J. Reprod. Fert., Suppl. 33 (1985), 1–19

Printed in Great Britain

Basic physiology of follicular maturation in the pig

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Summary. The pig is an excellent animal in which to study the control of folliculogenesis in a polytocous species, and particularly to examine the inter-relationships between follicles from the same animal. Follicle recruitment occurs from the proliferating pool, and various studies suggest that this recruitment occurs between Days 14 and 16 of the oestrous cycle. The growth of follicles selected for ovulation is associated with rapid atresia of smaller follicles and a block to their replacement in the proliferating pool. However, there is a considerable range in the morphological and biochemical development of the dominant follicles in the early follicular phase, suggesting that follicles are recruited at markedly different stages of development, or that recruitment continues into the follicular phase. A significant and predictable relationship has been established between follicular diameter and follicular fluid volume, and a comparison of these two characteristics demonstrates a gradual increase in follicular tissue volume as a proportion of total volume. Growth of follicles from 2 to 4 mm is associated with a proportional increase in granulosa cell numbers, but above 4 mm the relationship is very variable even in selected follicles that are steroidogenically active. Therefore, the number of granulosa cells cannot be used as an indicator of atresia in pig follicles.

LH receptors are present in thecal tissue throughout development, reaching maximal levels on Day 20 of the oestrous cycle and declining on Day 21. Granulosa cells possess receptors for LH only in the later stages of maturation, and again these are maximal on Day 20. The pattern of steroidogenesis in pig follicles is consistent with the two-cell theory of steroidogenesis in that androgen produced by the theca is aromatized to oestrogen by the granulosa cells, However, in contrast to that of many other species, the theca of the pig also produces oestradiol in quantities comparable to those secreted by the granulosa. As with morphological development, the selected population of preovulatory follicles shows a considerable range of biochemical development and follicles of identical size may show great dissimilarity in follicular fluid steroid concentrations and LH binding. Androgen availability rather than aromatase activity appears to be the limiting factor for steroidogenesis. There are also several non-steroidal factors which have been isolated from porcine tissue and play some role in follicular maturation.

Although exogenous gonadotrophins are effective in promoting follicular development, other factors of extra- or intra-ovarian origin may limit follicular responsiveness to gonadotrophins.

Finally, it is suggested that the interfollicular relationships in polytocous animals may differ from those of monotocous animals and in the pig the dominant follicles may promote the maturation of the smaller follicles, in contrast to their inhibitory effect in other species. This may be achieved by oestrogens secreted from the dominant follicles passing to the ovarian artery via a sub-ovarian countercurrent exchange mechanism.

Introduction

Ovarian tissue from the gilt and sow has been extensively used to study many aspects of follicular

development, oocyte maturation and the luteinization of theca and granulosa tissue. However, the ovarian tissue used has frequently originated from abattoirs and therefore from animals of uncertain physiological status. Follicles dissected from such ovaries have sometimes, but not always, been classified as being atretic or non-atretic and generally pooled on the basis of size before study *in vitro*. Although these studies have established many of the characteristics of follicle development in pigs, considerable refinement in the collection and classification of follicles seems necessary to permit accurate description of the factors controlling follicle maturation and the intra-follicular mechanisms involved. This objective has been partly achieved by studying follicles obtained from gonadotrophin-treated prepubertal gilts (Ainsworth, Tsang, Downey, Marcus & Armstrong, 1980; Evans, Dobias, King & Armstrong, 1981).

There is nevertheless a paucity of data describing the relative morphological and biochemical development of follicles within the same ovary, yet an understanding of the inter-relationships between follicles is crucial in a polytocous species, such as the pig, in any attempt to control reliably the time of ovulation or ovulation rate. This paper reviews published information on follicular development in the pig in the light of a model for follicular maturation developed in laboratory species. Recent data on follicular inter-relationships obtained from the study of cyclic gilts or lactating and weaned sows are also summarized.

A model of follicular maturation derived from studies of laboratory species

The initiation of primordial follicle growth and the formation of a small growing primary follicle occurs independently of pituitary gonadotrophins and is subject only to intraovarian controls (Peters, Byskov, Himelstein-Braw & Faber, 1975). At about the time the growing primary follicle acquires theca interna cells, further growth and maturation of the follicle become completely dependent on pituitary gonadotrophins and development involves both steroid and protein hormone regulation of granulosa–theca cell proliferation, differentiation and function. Autoradiographic studies with rat ovarian tissue indicate that LH binds to thecal tissue and to receptors that develop on granulosa cells during the final stages of follicular maturation (Richards & Midgley, 1976). The binding capacity of rat thecal tissue for LH is greater in preovulatory follicles than small follicles (Richards, 1979) and LH stimulates the production of androgens, principally androstenedione and testosterone. FSH binds exclusively to the granulosa cells (Richards & Midgley, 1976) and induces the aromatase enzymes that convert androgens to oestradiol. Although the number of granulosa cells increases as the follicle develops, the number of FSH receptors per cell and binding affinity do not change (Nimrod, Erickson & Ryan, 1976).

It has been demonstrated that oestradiol acts on the granulosa cells of hypophysectomized rats to increase the number of receptors for oestradiol (Richards, 1975) and FSH can act to increase the number of receptors for FSH. After priming with oestradiol, FSH induces receptors for LH on large antral follicles, and therefore only the granulosa cells of large preovulatory follicles possess receptors for both LH and FSH (Richards & Midgley, 1976). The sequence of events involved in follicular maturation in the rat is summarized in Fig. 1 and the mechanism by which FSH and oestradiol act to stimulate the production of LH receptors has been most thoroughly studied in this species. Oestradiol enhances the ability of FSH to stimulate cyclic AMP production and also increases intracellular concentrations of cyclic AMP-binding proteins in rat granulosa cell cytosol (Richards, 1979). It is this amplification of the actions of FSH by oestradiol which, at least in the rat, appears necessary to promote the increase in granulosa cell LH receptor formation.

Richards, Rao & Ireland (1978) proposed that changes in the endogenous production of oestradiol and/or the response of follicular cells to oestrogen may affect the rate of follicular growth and the differentiation of follicular cells, including the number of receptors for FSH and/or LH in the granulosa and theca. The accumulation of FSH and oestradiol-17β by the developing follicle is essential to ensure maximum proliferation of follicular granulosa cells in the rat

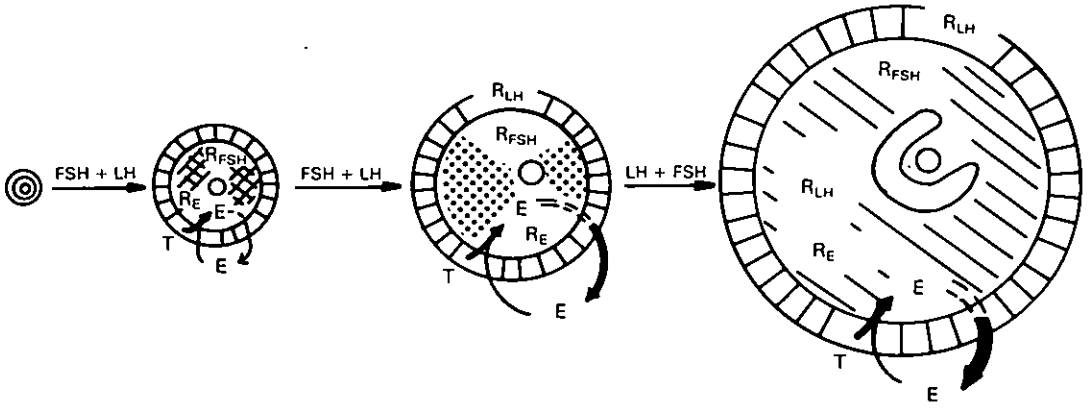

Fig. 1. A model for progressive developmental changes in the response of rat granulosa cells to hormones. R_{FSH}, R_{LH}, R_E, receptors for FSH, LH and oestradiol (E); T, testosterone. □, ▨, ⊡, ▧ represents successive stages of granulosa cell differentiation. (Redrawn from Richards *et al.*, 1978.)

(Goldenberg, Vaitukaitis & Ross, 1972) and in the human (McNatty & Sawers, 1975). Interaction with its specific nuclear receptor probably mediates the mitotic activity of oestradiol-17β (Richards, 1975).

Therefore, the initiation of follicular oestradiol production and the ability of the granulosa cells to respond to oestradiol appears to determine whether a given follicle goes on to ovulate or becomes atretic.

Follicle maturation in the pig

Morphological development

Studies with the late prepubertal gilt (see Christenson, Ford & Redmer, 1985), cyclic gilt (Robinson & Nalbandov, 1951; Kirkpatrick, Howland, First & Casida, 1967; Dailey, Clark, First, Chapman & Casida, 1972; Clark, Edey, First, Chapman & Casida, 1973; S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations) and the lactating and weaned sow (see Britt, Armstrong, Cox & Esbenshade, 1985) suggest that, except in the follicular phase, continuous development and subsequent atresia maintain a proliferating pool of follicles of 1–6 mm in diameter. Estimates of the numerical size of this pool vary, depending on the classification system used, but in his review of follicular development in the cyclic gilt Anderson (1980) suggested that the proliferating pool contained approximately 50 follicles between 2 and 5 mm in diameter. It has also been reported, however, that the number of follicles within a specified size range was greater in breeds with high ovulation rates (Kirkpatrick *et al.*, 1967; Clark *et al.*, 1973) and could therefore be a potential determinant of eventual ovulation rate and litter size, as has been suggested for some breeds of sheep (Lahlou-Kassi & Mariana, 1984). Thus an understanding of the physiological mechanisms that control the size of the proliferating pool could be of practical significance also in the pig.

Given an appropriate stimulus, recruitment occurs from the proliferating pool, which establishes a group of selected follicles destined to ovulate. The time at which selection (or recruitment) occurs has been extensively studied in the cyclic gilt and data from experiments involving treatment with exogenous gonadotrophins (Phillippo, 1968; Hunter, 1972; Hunter, Cook & Baker, 1976), electrocautery of follicles (Clark, Kelly, Orr & Tribble, 1979) and unilateral ovariectomy (Coleman & Dailey, 1979; Clark, Brazier, Wiginton, Stevenson & Tribble, 1982) suggest that

recruitment occurs between Days 14 and 16 of the oestrous cycle. After Day 16 incomplete ovarian compensation was observed after electrocautery or unilateral ovariectomy, and treatment with exogenous gonadotrophins did not induce superovulatory responses. Similarly, the beneficial 'flushing' effects of providing additional nutrients to the gilt or sow are only consistently observed when 'flushing' starts some 4–6 days before oestrus (Kirkpatrick *et al.*, 1967; Dailey *et al.*, 1972).

The growth of selected preovulatory follicles during the follicular phase is associated with rapid atresia of smaller follicles and a block to their replacement in the proliferating pool (Robinson & Nalbandov, 1951; Anderson, 1980; Clark *et al.*, 1982; S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations), as illustrated in Fig. 2. Recent studies from our own laboratory in both the weaned sow (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations) and cyclic gilt (S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations) raise other points of interest. Firstly, the number of follicles >6 mm in diameter in the early follicular phase is frequently below the expected ovulation rate and there is a considerable range in the morphological and biochemical development of the dominant follicles at this stage (see Fig. 3). Therefore, if recruitment of all destined ovulatory follicles occurs at one specific time, then the follicles are recruited at markedly different stages of development. Alternatively, the recruitment process may continue into the follicular phase, as has been proposed for at least one prolific breed of sheep (Driancourt, Cahill & Bindon, 1985); in this case the stimulus for recruitment, at least in terms of circulating gonadotrophins and steroids, will change as recruitment proceeds. Secondly, a comparison in both the cyclic gilt and weaned sow between the number of

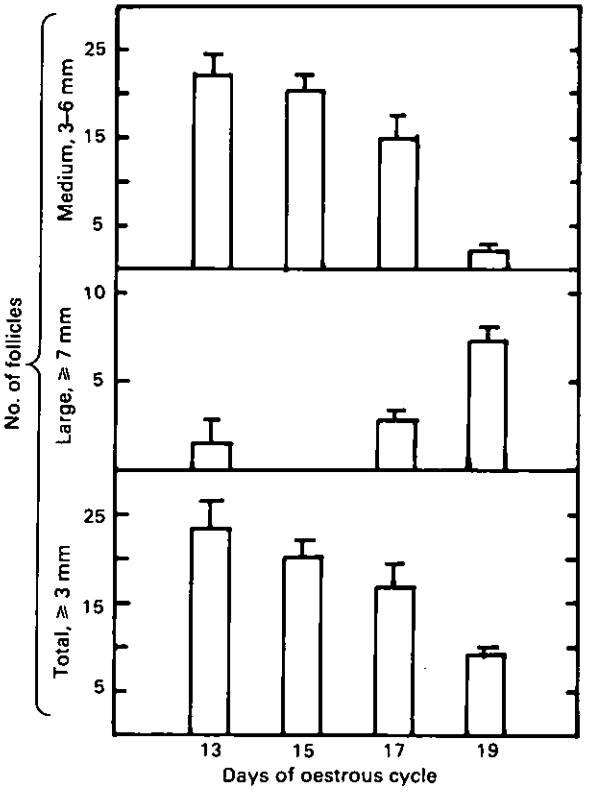

Fig. 2. Mean (+ s.e.m.) number of medium and large follicles and the total number of follicles in the right ovary of the pig on Days 13, 15, 17 and 19 of the oestrous cycle. (From Clark *et al.*, 1982.)

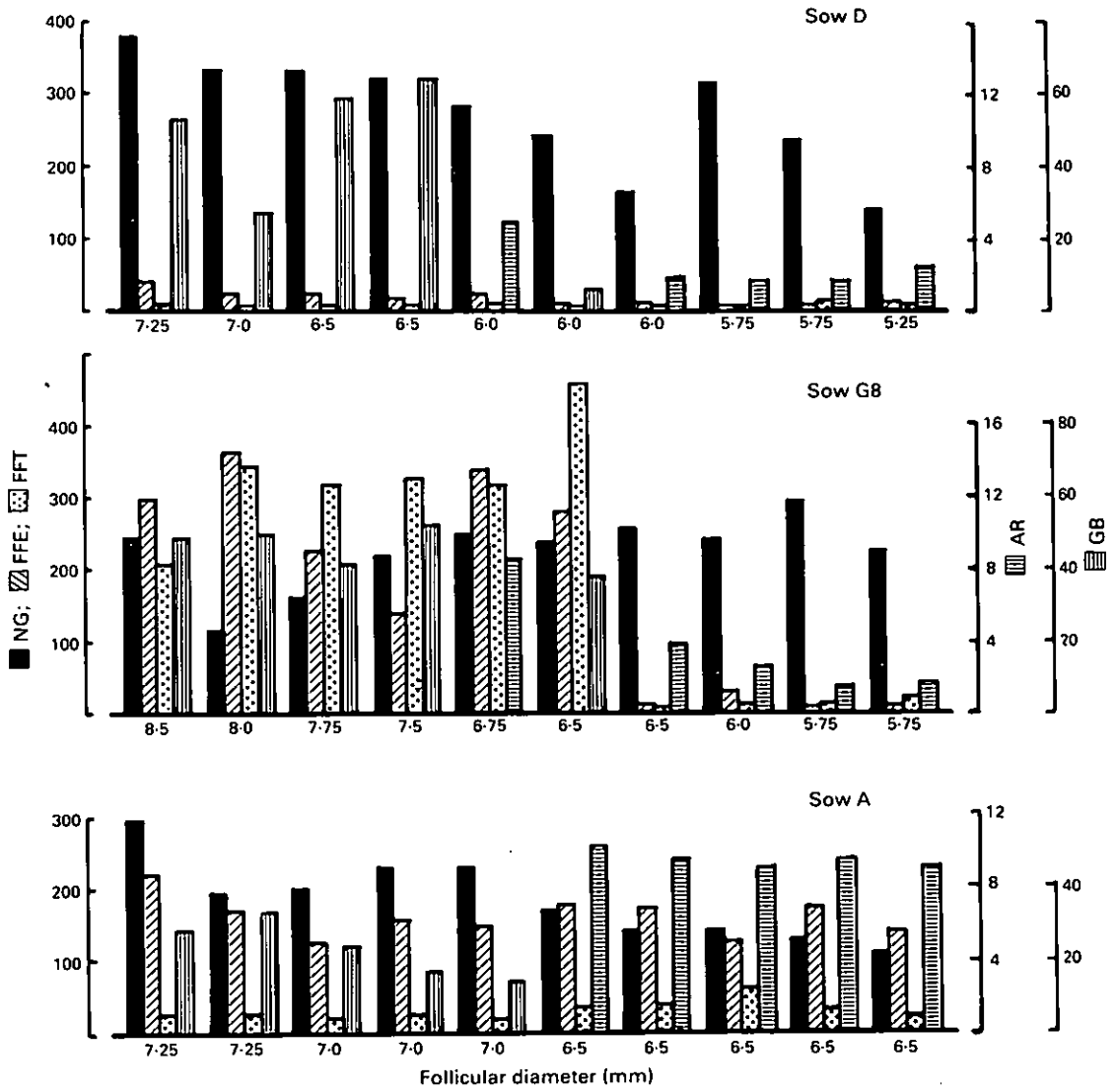

Fig. 3. Follicular maturation assessed *in vitro* in the 10 largest follicles dissected from sows 48 h after weaning. ■, Number of granulosa cells per follicle (NG), × 10^4; ▨, follicular fluid oestradiol (FFE) (ng/ml); ⊡, follicular fluid testosterone (FFT) (ng/ml); ▥, granulosa cell aromatase activity (AR), ng oestradiol/follicle/2 h incubation; ▤, granulosa cell binding (GB) of ^{125}I-labelled hCG, c.p.m. × 10^3/follicle. AR and GB measured in the 5 largest and 5 smallest follicles per sow. Values below histograms, follicle diameter (D), mm. (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations; for details see text.)

follicles >6 mm in diameter during the mid- to late-follicular phase, and the anticipated number of ovulations in comparable animals, suggests that the incidence of atresia at this time is low. Although in his review of follicular atresia Ryan (1981) reached a different conclusion, the 30–40% atresia reported in the large 7–10 mm follicles studied may have been related to the uncertain status of the animals from which the ovarian tissue was obtained.

As in other species (Carson, Findlay, Clarke & Burger, 1981; England, Dahmer & Webb, 1981) a predictable positive relationship was established in weaned sows and cyclic gilts between follicular diameter and follicular fluid volume (Fig. 4); as shown, the difference between follicular fluid volume and the calculated total volume of the follicle represents follicular tissue volume. As a proportion of total volume, tissue volume gradually decreased from ~0·57 at 3 mm to 0·40 at 9 mm in diameter. However, in the late follicular phase a dramatic increase in tissue volume was apparent (S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations) and only a small volume of viscous follicular fluid was present in 10–11 mm follicles dissected from the ovaries of cyclic gilts on the 2nd day of oestrus (about 12–16 h before ovulation). Histological examination of such follicles showed considerable infolding of both the granulosa and theca which to our knowledge has only previously been described in preovulatory follicles of most carnivores (see Brambell, 1956).

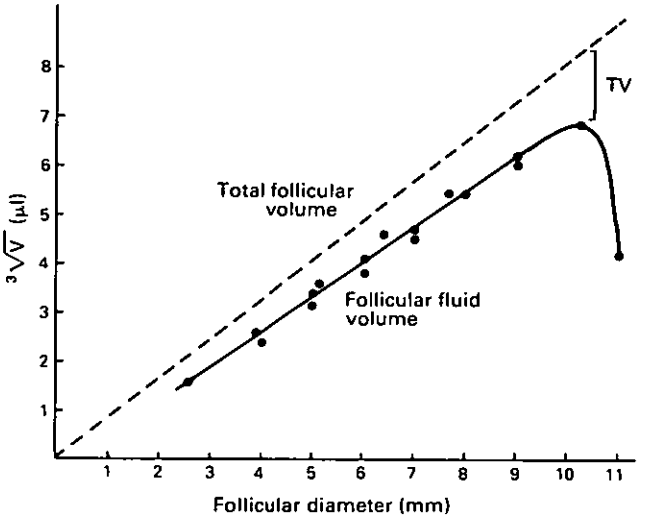

Fig. 4. Overall relationship between total follicular volume and the volume of aspirated follicular fluid in pig follicles; derived from studies of lactating sows (Shaw, 1984), sows 48 h after weaning (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations) and cyclic gilts (S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations). TV, estimated tissue volume. Data points are representative of the different sets of follicles from which the overall relationship was established.

The overall relationship between follicular diameter and granulosa cell numbers is shown in Fig. 5 and has some of the characteristics described for sheep and human follicles (McNatty, 1982). Given the similarity in maximal granulosa cell numbers when follicles of the three species approach ovulatory size, and the much lower ovulation rate in sheep and women, it is clear that the preovulatory follicles of the pig collectively contain a greater total mass of granulosa tissue and this is probably functionally related to the higher peripheral concentrations of ovarian steroids in this species. At least in the weaned sow the great variability in granulosa cell numbers in follicles of similar diameter was not necessarily related to atresia, as follicles with both high and low cell numbers were found to be equally active oestrogenically (see Fig. 5). The numbers of granulosa cells cannot therefore be used as an indicator of atresia in pig follicles.

Biochemical development

Gonadotrophin and prolactin receptors. It has been found that, as in the rat, thecal tissue from large follicles binds more LH/hCG than small follicles (Channing & Kammerman, 1974; Daguet, 1979; S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations; see Fig. 7) and granulosa cells bind LH/hCG with an increasing number of sites per cell as the follicle matures (Channing & Kammerman, 1974; Kammerman & Ross, 1975; Lee, 1976; Stouffer, Tyrey & Schomberg, 1976; Nakano, Akahori, Katayama & Tojo, 1977; Daguet, 1979; S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations; see also Fig. 7). FSH has been reported to bind only to granulosa cells and to show a different pattern of binding from LH in that, as in the rat, there is no increase in the number of FSH receptors per cell as the follicle enlarges (Nakano *et al.*, 1977).

With regard to steroid–protein hormone interactions in terms of receptor induction, it has been reported that follicular fluid oestradiol concentration increases as pig follicles mature from Day 17 to Day 20 although there is considerable variation between animals (Hunter *et al.*, 1976). Daguet (1979) also measured high follicular fluid oestradiol concentrations just before high LH binding by the granulosa cells and suggested that, as in the rat, oestradiol may be involved in the induction of

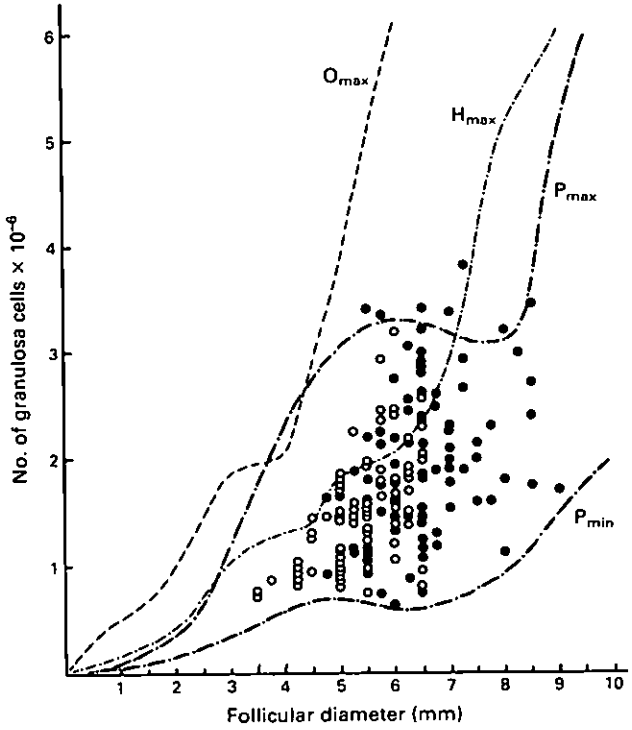

Fig. 5. Semi-schematic representation of the relationship between follicular diameter and granulosa cell numbers in pig follicles. P_{max}, P_{min}, approximate maximal and minimal cell numbers recovered from follicles from lactating sows (Shaw, 1984), sows 48 h after weaning (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations) and from cyclic gilts (S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations). ●, ○ actual data points from the 48 h weaned sow study; closed circles represent follicles with greater than, and open circles less than, 100 ng oestradiol/ml follicular fluid. For comparison, maximal granulosa cell numbers recovered from sheep (O_{max}) and human (H_{max}) follicles are shown (from McNatty, 1982).

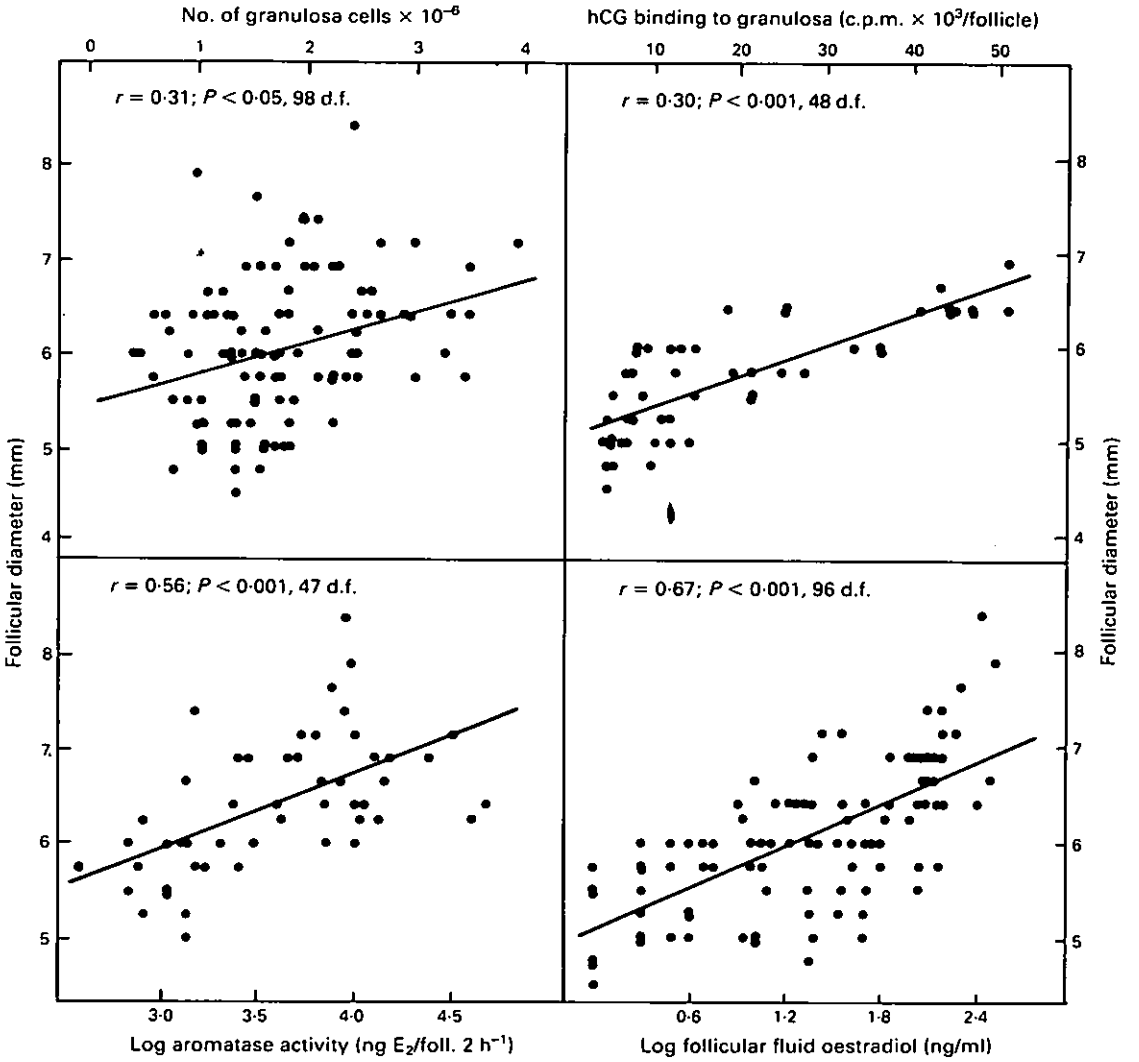

Fig. 6. Correlations between follicular diameter and granulosa cell numbers, follicular fluid oestradiol (E_2) concentrations, granulosa cell aromatase activity and granulosa binding of ^{125}I-labelled hCG, established in follicles recovered from sows 48 h after weaning. (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations.)

LH receptors. There is some in-vitro evidence for the involvement of steroids in receptor induction/responsiveness because Veldhuis, Klase, Strauss & Hammond (1982) reported that oestradiol amplifies the stimulatory effects of FSH on steroid production by pig granulosa cells. It has also been demonstrated that FSH plus insulin, cortisol and thyroxine stimulate de-novo synthesis of LH receptors in granulosa cells from small (< 2 mm) pig follicles (Loeken & Channing, 1985).

Specific receptors for prolactin in luteal and granulosa tissue from pig follicles have been described (Rolland, Gunsalus & Hammond, 1976; Veldhuis, Klase & Hammond, 1980) and the level of specific binding is higher in small (1–2 mm) than in large (> 6 mm) follicles. In-vitro cultures of granulosa cells with levels of prolactin that are within the range reported for human follicular fluid *in vivo* (McNatty, Hunter, McNeilly & Sawers, 1975; Soto, Tureck & Strauss, 1985),

produced an inhibitory effect on progesterone accumulation in cells from small follicles but a stimulatory effect on granulosa tissue from large follicles (Veldhuis *et al.*, 1980); a physiological role for prolactin, acting at the ovarian level to control follicular steroidogenesis, was therefore proposed.

Steroidogenesis. Studies on steroidogenesis in pig follicles support the 'two-cell' theory of steroidogenesis as originally proposed for the rat (Falck, 1959) and subsequently confirmed for the pig (Haney & Schomberg, 1981; Evans *et al.*, 1981). The granulosa cells are the predominant site of progesterone synthesis and are responsive to LH, FSH (Evans *et al.*, 1981; Stoklosowa, Gregoraszczuk & Channing, 1982) and prolactin (Veldhuis *et al.*, 1980). Although pig granulosa cells lack the 17α-hydroxylase enzymes necessary to synthesize androgens from progesterone or pregnenolone (Bjersing & Carstensen, 1967), substantial androgen synthesis by the thecal tissue of pig follicles has been demonstrated (Stoklosowa, Bahr & Gregoraszczuk, 1978; Tsang, Leung & Armstrong, 1979; Evans *et al.*, 1981; Stoklosowa *et al.*, 1982). The major androgen produced by the theca tissue is androstenedione (Evans *et al.*, 1981) which, after transfer to the granulosa, is converted to testosterone and then aromatized to oestradiol.

In contrast to many other species, however, the pig theca interna tissue produces oestradiol in quantities comparable to those of the granulosa, to which it also provides exogenous aromatizable substrate (Evans *et al.*, 1981; Haney & Schomberg, 1981; Stoklosowa *et al.*, 1982). Stoklosowa *et al.* (1982) reported considerable oestrogen synthesis by both granulosa and thecal tissue during monolayer culture, but significantly more oestradiol was secreted when the two cell types were cultured together. In terms of oestradiol production by granulosa cells, Stoklosowa *et al.* (1982) reported significant stimulation by both LH and FSH, but Evans *et al.* (1981) could find no effect of the gonadotrophins. This discrepancy may be accounted for by different sources of tissue (random slaughterhouse tissue or PMSG-treated prepubertal gilts) or by different culture techniques. In many other species including the rat (Falck, 1959) aromatase activity is confined to the granulosa cells and thus in immature follicles is controlled by the binding of FSH to granulosa cells (Richards & Midgley, 1976; Armstrong & Dorrington, 1977; Erickson & Hsueh, 1978). As the thecal tissue from the pig also possesses an aromatase enzyme system (Evans *et al.*, 1981; Stoklosowa *et al.*, 1982) then the possibility exists that FSH, as well as LH, may activate aromatase in this tissue. Thecal tissue was responsive to LH (but not FSH) in the production of androgens, and this production increased with development of the follicle (Evans *et al.*, 1981).

Intraovarian interactions during follicular maturation. The maturational characteristics of individual follicles from within the same ovary were initially studied in our laboratory in ovarian tissue recovered from sows 48 h after weaning (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations), when sows were considered to be in the early- to mid-follicular phase of the first oestrous cycle after weaning. The largest 10 (presumed ovulatory) follicles were dissected from the ovaries of each sow and studied *in vitro* to determine follicular diameter, follicular fluid volume, follicular fluid concentrations of oestradiol-17β and testosterone, the number of granulosa cells, granulosa cell aromatase activity and the binding of ^{125}I-labelled hCG to theca and granulosa tissue. Figure 6 illustrates the significant positive correlations established between follicular diameter and the other measures and these confirm predictable relationships between morphological development, the development of aromatase activity and LH binding within granulosa tissue, and, as a consequence, the secretion of oestradiol into follicular fluid. However, a greater appreciation of follicular interactions was obtained from a study of the variation in follicular maturation both within and between animals, as shown in Fig. 3.

In Sow D, in which no follicles were classified as being oestrogenic (follicular fluid oestradiol >100 ng/ml) and hCG binding to granulosa tissue was low, aromatase activity was nevertheless high, suggesting that the development of aromatase activity does not limit oestrogen synthesis in the early follicular phase. This is consistent with the observations of Shaw (1984) in lactating sows; compared to control females a significant increase in granulosa cell aromatase activity occurred

Table 1. Mean follicular data for subsets of follicles segregated on the basis of known oestrogenic activity 48 h after weaning in the sow

	Sows with oestrogenic follicles (N = 5)		Sows with no oestrogenic follicles (N = 5)	
	Subset 1 (oestrogenic follicles)	Subset 2 (non-oestrogenic follicles)	Subset 3 (all follicles)	s.e.d.
Diameter (mm)	6·87	5·64	5·90	0·16
Follicular fluid oestradiol (ng/ml)	173·5	30·6	17·8	10·1
Follicular fluid testosterone (ng/ml)	119·9	48·5	15·6	18·9
No. of granulosa cells ($\times 10^6$)	1·96	1·60	1·84	0·17
Granulosa aromatase activity (ng/follicle/2 h)	7·31	3·06	9·90	2·64
hCG binding to theca (c.p.m. $\times 10^3$/follicle)	13·87	9·81	8·90	1·25
hCG binding to granulosa (c.p.m. $\times 10^3$/follicle)	42·73	16·06	15·22	2·98

after a reduction in the suckling stimulus in the absence of any increase in follicular fluid testosterone or oestradiol concentrations. In Sow G8, 6 of 10 follicles dissected were classified as oestrogenic, with follicular fluid oestradiol:testosterone ratio of approximately unity. Although LH binding to granulosa tissue was not studied in all follicles, a lack of binding in the 4 smallest follicles was associated with low follicular fluid oestradiol and testosterone values, whereas in the 5th largest follicle oestradiol and testosterone and binding of ^{125}I-labelled hCG to granulosa cells were elevated. Similarly, in Sow E in which even the smallest follicles were oestrogenic, hCG binding to granulosa tissue was again high. Overall significant correlations ($P < 0{\cdot}001$) were established between follicular fluid oestradiol and binding of ^{125}I-labelled hCG to the theca and granulosa, suggesting a functional relationship between LH binding and the activation of oestrogen synthesis, probably acting via the availability of androgen precursors. This suggestion is consistent with the lack of any significant correlations between ^{125}I-labelled hCG binding to theca or granulosa cells and follicular fluid testosterone; as androgen precursors for the theca became available to follicles already possessing active aromatase systems in both the granulosa and also probably in the theca itself (see Evans *et al.*, 1981), rapid conversion to oestradiol would be expected and would deplete androgen levels in follicular fluid whilst oestradiol accumulated. This pattern of events is certainly reflected in the follicular data in Fig. 3 and in this situation increasing levels of LH binding, promoting an increase in androgen synthesis, would not necessarily be associated with increasing follicular fluid testosterone levels.

As with morphological development, the range of biochemical development in the selected population of preovulatory follicles should again be emphasized. As shown in Fig. 3, follicles of identical size and with equal numbers of granulosa cells may show great dissimilarity in follicular fluid steroid concentrations and in LH binding. The importance of the intraovarian environment in which follicles are developing is shown by the data in Table 1, in which follicle subsets were created by classifying sows, and follicles within sows, on the basis of high or low (> or < 100 ng/ml) follicular fluid oestrogen concentrations. In follicle subset 3 from sows with no oestrogenic follicles, maximal aromatase activity was associated with low theca and granulosa hCG binding and basal levels of follicular fluid steroids. In subset 2, from sows in which the dominant follicles were oestrogenic, although follicular diameter, number of granulosa cells and LH binding to thecal and

granulosa cells were similar to those of subset 3, aromatase activity was significantly ($P<0.001$) lower, whilst follicular fluid oestradiol and testosterone were significantly ($P<0.05$ and 0.01, respectively) higher. Thus the pooling of follicles from within the same ovary on the basis of size, in addition to the pooling of similar sized follicles from different animals before in-vitro study, is likely to confound the interpretation of the data obtained.

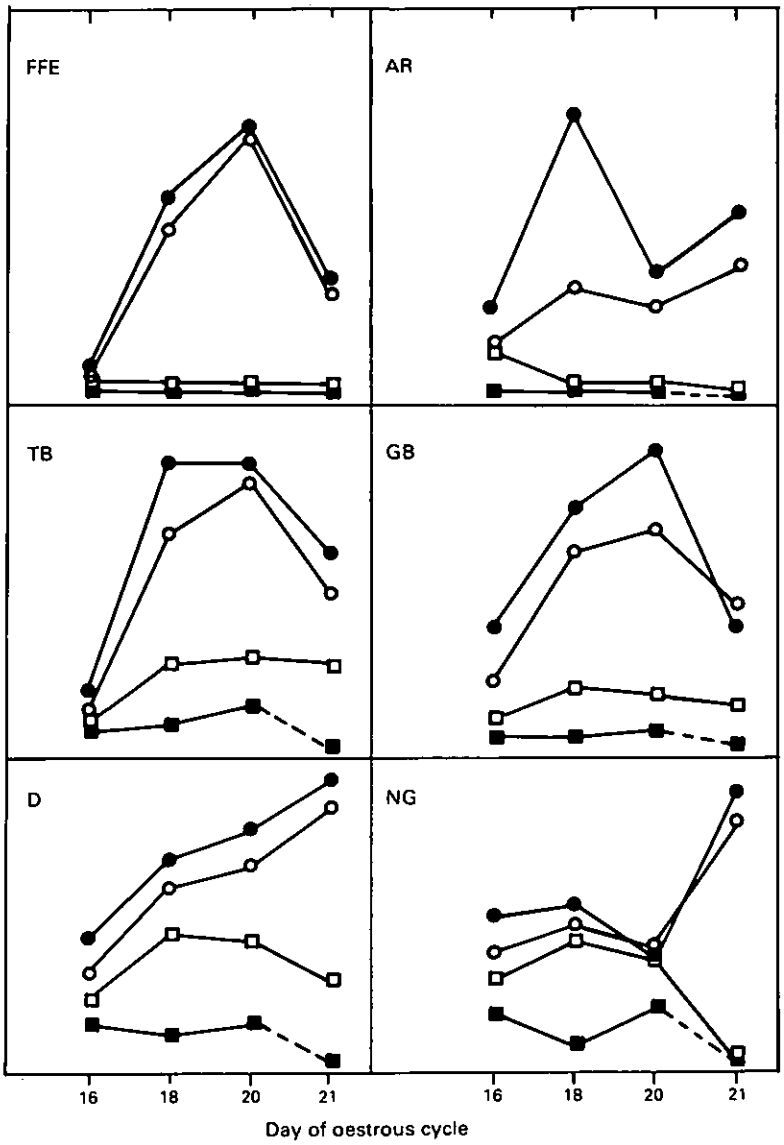

Fig. 7. Relative differences in pig follicular maturation assessed *in vitro* in sets of follicles within the ovarian hierarchy of individual gilts in the late-luteal and follicular phase of the oestrous cycle. ●, Dominant, presumed ovulatory follicles; ○, smaller, presumed ovulatory follicles; □, largest non-ovulatory follicles; ■, smallest non-ovulatory follicles (>2 mm in diameter): when the last set of follicles was absent a broken line connects the datum points. FFE, follicular fluid oestradiol concentration; AR, granulosa cell aromatase activity; TB, theca binding and GB, granulosa binding of ^{125}I-labelled hCG; D, diameter; NG, numbers of granulosa cells. (S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations.)

Further information on follicular maturation has been obtained from a recent study of follicles recovered from the ovaries of cyclic gilts on Days 16, 18, 20 and 21 of the oestrous cycle (S. A. Grant, M. G. Hunter & G. R. Foxcroft, unpublished observations). In this experiment all follicles $\geqslant 2$ mm in diameter were dissected from the ovaries and studied *in vitro*. A preliminary descriptive assessment of the data is presented in Fig. 7 in which means were calculated for four subsets of follicles. Set 1 was the largest 5 follicles in the presumed ovulatory population; Set 2 was the last 5 follicles ranked by size that were considered to be in the 'selected' preovulatory population. For Day 16 and 18 follicles the designation of assumed preovulatory status was based on characteristically higher aromatase activity and steroid accumulation in follicular fluid; on Days 20 and 21 the designation was made on the basis of distinct size differences in addition to a number of biochemical characteristics. Set 3 comprised the 5 largest follicles that were not in the assumed ovulatory population and when present, Set 4 consisted of all other follicles $\geqslant 2$ mm. Assuming that the dominant follicles on Day 16 do not undergo atresia and would remain as the dominant follicles during the remainder of the follicular phase (a supposition that requires experimental confirmation), it is evident that the more mature follicles within the proliferating pool at the time of selection are those that are most likely to ovulate. These data also suggest that the dominant follicles at Day 16 not only have elevated granulosa aromatase activity and LH binding to granulosa and theca cells but that they are also already synthesizing oestradiol. Within the ovulatory Set 1 and Set 2 follicles, follicular fluid oestradiol and granulosa and theca LH binding increase substantially to reach maximal values on Day 20 and then decline by Day 21, presumably due to down-regulation of LH receptors as described for other species (Webb & England, 1982; Ireland & Roche, 1982) and hence a reduction in both the synthesis of androgen precursors and in aromatase activity. A similar precipitous decline in follicular fluid oestradiol has also been recorded in pig follicles after the endogenous LH surge (Eiler & Nalbandov, 1977) and after hCG treatment *in vivo* (Ainsworth *et al.*, 1980). Aromatase activity in the selected follicles in fact declined between Day 18 and 20 in the presence of increasing follicular fluid oestradiol, again suggesting that aromatase activity is unlikely to be a limiting factor in oestrogen biosynthesis. The considerable range in development of follicles within the ovulatory population is further emphasized in the disparity between Set 1 and Set 2 follicles for all parameters studied throughout the follicular phase.

Some of the characteristics of atretic, 'unselected' follicles are also apparent from the data in Fig. 7. Although the less mature follicles continue to develop morphologically beyond Day 16, the immediate decline in aromatase activity by Day 18 undoubtedly prevents the developing granulosa cells from synthesizing oestradiol, even if androgen precursor availability were not already limited by low levels of LH binding to both theca and granulosa. As an increase in intrafollicular oestrogen is reported to be essential to the development of a full complement of LH and FSH receptors, lack of aromatase activity would prevent the unselected follicles from maturing further. The precise mechanism controlling the decrease in aromatase activity in unselected follicles is uncertain but these follicles might be expected to develop low ratios of oestradiol:testosterone, as in atretic follicles of other species (McNatty & Baird, 1978), if the synthesis of androgen precursors were initially stimulated in the absence of an active aromatase system.

The potential roles of other intraovarian regulators or cybernins (Guillemin, 1981) in the control of oocyte and follicle maturation seem at present to be almost unlimited (see Ledwitz-Rigby & Rigby, 1981; Channing *et al.*, 1982; Ward, Glenn, Liu & Gordon, 1983). To date oocyte maturation inhibitor, granulosa cell maturation and luteinization stimulator, granulosa cell luteinization inhibitor, LH receptor binding inhibitors, gonadocrinin and, of course, inhibin, have all been described as components of pig ovarian tissue. Oocyte maturation inhibitor is probably a polypeptide of <2000 molecular weight, secreted by pig granulosa cells, and plays a role in keeping the oocyte in the arrested dictyate state before ovulation (Channing & Pomerantz, 1981). Many instances have been reported of spontaneous oocyte maturation on isolation from the follicular fluid (Chang, 1955; Edwards, 1965; Tsafriri & Channing, 1975; Tsafriri, Channing, Pomerantz & Lindner, 1977).

The stimulatory actions of pig follicular fluid on granulosa cells include morphological

maturation, progesterone and oestrogen secretion, enhanced responsiveness to LH and FSH and activation of ornithine decarboxylase (Veldhuis, Demers & Hammond, 1979; Ledwitz-Rigby & Rigby, 1981). The inhibitory actions include morphological maturation, progesterone secretion, prostaglandin F-2α secretion, responsiveness to LH, FSH and prostaglandins, FSH binding and adenylyl cyclase activity (Ledwitz-Rigby *et al.*, 1977; Ledwitz-Rigby & Rigby, 1981).

Pig corpus luteum extract has been shown to contain increasing amounts of LH receptor binding inhibitor as the corpus luteum ages (Sakai, Engel & Channing, 1977; Yang *et al.*, 1981), and an inhibitor of FSH binding has been isolated from cow follicular fluid (Darga & Reichert, 1978). Gonadocrinin, a peptide with LHRH-like activity produced by the granulosa cells of the follicle has also been extracted from pig follicular fluid (Ying & Guillemin, 1981).

Inhibin is also produced by pig granulosa cells, and Lorenzen, Channing & Schwartz (1978) detected higher concentrations of inhibin-like activity in small as opposed to large follicles. However, it has also been shown that atresia influences follicular inhibin production (Henderson, Franchimont, Charlet-Renard & McNatty, 1984).

Although all these non-steroidal factors undoubtedly play some role in follicular maturation, they have mainly been isolated from pooled tissue from animals of unknown physiological states and more study is required on individual follicles from animals of a known history.

Finally, the interaction of other, even non-reproductive, hormones, at the ovarian level will need consideration in the evolution of a complete model for follicular maturation in the pig ovary. A possible role for prolactin has already been discussed; in addition specific effects of insulin on granulosa cells *in vitro* have been reported (May & Schomberg, 1981; Veldhuis, Kolp, Toaff, Strauss & Demers, 1983). Although other hormones such as thyroxine and cortisol have no direct stimulatory effect on ovarian tissue, their addition to culture media significantly enhanced the effects of other hormones (Loeken & Channing, 1985), suggesting ways in which the metabolic status of the animal may affect the potential for normal follicular development.

Gonadotrophic stimulation of follicular development

As reviewed by Paterson (1982) and Christenson *et al.* (1985), available data suggest that, except in the early neonatal period, follicular development can be stimulated in the prepubertal gilt by treatment with exogenous gonadotrophins. Indeed, responsiveness to exogenous gonadotrophins, or to GnRH-induced endogenous LH release, has been described for most phases of the reproductive cycle in which follicular development is suppressed. Neill & Day (1964) and Caldwell, Moor, Wilmut, Polge & Rowson (1969) were able to induce supplementary corpora lutea during the luteal phase of the oestrous cycle using exogenous PMSG and hCG, and the same hormones also induced follicular growth, ovulation and functional accessory corpora lutea during gestation (Christenson & Day, 1971; Coggins & First, 1973; Martin, Norton & Dziuk, 1977). Similarly, Britt *et al.* (1985) review evidence for ovarian responsiveness to gonadotrophins during lactation and after weaning.

It seems logical to suggest, therefore, that a lack of gonadotrophic stimulation is the prime cause of arrested follicular development in the gilt and sow; however, as discussed previously (Foxcroft & van de Wiel, 1982), consistent experimental evidence for characteristic increases in gonadotrophin secretion at the initiation of the follicular phase is lacking. There is certainly evidence that an actual increase in FSH secretion (which treatment with exogenous PMSG might be thought to mimic) is not an absolute pre-requisite for follicular recruitment in the cyclic gilt (see Foxcroft & van de Wiel, 1982) or weaned sow (Shaw & Foxcroft, 1985; G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations). However, plasma FSH concentrations are consistently elevated at these times of follicular recruitment; assuming that some interaction exists between the gonadotrophic effects of LH and FSH, then changes in LH in the presence of stable or even declining levels of FSH still constitutes an adequate stimulus for follicular maturation (for detailed discussion, see Shaw & Foxcroft, 1985). Whether PMSG really acts

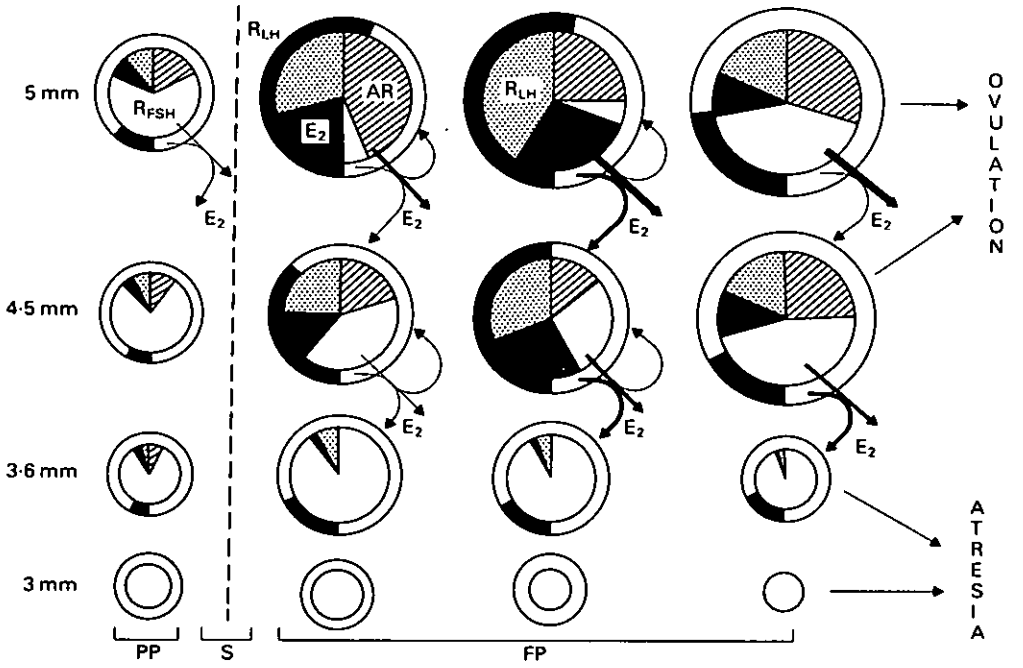

Fig. 8. A model for the sequential maturation or atresia of pig follicles. Sizes (mm) represent initial diameter of follicles within the proliferating pool (PP) before selection (S). Probable subsequent development or atresia of each size class in the follicular phase (FP) is then shown. The final stages represent initial follicular responses to the preovulatory LH surge. Outer zone of follicles represents theca and R_{LH} (solid sector) the relative amount of theca LH binding. Inner zone represents granulosa tissue and follicular fluid: R_{FSH} (clear areas), presence of, but not relative differences in, granulosa FSH binding; R_{LH} (stippled area), relative differences in granulosa LH binding and AR (cross-hatching), relative differences in maximal demonstrable aromatase activity *in vitro;* E_2 (solid sector) relative concentrations of follicular fluid oestradiol-17β. Arrows and E_2 signify origin and relative amounts of oestradiol-17β secretion from follicles and possible intraovarian as well as peripheral effects of E_2.

in an FSH-like manner to stimulate follicular recruitment should perhaps be questioned in the light of these observations. Nevertheless, a longer-term increase in FSH secretion in the prepubertal period (see Christenson *et al.*, 1985) and during lactation (see Britt *et al.*, 1985) is associated with increased follicular development within the proliferating pool and, as discussed earlier, Shaw (1984) has established some relationships between plasma FSH levels during lactation and follicular development assessed *in vitro*. The report of a significant positive correlation between FSH levels after weaning and ovulation rate (Shaw & Foxcroft, 1985) raises the possibility that FSH may be involved in determining the final number of follicles within the ovulatory population.

The evidence that an increase in LH episode frequency or baseline acts as an effective stimulus for follicular recruitment and the initiation of the follicular phase is particularly convincing in the lactating and weaned sow (see Britt *et al.*, 1985). However, we have observed that normally adequate increases in both episodic and basal LH release after weaning have not been associated with the immediate follicular response seen in other sows (G. R. Foxcroft, H. J. Shaw, M. G. Hunter, P. J. Booth & R. T. Lancaster, unpublished observations). In some situations, therefore, factors of extra- or intra-ovarian origin (discussed earlier) may limit follicular responsiveness to what in other situations would be stimulatory levels of gonadotrophins. Indeed, a situation in

which one or more factors exerted either inhibitory or stimulatory activity at both the hypothalamo–hypophysial and at the ovarian level would have considerable merit as a fail–safe control system.

A model for follicular maturation in the pig

The morphological and biochemical studies on pig ovarian tissue reviewed in this paper suggest that in many respects the development of steroidogenic activity involves mechanisms similar to those described for the rat. However, the capacity of the theca for oestrogen synthesis requires further investigation to determine the relative contribution of the theca and granulosa to oestrogenic stimulation within the follicle and in more peripheral target tissues.

Preliminary data from the study of individual follicles within the ovarian hierarchy of individual animals suggest that the interfollicular relationships of polytocous animals may differ considerably from those of monotocous animals. A schematic representation of some of these inter-relationships is presented in Fig. 8, which depicts the morphological and biochemical characteristics of follicles within the proliferating pool and the likely destiny of these follicles once selection has occurred and the follicular phase is established. As discussed earlier, in the pre-selection population the mechanisms controlling the numbers of more mature follicles may be one crucial determinant of ovulation rate; yet not all destined ovulatory follicles are at the same stage of maturity at the time of recruitment. An appreciation of the minimal level of maturation needed to ensure selection and successful development within the follicular phase would assist the analysis of applied experiments in which techniques were used to promote follicular growth. The interactions between the gonadotrophins and other extra- or intra-ovarian factors in establishing a population of follicles able to be recruited from the proliferating pool are poorly understood. Further studies of the prepubertal gilt, the cyclic gilt or sow and of the lactating sow are therefore needed; as in recent data reported for the sheep (Lahlou-Kassi & Mariana, 1984; Driancourt *et al.*, 1985), a comparison of follicular development in breeds or strains with a high and low ovulation rate may be of value.

The stimulus for recruitment into the follicular phase at any time is also uncertain but probably involves adequate levels of FSH, an increase in episodic and basal LH secretion and the absence of local inhibitory mechanisms within the ovary. Even in the preovulatory population of follicles within the follicular phase, a range of development exists; as a consequence, although all the follicles within this population show similar patterns of development (see Fig. 7), the difference in maturity at the time of selection persists and the smaller selected follicles never attain the maturity of the dominant follicles in the hierarchy. Whether this difference in maturity has any consequence for the later development of the oocyte, or for subsequent luteal function is unclear, but Rao & Edgerton (1984) have described different populations of functional corpora lutea in the pig.

One of the most obvious consequences of the asynchrony in development of destined ovulatory follicles is the difference in time at which high concentrations of oestradiol first appear in follicular fluid, as clearly shown in Sow G8 in Fig. 3. The intraovarian effects of increased oestrogen synthesis have been described earlier but an increase in oestrogen concentrations in the ovarian venous outflow would also be expected to act as a positive feedback signal for the preovulatory surge of LH. Thus the oestrogenic activity of the dominant follicles would determine the timing of the LH surge and hence ovulation. The final ovulation rate would then be dependent on the number of smaller follicles that could attain an appropriate stage of maturity to respond to the LH surge. Oestrogens secreted by the dominant follicles could also act at the ovarian level to promote the maturation of smaller follicles in the hierarchy, in contrast to the inhibitory effects of the dominant follicles proposed in other species (Baird, 1983; Ireland & Roche, 1983). The accumulation of oestradiol in the follicular fluid of the subset 2 follicles in Table 1 (which themselves lacked high aromatase activity and enhanced binding of hCG to theca and granulosa) is consistent with the hypothesis of an extrafollicular source of oestradiol to less mature, non-oestrogenic follicles. Furthermore, Krzymowski,

Kotwica, Stefanczyk, Debek & Czarnocki (1981, 1982) and Krzymowski, Kotwica, Stefanczyk, Czarnocki & Debek (1982) have described a sub-ovarian countercurrent exchange mechanism for the transfer of oestradiol and other steroids from the ovarian pedicle to the ovarian artery; this would greatly enhance any effects of ovarian secretion by dominant follicles on smaller follicles within the ovarian tissue. Extensive interfollicular interactions are therefore probably an essential component of a model for follicular maturation in the pig and may involve factors other than ovarian steroids. As all the follicles initially 'selected' at the onset of the follicular phase do not necessarily proceed to ovulation, the mechanisms controlling the later stages of follicular maturation, particularly in the less dominant follicles, may also have a crucial influence on ovulation rate and final litter size.

Parts of the work reported in this paper received financial support from the Agricultural and Food Research Council, the Ministry of Agriculture, Fisheries & Food and the Meat and Livestock Commission.

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