J. Reprod. Fert., Suppl. 40 (1990), 163–177

Printed in Great Britain

Evidence for and implications of follicular heterogeneity in pigs

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Summary. Follicular heterogeneity has been demonstrated in both naturally cyclic and PMSG-stimulated immature gilts in that follicles in the selected ovulatory population differ in size by up to 2 mm and show marked variability in steroid content and gonadotrophin binding ability. This biochemical and morphological asynchrony continued into the immediate preovulatory phase and changes in response to the LH surge did not always occur simultaneously even in the same follicle or in all follicles within an ovary. It is suggested that these differences in follicular maturation immediately before ovulation may result in heterogeneity in the population of corpora lutea. Following oestrus or hCG administration, oocyte maturation was again not completely synchronous in all follicles within an animal. It is proposed that follicular heterogeneity has implications for oocyte maturation and early embryonic development.

Keywords: follicles; heterogeneity; pigs

Introduction

Follicle recruitment in the gilt occurs from the proliferating pool after which a process of selection during the follicular phase establishes a group of follicles destined to ovulate. It has previously been suggested (Foxcroft & Hunter, 1985) that the inter-follicular relationships in a polytocous animal such as the pig may differ from those in monotocous animals, and moreover that within the follicular hierarchy of the pig there are both dominant and smaller presumed ovulatory follicles (Grant *et al.*, 1989). Therefore, at the time of the LH surge and subsequent ovulation, follicles may be at markedly different stages of morphological and biochemical maturation.

The purpose of this paper, therefore, is to review the evidence that follicular heterogeneity occurs in the pig and to discuss the implications this may have for subsequent luteal function, oocyte maturation and embryo development.

Follicle recruitment and selection

The time of follicular recruitment from the proliferating pool of antral follicles ranging from 1 to 6 mm in size has been extensively studied in the cyclic gilt and the results from several experiments (Phillippo, 1968; Hunter *et al.*, 1976; Clark *et al.*, 1979, 1982) have suggested that recruitment occurs between Days 14 and 16 of the oestrous cycle. It appears that a continual process of growth and atresia occurs within this proliferating pool and that the rate of atresia is highest in the smaller sized follicles. If recruitment does not occur then the larger follicles also inevitably become atretic, and they will be replaced with smaller, non-atretic follicles from the proliferating pool. Clark *et al.* (1973) suggested that the composition of the proliferating pool could be genetically determined and may therefore be an important factor controlling potential ovulation rate.

Once recruitment has occurred, a number of processes may interact to determine final ovulation rate. The smaller follicles present at the time of recruitment either grow or become atretic, and so at the end of the follicular phase all follicles are in the larger size categories (Clark *et al.*, 1982; Grant *et al.*, 1989; T. Wiesak & M. G. Hunter, unpublished observations). Hence by Day 21 only the ovulatory population remains. The number of smaller sized follicles present at the time of recruitment that escape atresia, and are sufficiently mature to respond to the preovulatory LH surge, therefore becomes the ultimate determinant of ovulation rate.

Evidence for an essential role for the gonadotrophic hormones in the control of follicular maturation comes from experiments in which the suppression of endogenous gonadotrophins appears to be the primary cause of arrested follicular development (see Britt *et al.*, 1985; Christenson *et al.*, 1985). Treatment with exogenous gonadotrophins or GnRH has resulted in ovarian responses and supports the concept that the suppression of episodic LH release in particular can be a primary cause of arrested follicular development. Nevertheless, the stimulation of follicular maturation in individual weaned sows has been reported in the absence of significant changes in LH and FSH secretion (Foxcroft *et al.*, 1987), suggesting other factors may regulate ovarian follicular development.

Follicle maturation in the gilt

A model for follicular maturation has been described by Foxcroft & Hunter (1985), based mainly on studies in the rat (Richards & Midgley, 1976; Richards, 1979) and supported by experiments in the pig (Ainsworth *et al.*, 1980; Evans *et al.*, 1981; Veldhuis *et al.*, 1982). Evidence that the two-gonadotrophin two-cell theory of follicular maturation initially proposed for other species is also applicable to the pig is extensively reviewed by Ainsworth *et al.* (1990).

In a recent study by Grant *et al.* (1989) of follicle development in cyclic gilts on Days 16, 18, 20 and 21, the progressive decrease in follicle number per animal from Day 16 to Day 21 (Fig. 1) supports the previous suggestion of Clark *et al.* (1973) that during the follicular phase there is a physiological block to the replacement of atretic follicles in the proliferating pool. This decrease in number was accompanied by an increase in the size of the follicles present and by Day 21 virtually all the follicles present were >8 mm in diameter. Various characteristics of follicular maturation were measured in all (or alternate) follicles $\geqslant 2$ mm in diameter ($n = 571$) and are summarized in Table 1. Mean follicular fluid oestradiol, testosterone and binding of ^{125}I-labelled hCG increased as the follicles grew until Day 20 but decreased on Day 21. It is likely that this fall was a consequence of the LH surge as reported for other species (sheep: Moor, 1974; Webb & England, 1982; cow: Staigmiller *et al.*, 1982). However, aromatase activity in the granulosa cells *in vitro* actually declined between Days 18 and 20 despite a rise in follicular fluid oestradiol concentrations, suggesting that granulosa aromatase activity is not the limiting factor in the control of oestradiol synthesis at this time. The precipitous decline in follicular fluid oestradiol which occurred on Day 21 in the presence of maximal aromatase activity further supports this theory. The availability of androgen substrate from the theca therefore appears to be of critical importance in maintaining the synthesis of oestradiol by the developing pig follicle.

In contrast to the limited data obtained from naturally cyclic gilts, many studies have been carried out on follicles from immature gilts stimulated with PMSG and hCG (see Ainsworth *et al.*, 1990). Although the overall pattern of change in follicular fluid steroid concentrations appears similar between naturally cyclic and PMSG-stimulated gilts there are some distinct differences. Ainsworth *et al.* (1980) reported that follicular fluid androgen concentrations remained relatively constant during PMSG-stimulated follicular development, but there was a distinct rise and fall in the naturally cyclic animals. Also, progesterone was the predominant steroid in the follicular fluid 72 h after PMSG injection (before the hCG injection), whereas in the cyclic gilts the increase in progesterone did not occur until Day 21, i.e. after the LH surge. Furthermore, Tsang *et al.* (1985)

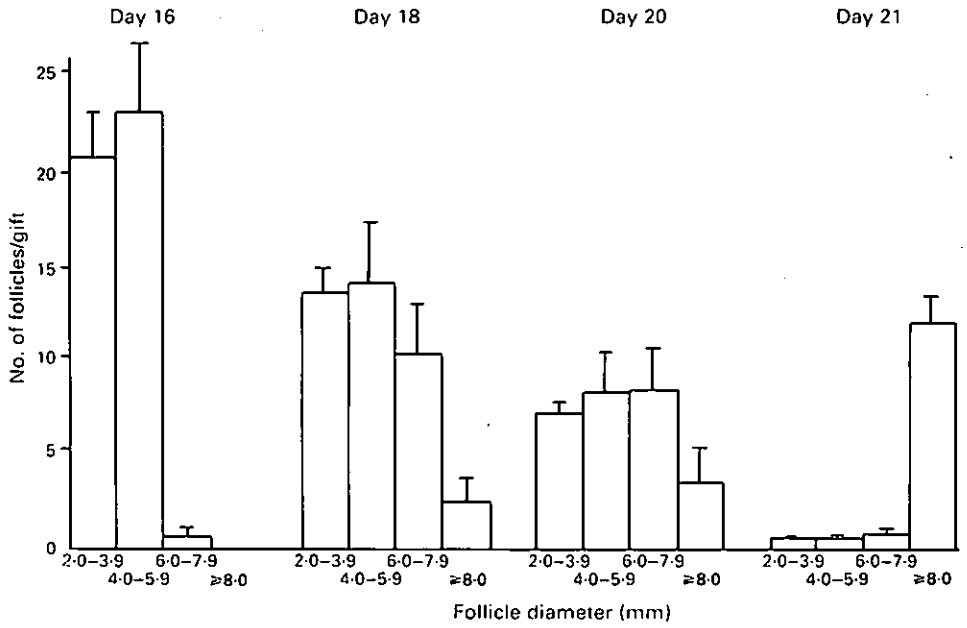

Fig. 1. Mean number (±s.e.m.) of follicles recovered from individual gilts on 4 days of the oestrous cycle (5 gilts/day) and divided into 4 size categories (from Grant *et al.*, 1989).

Table 1. Overall means and s.e.d. of follicle diameter, granulosa cell number, ^{125}I-labelled hCG binding to granulosa and theca tissue, follicular fluid concentrations of oestradiol, testosterone and progesterone, testosterone content of theca tissue and granulosa cell aromatase activity in pig follicles recovered during the oestrous cycle (from Grant *et al.*, 1989)

Variables measured	Day 16 (n = 180)	Day 18 (n = 164)	Day 20 (n = 110)	Day 21 (n = 57)	s.e.d.
Diam. (mm)	3·99	4·91	5·50	8·80	0·47
No. of granulosa cells/follicle ($\times 10^{-4}$)	121·1	145·7	146·8	376·5	44·2
Granulosa cell aromatase activity (ng oestradiol/2 h/follicle)	1·13	3·70	1·73	4·65	2·04
^{125}I-labelled hCG binding to granulosa cells (c.p.m. bound $\times 10^{-3}$/follicle)	21·2	52·1	82·9	59·7	23·5
^{125}I-labelled hCG binding to theca tissue (c.p.m. bound $\times 10^{-3}$/follicle)	7·7	24·2	38·8	53·0	14·3
Oestradiol in follicular fluid (ng/ml)	22·0	110·6	235·5	102·6	89·8
Testosterone in follicular fluid (ng/ml)	22·3	55·8	75·7	34·3	31·4
Progesterone in follicular fluid (ng/ml)	102·1	83·4	76·2	497·2	175·8
Testosterone content of theca tissue (ng/follicle)	0·06	0·69	1·76	2·12	1·36

s.e.d. = Standard error of difference for comparing between means.

reported that androgen production was maintained and aromatase activity was decreased by the hCG injection after PMSG stimulation. The endogenous LH surge in the naturally cyclic gilts clearly did not have similar effects, since testosterone in follicular fluid had declined in these animals on Day 21 although aromatase activity increased. Babalola & Shapiro (1988) measured the concentrations of several steroids in follicular fluid from cyclic animals, and concluded that they

differed from those of the PMSG/hCG-stimulated ovary. Clearly therefore, although the PMSG-hCG-treated prepubertal pig is a well established model for the study of follicular development, the sequence of maturational changes which occurs is not exactly the same as that in a naturally cyclic animal.

Intra-ovarian regulation

It has previously been suggested (Foxcroft & Hunter, 1985) that the inter-follicular relationships in polytocous animals may differ from those of monotocous animals, and that in the pig the dominant follicles may promote the maturation of the smaller follicles, in contrast to their inhibitory effect in other species.

To date, a vast array of 'intra-ovarian regulators' has been described for the pig, including steroids, peptides, proteins and prostaglandins (see reviews by Channing *et al.*, 1982; Ellendorff & Parvizi, 1987; Tonetta & diZerega, 1990; Ainsworth *et al.*, 1990) and have been reported to act either independently or in association with gonadotrophins. As the precise roles of many of these factors have not yet been fully elucidated (e.g. oocyte maturation inhibitor, granulosa cell luteinization stimulator and inhibitor, inhibin, gonadocrinin, LH and FSH binding inhibitors) only those for which a specific function has been postulated during follicular recruitment and maturation will be further discussed.

Follicle regulatory protein (FRP) has been proposed as a major regulator of follicular development in the pig and as its actions have already been discussed in detail by Tonetta & diZerega (1990) it will not be further reviewed here. However, there have also been reports that steroids have both inhibitory and stimulatory effects on their own synthesis and on the synthesis of other steroids in several species (pig: Haney & Schomberg, 1978; Veldhuis, 1985; Hunter & Armstrong, 1987; cow: Williams & Marsh, 1978; Fortune & Hansel, 1979; rat: Fanjul *et al.*, 1984). Many studies have described the bipotential effects of oestrogen, such that, under different conditions, oestradiol will either stimulate or inhibit steroid production by granulosa (Veldhuis, 1985) and theca (Hunter & Armstrong, 1987; Tonetta *et al.*, 1986) cells. These effects have also been described as being dose-dependent in that low doses enhance steroidogenesis and high doses are inhibitory (Tonetta, 1987; M. G. Hunter, unpublished observations), but any clear relationship between sensitivity of response and maturity of the follicles from which the cells were obtained has yet to be established. Nevertheless, it is possible to postulate both an intra- and inter-follicular role for oestradiol, particularly in a polyovulatory species such as the pig. Oestradiol secretion from the more dominant oestrogenic follicles within the ovulatory population may promote the maturation and steroidogenesis of the less mature follicles, while at the same time remaining themselves in a 'resting state' due to an inhibitory effect of the very high intra-follicular concentrations of oestradiol. The stimulatory effects of oestradiol may also act via the sub-ovarian countercurrent exchange pathways (Krzymowski *et al.*, 1982) as previously suggested by Foxcroft & Hunter (1985).

It has been demonstrated that insulin-like growth factor 1 (IGF-I) is present in pig follicular fluid (Hammond *et al.*, 1985) and also that it is secreted by pig granulosa cells in culture in response to gonadal and pituitary hormones (Hsu & Hammond, 1986). Furthermore, it has been suggested that IGF-I may amplify the actions of gonadotrophins on the ovary (Veldhuis *et al.*, 1985) and gonadotrophin administration *in vivo* increased ovarian concentrations of IGF-I (Hammond *et al.*, 1988). Since concentrations of IGF-I in follicular fluid increased in parallel with oestradiol as the follicles increased in size, it is also possible to speculate on a similar inter-follicular role of IGF-I.

Follicular heterogeneity in cyclic gilts

Morphological and biochemical

Although the results presented in Table 1 represent the overall development of the follicular population $\geqslant 2$ mm in diameter during Days 16–20 of the oestrous cycle, it was not possible to

identify the selected ovulatory population until Day 20. Up to this time, i.e. on Days 16 and 18, as many as 50 follicles $\geqslant 2$ mm diameter could be present in the ovaries, but this population consisted of a continuum in terms of morphological and biochemical characteristics (Grant, 1989). It was only by Day 20, that a subset of 14–16 large, presumed preovulatory follicles was readily distinguishable from the remaining smaller, less oestrogenically active population in each animal (Table 2), and yet consistently within these subsets there was a difference in the range in follicular diameter of around 2 mm. A similar range was also noted in the other characteristics measured and follicles of similar size could show marked differences in follicular fluid steroid concentration. A marked variation in the concentration of oestrogen in follicular fluid in individual follicles from Day 17 to 20 was also reported by Hunter *et al.* (1976). Foxcroft & Hunter (1985) noted that the number of granulosa cells per follicle was very variable, especially in the larger follicles and even in those which were oestrogenically active.

The range of morphological and biochemical development present from Day 16 onwards (and probably before Day 16) indicates that follicles destined to ovulate were not all at the same stage of maturation at the time of recruitment and selection. Presumably the high oestrogenic activity of the most mature follicles would provide the trigger for the preovulatory LH surge, and since the reported timing between oestrogen stimulation and the LH surge in the pig is about 55 h (Edwards & Foxcroft, 1983) then it would be expected that the activation of the surge mechanism would occur on Day 18. At this time the selection of the ovulatory follicles would still be continuing and so the time interval between the final selection of the least mature ovulatory follicles and the dramatic maturational changes induced by the LH surge, including the inhibition of mitosis in the granulosa cells, must be very short. This suggests that at the time of ovulation (assumed to be $\simeq$36–40 h after the LH surge) all the follicles would not be at an identical stage of maturation. This hypothesis is supported by the fact that follicles recovered on Day 21 (i.e. between the LH surge and ovulation) also had a range of morphological and biochemical development (Grant, 1989). Furthermore, in a study of follicles from gilts on Day 22 (Day 1) just before ovulation, many of the follicles recovered had become flaccid and this was associated with an increase in follicular fluid viscosity; however, there was still a considerable range in follicle diameter and follicular fluid steroid concentrations (Hunter *et al.*, 1989). Marked infolding of both the granulosa and theca tissue in a proportion of the follicles suggested early luteinization (see Fig. 2), although these morphological changes did not necessarily occur simultaneously in the same follicle, or in all follicles within an ovary. Moreover, such changes were not consistently related to characteristic differences in the concentration of follicular fluid steroids. Collectively, these results demonstrate that both morphological (histological) and biochemical asynchrony continued into the immediate preovulatory period and that follicles responded in different ways to the LH surge signal, probably reflecting their maturational status at that time.

Heterogeneity in the granulosa cell population within individual follicles has been reported for the pig (Lahteenmaki *et al.*, 1982), the rat (Amsterdam *et al.*, 1975; Zoller & Weitz, 1978) and the monkey (diZerega & Hodgen, 1980), showing that heterogeneity occurs within as well as between follicles.

Oocyte maturation

It is well documented that from birth the pig oocyte remains arrested in the dictyate stage of meiosis until just before the time of ovulation (Spalding *et al.*, 1955; Brambell, 1956; Hunter & Polge, 1966). The LH surge is the signal for the resumption of meiosis *in vivo*, but oocytes cultured *in vitro* initially undergo spontaneous maturation (Edwards, 1965), although this process is incomplete (Motlik & Fulka, 1974) and the necessary competence for embryonic development may not be achieved (Thibault *et al.*, 1976). The resumption of meiosis can also be induced in immature or adult animals by the injection of gonadotrophins (Edwards, 1962; Hunter & Polge, 1966; Cran, 1985). The stage of meiosis of many of the oocytes at particular times after gonadotrophin injection

Table 2. Individual follicular characteristics measured in the 19 follicles recovered from a gilt on Day 20 of the oestrous cycle (from Grant *et al.*, 1989)

Follicle no.	Diam. (mm)	No. of granulosa cells ($\times 10^{-4}$)	Aromatase activity (ng oestradiol/ 2 h/follicle)	^{125}I-labelled hCG binding (c.p.m. bound $\times 10^{-3}$/follicle)		Follicular fluid			Thecal testosterone (ng/follicle)
				Granulosa	Theca	Oestradiol (ng/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)	
1	9·0	203	3·1	—	—	1245	182	173	1·29
2	9·0	172	—	221	107	968	279	270	—
3	8·8	148	4·4	—	—	1251	253	331	1·45
4	8·5	132	—	57	103	1082	188	122	—
5	8·5	54	4·2	—	—	1029	329	179	1·34
6	8·3	197	—	234	104	538	207	223	—
7	8·3	135	2·5	—	—	1152	295	214	1·52
8	8·2	205	—	149	79	618	173	94	—
9	8·1	205	5·0	—	—	626	197	137	1·96
10	8·1	168	—	123	89	648	375	199	—
11	8·1	131	5·4	—	—	738	317	246	1·56
12	7·8	134	—	133	80	1038	169	86	—
13	7·8	41	4·6	—	—	874	301	172	1·21
14	7·1	258	—	44	24	54	26	16	—
15	3·9	89	0·1	—	—	14	13	69	0·07
16	3·7	150	—	28	6	<2	3	47	—
17	3·0	95	0·1	—	—	<3	14	118	0·06
18	3·0	60	—	3	1	<7	<8	54	—
19	2·9	117	0·3	—	—	<3	<8	64	0·16

— Not determined.

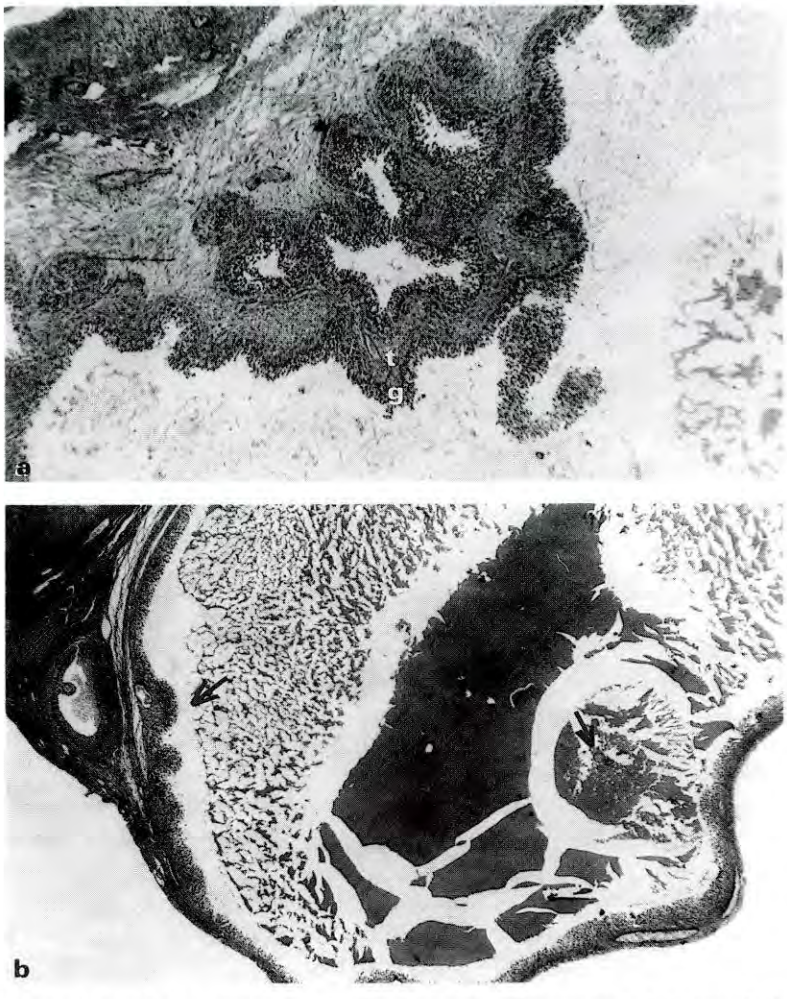

Fig. 2. Sections of pig follicles recovered during the periovulatory period: (a) marked infolding of both the granulosa (g) and theca (t) layer in a flaccid follicle (× 200); (b) the oocyte (marked with an arrow) is present in a flaccid follicle along with some infolding (also marked with an arrow) which is not associated with the oocyte (× 200).

(Hunter & Polge, 1966; Ainsworth *et al.*, 1980; Cran, 1985) or following the onset of oestrus (Spalding *et al.*, 1955) has been reported. Hancock (1961) reported that, of 1677 eggs examined after spontaneous ovulation, only 3 were immature primary oocytes. A study has also been conducted in which the stages of oocyte maturation as well as other morphological and biochemical characteristics of individual follicles were assessed (M. G. Hunter & T. Wiesak, unpublished observations). The results confirmed that resumption of meiosis did not begin until a considerable time after the onset of oestrus and that, although the steroidogenic output of the follicles had changed from predominantly oestrogen to progesterone, in some animals all the oocytes were still in the intact germinal vesicle stage (GV). In general, germinal vesicle breakdown (GVBD) had occurred in those gilts from which ovaries were recovered on the 2nd day of oestrus, and in all animals (N = 6) follicular fluid oestradiol concentration was low and progesterone was high. Nevertheless, Gerard *et al.* (1979) reported that high progesterone concentrations did not appear to be necessary for the nuclear maturation of oocytes *in vitro*. In the above study (M. G. Hunter &

T. Wiesak, unpublished observations) all the oocytes identified in some of the animals were at similar stages of maturation as shown in Fig. 3(a): all 11 oocytes identified in this gilt were in the prometaphase/metaphase stage. These ova were recovered from follicles ranging from 7·0 to 9·0 mm in diameter and with concentrations of progesterone in follicular fluid ranging from 370 to 796 ng/ml. The amount of progesterone secreted during incubation of the follicles is also shown in Fig. 3(a), and again was variable, with low secretion from the small, presumed atretic, follicles which contained oocytes still in the intact germinal vesicle stage. As maturation progressed (Fig. 3b), the stages of oocyte maturation ranged from intact germinal vesicle to telophase 1 with follicular fluid progesterone concentrations ranging from 471 to 1220 ng/ml. Figure 3(c) shows the oocytes recovered from one animal which were all past the metaphase 1 stage; follicular fluid progesterone concentrations ranged from 225 to 586 ng/ml. Follicles in which the oocyte had undergone germinal vesicle breakdown in Fig. 3(c) were all 9–11 mm in diameter, whereas those in Figs 3(a) and 3(b) ranged from 6 to 9 mm in diameter. This demonstrates that considerable variation in terms of follicular size also occurred between as well as within animals. When data on all the oocytes recovered ($n = 60$) were analysed, the stage of oocyte maturation was correlated ($r = 0{\cdot}33$, $P < 0{\cdot}05$) with follicular fluid volume and closely approached significance with both follicular diameter and oestradiol secreted during incubation of the collapsed follicle. There was no significant correlation with progesterone in follicular fluid or secreted during incubation. Of course, it was not possible to determine to what steroidogenic (oestrogenic) environment the follicles had previously been exposed. This also applies to other non-steroidal regulatory factors such as oocyte maturation inhibitor (OMI) which are present in the follicle and influence oocyte maturation (Tsafriri *et al.*, 1982; Mattioli *et al.*, 1988).

Follicular heterogeneity in PMSG/hCG-primed gilts

Morphological and biochemical heterogeneity

As previously mentioned, the treatment of immature gilts with PMSG followed by hCG has frequently been used to induce follicular growth and ovulation (Baker & Coggins, 1968; Ainsworth *et al.*, 1980; Cran, 1985). Although it was stated that this treatment synchronized the production of a population of uniformly developing follicles (Ainsworth *et al.*, 1980; Hammond *et al.*, 1988), in both studies the diameter of follicles recovered at different times after PMSG/hCG administration was reported to vary by up to 2 mm. This may partly be accounted for by variation between animals. Nevertheless, when follicles within individual animals were investigated (M. G. Hunter & T. Wiesak, unpublished observations), a similar range in follicular diameter and follicular fluid steroid concentrations was noted at 36 and 72 h after PMSG administration (see Table 3). At 72 h after PMSG treatment a subset of large steroidogenic follicles was distinguishable from the smaller, less steroidogenic follicles as described previously for follicles recovered on Day 20 of the oestrous cycle.

Analysis of follicular characteristics in naturally cyclic (Days 16–18) and immature, PMSG (36 h)-stimulated animals showed that the mean follicle diameter and oestradiol and testosterone content of follicular fluid were all similar, whereas progesterone concentration in follicular fluid and oestradiol and testosterone secretion rates during incubation were all higher ($P < 0{\cdot}05$) in the PMSG-primed than the cyclic animals. Also, the variance in the follicular population within an animal in each group, in terms of follicle diameter, progesterone, oestradiol and progesterone secretion, was greater in PMSG-treated than in cyclic animals ($P < 0{\cdot}05$). Any differences ($P < 0{\cdot}05$) in, for example, oestradiol and testosterone secretion during incubation between follicles recovered from Day-20 cyclic gilts and 72 h after PMSG treatment of immature gilts again showed follicles from PMSG-treated animals to be more variable. This suggests that the developing follicular population was more heterogeneous in PMSG-stimulated immature gilts than in naturally cyclic animals.

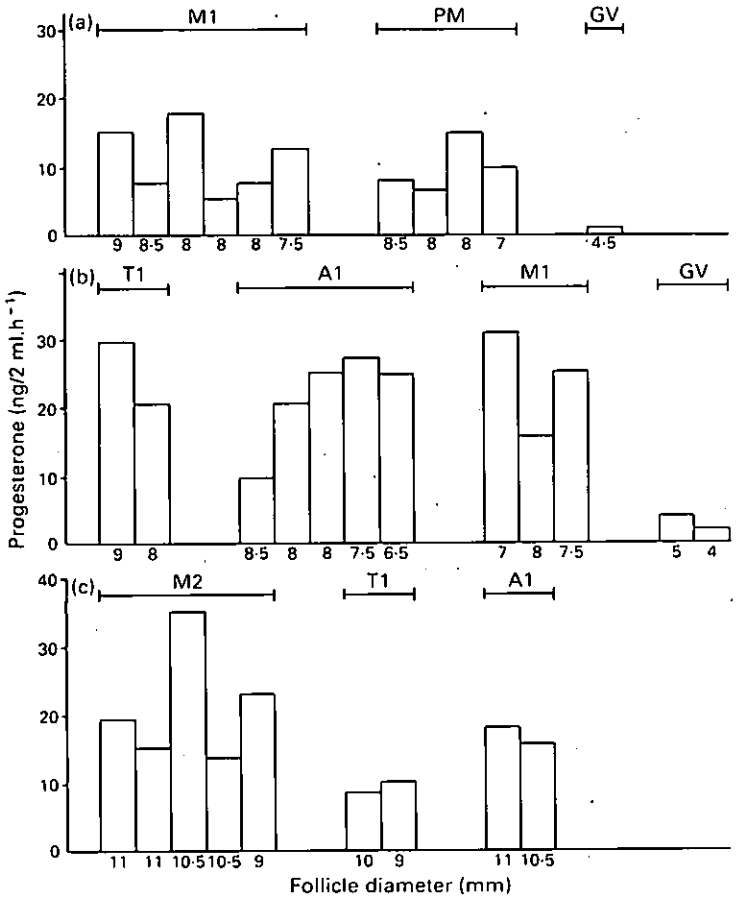

Fig. 3. Follicle diameter, progesterone secretion by the collapsed follicle during incubation and stage of oocyte maturation of individual follicles recovered from 3(a, b, c) naturally cyclic gilts during the peri-ovulatory period. Stages of oocyte maturation: GV, germinal vesicle; PM, prometaphase; M1, first metaphase; A1, first anaphase; T1, first telophase; M2, second metaphase. (M. G. Hunter & T. Wiesak, unpublished observations.)

Oocyte maturation

The time sequence of oocyte maturation in PMSG/hCG-treated animals or cyclic gilts treated with hCG only has been well documented (Hunter & Polge, 1966; Ainsworth *et al.*, 1980; Cran, 1985; M. G. Hunter & T. Wiesak, unpublished observations). For a period of approximately 18–20 h after hCG, the oocytes remain in the intact germinal vesicle stage. Following this, resumption of meiosis occurs and appears to be completed by $\simeq$40 h (Cran, 1985; M. G. Hunter & T. Wiesak, unpublished observations). Although many of the oocytes may be at the same stage of maturation at the same time, this is certainly not true for all oocytes. Hunter & Polge (1966) reported that the maturation of only 71% of oocytes examined conformed to their predicted stage of development; such differences between observed and expected maturational stages occurred both within and between animals. Ainsworth *et al.* (1980) found large percentages of oocytes to be in one of three maturational stages (intact germinal vesicle, germinal vesicle breakdown, metaphase I) at 24, 30 or 36 h after hCG. In a study by Cran (1985) only a limited number of oocytes was examined but at 30 h after hCG he noted that oocytes could be at the metaphase I or II stage. The asynchrony in the timing of oocyte maturation in individual animals which were treated with

Table 3. Individual follicular fluid concentrations of hormones in follicles recovered from 2 immature gilts 36 or 72 h after PMSG (750 i.u.) injection (T. Wiesak & M. G. Hunter, unpublished observations)

36 h after PMSG					72 h after PMSG				
Follicle no.	Diam. (mm)	Oestradiol (ng/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)	Follicle no.	Diam. (mm)	Oestradiol (ng/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)
1	8·5	51	9	321	1	8·5	81	35	240
2	8·0	108	9	886	2	8·5	50	41	835
3	8·0	34	26	153	3	8·0	108	47	192
4	7·0	68	26	224	4	8·0	94	69	301
5	7·0	22	92	49	5	8·0	132	32	436
6	7·0	52	20	168	6	8·0	109	41	230
7	7·0	60	36	176	7	7·5	52	50	229
8	6·5	56	35	174	8	7·5	125	48	241
9	6·0	88	13	398	9	7·0	103	43	241
10	6·0	67	20	255	10	6·5	15	36	45
11	6·0	35	54	126	11	5·5	5	10	61
12	6·0	89	95	102	12	5·0	5	10	88
13	6·0	38	60	79	13	5·0	3	8	85
14	6·0	25	143	75					
15	5·5	13	115	101					
16	5·0	18	121	56					
17	5·0	48	36	84					
18	5·0	13	41	29					
19	5·0	12	26	3					
20	4·5	16	27	175					
21	4·0	41	142	163					
22	4·0	20	101	73					
23	4·0	8	14	49					
24	3·5	16	68	22					

PMSG followed 72 h later by hCG and slaughtered after 35 or 45 h is shown in Fig. 4 (M. G. Hunter & T. Wiesak, unpublished observations). Significant correlations between stage of oocyte maturation and both follicular diameter ($r = 0{\cdot}24$, $P < 0{\cdot}05$) and progesterone secretion during incubation ($r = 0{\cdot}31$, $P < 0{\cdot}01$) were observed when all oocytes ($n = 121$) were considered. By 45 h (Fig. 4b) all the oocytes identified were at metaphase II. This confirms that before ovulation most oocytes undergo a second meitotic division and such data are consistent with previous reports of a low incidence of primary oocytes being released during spontaneous ovulation (Hancock, 1961) or following ovulation induced during the follicular phase (Spalding *et al.*, 1955; Polge & Dziuk, 1965). Asynchrony in the timing of maturation of oocytes cultured in follicles *in vitro* has also been reported (Gerard *et al.*, 1979).

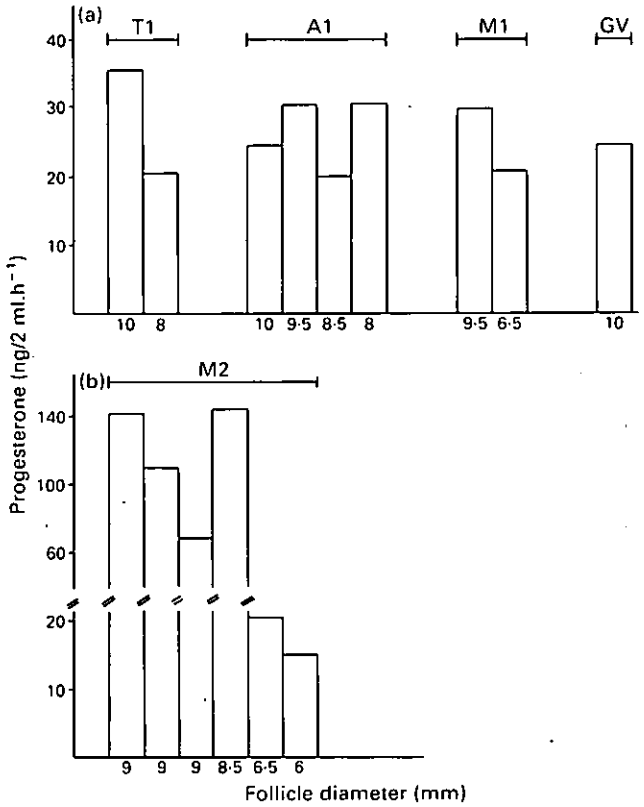

Fig. 4. Follicle diameter, progesterone secretion by the collapsed follicle during incubation and stage of oocyte maturation of individual follicles recovered from 2 immature gilts which had been stimulated with PMSG (750 i.u.) for 72 h followed by hCG (500 i.u.) for (a) 35 h or (b) 45 h. Stages of oocyte maturation: GV, germinal vesicle; PM, prometaphase; M1, first metaphase; A1, first anaphase; T1, first telophase; M2, second metaphase. (M. G. Hunter & T. Wiesak, unpublished observations.)

Implications of follicular asynchrony

Luteal function

Since both morphological and biochemical follicular heterogeneity continues into the immediate preovulatory period, it is logical to predict that this will have consequences for corpus

luteum formation. To the authors' knowledge, only two papers have been published on this topic but different conclusions were reached. While Rao & Edgerton (1984) reported dissimilarity between corpora lutea (CL) within the same ovary and also between left and right ovary, Ottobre *et al.* (1984) concluded that there was low variability between CL, and that pig ovaries contained a homogeneous population of CL. There are several reasons why the conclusions should be different. Firstly, Ottobre *et al.* (1984) obtained tissue recovered on known days of the oestrous cycle, and it is quite possible that the extent of heterogeneity may vary with age of the tissue. Secondly, although both studies investigated luteal progesterone content and LH/hCG binding, Rao & Edgerton (1984) also analysed mitochondrial, lysosomal and cytosolic enzyme activity. Thirdly, statistical analysis of the data differed, and although Ottobre *et al.* (1984) concluded that there was more variation between than within animals, this may well be because tissue from animals at different stages of the luteal phase was being compared. Heterogeneity in the luteal population in the pig in terms of DNA and progesterone content and acid phosphatase, cytochrome oxidase and N-acetyl glucosaminidase has also been noted by T. A. Bramley (personal communication). Our own preliminary observations (T. Wiesak & M. G. Hunter, unpublished observations) on weights of individual CL on Day 12 and during pregnancy (Days 12, 20 and 30) have shown that weight can vary by at least 2-fold within an animal, and also that this variation was greater ($P < 0{\cdot}05$) on Day 30 than earlier in pregnancy or during the cycle. Indeed, it may be that some CL are functionally 'inadequate' and this results in a failure to maximize progesterone production in early pregnancy which may contribute to embryonic loss. Furthermore, induced CL from PMSG and hCG-treated immature gilts have been shown to be less sensitive to gonadotrophins than the spontaneous CL of mature gilts (Kineman *et al.*, 1987a, b). These differences may contribute to abnormal function of the induced CL and thus to the inability of the immature gilt to maintain pregnancy. Luteal heterogeneity has also been described for sheep, a monovulatory species, following superovulation with PMSG and hCG (Hunter & Southee, 1989). Intra- as well as inter-luteal heterogeneity had earlier been reported, in terms of progesterone content and gonadotrophin binding (Estergreen *et al.*, 1968; Niswender *et al.*, 1976) and immunocytochemical staining for relaxin (P. A. Denning-Kendall, personal communication).

Therefore, evidence suggests that luteal heterogeneity does occur in a polyovulatory species such as the pig, and we suggest that this is a consequence of variation in the extent of follicular development before the LH surge.

Embryo development

There is evidence that follicle somatic cells play a vital role in programming the maturation of the oocyte (Moor *et al.*, 1983) and as such that follicle cells can also influence the developmental competence of the oocyte (Staigmiller & Moor, 1984; Mattioli *et al.*, 1988). The inter-cellular coupling between the oocyte and cumulus cells is essential for normal oocyte maturation and although oocytes cultured *in vitro* in the absence of follicular tissues initially undergo spontaneous maturation, this process is incomplete (Motlik & Fulka, 1974). Non-steroidal components of pig follicular fluid or produced during co-culture of follicles and oocytes have been reported both to inhibit oocyte maturation (OMI) (Tsafrifi *et al.*, 1982) and to promote nuclear and cytoplasmic maturation of oocytes (Naito *et al.*, 1988; Ding *et al.*, 1988). Indeed, the high incidence of abnormality associated with oocytes cultured outside the follicle (Thibault & Gerard, 1973; Motlik & Fulka, 1974) can be overcome by culturing oocytes within intact isolated follicles (Moor & Warnes, 1978). This information demonstrates that the follicular environment to which the oocyte is exposed before ovulation has a critical role in the maturation and 'fertilizability' of the oocyte and thus subsequent early embryonic development. Since we have already demonstrated that considerable follicular heterogeneity continues even until the immediate preovulatory period (and probably also during the luteal phase), then it is logical to deduce that the oocytes had been exposed to various intra-follicular environments before ovulation. Although the heterogeneity described was

based on observations of morphology, steroid production and gonadotrophin binding sites, it is also likely that the production of the 'regulatory factors' described above would vary among the follicles. The fact that the timing of the maturation of the oocytes *in vivo* is not completely synchronous would support this suggestion.

It is also possible that this asynchrony may have consequences for, and be reflected in, early embryonic development and survival. This is supported by the report that conceptus development in the highly prolific Meishan breed of pig was more uniform than in Large White gilts and associated with greater embryonic survival (Bazer *et al.*, 1988). Furthermore, Pope *et al.* (1990) have established a relationship between time of ovulation and morphological variation of Day-11 pig embryos. The elimination of the later ovulating follicles resulted in more uniform embryonic development than when all follicles were allowed to ovulate, indicating that the later protracted ovulations resulted in lesser developed embryos. These results further demonstrate that follicles do not respond synchronously to the LH surge in terms of ovulation and thus agree with findings that the morphological and biochemical luteinization of follicles in response to the LH surge did not occur simultaneously in all follicles within an ovary.

Collectively, these data show that follicular heterogeneity continues during the peri-ovulatory period and probably also through the luteal phase and as a likely consequence will influence oocyte maturation and subsequent early embryonic development.

Financial support from the Agricultural and Food Research Council is acknowledged. T.W. was in receipt of a Research Fellowship from the Journals of Reproduction & Fertility Ltd.

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