

## Uterine Space Affects Placental Protein Secretion in Swine<sup>1</sup>

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### ABSTRACT

The effect of altering uterine space available to developing conceptuses on placental and endometrial protein secretion and endometrial <sup>3</sup>H-thymidine incorporation on Days 25 and 35 of gestation was tested. Gilts bred at estrus (Day 0) were laparotomized on Day 4, and the uterine horn ipsilateral to the ovary with the most ovulations was ligated midway between the uterine tip and uterine bifurcation to create a crowded and a roomy uterine environment. On Day 25 (n = 7) or 35 (n = 6), gilts were killed and the reproductive tract was dissected from each. Each placenta and fetus was weighed, and a sample of endometrium adjacent to each placenta was collected. Within each uterine environment, tissues (500 mg) from the heaviest and lightest (by weight) placentae and adjacent endometrial tissue were cultured for 24 h at 37°C in 15 ml Minimal Essential Medium having 0.1 times the normal amount of leucine plus 50 µCi <sup>3</sup>H-leucine. Aliquots (2 ml) of dialyzed medium were subjected to two-dimensional PAGE and fluorography to examine proteins with molecular weight ranges of approximately *M*<sub>r</sub> 20 000–130 000, and a further aliquot was subjected to SDS-PAGE to examine low molecular weight proteins (*M*<sub>r</sub> 2000–20 000). In addition, endometrium (200 mg in duplicate) adjacent to the heaviest and lightest placentae within each uterine environment was incubated for 4 h in 5 ml MEM plus 1 µCi <sup>3</sup>H-thymidine to measure DNA synthesis. After culture, <sup>3</sup>H-thymidine incorporation and total DNA were determined. Fetal survival in the crowded uterine environment decreased (*p* < 0.05) on Day 35 compared to Day 25. On both days, the heaviest and lightest placentae from the crowded uterine environment weighed less (*p* < 0.01) than the heaviest and lightest placentae from the roomy uterine environment. The fetuses associated with the heaviest and lightest placentae from the crowded uterine environment also weighed less (*p* < 0.05) than fetuses associated with the heaviest and lightest placentae from the roomy uterine environment. Nondialyzable radioactivity in medium per gram of tissue cultured from placental cultures decreased (*p* < 0.05) and from endometrial culture increased (*p* < 0.05) between Days 25 and 35. Placental secretion of a protein with *M*<sub>r</sub> 46 000, pI 5, decreased between Days 25 and 35. Endometrial secretion of three proteins (*M*<sub>r</sub> 35 000, pI 6.2; *M*<sub>r</sub> 25 000, pI 4.9; and *M*<sub>r</sub> 14 000, pI unknown) increased, and secretion of three other proteins (*M*<sub>r</sub> 24 000, pI 6.5 doublet; *M*<sub>r</sub> 22 000, pI 7.5, and *M*<sub>r</sub> 7000, pI unknown) decreased from Day 25 to Day 35 of pregnancy. Endometrial <sup>3</sup>H-thymidine incorporation per microgram of total DNA decreased (*p* < 0.1) between Days 25 and 35 and was also less (*p* < 0.1) for endometrium adjacent to the lightest placentae within each uterine environment than for endometrium adjacent to the heaviest placentae.

The amount of uterine space available to conceptuses had no detectable effect on endometrial <sup>3</sup>H-thymidine incorporation or endometrial protein secretion. Placental secretion of two proteins (*M*<sub>r</sub> 36 000, pI 4, and *M*<sub>r</sub> 46 000, pI 4) was increased (*p* < 0.05), and secretion of another protein (*M*<sub>r</sub> 46 000, pI 5) was decreased when the amount of uterine space available decreased. These results indicate that the amount of uterine space available to conceptuses affects placental protein secretion on Days 25 and 35 of pregnancy.

### INTRODUCTION

Factors affecting litter size in swine include ovulation rate, fertilization rate, early embryonic mortality (loss that occurs before Day 30 of gestation), and uterine capacity [1, 2]. Ovulation rate may be increased by selection [3] or by hormonal means [4]. Fertilization rate has been estimated to be 95% or greater in swine [5]. Although embryonic mortality accounts for 20–30% of loss in normal pregnant pigs [6], studies show that an increase in the number of embryos that survive until Day 30 of gestation can be obtained either by superovulation [4, 7] or by selection [3]. This increase in number of conceptuses at Day 30 is generally reduced during later gestation as a result of the limited ability of the

uterus to maintain fetuses to term. Thus, of the factors affecting litter size in swine, uterine capacity is most limiting to litter size.

Using superovulation to attempt to increase litter size, a treatment that results in crowding of conceptuses, Longenecker and Day [4] reported that more conceptuses survived in superovulated pigs as late as Day 40 of gestation. However, increased litter size at farrowing was not observed. Knight et al. [8], using unilateral hysterectomy and ovariectomy to study conceptus development, a treatment that also causes crowding of conceptuses, reported decreased survival of fetuses in unilaterally hysterectomized pigs by Day 40 of gestation. Wu et al. [9] reported that minor crowding of conceptuses caused fetal death by Day 50 of gestation, while severe crowding caused fetal death as early as Day 25. These reports indicate that conceptuses are susceptible to uterine crowding during early gestation (Days 30–50). An examination of the effects of crowding on placental and endometrial function during the period immediately prior to loss of the extra fetuses may suggest reasons for the demise of these fetuses.

Accepted October 23, 1992.

Received July 27, 1992.

<sup>1</sup>Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Little is known about the effects of available uterine space on the physiology and biochemistry of the conceptus and endometrium. It may be speculated that uterine space available to developing conceptuses may cause changes in either conceptus or endometrial function or both. Placental nutrient uptake, nutrient utilization by the placenta or fetus, placental secretion of factors that stimulate endometrial growth or function, endometrial growth or secretion of either histotroph or growth factors, and other functions might all be influenced by the available uterine space. All of these functions may involve an alteration in protein secretion by either the placenta or the endometrium. An examination of proteins secreted by the placenta or endometrium when conceptuses are in uterine environments that differ in the amount of space available to the conceptus could provide clues to which of the above processes, if any, are influenced by uterine space. Increased growth of the endometrium could also be directly measured as DNA synthesis.

The objectives of this study were to 1) determine whether the uterine space available to conceptuses on Days 25 and 35 of gestation affects placental or endometrial protein secretion and 2) determine whether uterine space available to conceptuses on Days 25 and 35 of gestation affects endometrial growth as measured by  $^3\text{H}$ -thymidine incorporation.

## MATERIALS AND METHODS

### *Animals*

All experimental procedures involving animals were approved by the Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee. Crossbred (1/4 Yorkshire, 1/4 Large White, 1/4 Chester White, 1/4 Landrace) gilts (age range 239–294 days) were bred at second or third estrus (Day 0) after a normal estrous cycle. On Day 4 of pregnancy, gilts were laparotomized, and the uterus and ovaries were exposed. Ovulation points on each ovary were counted, and the uterine horn ipsilateral to the ovary with the most ovulation points was double-ligated with umbilical tape approximately midway between the uterine tip and the uterine bifurcation. Gilts were then assigned to slaughter on either Day 25 ( $n = 7$ ) or Day 35 ( $n = 6$ ) of pregnancy. At slaughter, reproductive tracts were collected and placed into a sterile pan, and each conceptus was dissected from its adjacent endometrium. To minimize loss of tissue viability, all placentae excluding the necrotic tips were placed into preweighed 50-ml centrifuge tubes containing 20 ml cold Minimal Essential Medium (MEM) with one tenth the normal concentration of leucine. The MEM also contained added glucose to make four times the normal concentration. Finally, 10 ml 100-strength nonessential amino acid solution (Cat. No. M7145), 10 ml 100-strength vitamin solution (Cat. No. M6895), 10 ml glutamine solution (200 mM; Cat. No. G7513), and 10 ml 100-strength antibiotic antimycotic solution (Cat. No. A9909) were also added per liter

of MEM. All added solutions were from Sigma Chemical Co., St. Louis, MO. Placenta that were clearly necrotic (brown color, lack of blood in blood vessels) or were associated with visibly dead fetuses (blood in abdomen, pale or brown color) were excluded at the time of sampling. Large placentae were divided among several tubes. Endometrium adjacent to each placenta was also collected randomly across each placenta into 20 ml cold MEM. Placental weight was determined by subtracting the initial weight of the tube(s) and medium alone from the total weight. Fetal weights were recorded.

### *Tissue Culture*

Placental and adjacent endometrial tissue (500 mg) for the heaviest and lightest (by weight) placentae from the crowded and roomy uterine environments were prepared for culture. No minimum difference between the two selected for culture was required, and the range in weight differences between the heaviest and lightest placentae selected was 2.3–10.6 g for the crowded uterine environment and 1.4–9.0 g for the roomy uterine environment on Day 25 and 14.8–33.1 g for the crowded uterine environment and 15.2–39 g for the roomy uterine environment on Day 35. Chorioallantoic tissue (cut into 1–2-mm pieces with scissors) taken at random was cultured in 15 ml MEM with 0.1 times the normal concentration of leucine plus 50  $\mu\text{Ci}$   $^3\text{H}$ -leucine in 100-mm-diameter culture plates at 37°C in an atmosphere of 50% nitrogen: 45% oxygen: 5%  $\text{CO}_2$  for 24 h, substantially as described by Basha et al. [10]. The interval from slaughter to culture was less than 2 h. After incubation, conditioned medium was collected after centrifugation and frozen at  $-20^\circ\text{C}$  until processed for electrophoresis. In addition, endometrial tissue (200 mg) adjacent to the heaviest and lightest placentae were cultured in duplicate for 4 h in 5 ml complete MEM plus 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine under the conditions described by Basha et al. [10]. After incubation, tissue and a 200- $\mu\text{l}$  aliquot of conditioned medium were frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  until analyzed for DNA and  $^3\text{H}$ -thymidine incorporation.

### *Electrophoresis of Conditioned Medium*

Conditioned media from cultures of placenta and endometrium with  $^3\text{H}$ -leucine were dialyzed (3500  $M_r$  cutoff; Fisher Scientific, Pittsburgh, PA) against three changes of four liters of 10 mM Tris, 0.02% sodium azide, pH 8.2, to remove unincorporated radioactivity. Incorporation of radioactivity into nondialyzable macromolecules was determined by scintillation counting. Two 2-ml aliquots of dialyzed medium from each culture were lyophilized; one aliquot was subjected to two-dimensional (2D)-PAGE as described by Roberts et al. [11]. The other aliquot was subjected to SDS-PAGE for the separation of low molecular weight proteins as described by Schagger and Von Jagow

[12]. Gels were stained with Coomassie blue R250 dye, destained, soaked in water (30 min) followed by 1 M sodium salicylate (30 min), and then dried and placed with Kodak (Rochester, NY) XAR-5 film (28-day exposure).

To determine effects of uterine space available to conceptuses on placental secreted proteins visualized by 2D-PAGE and fluorography, proteins of interest were punched from the gel and solubilized, and the radioactivity in the gel for each protein was determined by scintillation counting as described by Vallet et al. [13].

#### *Incorporation of $^3\text{H}$ -Thymidine*

Endometrial tissue was thawed, and 1 ml 10 mM Tris, 20 mM NaCl, 5 mM EDTA, pH 7.4 (buffer A) was added to each sample. Tissue was then homogenized by means of a Polytron homogenizer (Brinkman Instruments, Westbury, NY). After homogenization, samples were centrifuged ( $2500 \times g$ ) for 10 min, and the supernatant was collected. Samples of medium from each culture were also diluted by adding 1 ml buffer A. Thymidine incorporated into DNA was determined through a modification of the method of Bird et al. [14]. Briefly, 100  $\mu\text{l}$  of each supernatant was spotted onto Whatman (Clifton, NJ) 540 filter paper discs in duplicate. To quantify nonspecific binding of  $^3\text{H}$ -thymidine to filters, 100  $\mu\text{l}$  of diluted medium from each culture was similarly treated. Filter paper discs were dried under a heat lamp and then washed sequentially (20 min each) with 20% trichloroacetic acid (TCA), 10% TCA, absolute ethanol, diethyl-ether, and then absolute ethanol (300 ml/50 filters) and then dried again under a heat lamp. Filters were then placed into 20-ml scintillation vials, 0.5 ml of 0.1 M NaOH was added, and the filters were incubated overnight. Then, 0.5 ml of 1 M Tris, pH 7.0, was added to neutralize the NaOH, and the samples were subjected to scintillation counting. Nonspecific binding was subtracted from all values. Preliminary trials indicated that measurement of  $^3\text{H}$ -thymidine incorporation was linear up to 100  $\mu\text{l}$  of sample. Finally, DNA was measured in each sample by the procedure of Labarca and Paigen [15]; data were expressed as dpm/ $\mu\text{g}$  DNA.

#### *Statistical Analysis*

All data were analyzed by analysis of variance using the Statistical Analysis System (SAS Inc., Cary, NC). Mean placental and fetal weights and variances changed drastically between Days 25 and 35, making analysis of the combined data inappropriate. These data were therefore analyzed by analysis of variance separately by day of pregnancy. Data from the placentae that were subsequently chosen for culture from each pig were analyzed separately from the data from all the placentae combined. The model used for the cultured placentae included effects of pig, uterine space available to the conceptuses, the interaction of pig and uterine space, placental weight (heaviest or lightest), the inter-

action of placental weight and pig, and the interaction of uterine space with placental weight. The interactions of main effects with the effect of pig were used as error terms for the main effects and the interaction of each main effect with day. Placental and fetal weights using the data from all placentae were analyzed separately by day. The model used included the effect of pig and uterine space available to conceptuses; and the interaction of uterine space with pigs was used as error term. Analysis of variance was used to analyze nondialyzable radioactivity in the conditioned medium from placental and endometrial cultures, radioactivity in 2D-PAGE gels corresponding to proteins 1–5 (see *Results*), DNA/g endometrial tissue, and  $^3\text{H}$ -thymidine incorporation into endometrial tissue per  $\mu\text{g}$  DNA. The model included the effects of day of pregnancy, pig within day of pregnancy, effect of uterine space available to conceptuses, the interaction of uterine space and day of pregnancy, the interaction of uterine space and pig within day of pregnancy, effect of placental weight (i.e., heaviest or lightest), the interaction of placental weight and day of pregnancy; the interaction of placental weight and pig within day of pregnancy; the interaction of uterine space and placental weight; and the interaction of uterine space, placental weight, and day of pregnancy. Heterogeneity of variance due to a scale effect was detected in the nondialyzable radioactivity data from endometrial cultures and in the data resulting from the radioactivity incorporated into placental proteins 1–5, so these data were analyzed after log transformation, which alleviated the heterogeneity of variance.

Three pigs had more embryos in the roomy uterine environment than corpora lutea (CL) present on the ipsilateral ovary, suggesting that either some CL were missed during counting or some embryos passed through the ligatures on the crowded horn. This made estimates of conceptus survival within each uterine horn based on ipsilateral CL number suspect. To examine conceptus survival within each uterine environment on different days of the cycle, the number of embryos within each uterine environment was expressed as a percentage of the total number of CL on both ovaries. These data were then arc sine transformed and then subjected to a separate analysis of variance for each uterine environment using the effect of day as the model. Any differences within each uterine environment between the days of the experiment should be attributable to conceptus survival as long as CL number was the same within uterine environment between days. Possible missed CL would be expected to be randomly distributed among the pigs, and intrauterine migration ends by Day 13 [16]; thus both problems would effect pigs on each day equally.

## RESULTS

#### *Conceptus Survival*

Mean CL number was not different within uterine environment on each day but was greater ( $p < 0.05$ ) for the

TABLE 1. Mean fetal and placental weights for all conceptuses and for conceptuses having the heaviest and lightest placentae in the crowded or roomy uterine environments.

| Day* | Size of placenta | Uterine environment | Placental weight (g)      | Fetal weight (g)           |
|------|------------------|---------------------|---------------------------|----------------------------|
| 25   | Heaviest         | Roomy               | 15.0 ± 1.0 <sup>ace</sup> | 0.51 ± 0.029 <sup>ad</sup> |
| 25   | Lightest         | Roomy               | 9.7                       | 0.49                       |
| 25   | Heaviest         | Crowded             | 8.4                       | 0.47                       |
| 25   | Lightest         | Crowded             | 2.0                       | 0.35                       |
| 25   | All placentae    | Roomy               | 12.2 ± 1.4 <sup>a</sup>   | 0.51 ± 0.034 <sup>b</sup>  |
| 25   | All placentae    | Crowded             | 4.6                       | 0.41                       |
| 35   | Heaviest         | Roomy               | 69.3 ± 7.5 <sup>ad</sup>  | 4.4 ± 0.17 <sup>bd</sup>   |
| 35   | Lightest         | Roomy               | 46.5                      | 3.9                        |
| 35   | Heaviest         | Crowded             | 38.2                      | 3.9                        |
| 35   | Lightest         | Crowded             | 14.5                      | 3.1                        |
| 35   | All placentae    | Roomy               | 59.0 ± 7.3 <sup>a</sup>   | 4.2 ± 0.28 <sup>b</sup>    |
| 35   | All placentae    | Crowded             | 25.9                      | 3.5                        |

\*Means are the result of data from 7 pigs on Day 25 and 4 pigs on Day 35.

<sup>a</sup>Effect of uterine space available to conceptuses was significant ( $p < 0.01$ ).

<sup>b</sup>Effect of uterine space available to conceptuses was significant ( $p < 0.05$ ).

<sup>c</sup>Effect of placental weight was significant ( $p < 0.01$ ).

<sup>d</sup>Effect of placental weight was significant ( $p < 0.05$ ).

<sup>e</sup>SEM from analysis of variance for each mean in a group is indicated for the first mean in that group.

crowded side of the uterus on both Day 25 ( $6.85 \pm 0.46$ ) and Day 35 ( $6.75 \pm 0.64$ ) compared to that for the roomy side of the uterus ( $4.14 \pm 0.46$  and  $5.0 \pm 0.64$ , respectively). Because of this, it was not possible to compare survival in the two uterine environments; however, comparisons within uterine environments should be valid. In the crowded uterine environment, the proportion of live embryos compared to the total possible in both environments decreased ( $p < 0.05$ ) from Day 25 ( $46.7 \pm 6.2\%$ ;  $n = 7$ ) to Day 35 ( $18.3 \pm 6.8\%$ ;  $n = 6$ ), whereas in the roomy uterine environment, no decrease from Day 25 to Day 35 ( $34.3 \pm 4.6\%$  versus  $43.5 \pm 5.0\%$ ) was observed.

#### Fetal and Placental Weights

Two pigs had no embryos in the crowded uterine horn on Day 35 of pregnancy, so these were excluded from fur-

ther analysis since no comparisons between uterine environments could be made. Mean fetal and placental weights for the conceptuses selected for culture and for all conceptuses are reported in Table 1. No pattern in the location of the heaviest and lightest placentae in each environment was observed. As expected, statistical analysis confirmed that within uterine environment (crowded or roomy), the lightest placentae weighed significantly less than the heaviest placentae on both Day 25 ( $p < 0.01$ ) and Day 35 ( $p < 0.05$ ) of pregnancy. Also, within the crowded or roomy uterine environments, fetuses associated with the lightest placentae weighed less ( $p < 0.05$ ) than fetuses associated with the heaviest placentae on both Days 25 and 35 of pregnancy. The heaviest and lightest placentae in the crowded uterine environment weighed less ( $p < 0.01$ ) than the heaviest and lightest placentae in the roomy uterine envi-

TABLE 2. Means ( $\pm$  SEM from analysis of variance) for placental and endometrial nondialyzable  $^3\text{H}$ -leucine radioactivity (dpm/g tissue  $\times 10^{-6}$ ), DNA ( $\mu\text{g/g}$  tissue) endometrial incorporation of  $^3\text{H}$ -thymidine (dpm/ $\mu\text{g}$  DNA) for conceptuses with the heaviest and lightest placentae in crowded and roomy uterine environments on Day 25 or Day 35 of pregnancy.

| Day* | Size of placenta | Uterine environment | Placental $^3\text{H}$ -leucine | Endometrial $^3\text{H}$ -leucine | DNA                     | $^3\text{H}$ -thymidine   |
|------|------------------|---------------------|---------------------------------|-----------------------------------|-------------------------|---------------------------|
| 25   | Heaviest         | Roomy               | 12.97 ± 1.47 <sup>ad</sup>      | 13.74 ± 0.75 <sup>a</sup>         | 48.9 ± 2.5 <sup>b</sup> | 152.2 ± 18.5 <sup>e</sup> |
| 25   | Lightest         | Roomy               | 18.81                           | 14.07                             | 57.5                    | 127.8                     |
| 25   | Heaviest         | Crowded             | 16.26                           | 14.41                             | 51.1                    | 162.8                     |
| 25   | Lightest         | Crowded             | 18.10                           | 14.91                             | 48.0                    | 128.3                     |
| 35   | Heaviest         | Roomy               | 9.64 ± 2.08                     | 21.32 ± 1.06                      | 59.5 ± 2.8              | 112.6 ± 26.2              |
| 35   | Lightest         | Roomy               | 7.85                            | 24.54                             | 59.5                    | 109.2                     |
| 35   | Heaviest         | Crowded             | 9.68                            | 24.63                             | 59                      | 122.9                     |
| 35   | Lightest         | Crowded             | 8.60                            | 25.61                             | 65                      | 88.1                      |

\*Number of observations for each mean was 7 for Day 25 and 4 for Day 35.

<sup>a</sup>Effect of day of pregnancy was significant ( $p < 0.01$ ).

<sup>b</sup>Effect of day of pregnancy ( $p < 0.1$ ) and the interaction of day of pregnancy, placental weight and uterine space ( $p < 0.05$ ) were significant.

<sup>c</sup>Effects of day of pregnancy and size of placental weight (heaviest or lightest) were significant ( $p < 0.1$ ).

<sup>d</sup>SEM from analysis of variance for all means in a group is indicated for the first mean in each group.

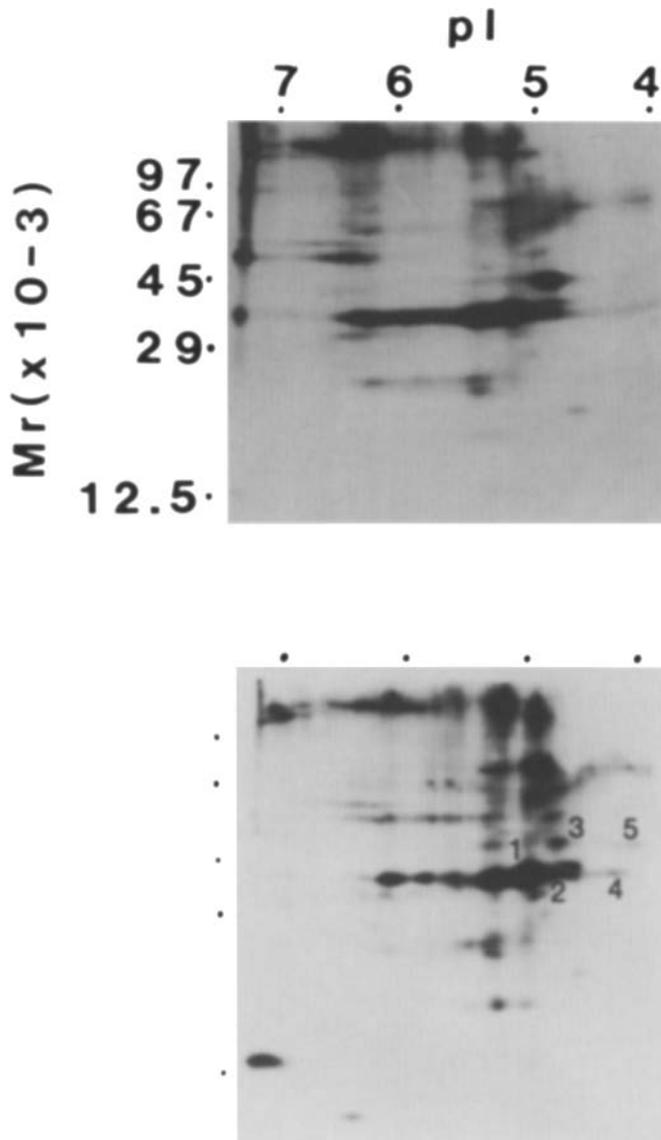


FIG. 1. Fluorographs of a 2D-PAGE gel of proteins secreted in culture by placental tissue from the lightest placenta from a roomy uterine environment (top) and the lightest placenta from a crowded uterine environment (bottom). For each sample, the five proteins indicated were punched from the gel and solubilized, and the radioactivity in the gel was determined by scintillation counting. Protein 1 is below and to the right of the number, protein 2 is immediately to the left of the number, protein 3 is below and to the left of the number, protein 4 is above the number, and protein 5 is below the number. Secretion of proteins 1–5 from the different treatments is reported in Table 3.

ronment on both Day 25 and Day 35 of pregnancy. Finally, fetuses associated with the heaviest and lightest placentae in the crowded uterine environment weighed less than fetuses associated with the heaviest and lightest placentae in the roomy uterine environment on both Day 25 ( $p < 0.01$ ) and Day 35 ( $p < 0.05$ ). For the combined data, placental ( $p < 0.01$ ) and fetal ( $p < 0.05$ ) weights were less in the crowded uterine environment compared to the roomy uterine environment on both days.

#### Placental and Endometrial Protein Secretion

Mean nondialyzable radioactivity in the medium from placental and endometrial cultures is reported in Table 2. Incorporation of  $^3\text{H}$ -leucine into macromolecules was greater ( $p < 0.01$ ) for placental tissue collected on Day 25 than on Day 35 of pregnancy. In contrast, incorporation of  $^3\text{H}$ -leucine into macromolecules by endometrial tissue increased ( $p < 0.01$  using log transformed data) from Day 25 to Day 35 of pregnancy. No effect of placental weight or uterine space available to the conceptuses was detected on  $^3\text{H}$ -leucine incorporation for either placenta or endometrium.

A representative 2D-PAGE fluorograph of proteins secreted by placental tissue in culture is shown in Figure 1. Visual inspection of fluorographs generated with medium from cultures from the different experimental categories revealed a high degree of variability in the patterns of proteins obtained. Nevertheless, by visual inspection, secretion of proteins 2–5 appeared to vary according to whether the placenta was from a crowded or roomy uterine environment. To confirm the effect of uterine space available to the conceptuses on secretion of these proteins, radioactivity incorporated into each protein was determined. Protein 1 was included as a negative control (i.e., by visual inspection, this protein did not appear to be affected by the uterine space available to conceptuses).

Mean radioactivity incorporated into each protein per gram of tissue is reported in Table 3. For protein 1, ( $M_r$  36 000, pI 5.2) only an effect of day ( $p < 0.01$  using log transformed data) was observed. Thus, secretion of this protein was greater on Day 25 than on Day 35. Placental weight or uterine space available to the conceptuses did not effect secretion of this protein.

For protein 2 ( $M_r$  32 000, pI 5.2), only the interaction of placental weight with uterine space available to conceptuses was significant ( $p < 0.05$  using log transformed data). Examination of the data reveals that in the roomy uterine environment, secretion of protein 2 was greater by the lightest than by the heaviest placentae. However, in the crowded uterine environment, there was no difference in secretion of protein 2 by the heaviest and lightest placentae, and secretion was intermediate to that of the heaviest and lightest placentae from the roomy uterine environment. The lack of an interaction with day indicates that this relationship occurred on both Day 25 and Day 35 of pregnancy.

For protein 3 ( $M_r$  46 000, pI 5), the effect of day ( $p < 0.01$  using log transformed data) and the effect of uterine space available to conceptuses ( $p < 0.05$  using log transformed data) were significant. These results indicate that secretion of protein 3 by placenta was less for conceptuses in the crowded uterine environment than for those in the roomy uterine environment. Results also indicate that secretion of protein 3 from placenta was greater on Day 25 than on Day 35 of pregnancy.

TABLE 3. Mean ( $\pm$  SEM from analysis of variance) for radioactivity (dpm/g tissue cultured  $\times 10^{-3}$ ) in 2D-PAGE gels for placental proteins 1–5 (see Fig. 1).

| Day* | Size of placenta | Uterine environment | Protein                       |                             |                             |                            |                            |
|------|------------------|---------------------|-------------------------------|-----------------------------|-----------------------------|----------------------------|----------------------------|
|      |                  |                     | 1                             | 2                           | 3                           | 4                          | 5                          |
| 25   | Heaviest         | Roomy               | 286.7 $\pm$ 37.3 <sup>a</sup> | 16.5 $\pm$ 3.5 <sup>b</sup> | 56.0 $\pm$ 8.3 <sup>c</sup> | 2.9 $\pm$ 5.9 <sup>d</sup> | 3.0 $\pm$ 1.7 <sup>d</sup> |
| 25   | Lightest         | Roomy               | 530.5                         | 32.5                        | 55.8                        | 9.8                        | 3.8                        |
| 25   | Heaviest         | Crowded             | 313.3                         | 29.0                        | 26.4                        | 17.0                       | 6.9                        |
| 25   | Lightest         | Crowded             | 318.4                         | 28.2                        | 23.0                        | 10.5                       | 7.7                        |
| 35   | Heaviest         | Roomy               | 172.0 $\pm$ 52.7              | 18.1 $\pm$ 5.0              | 13.5 $\pm$ 11.8             | 7.4 $\pm$ 8.3              | 4.1 $\pm$ 2.3              |
| 35   | Lightest         | Roomy               | 95.0                          | 21.2                        | 8.3                         | 6.3                        | 3.7                        |
| 35   | Heaviest         | Crowded             | 91.9                          | 18.9                        | 6.5                         | 11.6                       | 6.6                        |
| 35   | Lightest         | Crowded             | 73.8                          | 16.8                        | 6.9                         | 26.9                       | 6.5                        |

\*Number of observations for each mean was 7 on Day 25 and 4 on Day 35.

<sup>a</sup>Effect of day of pregnancy ( $p < 0.01$  using log transformed data) was significant.

<sup>b</sup>The interaction of size of placental weight and uterine space available to conceptuses was significant ( $p < 0.05$  using log transformed data).

<sup>c</sup>Effects of day ( $p < 0.01$  using log transformed data) and uterine space available to conceptuses were significant.

<sup>d</sup>Effect of uterine space available to conceptuses was significant ( $p < 0.05$  using log transformed data).

\*SEM from analysis of variance for all means in a group is indicated for the first mean in each group.

Secretion of protein 4 ( $M_r$  36 000, pI 4) and protein 5 ( $M_r$  46 000, pI 4) was greater ( $p < 0.05$  using log transformed data) for conceptuses in a crowded uterine environment as compared to a roomy uterine environment.

Representative fluorographs of radioactive proteins from endometrial cultures are shown in Figure 2. Endometrial secretion of a  $M_r$  35 000, pI 6.2 protein and a  $M_r$  25 000, pI 4.9 protein increased, and secretion of a  $M_r$  24 000, pI 6.5 doublet of proteins and a  $M_r$  22 000, pI 7.5 protein decreased from Day 25 to Day 35. No consistent effect of placental weight or uterine space available to conceptuses was observed. No consistent changes in any other proteins were observed.

Representative fluorographs of low molecular weight radioactive proteins observed on SDS-PAGE gels are illustrated in Figure 3. For proteins from placental culture, no consistent differences due to day, placental weight, or uterine space available to conceptuses were observed. Secretion of an endometrial protein with a  $M_r$  14 000 was less on Day 25 compared to Day 35 while secretion of another endometrial protein with a  $M_r$  7000 was greater. No effect of size of placenta or uterine space available to conceptuses on endometrial secretion of low molecular weight proteins was observed.

### <sup>3</sup>H-Thymidine Incorporation and DNA in Endometrium

Mean DNA ( $\mu\text{g/g}$  tissue) and <sup>3</sup>H-thymidine incorporation (dpm/ $\mu\text{g}$  DNA) are reported in Table 2. Endometrium collected on Day 35 of pregnancy had more DNA ( $p < 0.1$ ) per gram of tissue compared to Day 25 of gestation. Also, the interaction of day of pregnancy, placental weight, and uterine space available was significant ( $p < 0.05$ ). Examination of the data indicated that on Day 25 endometrium adjacent to the lightest placenta in the roomy uterine environment had more DNA/g tissue than the other groups, while on Day 35, endometrium adjacent to the lightest pla-

centa in the crowded uterine environment had more DNA/g tissue compared to the other groups. Endometrial tissue adjacent to the lightest placentae incorporated less ( $p < 0.1$ ) <sup>3</sup>H-thymidine than endometrial tissue adjacent to the heaviest placentae regardless of whether the conceptuses were from a crowded or roomy environment. Also, incorporation of <sup>3</sup>H-thymidine per  $\mu\text{g}$  DNA was greater ( $p < 0.1$ ) on Day 25 than on Day 35 of pregnancy.

## DISCUSSION

This study indicates that uterine space available to conceptuses has a significant effect on the conceptus, because a crowded uterine environment not only decreased the size of the heaviest and lightest placenta present on both Day 25 and Day 35 of gestation, but also decreased fetal weights on both days. Furthermore, results of this experiment suggest that fetal survival decreased between Day 25 and Day 35 of pregnancy only in the crowded uterine environment. Results of this study also indicate that secretions of specific placental proteins differ in a crowded uterine environment compared to a roomy uterine environment. Secretions of four of the five placental proteins examined were affected by uterine space available to conceptuses. Identification of these proteins and their functions may increase our knowledge of how the pig conceptus adjusts to variations in the amount of uterine space available for development. Determining the extent to which these proteins are secreted during normal pregnancy and their possible function during normal pregnancy was not an objective of this experiment, and further experiments will be required to ascertain the normal functions of these proteins.

Placental protein secretion in general and the placental response to variations in uterine space were variable. There are several explanations for this. The first is that each fetus has variable requirements for nutrients provided via the placenta. For example, a given amount of uterine space may

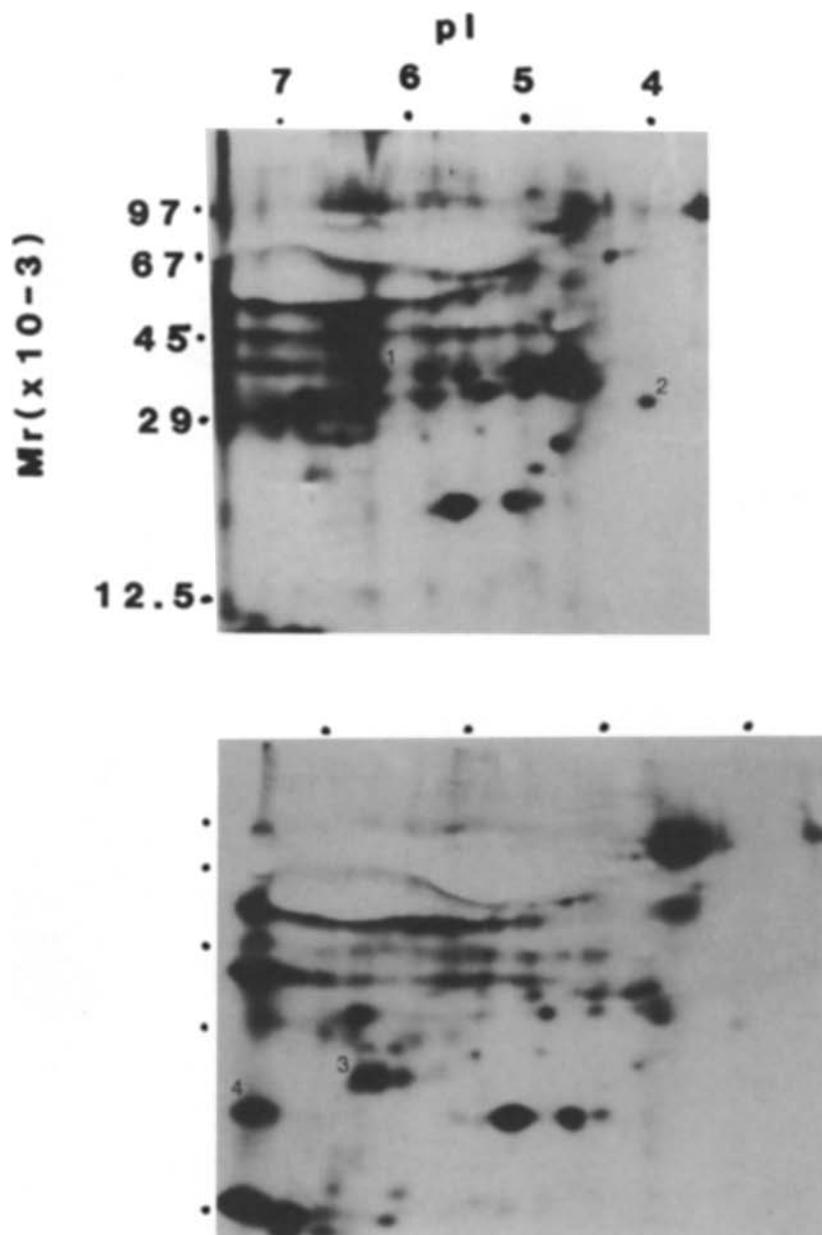


FIG. 2. Representative fluorographs of 2D-PAGE gels of proteins secreted by endometrial tissue collected on Day 25 (top) and Day 35 (bottom) of pregnancy. According to visual inspection of the fluorographs from all treatments, secretion of proteins 1 and 2 was consistently greater and that of proteins 3 and 4 was consistently less on Day 25 than on Day 35 of pregnancy. No other differences between these two fluorographs occurred consistently among the fluorographs from the experiment.

be sufficient for some fetuses and detrimental to fetuses whose genetic makeup disposes them to requiring large amounts of nutrient, either because of their overall growth rate (see below) or because of their inherent efficiency of nutrient utilization. The second explanation is that uterine delivery of nutrients may vary between dams so that a given amount of uterine space may be sufficient for fetuses in one pig and detrimental in another. Finally, in this experiment, uterine space available varied according to the num-

ber of conceptuses present in the crowded uterine environment and the size of the crowded uterine environment in each case. Since we did not quantify uterine space per conceptus, we could not account for this variability. Using a more sophisticated method such as that used by Wu et al. [9] to cause a crowded uterine environment might eliminate this variability. Nevertheless, this study demonstrated a statistically significant effect of uterine space on placental secretion of four proteins.

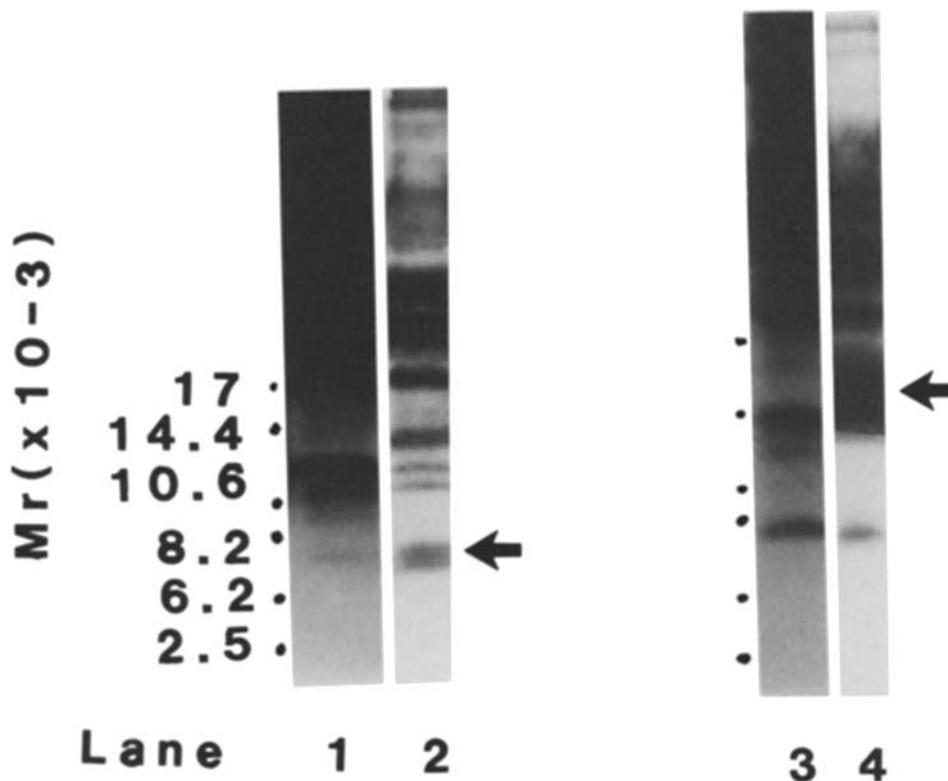


FIG. 3. Representative fluorographs of SDS-PAGE gels of low molecular weight proteins secreted by placental tissue on Day 25 (lane 1) and Day 35 (lane 3) and by endometrial tissue on Day 25 (lane 2) and Day 35 (lane 4). According to visual inspection, no consistent differences in secretion of low molecular weight proteins by placenta or endometrium were observed for tissue from crowded versus roomy uterine environments or from the heaviest or lightest placentae. Secretion of proteins by placenta did not differ with day of pregnancy (the apparent changes in the two fluorographs illustrated were not consistent among all samples). Secretion of a  $M_r$  14 000 endometrial protein was consistently greater and of a  $M_r$  7000 endometrial protein was consistently less (see arrows) for endometrial tissue in culture collected on Day 35 compared to Day 25 of pregnancy.

The question arises whether the differences in protein secretion observed in this experiment are due to effects of uterine space independent of the effect of uterine space on placental weight (i.e., would placentae of similar weights differ in secretion of the above proteins if uterine space available was different?). A partial answer to this question may be obtained from this experiment. The means for placental weight for the lightest placentae from the roomy uterine environment and the heaviest placentae from the crowded uterine environment were not different on either day examined. However, secretion of proteins 3, 4, and 5 was altered for the heaviest placentae from the crowded uterine environment compared to the lightest placentae from the roomy uterine environment. This conclusion is indicated by the presence of a main effect of uterine space on secretion of these three proteins and the lack of a main effect of placental weight or an interaction of uterine space with placental weight. Thus, secretion of these proteins would seem to be affected by uterine space independent of placental weights. However, a better answer to this question may be obtained once these proteins are identified and studied further.

In contrast to placental protein secretion, endometrial protein secretion appeared unaffected by uterine space, as did  $^3\text{H}$ -thymidine incorporation. From these results, one might speculate that the endometrium has little or no ability to respond to changes in uterine space per conceptus. The endometrium may deliver nutrients at its maximum capability at this stage of pregnancy and therefore may be unable to respond further when conceptuses are crowded. However, only protein secretion and  $^3\text{H}$ -thymidine uptake were examined in this study; other changes in endometrial physiology may occur when conceptuses are crowded, and this topic warrants further study.

Secretion of several endometrial proteins including two low molecular weight proteins changed during the period examined in this experiment. Several changes in placental and endometrial physiology are known to occur during this period. Between Days 25 and 35 of pregnancy, estrogen secretion by the placenta reaches a peak and then decreases [17]. Also, formation of the placental aureolae occurs [18]. Furthermore, as can be seen from the data presented in Table 1, both the fetus and the placenta undergo rapid growth. Changes in secretion of endometrial proteins

suggest that these proteins may be involved in some of these processes.

Results from this study show that a significant loss of crowded conceptuses occurred between Day 25 and Day 35 of pregnancy. Yet other experiments using other methods of altering uterine space per conceptus have shown that significant loss occurs before Day 25 [9]; still others indicate that when the amount of uterine space per conceptus is limited, extra conceptuses can survive to as late as Day 40 of gestation [4, 8]. An explanation for the death of crowded conceptuses must account for the fetal loss between Day 25 and 35 in this experiment and the difference between the earlier and later loss of conceptuses in different experiments. A possible explanation may come from comparisons of the rates of growth of the placentae and fetuses in this experiment and the difference in weights of the placentae and fetuses between treatments. In Table 1, average placental weights on Day 25 of pregnancy for the lightest and heaviest placentae from the crowded uterine environment and the lightest placentae from the roomy uterine environment are 13, 56, and 65% of the average weight of the heaviest placentae from the roomy uterine environment. Yet fetal weights associated with these placentae are 69, 92, and 96% of the weight of fetuses associated with the heaviest placentae from the roomy uterine environment. This suggests that fetal growth is less sensitive to the effects of decreased uterine space per conceptus than placental growth. This is not surprising because the weight of the placenta is limited by both the initial uterine space acquired at elongation and subsequent placental nutrient uptake while fetal weight is only limited by placental nutrient uptake. Also, fetal weights for all treatment groups increase 8–9 times between Days 25 and 35 of pregnancy. By contrast, placental weights in the heaviest and lightest placentae from the roomy uterine environment and the heaviest placentae from the crowded uterine environment increase about 4.5 times between Days 25 and 35 of pregnancy, whereas placental weights of the lightest placentae from the crowded uterine environment increase over 7 times. The difference in placental growth rates between the lightest placentae from the crowded uterine environment and the other groups from Day 25 to Day 35 is probably best explained by the loss of small conceptuses between the two time periods, rather than an increase in the growth rate of small placentae.

The difference between placental growth rate and fetal growth rate suggests a mechanism for the loss of conceptuses in a crowded uterine environment. The results in Table 1 indicate that placental and fetal growth both occur at well-controlled rates, but fetal growth occurs relatively faster than placental growth. Crowding limits the initial size of the placenta by decreasing the uterine space available to each conceptus during the blastocyst elongation phase. Because of the difference in fetal and placental growth rates, the fetus becomes an increasing burden on the placenta. Pre-

sumably, there is a threshold fetal burden beyond which the placenta can no longer maintain the fetus, and this threshold may be relatively low until the placenta reaches full growth and maturation at approximately mid-gestation. The threshold fetal burden of the placenta probably increases during later pregnancy, since placental weight does not increase appreciably after Day 60–70 of gestation even though fetal weight continues to increase [8]. When the fetal burden exceeds the placental threshold for that stage of pregnancy, the placenta can no longer meet the requirements of the fetus and the fetus dies. Under normal conditions, the fetal burden does not reach the placental threshold. However, the effect of decreasing uterine space on placental size may create a situation where the fetal burden exceeds the threshold during early gestation. Thus severe crowding like that reported by Wu et al. [9] causes earlier loss because each placenta is smaller and the threshold fetal burden is reached earlier. Minor crowding like that obtained by Knight et al. [8] would have the opposite characteristic: the fetal burden would not exceed placental threshold until later in pregnancy. This hypothesis predicts that decreasing the disparity between fetal and placental growth by slowing fetal growth or increasing placental growth during early pregnancy (i.e., between Days 30 and 60 of gestation) would allow for the maintenance of the fetuses and an increase in litter size.

In conclusion, the effects of uterine space available to conceptuses on placental and endometrial protein secretion and endometrial  $^3\text{H}$ -thymidine incorporation have been examined during a period in pregnancy when loss of conceptuses due to decreased uterine space per conceptus occurred. Results have shown that the secretions of four placental proteins are altered when uterine space per conceptus is altered, while protein secretion and  $^3\text{H}$ -thymidine incorporation by endometrium are not changed. Identification of the proteins and determination of their function, if any, during normal pregnancy and during pregnancy when the number of conceptuses exceeds uterine capacity may provide clues to ways of increasing litter size in swine.

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