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Sperm Distribution in the Porcine Oviduct in Relation to Spontaneous Ovulation and Stress

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Abstract

Due to the long interval from onset of standing oestrus to ovulation in the pig, a reservoir is needed in which spermatozoa can be stored prior to ovulation.

The main objectives of the work described in this thesis were to i) determine whether it would be possible to use information from a previous oestrus to predict the time of ovulation in the next oestrus, ii) investigate how sperm distribution and membrane integrity of spermatozoa present in the utero-tubal junction (UTJ) and isthmus, are affected by ovulation, iii) examine changes in morphology and localisation of spermatozoa in vasculary perfusion-fixed UTJ and isthmus around ovulation, iv) study the effects of food deprivation during the post-ovulatory period on cleavage rate, numbers of accessory spermatozoa in the zona pellucida and hormonal profiles in multiparous sows.

In the 44 multiparous sows studied ovulation was determined by transrectal ultrasonography. Time lapsed from onset of standing oestrus to ovulation in two consecutive oestruses did not differ significantly. Ovulation during the second oestrus after weaning could therefore be predicted using the onset of standing oestrus as a reference point.

The sows were inseminated with neat semen 18 h prior to expected ovulation. The distribution pattern, number and viability of spermatozoa in the UTJ and isthmus were related to ovulation in flushed and fixed oviducts. Sperm numbers diminished gradually along a gradient from UTJ to the isthmus during the pre-ovulatory period. Ovulation reduced the numbers of viable spermatozoa in the UTJ and isthmus, and was related to the relocation of spermatozoa and reduction of intraluminal fluid masses. Scanning electron microscopy revealed the presence of two populations of spermatozoa in the reservoir, one with epithelial contact and the other without. Transmission electron microscopy studies showed that spermatozoa attached to the epithelium had intact plasma membranes prior to ovulation, whereas they were broken afterwards.

Food deprivation for about 48 h, starting immediately after ovulation, reduced the numbers of viable spermatozoa in the UTJ and isthmus, measured indirectly by counting the numbers of accessory spermatozoa in the zona pellucida, and lowered the cleavage rate of fertilized ova recovered at a mean of 79 ± 3.4 h after ovulation. During the period of food deprivation, plasma levels of insulin were low, while those of cortisol, progesterone and prostaglandin $F_{2\alpha}$ metabolite (PG-metabolite) were elevated. Thus, food deprivation was associated with changes in the secretion of metabolic and reproductive hormones, which might have modulated the oviductal environment affecting the embryos as well as the spermatozoa in the oviduct.

Key words: oestrus, ovulation ultrasonography, utero-tubal junction, isthmus, sperm distribution, membrane integrity, ultrastructure, embryo development, food deprivation, sow.

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You gave me your shield of victory, and your right hand sustains me.
Psa, 18:35

To Sarah, Ken, Susie and Tony

Abstract

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Contents

Appendix

Papers I - IV:

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Mburu, J. N., S. Einarsson, A-M. Dalin and H. Rodriguez-Martinez, 1995: Ovulation as determined by transrectal ultrasonography in multiparous sows: Relationships with oestrous symptoms and hormonal profiles. J. Vet. Med. A 42, 285-292.

II. Mburu, J. N., S. Einarsson, N. Lundeheim and H. Rodriguez-Martinez, 1996: Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. Anim. Reprod. Sci. 45, 109-121.

III. Mburu, J. N., H. Rodriguez-Martinez and S. Einarsson, 1997: Changes in sperm ultrastructure and localisation in the porcine oviduct around ovulation. Anim. Reprod. Sci. 47, 137-148.

IV: Mburu, J. N., S. Einarsson, H. Kindahl, A. Madej and H. Rodriguez-Martinez, 1997: Effects of post-ovulatory food deprivation on oviductal sperm concentration, embryo development and hormonal profiles in the pig. Manuscript.

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Introduction

Transrectal ultrasonography has been found to be a highly suitable non-surgical method for studying follicular development and, possibly, for determining the moment of ovulation (Soede et al., 1991; 1992). In a study of the oestrous cycle in the sow in which hormonal patterns were related to ovarian activity detected by transrectal ultrasonography, ovulation was shown to consistently take place about seven-tenths of the way through the total standing oestrus (Soede et., al. 1994).

The interval between the onset of oestrus and ovulation is longer in the pig than in several other domestic animals. In the pig, billions of spermatozoa enter the female reproductive tract during mating or artificial insemination. However, out of those billions of spermatozoa, only a few thousand reach the lower oviduct and even fewer reach the fertilisation site.

A sperm reservoir in the pig develops in the utero-tubal junction (UTJ) and the ad-uterine segment of the isthmus during the long pre-ovulatory oestrus (Viring et al., 1980; Hunter, 1981; 1984) where spermatozoa maintain a normal ultrastructure (Rodriguez-Martinez et al., 1990). Similar sperm reservoirs have been described in rabbit (Overstreet et al., 1978), sheep (Hunter et al., 1980), cow (Hunter and Wilmut, 1984), hamster (Smith et al., 1987), mice (Suarez, 1987) and guinea pigs (Yanagimachi, 1976). In these species spermatozoa are stored in the reservoir until just before ovulation when they move towards the ampulla, where fertilisation takes place. The reservoir is also important in regulating the number of ascending spermatozoa, thereby reducing the numbers reaching the fertilisation site (Thibault, 1973). As a result the number of spermatozoa interacting with the oocyte is limited, thus helping to avoid polyspermy (Hunter and Leglise, 1971; Hunter and Nichol, 1988; Hunter, 1991). It has been thought that the reservoir provides a favourable environment for sperm capacitation (Hunter and Hall, 1974; Ellington et al., 1993). In vitro studies with bovine frozen-thawed spermatozoa and oviductal epithelial cells led to a suggestion by Pollard et al. (1991) that the oviduct not only stores spermatozoa but also maintains their viability and fertilising capacity before ovulation.

Some characteristics of the UTJ and lower isthmus suggested to facilitate their function as a reservoir, include the ability of their epithelium to bind spermatozoa and the presence of pre-ovulatory mucous material that entraps the spermatozoa (Raychoudhury and Suarez 1991). However, some studies suggest that spermatozoa are sequestered in the reservoir merely because it is the first oviductal region they encounter (Lefebvre et al., 1995). The fact that spermatozoa attach to the oviductal epithelium has been revealed by translumination of excised whole oviducts in hamster (Katz and Yanagimachi, 1980), in some marsupials (Bedford and Breed, 1994) and mice (Suarez, 1987).
It has been thought that the transport and storage of spermatozoa in the pig oviduct is controlled in part by the local endocrine environment of the oviduct (Hunter et

al., 1983; Raychoudhury and Suarez, 1991). Little information is available on the effect of spontaneous ovulation (precisely determined) on the distribution and viability of spermatozoa in the oviduct of the pig.

The distribution of spermatozoa was indirectly assessed through the recovery of ova following surgical post-coital ligation and transection of the distal oviduct, and time of ovulation was not determined before surgery (Hunter, 1984). Based on the results of the latter study it was suggested that ovulation is synchronised with a redistribution of the spermatozoa from the reservoir in the pig.

Adequate information on the interaction between the tubal epithelium and the spermatozoa in the pig is lacking, although Fléchon and Hunter (1981) and Hunter et al. (1987) have studied these events, they estimated that ovulation occurred within a fixed time interval and tissues were fixed by immersion after opening the oviductal lumen with scissors, thereby risking the introduction of artefacts.

Some management practices carried out by farmers include grouping/mixing of sows immediately after mating, which is likely to result in aggressive interactions and a drop in food intake by the more submissive animals (Mendl et al., 1992; Tsuma, 1995). It may also lead to an increased return to service and a reduction in litter size (Bokma, 1990).

Food deprivation during the first trimester leads to pregnancy losses (Anderson, 1975) and can tend to reduce embryo survival (Tsuma et al., 1996). It has been suggested that activation of hypothalamic neuropeptides and modulation of the secretion of the related pituitary hormones could partly explain the food deprivation results (Polkowska, 1996; Maeda and Tsukamura, 1996). Food deprivation in sows around implantation induces an increase in maternal plasma concentrations of cortisol, prostaglandin $F_{2\alpha}$ metabolite (PG-metabolite) and progesterone (P_4) (Tsuma et al., 1996). Although corpora lutea have been demonstrated to be the main source of P_4 in the pregnant pig (Nara et al., 1981), the adrenals have been incriminated as another possible source in food deprived pregnant sows (Tsuma et al., 1996). Administration of ACTH has been shown to result in P_4 production in ovariectomised cows (Bolanos et al., 1997) and increase production in pregnant sows (Tsuma, 1995). Embryo mortality is highest before day 18 of pregnancy (Pope and First, 1985; van der Lende et al., 1994), whereas the early post-mating period has been suggested to be a critical window for nutritional effects on embryo survival (Dyck and Strain, 1983; Jindal et al., 1996). There is little data on the effects of food deprivation during early cleavage in sows where precise time of ovulation is known.

Aims of the study

The aims of this thesis were to:

- record the duration of oestrous symptoms and the blood plasma levels of luteinizing hormone (LH) and oestradiol-17b (E_2) and to relate them to the onset of standing oestrus and ovulation monitored by transrectal ultrasonography.

- determine whether the occurrence of spontaneous ovulation could be predicted during an oestrus using information obtained from the previous oestrus in individual sows.

- characterise any changes in the distribution, quantity and membrane integrity of spermatozoa present in the utero-tubal junction (UTJ) and isthmus that occur in connection with spontaneous ovulation in sows.

- examine changes in morphology and localization of spermatozoa in vascularly perfusion-fixed UTJ and isthmus around ovulation in sows, using scanning and transmission electron microscopy.

- determine the effect of food deprivation during the post-ovulatory period on cleavage rate, numbers of accessory spermatozoa in the zona pellucida and hormonal profiles in multiparous sows.

Materials and Methods

Animals and general management

Forty-four multiparous, crossbred sows (Swedish Landrace x Swedish Yorkshire) were included in the experiments. Twenty-eight out of them were used in the experiments described in papers I, II and III and other 16 were used in experiments covered in paper IV. The sows were brought from a commercial farm to the Department of Obstetrics and Gynaecology, SLU, on the day of weaning. Throughout the experimental period, they were kept in individual pens with adult boars nearby. The sows were fed a commercial ration in accordance with Swedish breeding stock standards (Göransson, 1984; Simonsson, 1994) i.e. the daily concentrate feed allowance was 2.9 kg until the first ovulation after weaning and 2.2 kg thereafter. The feed contained 14.9 % crude protein and 12.1 MJ ME /kg (papers I, II and III) or 12 % crude protein and 12.5 MJ ME/kg (paper IV). The concentrate ration was fed to the animals twice daily at 07.00 h and 15.00 h; straw was provided once daily, and water was supplied ad libitum. In the food deprivation experiment (paper IV) food and straw were withheld (a total of 4 meals) from the food-deprived group of sows (D-group), starting with the first morning meal after ovulation. Reproductive performance during the two oestruses after weaning was recorded in all experiments. Three sows from paper I were excluded from the experiment owing to a delayed oestrus after weaning (> 10 d), while one was removed from the study in paper IV owing to her failure to ovulate during the second oestrus (developed ovarian cysts).

Clinical investigations

Oestrous detection

Standing oestrous reflex in front of the boar was employed for detection of onset of oestrus. After weaning, the sows were carefully examined for signs of pro-oestrus twice daily (morning and evening). Detection of the onset of oestrus was performed every 4 h until sows showed standing reflex. The experimental design of all studies in this thesis required that the time at which standing oestrus started be accurately identified. The onset of standing oestrus was defined as being midway between the first occasion on which she showed standing oestrus and the time of the previous check, which was made 4 h earlier.

Blood collection and insemination

In all studies, indwelled, permanent silastic tubing for blood collection was inserted into a jugular vein under general anaesthesia (Rodriguez-Martinez and Kunavongkrit, 1983) between days 13 and 16 of the second oestrous cycle after weaning. In connection with the blood-collection events, care was taken to minimise any undue disturbances to the sows. Blood was collected at 12 h intervals

for 72 h before onset of standing oestrus and every 2 h thereafter until slaughter (paper I), or it was collected daily between insemination (every 2 h) and slaughter (paper IV). During the second oestrus after weaning (papers II and III) either artificial insemination with neat semen or natural mating once with one of three boars of proven fertility was performed about 18 h prior to expected ovulation (except for four sows, paper III). Sows in paper IV were inseminated once with 100 ml of Beltsville thawing solution (BTS)-extended fresh semen containing 10 x 109 spermatozoa, 20-10 h before expected ovulation.

Handling of blood samples

Blood was collected in heparinized tubes (10 ml blood sample) for analysis of oestradiol-17β (E_2), luteinizing hormone (LH), progesterone (P_4), cortisol, insulin and PG-metabolite (papers I and IV). The blood samples were centrifuged at room temperature immediately after collection whereupon the plasma was removed and stored at -20 ^{0} C until analysis.

Hormone analysis

E_2 and P_4 concentrations in plasma samples collected in the studies described in papers I and IV were determined by enzyme immunoassay (Amerlite, Kodak Clinical Diagnostics Ltd., Amersham, England). The kits were used in accordance with the manufacturer's instructions, with modifications as described by Rojkittikhun et al. (1993a). Samples collected from 72 h before the onset of standing oestrus until slaughter in paper I were quantified for LH using a heterologous radio-immunoassay validated for porcine plasma (Forsberg et al., 1993). Plasma samples collected every second hour between insemination and slaughter were analysed to determine cortisol concentrations using a luminescence immunoassay as described by Magnusson et al. (1994). Insulin levels in the plasma samples were measured by radioimmunoassay (Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden). The main initial blood plasma metabolite of prostaglandin $F_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, (PG-metabolite) was quantified by radioimmunoassay as described previously (Kunavongkrit et al., 1983).

Morphological investigations

Sows in paper IV were slaughtered 65 - 91 h post ovulation. All other sows (papers I, II and III) were slaughtered either 8 h before ovulation (pre-ovulatory group), during ovulation (peri-ovulatory group) or 8 h after ovulation (post-ovulatory group). In all cases (papers II and III), the internal genital organs were immediately removed and transferred to a prewarmed room (about 37 0 C). One oviduct per sow in papers II and III was chosen at random and vascularly perfused with a 3% solution of glutaraldehyde in 0.067 M sodium cacodylate buffer (pH 7.3, 500 mOsm/l). The remaining oviduct was divided up into four segments, i.e.

the utero-tubal junction (UTJ) and three equal isthmic segments (lower, middle and upper isthmus), and flushed with warm Beltsville thawing solution (BTS). The flushings from each segment were examined in search of spermatozoa whose membrane integrity was determined by staining with fluorochrome dyes (carboxyfluorescein diacetate and propidium iodide) in accordance with the method used by Ortman and Rodriguez-Martinez (1994). Sperm numbers in each segment were quantified using a haemocytometer (Burker chamber, 0.1 mm deep). After flushing, the four tubal segments were opened longitudinally and immersion fixed in a 3% solution of glutaraldehyde in 0.067 M sodium cacodylate buffer (pH 7.3, 500 mOsm/l). In paper IV, the oviducts and uterine segments were flushed separately with warm phosphate-buffered solution (PBS) for ova recovery. All flushings were collected in a glass beaker. Most of the fluid was filtered away leaving only a small volume to which a 3% solution of glutaraldehyde fixative was added. An inverted microscope (Olympus, Japan) with phase contrast optics was used at x 200 magnification to examine and count the blastomeres in the cleaved ova and the accessory spermatozoa in the zona pellucida (ZP).

Determination of sperm numbers and membrane integrity by electron microscopy

Fixed oviduct samples in papers II and III were divided up in the same way as the flushed oviduct samples. The samples were routinely prepared for scanning (SEM) and transmission electron microscopy (TEM) and examined in electron microscopes (Cambridge 150 SEM at 20 kV and Philips EM 201 TEM at 60-80 kV, respectively).

Statistical analyses

Statistical evaluations were performed using the analysis of variance (GLM procedure), t-test and Spearman correlation analyses included in the SAS software package (SAS Institute Inc. 1987; 1989). The frequency distribution of groups (paper II and IV) was compared using a chi-square test (Fisher's exact test). In papers II and III, the data were logarithmically transformed ($\log_e$). All statistical tests having a P-value of 0.05 or less were considered significant.

Results

Oestrus/oestrous symptoms , hormonal changes and their relationships to ovulation (paper I)

In 15 sows, oestrous period had a mean duration of 56 ± 7.9 h and ovulation took place about seven-tenths (mean of 68 ± 7.7 %) of the way through the standing oestrus. A positive correlation was found between the interval from onset of oestrus to ovulation and the length of oestrus ($r = 0.51$, $P = 0.05$). Total numbers of ovulatory follicles correlated significantly with the interval between the onset of pro-oestrus and the oestradiol-17 β (E_2) peak ($r = 0.62$, $P < 0.05$). In cases where the interval from weaning to onset of oestrus was short, the duration of oestrus tended to be long ($r = - 0.48$, $P < 0.07$). The interval from onset of pro-oestrus to E_2 peak was negatively related to the interval from the E_2 peak to ovulation, ($r = - 0.84$, $P < 0.05$). In sows with a short LH surge the period between the onset of the LH surge and ovulation was long ($r = - 0.73$, $P = 0.06$). The mean interval from onset of oestrus to E_2 peak was 1 h with a large amount of variation between sows (range: -10 to + 22 h). The interval from onset of oestrus to LH peak concentration was 12 h and in this case as well there was large variation between sows (range: -10 to +32 h).

Oestrus/oestrous symptoms and ovulation during two consecutive oestruses

After careful establishment of onset of standing oestrus, no significant differences were found between the intervals from onset of standing oestrus to ovulation after weaning and the corresponding interval during second oestrus in eleven sows ($P > 0.10$). The first pro-oestrus was shorter than the second pro-oestrus after weaning ($P < 0.001$). In all the sows studied (papers I and IV), no ovulation occurred within 24 h of the onset of standing oestrus (unpublished observations).

Distribution and number of spermatozoa in flushed and fixed oviducts (papers II and III)

Irrespective of the stage during oestrus, the utero-tubal junction (UTJ) and lower isthmus contained more spermatozoa than the middle and upper segments of the isthmus. A significant boar influence was seen in sperm distribution in the UTJ and lower isthmus ($P < 0.01$). The first flushing of the UTJ and the isthmus yielded more spermatozoa compared with the second flushing, especially during post-ovulation ($P < 0.05$). Sperm numbers in the UTJ and lower isthmus were significantly higher during the pre-ovulatory period than during the peri- and post-ovulatory periods ($P < 0.01$). The population of spermatozoa in the upper isthmus was higher during and after ovulation compared with the period before ovulation ($P< 0.05$). Before ovulation sperm numbers tended to decrease from the UTJ

towards the upper isthmus in both flushed and fixed oviducts. The quantification of spermatozoa before ovulation was hindered by the presence of thick intraluminal fluid (masses) and tightly closed primary folds of the endosalpinx in fixed oviducts. On the luminal surface of the flushed oviducts that were immersion-fixed no masses were observed, but spermatozoa were found inside the primary folds of the endosalpinx. Sperm numbers before ovulation were lower on the central mucosal surface of the UTJ than inside the crypts formed by the folded mucosa ($P < 0.01$), (Paper III). No differences were found between the numbers of spermatozoa on the central mucosal surface and the number inside the crypts during or after ovulation.

Morphology of epithelial lining and location of spermatozoa

Two populations of spermatozoa were identified in this study; a) those that established intimate head contact with microvilli and cilia on the surface of the mucosal folds or inside the crypts or interfolds, and b) those lacking epithelial contact but entangled in coagulated intraluminal masses (paper III). A characteristic presence of masses of spermatozoa trapped in intraluminal material was a common observation in vascularly perfusion-fixed specimens before ovulation (papers II and III) but was less pronounced during and after ovulation. Spermatozoa were observed singly or in groups on the central mucosal surface and with straight or slightly curved tails in clusters inside the crypts or interfolds, before ovulation.

Sperm plasma membrane integrity as studied with fluorochrome dyes, scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The proportion of spermatozoa from flushed oviducts which had intact plasma membranes in the UTJ after staining with fluorochrome dyes was higher ($P < 0.05$) before ovulation than afterwards (paper II). SEM, revealed that the numbers of spermatozoa with intact plasma membrane tended to be higher ($P = 0.06$) before ovulation compared with the period during or after ovulation (paper II). It was difficult to evaluate the membrane status especially inside the coagulated intraluminal masses using SEM. However, with TEM it was possible to see that most spermatozoa inside the coagulated intraluminal masses had damaged plasma membranes in the head domain, while those apposed to the epithelium had intact plasma membranes (paper III). The use of TEM on post-ovulatory sections showed that even some spermatozoa apposed to the epithelium had damaged plasma membranes and acrosomes (paper III).

Recovery and cleavage rate of ova, numbers of spermatozoa in the zona pellucida (paper IV)

Food-deprived and control sows did not differ in terms of their ova recovery rate, ovulation rate or fertilisation rate or in the length of the intervals between i) insemination and ovulation, ii) ovulation and start of food-deprivation or iii) the start of food-deprivation and slaughter. Embryos from food-deprived sows showed a lower cleavage rate and had fewer accessory spermatozoa in the ZP compared to those of the controls.

Peri-ovulatory changes in reproductive and metabolic hormones during food deprivation

In the D-group plasma insulin levels were lower during the food deprivation period than afterwards ($P < 0.01$). Peripheral blood cortisol was higher in the D-group sows than in the C-group sows ($P < 0.05$). The increase in plasma progesterone (P4) concentrations was more pronounced in the D-group sows than in the C-group sows, and the difference was significant during the second day of deprivation ($P < 0.05$). Plasma levels of PG-metabolite during the food deprivation period were higher in the D-group sows than in the C-group sows ($P < 0.05$).

General Discussion

Oestrus/oestrous symptoms , hormonal changes and their relationship to ovulation (paper I)

In cases where the duration from onset of standing oestrus to ovulation was long the duration of standing oestrus was also found to be long. These findings are comparable to those of Soede et al. (1994). Irrespective of the duration of standing oestrus, ovulation always occurred about seven-tenths (mean of 68 %) of the way through the standing oestrus as similarly reported by Soede et al. (1994).

The peri-ovulatory hormonal profiles of E_2 and LH observed were expected and related to follicular development and occurrence of ovulation, and were comparable with earlier studies by Soede et al. (1994) and Blair et al. (1994). Peak LH concentrations (paper I) occurred at a mean of 12 h after the onset of oestrus and there was a large amount of variation between sows (range: - 10 to + 32 h) as was also found by Soede et al. (1994) and Dalin et al. (1995). On average E_2 concentrations peaked around one hour after the onset of oestrus, and, again, there was large variation between sows (range: - 10 to + 22 h), in accordance with the findings of Soede et al. (1994). Wide variation between sows was also found in the lengths of the interval between the E_2 peak and ovulation, with a mean of 44 h (range 34 - 54 h), and from LH peak to ovulation with a mean of 35 h (range: 27 to 48 h). These findings were also similar to earlier results obtained by Soede et al. (1994) and Dalin et al. (1995) respectively. It might be difficult to estimate the time of ovulation based on the hormone parameters with standing oestrus as a reference point since some peaks occurred before the onset of oestrus, while others occurred afterwards.

Durations of pro-oestrus, and oestrus (paper I) were similar to those reported previously by Sterning et al. (1994). It is difficult to explain why the first pro-oestrus in sows after weaning was shorter than the second one. One possibility is that the difference is related partly to changes in hormonal patterns (E_2). The fact that weaning results in an elevation in gonadotropin secretion and lead to the occurrence of oestrus suggests that weaning results in a quick rise in E_2 levels. However, it seems unlikely that E_2 reaches high concentrations since Rojanasthien (1988) reported that E_2 levels remained low during the first 60 - 84 h after weaning. Alternatively, the concentration threshold above which E_2 initiates standing oestrus might be lower immediately after weaning owing perhaps to increased receptor sensitivity. In any case, further studies to elucidate this occurrence are needed.

Oestrus/oestrous symptoms and ovulation during two consecutive oestruses

The durations of the interval from onset of oestrus to ovulation for the two consecutive oestruses in the same sow did not show any significant differences, and the variations between sows were small in cases where the onset of standing oestrus during the first and second oestrus post-weaning had been accurately established. In another study where oestrous symptoms were carefully recorded in individual sows for two consecutive oestruses (Sterning et al., 1994), it was found that the pro-oestrus and external genital changes characterising the first oestrus after weaning were repeated during the second oestrus.

Once it had been documented that the onset-of-oestrus-to-ovulation intervals between the first and second oestrus after weaning did not differ significantly, it was possible to use the information gathered during the first oestrus after weaning to predict the time of ovulation and in retrospective calculate the time of insemination during the second oestrus. Improper establishment of the time of onset of standing oestrus might reflect a shortened duration to ovulation, however, in all cases where onset of oestrus was accurately established, ovulation were not detected for at least after 24 hours from standing oestrus.

Distribution and number of spermatozoa in flushed and fixed oviducts

Results obtained with regards to the proportional distributions of spermatozoa among the segments from the UTJ to the upper isthmus and among stages during oestrus were similar in connection with the two techniques used (paper II). Both techniques revealed that the number of spermatozoa diminished gradually along a gradient from the UTJ to the upper isthmus during the pre-ovulatory period. However, more spermatozoa were recovered with the flushing technique than with the SEM technique. This difference was attributed in part to difficulties in counting the spermatozoa when using SEM. In the fixed segments spermatozoa were trapped in a thick intraluminal fluid during the pre-ovulatory period and the folds were more closed with spermatozoa in the deep mucosal furrows. The situation changed with ovulation, where the intraluminal fluid was much less perhaps due to a reduction in viscosity and less intense folding of the endosalpinx.

Irrespective of the stage in oestrus, the UTJ and lower isthmus contained more spermatozoa than did the middle or upper isthmus (paper II). These results are in accordance with earlier studies (Viring et al., 1980; Hunter, 1981) showing these sites to be the sperm reservoir in the pig. Further, in the reservoir spermatozoa were more common inside the spaces formed by the folded endosalpinx (crypts in the UTJ and interfolds in the lower isthmus) than on the central mucosal surface (paper III) pre-ovulation. This observation was thought to result from continous removal of the spermatozoa on the mucosal surface together with the intraluminal

fluid by the ongoing contractions of the oviduct which pushes the luminal fluid towards the peritoneal cavity (paper III). Sperm numbers in the crypts and interfolds and those on the central mucosal surface were not different during ovulation and afterwards owing perhaps to a possible redistribution effected by ovulation (paper III).

The significant influence of boar on sperm distribution (paper II) is difficult to explain but might indicate that boars differ in terms of survival rates of their spermatozoa inside the female genital tract. Although all other semen parameters were within the accepted range of the values for boars, according to the semen laboratory at the Department of Obstetrics and Gynaecology (Mburu et al., 1996), the number of spermatozoa in the oviduct following insemination/mating was significantly lower for one of the boars in the oviduct. Spermatozoa from this boar also had a lower post-thaw motility in vitro (Eriksson, personal communication).

The fact that more spermatozoa were liberated during the first flushing than the second flushing during the post-ovulatory period (paper II) indicates that the spermatozoa were more loosely attached to the epithelium during this period. This observation was attributed to occurrence of ovulation (Paper III). Lack of differences in sperm numbers liberated between the first and second flushing before ovulation indicates that a firm attachment of boar spermatozoa to the epithelium exists similar to the one observed in the hamster (Smith and Yanagimachi, 1990).

The upper isthmus contained more spermatozoa during and after ovulation compared with before ovulation (paper II), which indicates that ovulation resulted in relocation of spermatozoa and, possibly, in a more co-ordinated adovarian movement (Hunter, 1984) of spermatozoa from the reservoir. The upper isthmus in most mammalian species is close to the fertilisation site. Thick intraluminal masses (papers II and III) were not characteristically present during or after ovulation, meaning that ovulation might have contributed to either their disappearance or loosening, therefore making it possible for spermatozoa to advance from the reservoir. In connection with ovulation a widening of the UTJ and isthmus lumen was observed (paper III) probably owing to the disappearance of oestrogen induced oedema and perhaps contributing to the relocation of spermatozoa from the reservoir. The reason why this relocation occurs in connection with ovulation has yet to be determined. One possibility is that the oocytes selectively attract spermatozoa with a strong fertilising capacity (Cohen-Dayag et al., 1994). Only a very small proportion of spermatozoa in the reservoir are able to advance to the upper isthmus (paper II). One hypothesis to explain why such a small number of spermatozoa advance is that a "dose-dependent message" stimulating advancement comes in or is activated in connection with ovulation, reaches the spermatozoa in the reservoir but only perceived by the small fraction of spermatozoa with intact plasma membranes. Follicular fluid has been shown to enter the porcine oviduct following ovulation, although in small quantities, (Hansen et al., 1991), and it is still not clear what role, if any, it might play in the relocation of spermatozoa.

Another possibility is that following ovulation, some follicular fluid might enter the oviduct and stimulate chemotactic and chemokinetic responses in the spermatozoa in the reservoir comparable to those shown by Ralt et al. (1994) in human. In the latter study only a small fraction of sperm population was responsive to the attractant. Hunter and Nichol (1986) reported that the caudal isthmus was cooler than the ampulla before ovulation, whereas no differences in temperature between these locations were found after ovulation. Thus it is possible that spermatozoa relocate once the reservoir has warmed up. Redistribution of spermatozoa from reservoirs related to occurrence of ovulation has been reported in other animals, i.e. Australian marsupial *Sminthopsis crassicaudata* (Bedford and Breed, 1994) and New World monkey *Cebus apella* (Ortiz et al., 1995).

Morphology of epithelial lining and location of spermatozoa (paper III)

The epithelial lining of vasculary perfused specimens in the present work revealed that, the apical side of the cilliated and non.ciliated cells were the same height in both SEM and TEM (Paper III). In earlier studies the non-ciliated cells were more prominent (Stalheim et al., 1975; Wu et al., 1976; Fléchon and Hunter, 1981; Hunter et al., 1987). Apical protrusion of non-ciliated cells was observed where gently flushed oviducts with physiological fluids prior to glutaraldehyde fixation was done (paper II). Protrusion of the epithelial lining was thought to be an artefact resulting from differences in osmotic sensitivity among epithelial cells following exposure of luminal environment (paper III).

It was possible to identify two populations of spermatozoa during the study. In one population, sperm heads made intimate contact with the microvilli and cilia of the epithelial cells either on the central mucosal surface or inside the crypts or interfolds. In the other population there was no epithelial contact, but spermatozoa were entangled in intraluminal masses. It is conceivable that the spermatozoa benefit in some way from their adherence/attachment to the epithelial cells, although it is difficult to suggest what benefits the spermatozoa could derive from the epithelial cells within the scope of this study. Smith and Yanagimachi (1990) showed that firm attachment of hamster spermatozoa to the epithelium increased their survival in the oviduct. In in vitro studies by Pollard et al. (1991), frozen-thawed spermatozoa were shown to maintain their motility and fertilising capacity when incubated together with bovine oviductal cells harvested between the onset of oestrus and ovulation. In vitro studies performed by Raychoudhury and Suarez (1991), pre-ovulatory levels of steroids were shown to enhance the binding of boar spermatozoa to the isthmic epithelium. It is speculated that broken/dead spermatozoa become entrapped in the coagulated material while intact/live spermatozoa attach to the epithelium (paper III).

The characteristic intraluminal masses observed before ovulation in the present study were perhaps a result of increased secretory activity (Johansson et al., 1997).

In the latter study increased electron-dense and lucent secretory granules were observed pre-ovulation. Further, in the same study mucus was more abundant prior to ovulation in the lumen of the lower isthmus. An increase in oviductal fluid resulting from secretion or transudation under the influence of high levels of oestrogens in the blood has been reported in sows (Iritani et al., 1974). It has been observed that small amounts of fluid are transported like a bolus as the luminal fluid is peristaltically propelled upwards from the UTJ to the ovarian end (Blandau and Gaddum-Rosse, 1974). The latter observation bears similarity to the observed masses of spermatozoa/coagulated intraluminal fluid (paper III). Studies in the cow revealed that pre-ovulatory spermatozoa in the caudal isthmus were located among the folds and in viscous intraluminal fluid (Hunter et al., 1991). Boar spermatozoa entrapped in mucus secretion was observed in pre-ovulatory isthmic explants by Raychoudhury and Suarez (1991) in vitro. Retention of spermatozoa in the reservoir might be partly resulting from presence of thick viscous secretion hindering forward progression of the spermatozoa.

Spermatozoa were observed to be packed in the crypts and interfolds of the reservoir before ovulation and had straight to slightly curved tails, indicating reduced activity. The lower temperature of the reservoir at this time, compared with that of the ampulla and with post-ovulatory temperature, could perhaps be one of the reasons why spermatozoal activity was reduced (Hunter and Nichol, 1986). A study in rabbits (Saksena and Harper, 1975), showed segmental variations in prostaglandin $F_{2\alpha}$ concentrations relative to ovulation. Further, in the same study prostaglandin $F_{2\alpha}$ was shown to be increased in the distal isthmus at ovulation whereas its levels were low in the ampulla. A peri-ovulatory role of Prostaglandins (PGs) of both F and E series might be worthy looking into in connection with relocation of spermatozoa in the pig in future.

Sperm plasma membrane integrity as studied with fluorochrome dyes, scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Fluorophore staining revealed that numbers of spermatozoa with intact plasma membranes in the UTJ were higher before ovulation than afterwards, and a similar tendency was found with SEM (paper II). Fluorochrome dyes were capable of staining spermatozoa with even small membrane disruptions which might not have been observable with SEM. Spermatozoa revealed by TEM to be in intimate contact with the epithelial cells of the UTJ and lower isthmus had intact plasma membranes before ovulation, and some had broken ones after ovulation. This observation suggests that plasma membranes of the spermatozoa undergo certain changes (acrosome-like reaction with disruption of the acrosome) in connection with ovulation.

Spermatozoa examined in TEM and in coagulated intraluminal mass had broken plasma membranes. Flushed-out spermatozoa that were intermingled with dead epithelial cells and other intraluminal material had broken plasma membranes (paper III). Attachment/adherence of spermatozoa to the oviductal epithelial cells before ovulation might contribute to their well being as indicated by the large numbers with intact plasma membranes before ovulation.

Spermatozoa capacitation in the bovine (one of the causes of membrane disruption) appears to be regulated by ovulation (Ellington, 1991). Studies on *in vivo* capacitation of bovine spermatozoa, showed that more spermatozoa were acrosome reacted in the ampulla of the oviduct ipsilateral to the ovulatory ovary (Herz et al., 1985). In the hamster, Yanagimachi (1969) noted that at ovulation, part of the follicular fluid is transported into the oviduct together with the cumulus oophorus containing the eggs. It has been shown by in vitro studies that follicular fluid can promote sperm capacitation and acrosome reaction in the hamster (Yanagimachi, 1969), and mouse (Iwamatsu and Chang, 1969). The follicular fluid of bovine was shown to contain compounds like proteoglycans which have ability to induce acrosome reactions (Lenz et al., 1982). Hansen et al. (1991) observed only small amounts of follicular fluid in the gilt oviduct at ovulation, where it was said to have a stimulatory but nonessential effect on the acrosome reaction. It is possible that during ovulation some follicular fluid was introduced which contained components that induced the observed membrane changes in the spermatozoa. Alternatively the oocytes, with their cumulus investments, or the follicular fluid, regardless of the amount, may have caused the epithelial cells to produce compounds that induce membrane changes in the spermatozoa.

Recovery and cleavage rate of ova, numbers of spermatozoa in the zona pellucida (paper IV)

Compared with the control group, the food-deprived group of sows had decreased numbers of viable spermatozoa in the oviduct reservoir as reflected in the numbers attached to the ZP. It is thought that food deprivation might have led to a change in the oviduct environment, hence reducing the viability of the spermatozoa or accelerating their elimination and therefore reducing the numbers attaching to the zona pellucida. The cleavage rate was lower in the embryos of the food-deprived group of sows than in those of the control group . A change in the oviductal environment induced by food deprivation was considered to be the most likely cause of the lowered cleavage rate. Reduced pregnancy rates (from 90 to 52%) and litter size were observed in a study by Wiebold et al. (1986) in mice stressed on days 1-3 of pregnancy, where abnormalities in the transport and development of the embryos as well as in the development of the corpus luteum (CL) were observed.

The main reproductive events during the first 2 days of pregnancy in the pig are the transport and cleavage of ova which occur in the oviduct. Any changes in the oviductal environment can influence both these events. Pratt and Lisk (1989)

suggested that in the rat, surbordinate females produce smaller litters via selective resorption or spontaneous abortion of males in utero. They went further to suggest that since the male offspring of those subordinate females were smaller compared with those of dominant or control females, the former were more susceptible in utero to the effects of maternal stress.

Peri-ovulatory changes in reproductive and metabolic hormones during food deprivation

Plasma insulin levels were low during the food-deprivation period in the D-group sows and increased again during the refeeding period, hence providing an objective indication that these sows had a reduced energy intake. Food deprivation has earlier been shown to reduce plasma insulin levels in lactating sows (Rojkittikhun et al., 1993b), gilts (Prunier et al., 1993) and horses (Buonomo and Baile, 1991). In the rat low insulin levels were shown (De-Hertogh et al., 1992) to cause a substantial reduction in the number of embryos and a cellular decrease at the blastocyst stage. In another study, a developmental delay was found in embryos recovered from diabetic mice (Diamond et al., 1989).

The plasma cortisol elevation seen in the D-group sows, compared with the C-group sows, might have been related to food deprivation. Increased cortisol levels in the peripheral blood following either deprivation or restriction of food have been reported in sows (Tsuma et al., 1996), gilts (Prunier et al., 1993), humans and monkeys (Alleyne and Young, 1967; Dubey et al., 1986). Behrens et al. (1993) reported that plasma cortisol can be transported into the uterine lumen where elevated levels may influence uterine environment and alter embryo development. It is not known whether the oviduct of food-deprived sows in our study contained elevated levels of cortisol, nor do we know how much of it was bound. Behrens et al. (1993) showed that 85% of the cortisol from the uterine flushings was unbound. Investigations in humans showed that women with high cortisol levels had reduced pregnancy rates (Demyttenaere et al., 1992).

The increase in plasma progesterone in both groups of sows was related to the development of corpora lutea, but the elevated increase seen in the D-group sows during deprivation, compared with the C-group sows, was related to fasting. Increases in plasma P_4 have also been reported during a 48-h period of food deprivation in sows (Tsuma et al., 1996) and in repsonse to a 50 % food restriction in gilts (Dyck and Kennedy, 1995). The reason(s) why food deprivation alters P_4 levels has yet to be identified. There has been some speculation that activation of the pituitary-adrenal axis results in an increase in plasma cortisol (Tsuma, 1995). In in vitro studies cortisol was shown to cause an increase in P_4 secretion from bovine granulosa cells (Kawate et al., 1993). Adrenal glands have been thought to contribute to P_4 increases during food deprivation; consequently, animals treated with ACTH have been shown to have increased P_4 plasma concentrations, i.e. sows (Tsuma, 1995), bos taurus cows (Alam et al., 1986), zebu cows (Bolanos et al., 1997), and white tailed deer (Plotka et al., 1983). Another probable cause of P_4

elevation is lowered metabolic clearance resulting from reduced blood flow to the liver (Symonds and Prime, 1989) during food deprivation. The possibility that some or all luteal cells increased production of P_4 owing to food deprivation should also be considered.

Variability in progesterone secretion has been thought to determine the synchrony between the uterine environment and embryo viability. In some studies it has been suggested that the synthesis or metabolism of progesterone affects the well being of the embryo in the pig (Pope, 1988; Pope et al., 1990). Progesterone variability in the latter study was thought to explain why changes in the uterine environment affected embryo viability. It is speculated that the same situation might have existed in the oviduct in this study. Pharazyn et al. (1991a) considered variability in progesterone secretion during early pregnancy in the gilt to be associated with changes in the concentration of P_4 perfusing through the oviductal vasculature. Changes in post-ovulatory progesterone concentrations have been thought to affect early embryonic survival through modification of the oviductal environment (Jindal et al., 1996). The mode of action through which P_4 influences the oviductal environment is not clearly understood, nor is it possible to know the type of effect, if any, increased P_4 might have on the fertilised ova. On the other hand, it is possible that the observed peripheral increases in P_4 in the present study were not reflected in the oviduct, especially if the source of the increased P_4 was extragonadal.

PG-metabolite profiles in control sows and before deprivation in food deprived sows were comparable to those observed by Shille et al. (1979) in gilts during early pregnancy. However, during food deprivation a marked elevation in PG-metabolite levels was seen in the D-group sows in the present study. Food deprivation has also been shown to cause elevated levels of plasma PG-metabolite in prepuberal gilts (Holst and Kindahl, 1995) and sows on days 10 and 11 of pregnancy (Tsuma et al., 1996). The increase in free fatty acids, including arachidonic acid, that occurred in response to food deprivation (Silver and Fowden, 1982) might have led to increased prostaglandin synthesis. Muscular contractility in the oviduct is the primary force propelling the ova along the isthmus (Gaddum-Rosse and Blandau, 1973; 1976) towards the uterus. The normal function of the rabbit oviduct in terms of embryo transport have been thought to be regulated by a balance between prostaglandins of F and E series (Spilman and Harper, 1973). It has been shown that in the pig, prostaglandin $F_{2\alpha}$ increases muscular contractility (Rodriguez-Martinez and Einarsson, 1985) and the frequency of phasic pressure fluctuations (Pettersson et al., 1993). The elevated levels of prostaglandin $F_{2\alpha}$ in this study, might have been responsible for an increase in oviductal contractility. However, in the present study it was not possible to determine whether this was the case. Studies in the rabbit showed that responsiveness to prostaglandin $F_{2\alpha}$ was reduced by half in progesterone-treated uterus (Porter and Behrman, 1971). In view of the elevated levels of progesterone in the present study, if the pig oviduct is affected in

the same way, then perhaps the contractility of the oviduct was not markedly increased.

Although in this study only the metabolite of prostaglandin $F_{2\alpha}$ was analysed, increased levels of other prostaglandins cannot be ruled out. The roles of prostaglandin E (PGE) include, maintenance of the corpora lutea during early pregnancy in the pig (Ford and Christenson, 1991) and thereby stimulation of luteal P_4 secretion (Christenson et al., 1994). On the other hand studies in the horse have shown that PGE is secreted by the horse embryos and is involved in initiating their oviductal transport to the uterus (Weber et al., 1991). In a study where a compound inhibiting metabolism of prostaglandins (PGs) (Poly I:C) was administered in rabbits, accelerated ovum transport was observed and was associated with increased PGs in the oviduct (Harper et al., 1981). More studies are essential to elucidate the relationships between PGE and PGF in the pig oviduct during post-ovulatory food deprivation.

The well being of the embryo and its ability to follow the internally programmed sequence apparently depend on there being a continuous supply of energy, hormones and growth factors. The transition from the fertilised-ova (one-cell) stage through the developmental stages of early cleavage involves many specific changes that are still not understood. As a result of hormonal changes, the oviductal environment in the food-deprived sows may not have been suitable for embryo development and the viability of spermatozoa.

General Conclusions

- Ovulation was shown to consistently take place about seven-tenths of the way through the standing oestrus. LH and E_2 peak concentrations in relation to the onset of standing oestrus and ovulation showed a large amount of variation between sows. Therefore it is not possible to use these hormone parameters to estimate the time of ovulation accurately while using the onset of standing oestrus as a reference point.

- It should be possible to use the information recorded in one oestrus, especially regarding the interval from onset of standing oestrus to ovulation, to the predict time of ovulation in the next oestrus, provided that the onset of oestrus is correctly established in both oestruses.

- The number and distribution of spermatozoa in the UTJ and isthmus varied in relation to occurrence of ovulation in the sow. Boar had an influence on the sperm distribution in the female genital tract (UTJ and isthmus). A re-location of spermatozoa from the reservoir to the upper isthmus, and hence towards the fertilisation site occurred in connection with ovulation. Ovulation also reduced numbers of spermatozoa with intact plasma membranes in the UTJ and lower isthmus.

- Spermatozoa are located at specific sites. Some are in contact with the oviductal epithelium while others are not, and the bulk of them reside inside the crypts or interfolds. The pre-ovulatory period was characterised by a narrowed lumen in the UTJ and isthmus and the presence of viscous intraluminal fluid, while the post-ovulatory period was characterised by a wider lumen and less intraluminal viscous fluid. Attachment/adherence of spermatozoa to the UTJ/isthmic epithelial cells prior to ovulation increased their viability, while after ovulation even the attached/adhered spermatozoa had broken plasma membranes. Spermatozoa inside the intraluminal masses were found to be less viable both before and after ovulation.

- Food deprivation in sows led to increased plasma concentrations of cortisol, progesterone and PG- metabolite and to a decreased concentration of plasma insulin. Food deprivation resulted in a lowered cleavage rate and a decreased number of viable spermatozoa in the oviduct (measured indirectly as the number of accessory spermatozoa in the zona pellucida) which was attributed to hormonal changes.

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