ cells or on the direct control of oestrogen on AMH synthesis. The promoter of the AMH gene contains an oestrogen response element (Guerrier *et al.*, 1990). In mice in which the oestrogen receptor has been knocked-out, the daily production of spermatozoa per testis was decreased by half, and the epithelium in many of the seminiferous tubules appeared to have been sloughed (Hess *et al.*, 1996). Moreover, in rats, aromatase was detected in germ cells (Nitta *et al.*, 1993). However, when spermatogenesis was disrupted by heating the testes (Hochereau-de Reviers *et al.*, 1993), in the absence of feedback control by the hypophysis, there was no modification of oestrogen plasma concentration in the venous testicular plasma (Setchell *et al.*, 1991). The

oestrogen concentration in the testicular vein appeared to be independent of the number of germ cells present.

In contrast to a previous study demonstrating that the *Fec^B* gene carriers, on average, exhibited higher plasma concentrations of FSH and LH and lower testosterone than non-carriers from 4 to 8 weeks of age, irrespective of the cross (Seck *et al.*, 1988), in this smaller sample of lambs, significant differences among genotypes in gonadotrophin or testosterone plasma concentrations were not observed. Consequently, the negative correlation observed at 4 weeks of age between FSH and AMH was not related to the presence of the *Fec^B* gene in half of the lambs of each cross. In blood samples from adult rams during sexual or non-sexual seasons, gonadotrophin, testosterone or oestrogen concentrations did not differ with genotype (Hochereau-de Reviers and Seck, 1990). Similarly, the secretion of AMH by adult Sertoli cells into the RTF did not differ significantly between carriers and non-carriers of the *Fec^B* gene. In terms of Sertoli cell function, the production of germ cells expressed per Sertoli cell did not differ according to the presence of the *Fec^B* gene (Hochereau-de Reviers and Seck, 1990). The presence of the *Fec^B* gene did not modify the secretion of AMH, or its relationship with oestrogen or germ cell production by the testis, and it seems unlikely that AMH is involved in *Fec^B* gene expression.

In conclusion, the AMH secretion by Sertoli cells could not be used as a predictor of the numbers of Sertoli cells. AMH secretions in prepubertal or adult rams did not vary with the presence of the *Fec^B* gene but may be considered to be inversely related to the daily production of primary spermatocytes.

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