J. Reprod. Fert. (1986) **76,** 471–480

Printed in Great Britain

Adenosine cyclic 3′,5′-monophosphate and steroid production by small ovarian follicles from Booroola ewes with and without a fecundity gene

K. P. McNatty, L. E. Kieboom, J. McDiarmid, D. A. Heath and S. Lun

Wallaceville Animal Research Centre, Research Division, Ministry of Agriculture and Fisheries, Private Bag, Upper Hutt, New Zealand

Summary. The tissue contents of adenosine cyclic 3′,5′-monophosphate (cAMP) in freshly dissected follicles (0·13–1·00 mm diam.) were significantly higher in Booroola ewes containing a major fecundity gene (FF and F+ ewes) compared to those values in Booroolas with no copy of the gene (++ animals; $P < 0·025$). After a 1 h incubation with LH + FSH, the respective proportions of follicles with a diameter of 0·13–0·52 mm ($n = 288$) and 0·53–1·00 mm ($n = 271$) that had synthesized $\geqslant 0·6$ pmol cAMP and $\geqslant 1·0$ pmol cAMP were significantly influenced by genotype (Booroola ewes homozygous for the F-gene, FF > heterozygous, F+ > ++; $P < 0·01$ for both follicle size ranges).

The contents of progesterone, androstenedione, testosterone and oestradiol-17β in minced ethanolic extracts of freshly dissected follicles ($n = 188$) were undetectable regardless of Booroola genotype. However, when follicles of 0·53–1·00 mm but not 0·13–0·52 mm diameter were cultured for 48 h with LH + FSH under 70 kPa of a 50% O_2, 45% N_2 and 5% CO_2 gas mixture, the proportions that synthesized high levels of progesterone ($\geqslant 4·0$ ng), androstenedione ($\geqslant 3$ ng), and oestradiol ($\geqslant 0·8$ ng) were significantly influenced by genotype (FF > F+ $\geqslant$ ++; $P < 0·05$ for each steroid). No significant genotypic differences were noted for testosterone synthesis.

Collectively, these results show that the Booroola F-gene has an influence on the maturation of ovarian follicles from an early stage of growth.

Introduction

High fecundity Booroola ewes contain a major gene which influences their ovulation rate (see Bindon, 1984, for review). Homozygous (FF), heterozygous (F+) and non-carriers (++) of the putative gene have tentatively been segregated on the basis of at least one ovulation rate recording of $\geqslant 5$, 3 or 4 and 1 or 2 respectively. For follicles of 3–4 mm diameter, the ovarian granulosa cells in F+ ewes were more responsive to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) compared to ++ ewes in terms of production of adenosine cyclic 3′,5′-monophosphate (cAMP) (Henderson, Kieboom, McNatty, Lun & Heath, 1985). Also, follicles in F+ ewes secreted their maximum amounts of oestradiol-17β and reached ovulatory maturity at a smaller diameter than in ++ ewes (F+, 3–4 mm diam.; ++, 5–7 mm diam.; McNatty *et al.*, 1985a).

The aim of the present study was to determine whether the Booroola F-gene was influencing ovarian follicles at earlier stages of development.

Materials and Methods

Animals and procedures. Booroola Merino ewes, 6–8 years of age, with repeated annual ovulation-rate recordings of $\geqslant 5$ (N = 13) and 3 or 4 (N = 16) were classified as FF and F+ carriers of the putative fecundity gene (Davis, Montgomery, Allison, Kelly & Bray, 1982). No comparably aged ++ ewes were available as controls. Instead, we used 4–5-year old Booroola Merino ewes (N = 18) that had repeated annual ovulation rate recordings of <3.

All animals were injected with cloprostenol (125 μg s.c.; Coopers Animal Health Laboratories, Upper Hutt, N.Z.) on Day 10 of the oestrous cycle (oestrus = Day 0) to induce luteolysis. At intervals after cloprostenol injection (0, 6, 12, 24, 36 or 48 h) each ewe was anaesthetized with thiopentone sodium (Intraval: May & Baker, Wellington, N.Z.) and both ovaries were removed for further study.

Follicle dissection and culture. All follicles between 0·13 and 1·00 mm from each ovary were carefully dissected free of extraneous tissue using a stereodissecting microscope (at × 30–80 magnification). Individual follicular diameters were measured by one operator using an eyepiece graticule (Type Ella, Graticules Ltd, Tonbridge, Kent, U.K.); the error associated with each measurement was $<0{\cdot}03$ mm. Although perpendicular diameters were recorded for all follicles it was found that most follicles (>90%) assumed a spherical configuration when freed of extraneous tissue.

For cAMP measurements each follicle was finely minced and placed in 1 ml Dulbecco's phosphate-buffered saline (Gibco, Grand Island, NY, U.S.A.) containing 0·1% (w/v) bovine serum albumin (DBS-BSA), LH (1 μg/ml, NIH-LH-S23) and FSH (1 μg/ml, NIH-FSH-S11). The total minced suspension of each follicle was then transferred to a 10 × 75 mm plastic test-tube, capped, and either heated in an 80°C water bath for 15 min (n = 89 from ++ ewes; n = 92 from F+ ewes; n = 66 from FF ewes) or incubated at 37°C for 1 h (n= 200 from ++ ewes; n = 224 from F+ ewes; n = 135 from FF ewes) in a shaking water bath (200 cycles/min) before being transferred to an 80°C water bath for 15 min. Thereafter, each sample was stored at −70°C until assayed for cAMP by radioimmunoassay (RIA).

To determine the steroid content of follicles at the time of isolation (t_o), entire follicles (n = 66 from ++ ewes; n= 64 from F+ ewes; n = 58 from FF ewes) were placed in 1·0 ml ethanol (BDH, Analar), finely minced with dissecting scissors and transferred to a 10 × 75 mm plastic test-tube, capped and stored at −20°C until assayed for steroid by RIA. To assess the steroidogenic potentials of individual follicles (n = 174 from ++ ewes; n = 108 from F+ ewes; n = 167 from FF ewes), a 48-h culture method similar to that described by Neal, Baker, McNatty & Scaramuzzi (1975) for mouse follicles was used. Briefly, individual entire follicles were placed on a strip of sterile filter paper and supported on a stainless-steel grid above 1·8 ml of culture medium consisting of Medium 199, Earle's salts, Hepes buffer (20 mM), 5% (w/v) calf serum, gentamycin sulphate (50 μg/ml), LH (NIH-LH-S23, 1 μg/ml) and FSH (NIH-FSH-S11, 1 μg/ml) in a 35 × 10 mm Petri dish (Falcon, Oxnard, CA, U.S.A.). The Petri dishes containing individual follicles were placed in a modified anaerobic jar (Oxoid Ltd, Basingstoke, Hampshire, U.K.). The air in the jar was evacuated and replaced with a mixture of 50% O_2, 45% N_2 and 5% CO_2 to 70 kPa pressure and placed in a 37°C incubator for 48 h. At the end of the culture period, the medium was collected, and stored at −20°C until assayed for steroid content. The only criterion used to select follicles for the various procedures (i.e. cAMP, steroid at t_o, 1 or 48 h) was that all ranges of follicle diameters were covered when possible.

cAMP. The minced follicular sample in DBS-BSA was centrifuged at 2000 g for 15 min and 40 μl of the supernatant were then added to 60 μl assay buffer (i.e. sodium acetate buffer (0·05 M, pH 6·5) containing 0·1% (w/v) bovine serum albumin (BSA)). These samples together with the cAMP standards in 100 μl of assay buffer were acetylated by the method of Harper & Brooker (1975).

The cAMP antiserum (ARL) was kindly supplied by Dr A. R. La Barbera (Northwestern University, Chicago, Ill, U.S.A.). The lyophilized material was made up in assay buffer and 100 μl

were added to the acetylated samples to a final dilution of 1:30 000. The cross-reactions of AMP, ADP, ATP and cGMP with the antiserum in our assays were all <0·00004% while that for dibutyryl cAMP was 0·0049%.

About 10 000 c.p.m. iodinated cAMP derivative in sodium acetate buffer (0·05 M, pH 6·5) containing 3% (w/v) BSA were added to each assay tube in 100 μl. The radioactive label was prepared by iodinating 2′,0-monosuccinyl adenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester (Sigma Chemicals, St Louis, Mo, U.S.A.) to a specific activity of 120–150 μCi/μg.

The assay tubes were incubated overnight at 4°C and the free and bound fractions were separated using 0·2% (w/v) Norit A charcoal in 0·1 M-potassium phosphate buffer (pH 6·3) with 0·25% BSA (w/v). The cAMP results were expressed as pmol cAMP/follicle. The intra- and inter-assay coefficients of variation were 8 and 14% respectively. The limit of detection was ⩽ 0·03 pmol/follicle.

Steroids. Progesterone, androstenedione, testosterone and oestradiol contents were all measured by previously published RIA procedures (McNatty, Gibb, Dobson, Thurley & Findlay, 1981; McNatty *et al.*, 1982). To assay the steroids in the small follicles that had been minced in ethanol, the samples were first centrifuged at 2000 *g* for 15 min and the supernatant was collected, evaporated to dryness and redissolved in 0·6 ml assay buffer (phosphate-buffered saline (PBS, 0·1 M, pH 7·2)). The steroids in the culture medium (1 ml) samples were extracted into 2 × 5 ml freshly distilled diethyl ether which was evaporated to dryness. Thereafter, the residues were redissolved into 0·5 ml assay buffer.

Further details regarding the assays, including the specifications of the progesterone (WA-26), androstenedione (WA-965), testosterone (WA-36) and oestradiol-17β (WA-27) antisera are provided elsewhere (McNatty *et al.*, 1981, 1982, 1984a). The only exception to the published RIA procedures was that [1,2,6,7,21-^{3}H(N)]progesterone (sp. act. 196 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) was used in the progesterone RIA to enhance assay sensitivity. Most (i.e. ⩾83%) of the steroid samples were assayed singly except for the internal standards; the intra- and inter-assay coefficients of variation for the latter for all the above steroid assays were <8% and <15% respectively. The results were expressed as ng/follicle and the limit of detection for progesterone, androstenedione, testosterone and oestradiol were, respectively, ⩽0·1 ng, ⩽0·06 ng, ⩽0·06 ng and ⩽0·06 ng/follicle.

Statistical procedures. The data were analysed using analysis of variance in conjunction with Newman–Keuls multiple range test or Student's *t* test or χ^2 as appropriate. Because there were no significant differences in the cAMP or steroid data between sheep within each genotype, the statistical comparisons between genotypes were based on the numbers of follicles for each genotype. All data were transformed to logarithms to equalize the variances before statistical analysis. The data are presented as geometric means together with 95% confidence limits. To examine the influence of genotype with respect to follicular diameter, data from follicles between 0·13 and 0·52 mm diameter were sometimes pooled as were those from follicles between 0·53 and 1·00 mm in diameter. For convenience follicles ⩽0·52 mm diameter were sometimes referred to as small follicles whereas those between 0·53 and 1·0 mm diameter were sometimes referred to as large follicles.

Results

The respective mean ± s.e.m. ovulation rates in FF, F+ and ++ ewes for the cycle under study were 5·2 ± 0·3 (N = 13), 2·9 ± 0·1 (N = 16) and 1·2 ± 0·1 (N = 18) (FF vs F+ vs ++; P <0·01, ANOVA). The geometric mean numbers (and 95% confidence limits) of ovarian follicles (0·13–1·0 mm diam.) per ewe did not differ between the genotypes: in FF (N = 13), F+ (N = 16) and ++ (N = 18) ewes, the respective numbers were 27 (17, 43), 26 (16, 46) and 26 (20, 34).

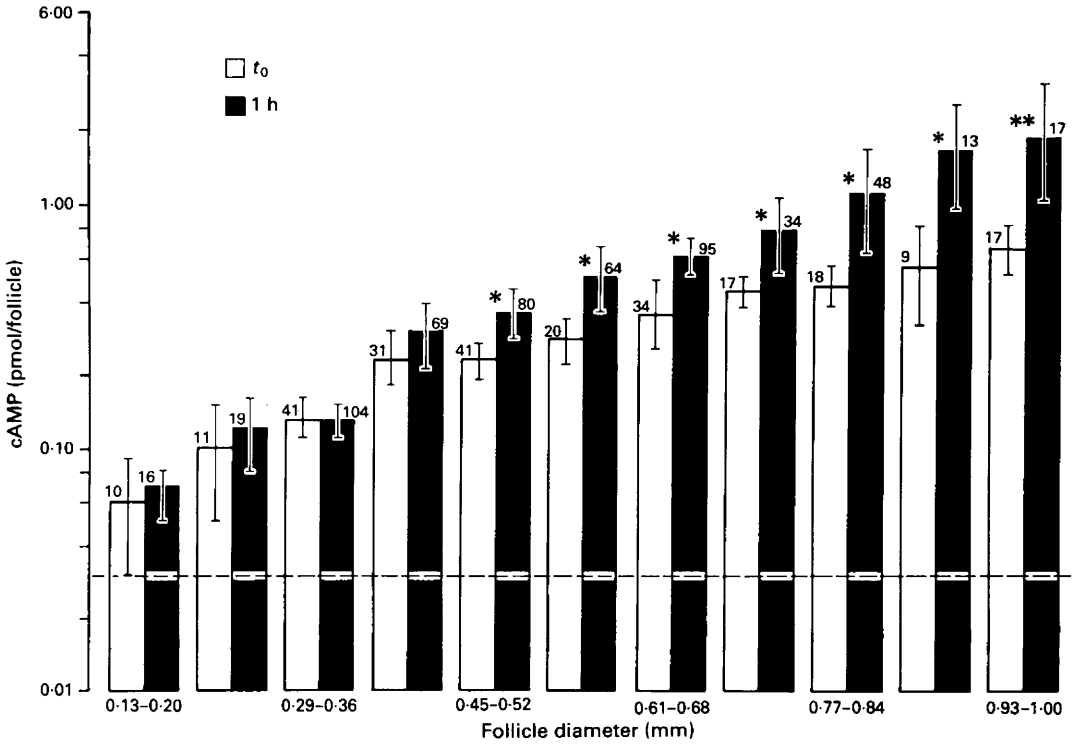

Fig. 1. cAMP content in ovarian follicles of Booroola ewes (data from all Booroola genotypes pooled) at t_0 and after a 1-h incubation in Dulbecco's modified phosphate-buffered saline + 0·1% BSA with LH (NIH-LH-S23; 1μg/ml) + FSH (NIH-FSH-S11; 1 μg/ml) with respect to follicular diameter. Values are geometric means and 95% confidence limits (vertical bars). Broken line indicates assay detection limit. Numbers refer to the number of follicles that were studied. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$ between t_0 and 1 h.

cAMP content and follicular diameter

The cAMP contents in all follicles (0·13–1·00 mm diam.) irrespective of genotype at t_0 and after a 1-h incubation with an LH + FSH enriched buffer with respect to follicular diameter are shown in Fig. 1. The contents at t_0 and after 1-h incubation both increased with increasing follicle size. In follicles between 0·13 and 0·44 mm diameter there was no significant difference in mean cAMP values after 1 h of incubation relative to those at t_0. However, in 0·45–1·00 mm follicles there was a 1·5 to 3-fold increase in the mean cAMP content after 1 h relative to that at t_0 ($P < 0{\cdot}05$, Student's t test on log transformed means).

cAMP content, Booroola genotype and follicular diameter

The cAMP contents in follicular tissue at t_0 and after a 1-h incubation with respect to Booroola genotype and follicular diameter are shown in Table 1. In the small follicles (0·13–0·52 mm diam.) the cAMP contents at t_0 were significantly influenced by genotype. Follicles from animals with the F gene contained higher cAMP contents than did follicles from the non-carriers. This difference was also apparent after a 1-h incubation but for ++ ewes there was no significant stimulation evident with respect to the values at t_0. When the cAMP values after 1-h incubation were analysed by contingency table analysis (Table 2), a significant effect ($P < 0{\cdot}01$) of genotype was noted on the

Table 1. cAMP content (pmol) in follicular tissue at zero time and after incubation with LH and FSH (both 1·0 μg/ml) for 1 h with respect to Booroola genotype and follicle diameter

Follicle diam. (mm)	Time (h)	Genotype		
		++	F+	FF
0·13–0·52	0	0·13 (0·09,0·16)[a]	0·17 (0·15,0·20)[b]	0·20 (0·16,0·24)[b]
(*n*)		(50)	(50)	(34)
	1	0·14 (0·12,0·16)[a]	0·26 (0·21,0·31)[c]	0·35 (0·23,0·48)[c]
(*n*)		(114)	(112)	(62)
0·53–1·00	0	0·40 (0·31,0·51)[b]	0·48 (0·39,0·57)[b]	0·40 (0·32,0·49)[b]
(*n*)		(39)	(42)	(32)
	1	0·32 (0·26,0·39)[b]	0·80 (0·67,0·95)[c]	1.14 (0·90,1·41)[d]
(*n*)		(86)	(112)	(73)

Values are geometric means (and 95% confidence limits); *n* = number of follicles.
For each follicle diameter, the values in rows or columns having a different superscript are significantly different from one another: a *vs* b, $P < 0{\cdot}025$; b *vs* c, $P < 0{\cdot}01$; c *vs* d, $P < 0{\cdot}01$.

Table 2. Contingency table showing effect of genotype on the number of small (0·13–0·52 mm diam.) or larger-sized (0·53–1·00 mm diam.) follicles which produced a certain level of cAMP after a 1-h incubation with an LH (NIH-LH-S23, 1 μg/ml) + FSH (NIH-FSH-S11, 1 μg/ml)-enriched medium

Follicle size	Genotype	cAMP content (pmol/follicle)	
		<0·6	⩾0·6
(a) Small	FF	52	10
	F+	100	12
	++	112	2
		<1·0	⩾1·0
(b) Large	FF	37	36
	F+	79	33
	++	80	6

Table 3. cAMP content (pmol) of ovarian follicles (0·53–1·00 mm diam.) with respect to Booroola genotype (++, F+, FF) and time after cloprostenol injection

Time after PG injection	Genotype		
	++	F+	FF
0–6 h	0·27 (0·20,0·35)[a]	0·51 (0·39,0·63)[b]	—
(*n*)	(19)	(17)	(0)
12–48 h	0·54 (0·40,0·68)[b]	0·48 (0·36,0·60)[b]	0·40 (0·32,0·49)[b]
(*n*)	(20)	(25)	(32)

Values are geometric means (and 95% confidence limits); *n* = number of follicles. The values in rows or columns having a different superscript are significantly different from one another: a *vs* b, $P < 0{\cdot}01$.

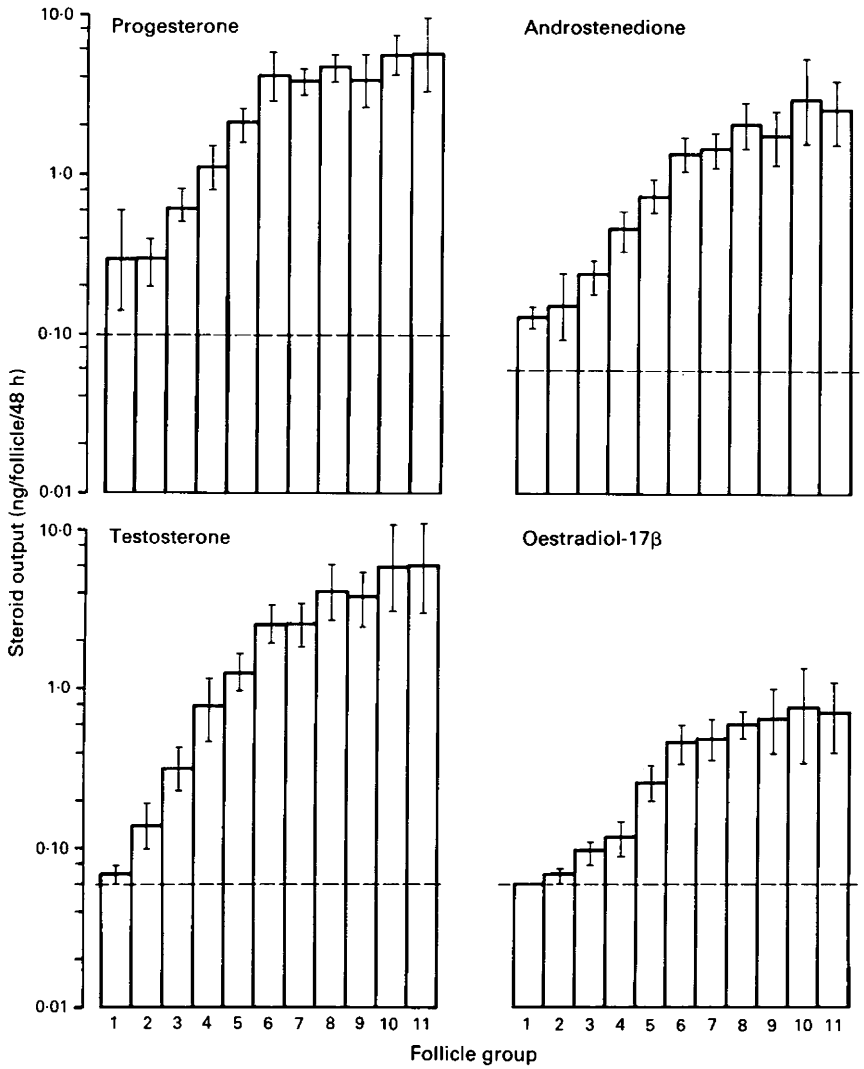

Fig. 2. Steroid output from ovarian follicles (data from all Booroola genotypes pooled) after 48-h culture in Medium 199, Earles salts, Hepes buffer (20 mM), containing LH (NIH-LH-S23; 1 μg/ml) + FSH (NIH-FSH-S11; 1 μg/ml) with respect to follicular diameter. Values are geometric means and 95% confidence limits (vertical bars). Broken lines indicate assay detection limits; the steroid contents at t_0 were below these limits. n = the number of follicles that were studied for all of the steroids. Follicle group numbers 1 to 11 refer to the follicle diameters: 1 = 0·13–0·20 mm (n = 11); 2 = 0·21–0·28 mm (n = 21); 3 = 0·29–0·36 mm (n = 78); 4 = 0·37–0·44 mm (n = 68); 5 = 0·45–0·52 mm (n = 76); 6 = 0·53–0·60 mm (n = 44); 7 = 0·61–0·68 mm (n = 59); 8 = 0·69–0·76 mm (n = 41); 9 = 0·77–0·84 mm (n = 28); 10 = 0·85–0·92 mm (n = 9); 11 = 0·93–1·00 mm (n = 14).

ability of an LH + FSH-enriched incubation medium to stimulate cAMP production. In Table 2a, the cut off point of ⩾0·6 pmol/follicle was selected on the grounds that this value was in excess of all recorded t_0 values. In small follicles (0·13–0·52 mm diam.) after a 1 h incubation, there was a significantly higher proportion of these follicles from F-bearing ewes which produced ⩾0·6 pmol cAMP.

In the larger sized follicles (0·53–1·00 mm diam.), there was no significant genotypic effect at t_0 but a genotypic effect was evident after a 1-h incubation; the F-gene animals contained follicles with ~1·7–2·9-fold higher mean values for cAMP content at this time (Table 1). These higher mean levels in the F-gene animals were reflected in a greater proportion of their follicles producing ⩾1·0 pmol cAMP (Table 2b).

In the small follicles (0·13–0·52 mm diam.), the mean cAMP content at t_0 was unaffected by time of ovariectomy after cloprostenol injection. However, in the larger-sized follicles (0·53–1·00 diam.), there was a significant influence of time after cloprostenol treatment on the cAMP values at t_0 for the ++ but not the F-gene animals (Table 3). At 0–6 h after cloprostenol injection, the mean cAMP content in F+ animals was 1·9-fold higher than that in ++ animals. However, at 12–48 h after cloprostenol injection, the mean cAMP content in ++ animals was 2·0-fold higher than that at 0–6 h, whereas the mean value in F+ animals was unchanged.

Steroid content and follicular diameter

The t_0 values for progesterone, androstenedione, testosterone and oestradiol in ovarian follicles (0·13–1·00 mm diam.) irrespective of genotype and follicular diameter (n = 188) were below the detection limits of the assays (i.e. progesterone ⩽0·1 ng/follicle; androstenedione, testosterone, oestradiol all ⩽0·06 ng/follicle). The steroid contents in the media after 48 h of culture with respect to follicular diameter are summarized in Fig. 2. All steroid contents increased with increasing follicular diameter until the follicles reached 0·53 mm. Thereafter, the steroid contents remained largely unchanged. In the smallest of the follicles (0·13–0·20 mm diam.), the mean progesterone and androstenedione contents were 3- and 2-times higher than the detection limits for these steroids whereas the testosterone and oestradiol contents were similar to or lower than the detection limits. The testosterone contents were measurable in almost all follicles ⩾ 0·2 mm diameter whereas the oestradiol contents were only detectable in most follicles when they were larger than 0·28 mm diameter.

Steroid content, Booroola genotype and follicular diameter

The steroid contents after 48 h culture with respect to Booroola genotype and follicular diameter are shown in Table 4. Regardless of diameter, no significant genotypic differences were discernible for any of the steroids except for progesterone in the larger-sized (0·53–1·00 mm diam.) follicles in which the follicles from FF ewes produced 1·7- to 1·9-fold more progesterone than did those from F+ and ++ ewes. In the small (0·13–0·52 mm diam.) and larger-sized (0·53–1·00 mm diam.) follicles, none of the steroids produced was influenced by time of ovariectomy after cloprostenol injection for any of the genotypes.

Table 4. Steroid accumulation (ng) from ovarian follicles after 48-h culture with respect to Booroola genotype (FF, F+, ++) and follicular diameter

Follicular diam. (mm)	Genotype	Progesterone	Androstenedione	Testosterone	Oestradiol-17β	No. of follicles
0·13–0·52	FF	1·2 (0·9,1·6)	0·5 (0·3,0·6)	0·7 (0·5,0·9)	0·2 (0·1,0·2)	85
	F+	1·1 (0·8,1·4)	0·4 (0·3,0·6)	0·8 (0·5,1·0)	0·1 (0·1,0·2)	49
	++	0·9 (0·8,1·1)	0·4 (0·3,0·4)	0·5 (0·4,0·6)	0·1 (0·1,0·2)	120
0·53–1·00	FF	5·7 (5·0,7·2)*	1·7 (1·4,2·1)	3·1 (2·3,3·9)	0·5 (0·4,0·6)	82
	F+	3·3 (2·5,4·4)	1·5 (1·1,1·9)	2·8 (2·2,3·4)	0·4 (0·3,0·6)	59
	++	3·0 (2·3,4·9)	1·4 (1·0,1·8)	2·2 (1·6,2·9)	0·5 (0·3,0·6)	54

Values are geometric means (and 95% confidence limits).
* $P < 0·01$ compared to values from F+ and ++ ewes.

For the small follicles (0·13–0·52 diam.), contingency table analysis revealed no significant genotypic effects ($P \geqslant 0{\cdot}1$) in the proportions of follicles which produced $\geqslant 1{\cdot}5$ ng progesterone/48 h, $\geqslant 1{\cdot}0$ ng androstenedione/48 h, or $\geqslant 0{\cdot}25$ ng oestradiol/48 h. However, there was a significant genotypic effect ($P < 0{\cdot}025$) in the proportions of small follicles which produced $\geqslant 0{\cdot}8$ ng testosterone/48 h; the respective proportions of follicles from FF, F+ and ++ ewes that produced $\geqslant 0{\cdot}8$ ng testosterone/48 h were 35% ($n = 86$ follicles), 46% ($n = 48$) and 24% ($n = 120$).

In the larger-sized follicles (0·53–1·00 mm diam.), contingency table analysis revealed significant effects in the proportions of follicles which produced $\geqslant 4$ ng progesterone/48 h ($P < 0{\cdot}01$), $\geqslant 3$ ng androstenedione/48 h ($P < 0{\cdot}05$) and $\geqslant 0{\cdot}8$ ng oestradiol/48 h ($P < 0{\cdot}025$) but not in the proportions which produced $\geqslant 3$ ng testosterone/48 h ($P > 0{\cdot}2$). For progesterone, the respective proportions of FF, F+ and ++ follicles which produced $\geqslant 4$ ng/48 h were 65% ($n = 82$ follicles), 47% ($n = 58$) and 38% ($n = 55$). For androstenedione, the respective proportions of FF, F+ and ++ follicles which produced $\geqslant 3$ ng/48 h were 40% ($n = 82$), 26% ($n = 58$) and 22% ($n = 55$). For oestradiol, the respective proportions of FF, F+ and ++ follicles which produced $\geqslant 0{\cdot}8$ ng oestradiol/48 h were 39% ($n = 82$), 26% ($n = 58$) and 25% ($n = 55$).

Discussion

These data show that there are significant differences attributable to the Booroola F-gene with respect to the cAMP contents of small ovarian follicles (0·13–1·00 mm diam.) and to the proportions of these follicles which have an ability to synthesize high levels of cAMP or steroid.

It seems unlikely that the age difference between the FF/F+ ewes and ++ ewes was a major factor contributing to the genotypic differences that were observed. The basal cAMP contents in small follicles (i.e. 0·13–1·00 mm diam.) in the 4–5-year-old ++ Booroola ewes were similar to those in Romney ewes of 2–6 years of age (K. P. McNatty & L. E. Kieboom, unpublished data). Moreover, the cAMP contents after a 1-h incubation with LH + FSH show differences in responsiveness between FF and F+ ewes as well as between FF/F+ ewes and ++ ewes (Tables 1 and 2). Likewise, genotypic differences in steroid output were often as great between FF and F+ ewes as those between FF/F+ ewes and ++ ewes.

In Booroola Merino ewes with or without the gene, histological studies reveal that the formation of an antral cavity is evident in some follicles around 0·20 mm in diameter and is a common feature of all follicles at 0·45 mm in diameter (Driancourt, Cahill & Bindon, 1985; K. P. McNatty & J. Fannin, unpublished data). The present studies therefore show that the Booroola F-gene has an influence on ovarian follicles from the very earliest stages of antrum formation. It remains to be determined whether the F-gene is influencing follicles at even earlier stages of follicular development. Driancourt *et al.* (1985) reported that Booroola Merino ewes had about 1·5–2 times more preantral follicles than did control Merino ewes, but whether this difference holds true between Booroolas with and without the F-gene is not known. In the present study, the mean numbers of follicles between 0·13 and 1·00 mm in diameter did not differ between the genotypes. It is possible that the lack of difference in follicle numbers was due to the ++ ewes being 2–3 years younger than the FF and F+ animals, notwithstanding the significant differences in the ovulation rates between the genotypes.

For all ewes (irrespective of genotype), the cAMP content of the follicles increased with increasing follicle size. Incubation of follicles for 1 h with an LH + FSH-enriched buffer did not lead to a significant increase in cAMP level above the t_0 values until the follicles reached 0·45 mm in diameter. One interpretation of these data is that the sensitivity of ovarian follicles to gonadotrophin stimulation is greater after antrum formation than in preantral follicles, or those in the process of forming an antrum. However, this interpretation can only be regarded as tentative since no attempt was made to examine the time course of cAMP accumulation after LH + FSH stimulation and, indeed, no follicles were incubated in an LH + FSH-free medium to serve as controls.

The presence in FF and F+ Booroola ewes of higher tissue contents of cAMP in both the small (0·13–0·52 mm diam.) and larger follicles (0·53–1·00 mm; i.e. at 0·6 h but not 12–48 h after cloprostenol injection) compared to those in ++ ewes is consistent with the notion that follicles in the F-gene carriers are more sensitive to gonadotrophin stimulation than those in ++ ewes (Bindon, 1984; Henderson *et al.*, 1985; McNatty *et al.*, 1985a). In hypophysectomized diethylstilboestrol-treated immature rats, cAMP is known to be the mediator of gonadotrophin action on granulosa and thecal/interstitial cells for de-novo synthesis of enzymes required for progestagen, androgen and oestrogen production (Wang, Hsueh & Erickson, 1982; Erickson & Magoffin, 1983). In cattle, the basal level of cAMP synthesis in theca interna tissue was positively correlated with C_{21} steroid 17β-hydroxylase and 17–20 desmolase enzyme activity (McNatty, Lun, Heath, Kieboom & Henderson, 1985b). Perhaps, therefore, the higher tissue contents of cAMP in small follicles of FF and F+ Booroolas relative to that in ++ Booroolas are causally related to the greater frequency of follicles (0·53–1·00 mm diam.) in the former which can produce high levels of progesterone, androstenedione and oestradiol.

The results from this study provide some insights into the ontogeny of steroidogenesis by sheep follicles. However, any interpretation of the data is limited to the extent that the culture conditions were probably suboptimal and because only one type of culture medium was tested with one dose of gonadotrophin. For example, it has been established that thecal steroidogenesis *in vitro* is markedly influenced by the flow of medium across the tissue. The in-vitro output of thecal androstenedione from bovine and ovine theca interna at a medium flow-rate of 1·4 ml/min is 7–50 times greater than that by theca interna in static cultures (McNatty *et al.*, 1984b, 1985a, b). Perhaps the unchanged 48-h output of steroid from follicles between 0·53 and 1·00 mm in diameter was due to the constraints of the culture system that was employed. Nevertheless, the present results indicate that the smallest follicles (0·13–0·20 mm diam.) that were examined were competent to synthesize progesterone and androstenedione but not oestradiol. These findings show that follicles clearly have the ability to synthesize progesterone and androgen before antrum formation but that appreciable oestradiol synthesis may not occur until antrum formation is underway. In the follicular fluid of 1–2·5 mm follicles from F+ and ++ Booroola ewes, the major steroids in descending order are testosterone, progesterone and oestradiol (McNatty *et al.*, 1985a); no data have yet been reported for androstenedione. The results of the present study together with those of McNatty *et al.* (1985a) suggest that developing follicles may first form a progesterone-enriched microenvironment and then evolve through an androgen-enriched state and finally, provided the follicle remains non-atretic, develop into an oestrogenic structure. With regard to genotypic differences in steroid accumulation, F+ ewes exhibit a greater capacity than ++ ewes to synthesize oestradiol but only in follicles between 2 and 4 mm in diameter (McNatty *et al.*, 1985a). The present results suggest that a significantly greater proportion of follicles in FF ewes between 0·53 and 1·0 mm in diameter develop the ability to synthesize $\geqslant$0·8 ng oestradiol/48 h compared to F+ and ++ ewes (see 'Results').

It has been shown that the capillary blood flow to the ovaries of anoestrous ewes can be enhanced by oestradiol provided the ovaries are first exposed to progesterone (Brown & Mattner, 1984). Perhaps at the level of individual follicles, the enrichment of the microenvironment with progesterone is an important prerequisite for an enhanced capillary blood flow when follicles start secreting oestradiol. The relative proportions of 0·54–1·00 mm diameter follicles with an ability to synthesize large amounts of progesterone (i.e. $\geqslant$4 ng/48 h) were significantly related to genotype (i.e. FF > F+ $\geqslant$ ++, see 'Results').

These findings therefore suggest that the Booroola F-gene has an influence on cAMP and steroid synthesis in ovarian follicles from a relatively early stage of development.

We thank our colleagues at the Invermay Agricultural Research Centre, Mosgiel, N.Z., and in particular Mr G. Davis, Ms J. Armstrong and Dr J. Owens for supplying the animals as well as details of their reproductive records; the National Institute of Arthritis, Metabolism and Digestive

Diseases, Bethesda, U.S.A., for the supply of ovine LH and FSH; Dr A. R. La Barbera, Northwestern University, Chicago, Illinois, U.S.A., for the cAMP antiserum; Dr K. M. Henderson for advice during preparation of this manuscript; and Miss N. Hudson, Mrs K. Ball and Mr P. Smith for care of the animals, documentation of oestrous cycle activity and surgery.

References

Bindon, B.M. (1984) Reproductive biology of the Booroola Merino sheep. *Aust. J. biol. Sci.* **37**, 163–189.

Brown, B.W. & Mattner, P.E. (1984) Effects of ovarian hormones on ovarian capillary blood flow in anoestrous ewes. *Aust. J. biol. Sci.* **37**, 389–392.

Davis, G.H., Montgomery, G.W., Allison, A.J., Kelly, R.W. & Bray, A.R. (1982) Segregation of a major gene influencing fecundity in progeny of Booroola sheep in New Zealand. *N.Z. Jl agric. Res.* **25**, 525–529.

Driancourt, M.A., Cahill, L.P. & Bindon, B.M. (1985) Ovarian follicular populations and preovulatory enlargement in Booroola and control Merino ewes. *J. Reprod. Fert.* **73**, 93–107.

Erickson, G.F. & Magoffin, D.A. (1983) The endocrine control of follicle oestrogen biosynthesis. *J. Steroid Biochem.* **19**, 113–117.

Harper, J.F. & Brooker, G. (1975) Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'1,0-acetylation by acetic anhydride in aqueous solution. *J. cyclic Nucleotide Res.* **1**, 207–218.

Henderson, K.M., Kiebom, L.E., McNatty, K.P., Lun, S. & Heath, D. (1985) Gonadotrophin stimulated cyclic AMP production by granulosa cells from Booroola × Romney ewes with and without a fecundity gene. *J. Reprod. Fert.* **75**, 111–120.

McNatty, K.P., Gibb, M., Dobson, C., Thurley, D.C. & Findlay, J.K. (1981) Changes in the concentrations of gonadotrophic and steroidal hormones in the antral fluid of ovarian follicles throughout the oestrous cycle of the sheep. *Aust. J. biol. Sci.* **34**, 67–80.

McNatty, K.P., Gibb, M., Dobson, C., Ball, K., Coster, J., Heath, D. & Thurley, D.C. (1982) Preovulatory follicular development in sheep treated with PMSG and/or prostaglandin. *J. Reprod. Fert.* **65**, 111–123.

McNatty, K.P., Hudson, N., Henderson, K.M., Lun, S., Heath, D.A., Gibb, M., Ball, K., McDiarmid, J.M. & Thurley, D.C. (1984a) Changes in gonadotrophin secretion and ovarian antral follicular activity in seasonally breeding sheep throughout the year. *J. Reprod. Fert.* **70**, 309–321.

McNatty, K.P., Heath, D.A., Lun, S., Fannin, J., McDiarmid, J.M. & Henderson, K.M. (1984b) Steroidogenesis by bovine theca interna in an *in vitro* perifusion system. *Biol. Reprod.* **30**, 159–170.

McNatty, K.P., Henderson, K.M., Lun, S., Heath, D.A., Ball, K., Hudson, N.L., Fannin, J., Gibb, M., Kieboom, L.E. & Smith, P. (1985a) Ovarian activity in Booroola × Romney ewes which have a major gene influencing their ovulation rate. *J. Reprod. Fert.* **73**, 109–120.

McNatty, K.P., Lun, S., Heath, D.A., Kieboom, L.E. & Henderson, K.M. (1985b) Influence of follicular atresia on LH-induced cAMP and steroid synthesis by bovine thecae interna. *Molec. cell. Endocr.* **39**, 209–215.

Neal, P., Baker, T., McNatty, K.P. & Scaramuzzi, R.J. (1975) Influence of prostaglandins and human chorionic gonadotrophin on progesterone concentration and oocyte maturation in mouse ovarian follicles maintained in organ culture. *J. Endocr.* **65**, 19–25.

Wang, C., Hsueh, A.J.W. & Erickson, G.F. (1982) The role of cyclic AMP in the induction of estrogen and progestin synthesis in cultured granulosa cells. *Molec. cell. Endocr.* **25**, 73–83.

Received 28 June 1985