EFFECTS OF INTRAUTERINE DYNAMICS ON STEROIDOGENESIS AND CONCEPTUS DEVELOPMENT IN THE PORCINE

By

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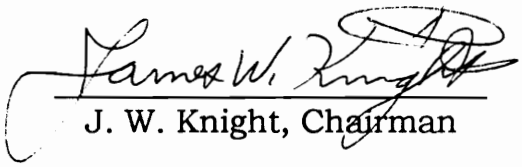

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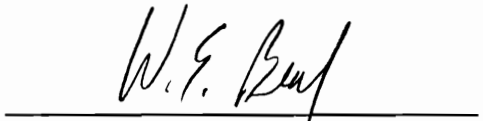

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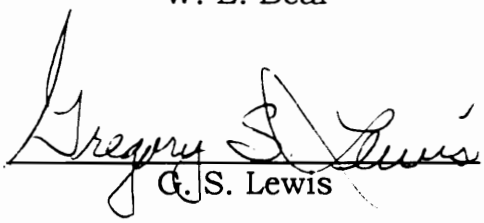

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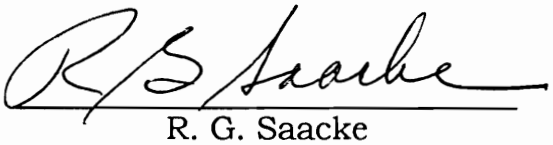

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EFFECTS OF INTRAUTERINE SPACE ON STEROIDOGENESIS AND CONCEPTUS DEVELOPMENT IN THEPORCINE

by

Charbel G. Tarraf

ABSTRACT

Intrauterine crowding and placental insufficiency are main reasons for prenatal losses in swine. Two studies were conducted to examine: 1) conceptus development and *in vitro* steroidogenic capability of three regions of the placenta (middle, inner, polar) at d 30, 50, 70, and 90 of gestation; and 2) the effects of intrauterine position and fetal sex on conceptus development and *in vitro* steroidogenic activity of the placenta and endometrium at d 40, 60, 80, and 100 of gestation All variables were examined in gilts that were uterine intact before hysterectomy (n=19) and in gilts unilaterally hysterectomized-ovariectomized (UHOX) before breeding (n=17) to induce intrauterine crowding. Placentas were combined according to the sex of the fetus associated with the placental unit (except at d 30). Placentas were sectioned into middle, inner and polar regions. Placental tissues were incubated, and release of progesterone (P_4) and estrone (E_1) was determined. Fetal survival rate was greater ($P< .01$, .05, .001 at d 50, 70 and 90, respectively) from intact versus UHOX gilts. Placental length and weight, fetal length and weight, and allantoic fluid volume were greater in intact compared to UHOX gilts. The polar region of the placenta released less P_4 than the other regions at d 50, 70 and 90. Uterine status ($P< .005$) affected P_4

release only at d 90. Sex of the fetus did not affect placental P_4 release. Region of the placenta affected E_1 release at d 30 ($P< .01$) and d 50 ($P< .06$). Uterine status did not affect E_1 release. Sex of the fetus affected ($P< .001$) E_1 release only at d 50. In the second study, a total of 45 gilts was used. Placentas and endometrium were combined based upon the intrauterine position of the associated fetus. Placental and endometrial tissues were incubated and release of P_4 and E_1 was determined. Uterine status (intact or UHOX) did not affect the variables measured. Intrauterine position affected fetal and placental weights ($P< .02$ and .01, respectively) at d 40 of gestation. No significant effect of intrauterine position was detected on placental and endometrial P_4 release. At d 100 of gestation, placentas associated with fetuses bordered in utero by fetuses of the same sex released more ($P< .01$) E_1 compared to placentas associated with fetuses bordered by fetuses of the opposite sex . Estrone release by the endometrium was not significantly affected by intrauterine position. Only trace amounts of testosterone and dehydroepi-androsterone sulfate were measured in the fetal fluids at all days of gestation. Intrauterine position had no effect on P_4, E_1 or androstenedione concentrations in fetal fluids. Collectively, the results indicated a) a differential release of P_4 and E_1 by the three regions of the placenta at certain days of gestation, b) no compensatory increase in placental steroidogenic activity per unit of tissue when total placental mass was reduced, and c) a limited effect of intrauterine position on placental and endometrial steroidogenesis at various days of gestation.

In memory of my father

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Chapter I

INTRODUCTION

Reproductive efficiency is an economically important component of swine production. In turn, litter size is an important component of reproductive efficiency, and prenatal mortality is one of the main factors that limits litter size. Prenatal mortality in pigs ranges from 20 to 46% (Pope and First, 1985). Genetics, nutrition, stress and various diseases may be associated with embryonal mortality. However, specific causes of prenatal mortality remain poorly understood.

The interaction between developing fetuses and the maternal system is important to successful maintenance of pregnancy. The inability of the conceptus to produce the appropriate products needed to control its local environment and(or) the asynchrony between the developing conceptus and the maternal endometrium are suggested to be the main reason for prenatal, and to a certain extent, even some postnatal death losses. There is mounting evidence that steroid hormones produced and metabolized by the feto-placental unit and the maternal endometrium play an essential role in this process. The steroidogenic capabilities of the blastocyst and early (up to day 25) conceptus in the porcine have been characterized. However, the steroidogenic capabilities and(or) limitations of the conceptus after day 25 and the endometrium throughout gestation have received relatively little attention. A thorough understanding of placental and endometrial

steroidogenesis and their effects on conceptus development is essential to better understand the underlying causes of prenatal losses in the porcine. This review will focus on the physiological events during the establishment of pregnancy and examine the effect of uterine space on conceptus development. Placental development and function, intrauterine steroidogenesis and the effect of intrauterine position on sexual differentiation and conceptus development in various species will also be described.

Chapter II

LITERATURE REVIEW

Establishment and Maintenance of Pregnancy

Sexually mature female pigs (Sus scrofa domesticus) are polyestrus with estrous cycles occurring every 18 to 21 days. Behavioral estrus lasts for 24 to 72 h and ovulation occurs 36 to 42 h after onset of estrus. The ovulation rate in swine ranges between 12 to 18 (Polge et al., 1966). After ovulation, the granulosa and thecalcell layers of the ovulated follicles become lutenized to form the corpora lutea (CL). Corpora lutea are well formed by day 4 or 5 of the estrous cycle and progesterone (P_4) secretion increases from that time to maximum concentrations between days 12 to 14 of the cycle (Guthrie et al., 1972). Unless interrupted with pregnancy or any dysfunction, luteal regression will begin at about day 15, and plasma P_4 concentrations will decrease to basal concentrations by day 17 to 18. However, if pregnancy is established luteal function persists for the whole period of gestation which is approximately 114 days.

Pre-attachment embryos lack direct access to the maternal vascular system. Normal development is therefore dependent upon the oviducal and uterine environments and the ability of the embryo to produce and(or) metabolize products for its own need (Niemann et al., 1989). The interaction between the developing conceptus and the maternal system is, however, crucial for the maintenance of successful

pregnancy (Bazer and First, 1983). The uterine milieu undergoes rapid changes due to the stimulation of the endometrium by the maternal steroids and by the local presence of the conceptus (Nieder et al., 1987; Morgan et al., 1987). Conceptuses developing slowly or out of phase with the mother may not be capable of coping with such change and will be lost. Pope (1988) considered the asynchrony between the needs of the developing embryos and the secretion of the uterus as the main cause of embryonic loss.

There are several key events in conceptus development during pregnancy. After fertilization, embryos spend a short period in the oviduct before entering the uterus about 48 to 56 h after ovulation (Dziuk, 1977). Embryos reach the blastocyst stage by day 5 and emerge (hatch) from the zona pellucida by day 6 to 7. Hatched blastocysts measure about .5 to 1 mm in diameter (Perry and Rowlands, 1962). Concomitant with hatching and until about day 12 of gestation, intrauterine migration of embryos (inherent characteristic of polyovular species to minimize mortality) takes place (Dhindsa et al., 1967). Increased synthesis of estradiol-17β (E_2) by the porcine embryo was found to occur concomitantly with migration of the embryos and increased myometrial activity in vitro (Pope et al., 1982a). Pope et al. (1982b) suggested that both E_2 and histamine are involved in the process of intrauterine migration of the porcine embryo.

Early pregnancy in pigs is a period of rapid biochemical, morphological and structural changes for both conceptus and uterus. Between days 10 and 12 of gestation, porcine blastocysts progress from

spherical (3 to 10 mm in diameter) to tubular (10 to 50 mm long), to elongated filamentous (>100 mm long) forms (Geisert et al., 1982a). Coincident with the morphological transformations, blastocysts acquire the capability to synthesize E_2 (Gadsby et al., 1980). The embryo signals its presence through E_2 which results in CL maintenance and continued endometrial development and secretory activity (Bazer et al., 1984). Estrogen produced by blastocysts is thought to be the substance responsible for maternal recognition of pregnancy in pigs. Injection of unmated pigs with estrogen between d 11 and 15 of the cycle induces a state of pseudopregnancy in which the CL are maintained for over 100 days (Frank et al., 1977; Bazer et al., 1982). The presence of fewer than five blastocysts within the uterus around the time of maternal recognition of pregnancy (day 12) results in termination of the pregnancy (Polge et al., 1966; Dziuk, 1985). However, pregnancy continues if the blastocysts are removed after day 12 (Dziuk, 1985).

Attachment of elongated blastocysts to the uterine epithelium via interlocking microvilli is initiated on d 13 and is completed by d 18 (Perry et al., 1962; Keys et al., 1986). Bazer and co-workers (1982) postulated that the structural changes in surface and glandular epithelium of the endometrium are temporally correlated with developmental events occurring in blastocysts. Geisert et al. (1982a,b) have shown that uterine glandular epithelium associated with spherical blastocysts accumulates clear vesicles that are released into the uterine lumen in concert with the elongation of blastocysts. The synchronized exocytosis of vesicle contents is postulated to be under the local control of

conceptus derived E_2. Coincidental with the secretion of the uterine secretory products (histotroph), there is also a marked intrauterine increase in calcium content which is probably induced by the E_2 produced by blastocysts (Geisert et al., 1982a). Similarly, there is an increase in the intrauterine content of prostaglandins ($PGF_2\alpha$ and PGE_2) due to the activation of the endometrial arachidonic cascade. Rubin and Laychock (1978) proposed that calcium released from the plasmallemma of the adrenal is involved directly with activation of phospholipase A_2 to enhance prostaglandin synthesis, and indirectly to induce exocytosis of secretory vesicles. Geisert et al. (1982a) suggested that the mechanism of induction of endometrial histotroph secretion occurs in a similar manner to that proposed for the adrenal cortex. Blood flow to the uterus is also increased during this period. Results presented by Ford and Christenson (1979) using a unilateral pregnant model, suggested a unilateral control of uterine blood flow by the early porcine conceptus. This increase in blood flow is apparently under the influence of conceptus produced E_2 and(or) PGE_2.

An increase in the amount of protein per conceptus occurs between day 12 (0.4 mg) and day 18 (17.5 mg) of gestation (Anderson, 1978). During the same period, the yolk sac begins to produce transferrin, α-fetoprotein and other plasma protein products (Godkin et al., 1985). This phase of growth and development has been suggested to be dependent upon the ability of trophoblasts to acquire nutrients from the uterine fluids (Roberts and Bazer, 1988). Pig conceptuses release

high amounts of plasminogen activator during the day 11 to 14 period (Mullins et al., 1980; Fazleabas et al., 1983).

The importance of the uterine secretions for proper conceptus development induced many researchers to focus on purification and characterization of these secretions. It is evident that the composition of the uterine secretions differs from that of the plasma (Roberts and Bazer, 1988). Uteroferrin is the best characterized component of pig uterine secretions. It is a 35,000 dalton glycoprotein that carries two bound atoms of iron per polypeptide chain (Roberts and Bazer, 1980; Roberts and Bazer, 1984; Roberts et al., 1986). Uteroferrin has a deep purple color, which arises from the coordination of one of the iron atoms with one or more of tyrosine residues (Gaber et al., 1979).

Uteroferrin secreted by the epithelial cells of the uterine glands ultimately enters the fetal circulation where the protein is distributed to sites of iron metabolism (liver and spleen), with the excess entering the allantoic sac (Renegar et al., 1982). Buhi et al. (1982, 1983) suggested that the allantoic fluid serves as a temporary site for iron storage and iron exchange through a mechanism that involves the donation of uteroferrin's iron to apotransferrin. Doi et al. (1986), however, failed to detect iron transfer from uteroferrin to transferrin.

Another group of proteins, the plasmin/trypsin inhibitors, has been also identified in the uterine secretions during pregnancy (Fazleabas et al., 1983). These compounds control proteolytic activity within the uterus.

An important substance released by the conceptus during the day 11 to 14 period is plasminogen activator (Mullins et al., 1980; Fazleabas et al., 1983). Plasminogen activator, in addition to other proteases, have been reported to be involved in the process of invasive implantation of the embryo (Strickland et al., 1976; Denker, 1980). Pig conceptuses have a noninvasive type of attachment within the uterus, but they show invasive characteristics when transplanted to ectopic sites where protease inhibitors may not be locally produced (Samuel, 1971; Samuel and Perry, 1972).

A number of additional proteins has been identified on electrophoretic gels when uterine flushings were analyzed. Lysozymes, a group of hydrolytic enzymes believed to play an antibacterial role, have been identified in the uterine secretions of pigs treated with P_4 (Roberts et al., 1976). Hansen et al. (1985) found appreciable amounts of two glycosidases (β-hexosaminidase and β-galactosidase) in uterine flushings derived from pseudopregnant gilts. Their function, however, is unclear.

An emerging concept is that steroid hormone actions in the uterus are mediated in part by autocrine/paracrine effects of growth factors (Simmen et al., 1990). One of the growth factors possibly involved in these processes is insulin-like growth factor-I (IGF-I). A member of a family of insulin-like peptides, IGF-I mediates the growth-promoting action of growth hormone (Foresch et al., 1985). Insulin-like growth factor-I has been detected in pig uterine extracts (Tavakkol et al., 1988) and luminal fluids (Simmen et al., 1989). The uterus appeared to be a major site of IGF-I synthesis as demonstrated by hybridization with

cloned IGF-I cDNA probes (Tavakkol et al., 1988). Letcher et al. (1989) have shown that endometrial production of IGF-I during early pregnancy was temporally correlated with maximal synthesis of estrogens by developing conceptuses. Simmen and co-workers (1990) have shown that E_2 and P_4 are involved in the normal regulation of uterine IGF-I synthesis and(or) secretion in the pig. Recently, Hofig et al., (1991) demonstrated the expression of IGF-I receptors in pig endometrial and myometrial tissues. However, they were not able to detect changes in uterine IGF-I receptors with different hormonal changes (E_2 and/or P_4) or pregnancy status.

As noted previously, E_2 produced by blastocysts is thought to be the substance responsible for maternal recognition of pregnancy in pigs. Geisert et al. (1984) reported that a single injection of estradiol benzoate on day 11 will allow CL maintenance to only 30 days after estrus.

Injections on day 11 and days 14-16 are required if pseudopregnancy is to persist for more than 60 days. Prostaglandin $F_{2\alpha}$ acts as a luteolytic agent when injected into the uterine lumen or intramuscularly in swine on day 12 or later in the estrous cycle (Bazer et al., 1982). Zavy et al. (1980), however, demonstrated that $PGF_{2\alpha}$ secretion by the endometrium is not affected by pregnancy, but E_2 seems to reduce the movement of $PGF_{2\alpha}$ from the uterine lumen into the utero-ovarian vasculature (Marengo et al., 1986). Concentrations of $PGF_{2\alpha}$ in utero-ovarian plasma and the $PGF_{2\alpha}$ metabolite (PGFM) in peripheral plasma are elevated during the period of luteolysis in non pregnant pigs, but they are not elevated between day 12-25 of pregnancy (Bazer et al.,

1984). In contrast, total recoverable PGF in uterine flushings was greater for pregnant than nonpregnant gilts on day 12 and 18. Lewis and Waterman (1982) however, suggested that PGF produced by pig blastocysts may contribute to the high levels of PGF in uterine flushings of pregnant compared to nonpregnant gilts. It is also possible that E_2 produced by the conceptus stimulates endometrial PGF production (Poyser, 1984).

Collectively, the aforementioned studies suggest that $PGF_{2\alpha}$ produced by the endometrium is responsible for luteolysis in the nonpregnant pig. In the pregnant pig, however, $PGF_{2\alpha}$ is secreted in an exocrine direction where PG from endometrium and conceptus origin is sequestered (Bazer et al., 1982; Bazer et al., 1984).

Another school of thought suggests that PGE_2 might be the signal for maternal recognition of pregnancy, but conflicting reports have been published so far. Schneider et al. (1983), demonstrated that intrauterine infusion of PGE_2 had no effect on interestrous interval in gilts. Akinlosotu et al. (1986), reported prolongation of estrous cycle length under their experimental conditions. Pseudopregnancy, however, was not maintained for more than 30 days. Recently, Laforest and King (1992) demonstrated that insertion of silastic beads containing either estradiol benzoate or PGE_2 into the uterine lumen of gilts slightly increased the length of the estrous cycle. No significant difference however, was shown between the treatment and control groups.

Prostaglandins may be critical for the establishment and maintenance of pregnancy. Prostaglandins exert their effect through

uterine blood flow, vascular permeability, fluid and electrolyte transport, cellular proliferation, steroid biosynthesis and immunoprotection of the conceptus (Bazer et al., 1982; Bazer et al., 1986).

Uterine Capacity and Conceptus Development

In the sow, 60 to 65% of the ovulated oocytes are represented by live pigs born at term. The major portion of the loss of potential pigs occurs by day 25 to 30 of gestation (Warthall, 1971; Biggers, 1969; Pomeroy, 1960). Attempts to increase the litter size of swine have met very little success. Use of superovulation (Longenecker and Day, 1968; Pope et al., 1968) or embryo transfer (Bazer et al., 1969 a,b) does not significantly increase litter size at term. Uterine capacity, which is defined as the ability of the uterus to support only a limited number of fetuses, has been suggested as a limit to the number of fetuses surviving (Dziuk, 1968; Bazer et al., 1969 a,b; Fenton et al., 1970; Webel and Dziuk, 1974). Those studies however, have shown that litter size is unrelated to uterine capacity before day 30 of gestation.

Brinkley et al. (1964) have shown that the remaining ovary of a unilaterally ovariectomized pig yields in approximately the same number of ovulations being produced as the total from both ovaries of an intact pig. To study the effect of quantity of uterus on uterine capacity of gilts, Fenton et al. (1970) compared the uterine capacity of unilaterally hysterectomized-ovarectomized (UHOX) to that of intact gilts. The results of their study suggested that the quantity of uterine tissue does not limit uterine capacity until after 25 days of gestation. The UHOX model has

been later justified by Christenson et al. (1987) as a method to induce intrauterine crowding. Knight et al. (1977), also using the UHOX model, reported decreased survival of fetuses in UHOX compared to intact pigs by day 40 of pregnancy. Wu et al. (1989), found that the restriction of embryos to 5 cm of initial uterine space caused fetal death as early as day 20, while increasing the initial uterine space to 30 cm caused fetal death by day 50. In a recent study, Vallet and Christenson (1993) reported a decrease in fetal survival between day 25 and day 35 of pregnancy in a crowded uterine environment. Wu et al. (1987) concluded that uterine length appears to be an important limiting factor to litter size when number of ovulations increases. A significant positive correlation was found between number of fetuses and uterine length. Also Wu et al. (1988) reported that a longer uterus had greater space per fetus, a larger number of live fetuses and a lower incidence of mummies where length of the uterine horns, litter size, and stage of gestation accounted for 12% of the incidence of mummies. Wu et al. (1988) also studied the influence of pig embryos on uterine growth. Results from their study showed that embryos or signals that they might give, apparently caused increases in length, weight and diameter of the uterine horn by day 18 but not before. The effect of initial length of uterus per embryo on fetal survival and development was studied by Wu et al. (1989). They concluded that each fetus requires at least 36 cm of initial uterine length to survive and develop. Recently, Chen and Dziuk (1993) reported that prenatal survival was highly correlated with initial assigned uterine space from 5 to 25 cm per CL (per potential embryo).

Prenatal survival, however, was not affected by uterine space when space was > 25 cm.

Knight et al. (1977) reported that all placental measurements were significantly greater in intact gilts compared to those from UHOX gilts. Observations from Knight et al. (1977) also indicated that intrauterine crowding and the associated decrease in endometrial surface area per fetus inhibited placental development that in turn resulted in decreased fetal development and survival. Dalton and Knight (1983) demonstrated that administration of a combination of P_4 and estrone (E_1) between days 20 and 30 of gestation augmented placental growth at day 50 gestation which may result in a reduction of secondary fetal death losses due to placental insufficiency. Vallet and Christenson (1993) found an alteration in four placental proteins when the uterine space per conceptus was reduced. The results of the same study also indicated that fetal growth occurs at a relatively faster rate than the placenta. This suggests that the disparity between fetal and placental growth (that increases under intrauterine crowded condition) will reach a point where the placenta can no longer support the development of the fetus.

Placental Development and Function

The term placenta was coined by Realdus Columbus (1516-1559). He described the placenta as an "affusion" of residual material in the form of a circular cake (placenta) in reference to the discoidal placenta of humans (Amoroso, 1952). Later, Mossman (1937) defined the placenta as "any intimate apposition or fusion of the fetal organs to the maternal

tissues for physiological exchange". Faber and Thornburg (1983) on the other hand, adopted a more restricted definition. They defined the human placenta as an organ that is formed by the fusion of maternally and embryologically derived tissue inside the uterus. Its main functions are: a) synthesis and secretion of hormones; b) the maintenance of an immunolgical barrier; and c) exchange of materials between the mother and the developing conceptus. The placenta is made of three fetal membranes, the chorion, allantois and the amnion. Development of fetal membranes in various species was first described early this century (Grosser 1909; Mossman, 1937; Patten, 1948; Amoroso, 1952). These descriptions remain valid. They recognized that the allantois develops as an extension from the hind gut of the embryo proper and extends almost the full length of the trophoblast with which it fuses to form the chorioallantois. In the pig, this process is completed by day 20 to 24 of gestation (Patten, 1948; Amoroso, 1952). As development proceeds, three zones develop on the chorionic surface: a) the placental zone that comprises the one-half to two-third central portion of the placenta and is characterized by the presence of numerous small folds on its surface; b) paraplacental zone peripheral to the placental zone which consists of the smooth chorionic surface; and c) the ischemic zone or necrotic tips where the tissues atrophy due to the occlusion of peripheral blood vessels (Brambel, 1933; Amoroso, 1952; Noden and De Lahunta, 1985)

Placentas are also classified according to shape. Pig placentas fall under the diffuse type where the apposition zones between the fetal and maternal tissues are distributed over most of the chorioallantois

(Amoroso, 1952; Noden and Du Lahunta, 1985). Furthermore, placentas are classified according to the type of attachment. As mentioned earlier, pig conceptuses have a noninvasive type of attachment. This is attributed to the epitheliochorial type of placentation by which the fetal membranes remain external to the uterine tissue and from which they can readily be separated from each other (Amoroso, 1952). Contact between the chorion and the uterine epithelium begins on day 13 of pregnancy (Crombie, 1970; Keys and King, 1990).

The folds of the placental zone interlock with corresponding endometrial folds (rugae). In apposition of the mouths of the uterine glands, the allantochorion is not attached to the endometrial epithelium but forms specialized structures known as areolae. Areolae first develop at about day 30 of pregnancy and are responsible for the absorption of uterine secretions before their transfer to the fetus (Brambel, 1933). The placental areolae appear initially in greatest concentration in the central portion of the placenta and then develop toward the polar portion so that by day 50 of gestation there is no significant difference in the number of areolae between the two regions (Brambel, 1933; Knight et al., 1977). No specific function for the areolae was assumed however, until Palludan et al. (1969) demonstrated that iron transfer is confined to this structure. As described earlier, the translocator of maternal iron to the fetal unit is the glycoprotein uteroferrin. Firth (1984) has shown that areolae are the main sites for the fetally directed active Na^+ transport of pig placenta.

Friess et al. (1981) studied the ultrastructure of the areolae and found that they display many morphological characteristics typical of

epithelia with high absorptive capacity, i.e., high columnar epithelium, long micro-villi, a well-developed apical tubular system, and numerous coated vesicles.

The epitheliochorial placenta has been considered to constitute the most complete morphological barrier because its six layers of tissue elements intervene between maternal and fetal blood streams (Grosser, 1909). Friess et al. (1980), however, demonstrated the presence of two different areas in the interareolar portion of the placenta. Those areas are characterized with different structure and function: a) the lateral and top sides of the chorionic ridges for exchange of gases; b) the base of the chorionic ridges where transport of blood borne nutrients takes place. This is facilitated by an intercellular channel system between the uterine epithelial cells.

Both amnion and allantoic sacs are filled with fluids that are considered evolutionarily as very important (Patten, 1948; Amoroso, 1952). It has been suggested that the allantoic and amniotic fluids are derived from fetal excretion, i.e. urine (Bremer, 1916; Davies 1952). McCane and Dickerson (1957), however, concluded that the allantoic fluid is derived from maternal origin. Their conclusion was supported by studies in sheep by Meschia (1955) and in pigs by Goldstein et al. (1980) who found that amniotic and allantoic fluid volumes decrease from about mid-gestation to term when the rate of fetal growth and metabolism is increasing.

Several studies in pigs (Choong and Raeside, 1974; Knight et al., 1977; Robertson et al., 1985), sheep (Carengie and Robertson, 1978) and

cows (Robertson and King, 1979) have shown that fetal fluids contain high concentrations of estrogens mainly E_1 and estrone sulfate(E_1SO_4). A peak of E_1SO_4 occurs in the pig around day 30 of gestation compared to day 45 in the ewe and day 135 in the cow (Robertson et al., 1985). As discussed earlier, placental insufficiency is one of the main reasons for fetal death. Allantoic fluid volume seems to play an important role in maximizing placental growth and expansion. Knight et al. (1977) found that a rapid increase in allantoic fluid concentrations of E_1 and E_2 between day 20 and 30 of gestation is associated with concurrent increase in allantoic fluid volume. This increase in allantoic fluid volume has been suggested to be involved in expanding the chorioallantoic membranes and thus enhancing fetal maternal interaction and consequently fetal development. Progesterone-estrogen treatments have been shown to alter allantoic fluid volumes in pigs (McGovern et al., 1981; Dalton and Knight, 1983) and sheep (Alexander and Williams, 1966, 1968).

Intrauterine Steroidogenesis

Sex steroids are classified into 3 major classes: progestins (C_{21}), estrogens (C_{18}) and androgens (C_{19}). Steroid production requires a very complex enzyme system (for review see, Solomon, 1988; Gore-Langton and Armstrong, 1988). Briefly, the C_{27} cholesterol is converted to the C_{21} product pregnenelone (P_5) through the enzymatic action of the cytochrome P-450 side chain cleavage located in the mitochondria. Pregnenelone is then either readily converted to progesterone (P_4) in the

presence of the 3β-hydroxysteroid dehydrogenase-isomerase or metabolized through the Δ^5 pathway to the androgen, dehydroepiandrosterone in the presence of 17α-hydroxylase. Progesterone in turn is converted to the androgen, androstenedione through the Δ^4 pathway in the presence of 17α-hydroxylase. Androstenedione can be converted to testosterone through the action of 17β-hydroxysteroid dehydrogenase. Androgens are converted to estrogens in the presence of the aromatase enzyme system.

In most mammals, the placenta is a major site for steroid biosynthesis during pregnancy. However, there are species differences in amounts and type of steroid produced (Gibori et al., 1988; Simpson and MacDonald, 1981). For example, in contrast to human and sheep placentas, rat and pig placentas secrete P_4 in quantities that are insufficient to sustain pregnancy in the absence of the ovaries (Casida and Warwick, 1945; du Mensil du Buison and Dauzier, 1957; Morrison et al., 1965; MacDonald and Matt, 1984). Gibori et al., (1979) found that androgen levels in maternal blood of pregnant rats increased dramatically from mid-pregnancy to term. This increase in circulating androgens was obliterated when the placentas were removed (Gibori and Sridaran, 1981). Subsequent investigations (Sridaran et al., 1983; Matt and MacDonald, 1984; Jackson and Albrecht, 1985; Warshaw et al., 1986) revealed that the rat placenta secretes principally androgen both in vitro and in vivo. Androgen biosynthesis, however, is limited to the fetal trophoblastic cells, which in primary culture accumulate a marked amount of androstenedione. Decidual (maternal) cells do not secrete

androgen (Sridaran, et al., 1983) nor do they express the message for the key rate-limiting enzyme (17α-hydroxylase) for androgen biosynthesis. The rat placenta acquires the capacity to secrete androgen only after day 11 of pregnancy (Sridaran et al., 1981; Matt and MacDonald 1984; Warshaw et al., 1986). The inability of placental cells to secrete androgen before day 11 of pregnancy is not due to the lack of the substrate (P_4) but rather to the absence of the enzyme 17α-hydroxylase. Recently, Durkee et al. (1992) reported that the trophoblastic tissue forming the rat placenta expresses the message for both cholesterol side chain cleavage enzyme and 17α-hydroxylase enzyme. They also showed that those enzymes are differentially expressed during the progression of pregnancy. The shift in androgen biosynthesis from the ovaries to the placenta at mid-pregnancy in the rat is accompanied by a decline in circulating levels of luteinizing hormone (Morshige et al., 1973; Taya and Greenwald, 1981). Warshaw et al.(1987) demonstrated a marked increase in placental androgen secretion through the prevention of decline in luteinizing hormone (LH) activity through the administration of human chorionic gonadotropin (hCG). Gibori et al. (1988) suggested that the effect of hCG on placental androgen biosynthesis is limited to the activity of the enzyme 17α-hydroxylase as shown for the luteal cells (Sridaran and Gibori, 1983; Khan et al. 1987). Durkee et al. (1992), however, reported that the expression of both cholesterol side chain cleavage enzyme and 17α-hydroxylase are down regulated by LH/hCG treatment.

The bovine placenta has been generally disregarded as a site of P_4 synthesis (Hoffman et al., 1979; Wendorf et al., 1983). Various studies however, have demonstrated that radiolabelled P_5 can be converted to P_4 by bovine placental tissues (Ainsworth and Ryan, 1967; Evans and Wagner, 1981; Shemesh et al., 1984; Reimers et al., 1985; Ullman and Reimers, 1989). Nevertheless, steroidogenic capability differs between the fetal component (cotyledon) and maternal component (caruncle) of the bovine placenta (Shemesh et al., 1984; Shalem et al., 1988; Shemesh et al., 1988). Recently, Izhar et al. (1992) reported that P_4 synthesis by bovine caruncular cells was low or undetectable in the first trimester but increased more than 10-fold in the second trimester of gestation. They suggested that the low P_4 levels synthesized by the caruncles was due to post-translational regulation of the enzyme complex. Relative to regulation of placental steroidogenesis in the bovine, Shemesh and his group (1988, 1989) have shown that P_4 biosynthesis is cyclic nucleotide (cAMP) independent, but Ca^{2+} dependent. It appears that placental steroidogenesis in the cow is activated by Ca^{2+} mobilization and(or) activation of protein kinase C. Both compounds act on the cholesterol side chain cleavage enzyme complex to increase P_5 production from cholesterol.

As previously mentioned, fundamental differences exist among species in the ability of the placenta to synthesize and metabolize steroids. In the ovine, beginning as early as day 50 of gestation the placenta is able to secrete enough P_4 to maintain pregnancy (Casida and Warwick, 1945). Rickets and Flint (1980) have reported an increase in P_4

synthesis by the ovine placenta after day 50 to plateau between day 65 and 95, and increase again after day 100. Results from Beal et al. (1986) and Power and Challis (1987) confirm the aforementioned report. Wango et al. (1990) demonstrated that sheep placental binucleate cells isolated between days 110 and 135 of gestation were almost as effective as isolated luteal cells in converting P_5 to P_4. Early studies (Ainsworth and Ryan, 1966; Pierrepoint et al., 1979) have shown that sheep placental tissues can form estrogens from radiolabelled androgen precursors. The mechanism that regulates placental steroidogenesis in the ovine is not well known. Koligian and Stormshak (1976) reported that LH supplementation caused an increase in P_4 output by day 55 sheep placental tissue suggesting that LH controls placental steroidogenesis in sheep. Beal et al. (1986) however, reported no increase in P_4 synthesis by day 80-115 placental tissues when exogenous hCG was supplemented in vivo or in vitro.

Removal of the ovarian source of P_4 at any stage of pregnancy in pigs results in the termination of pregnancy. This finding led to the inference that the porcine placenta is incapable of P_4 production. Early histochemical studies (Christie, 1968; Dufour and Raeside, 1969), however, have demonstrated that the porcine placenta has the enzyme system for P_4 production from the fourth week of gestation onward. Northern and Western blot analyses (Conley et al., 1991) have shown very low expression of the side chain cleavage and the 3β-hydroxysteroid dehydrogenase enzymes by the pig placenta, as well as the relative lack of the 17α-hydroxylase enzyme at different stages of gestation. Knight

and Kukoly (1990) reported that placental tissues collected throughout gestation can release large amounts of P_4 in vitro. Furthermore, they reported a biphasic pattern of estrone release that mimicked the pattern observed in maternal plasma. Fisher et al. (1985) found that chorion tissues collected between day 16 and 25 of gestation are active in estrone and estradiol production. In an experiment to study the activity of the enzymes aromatase, sulfotransferase and sulfatase in the pig placenta at 3 stages of gestation, Knight and Hopkins (1989) showed no change in aromatase and sulfotransferase activity but a decrease in sulfatase activity late in gestation. Faillace (1989) reported that ovarectomized gilts, injected daily with a non-aromatizble form of progestagen (medroxyprogesterone acetate) had adequate production of intrauterine estrogens to maintain pregnancy. Faillace (1989) also reported that the placenta of ovarectomized gilts can produce sufficient P_4 to maintain pregnancy and estrogens to support conceptus development if an adequate quantity of P_5 is available as a precursor. In another experiment, Knight and Jeantet (1991), found that the mechanism controlling placental steroidogenesis in pigs (as in ovine) does not involve Lh/hCG stimulation. However, cAMP supplementation to placental tissue enhanced P_4 release in vitro which indicates that cAMP is an effective second messenger.

In an early study, Knight et al. (1977) found a positive uterine arterial-venous difference in concentrations of P_4 in plasma of pregnant pigs indicating an uptake of P_4 by the pregnant uterus. Those results indicate that P_4 is a potential substrate for enzymes in the gravid uterus

(conceptus and/or endometrium) for metabolism to androgens and strogens. In an experiment to study the metabolism of tritiated P_4 to other metabolites, Dueben et al. (1979) found that endometrial tissues from day 14 to 18 of gestation are able to convert P_4 to estrogens in vitro. Later, Fisher et al. (1985) compared the metabolism of tritiated P_4 in placental and endometrial tissues collected between days 16 and 25 of gestation. Endometrial tissues were less active than those of the conceptus in converting P_4 to estrogens. In the same study, they found that endometrial tissues from pseuopregnant gilts were not able to metabolize P_4 to estrogens. Knight et al. (1988) also found that in the absence of prior exposure to feto-placental units, the porcine endometrium is severely restricted in its ability to synthesize P_4 in vitro. Knight and Hopkins (1989) reported that the aromatase activity of the endometrium remained stable at days 30, 60, and 90 of gestation. In a later study, Knight and Jeantet (1991), found that P_5 supplementation to endometrial tissue in vitro enhanced P_4 and E_1 production but to a much lesser extent than the placenta.

Sexual Differentiation and The Intrauterine Position Effect

Sexual differentiation in mammals is viewed by Jost (1972) as a sequential process that begins at fertilization with the formation of either XX (female) or XY (male) karyotype. Initially, male and female embryos develop in an identical fashion leading to the differentiation of the gonads into ovaries or testes (George and Wilson, 1988). It was found that in the absence of the gonads, regardless of the genetic sex of the fetus, the

genital tract becomes female. This implies that a testicular product is the "sex differeniator" of the genital tract. Three hormones, Mullerian Inhibiting Hormone (MIH), testosterone and dihydrotestosterone, are responsible for the formation of the male phenotype (Jost, 1972; Wilson et al., 1981; Bardin and Catterall, 1981; George and Wilson, 1988). H-Y antigen, which was discovered by Eichwald and Silmser (1955), was mistakenly proposed to induce testis formation (Ohno et al., 1979). Recently however, a gene, the sex determining region of Y (SRY), has been identified (Gubbay et al., 1990; Koopman et al., 1990, 1991), and the presence of a product of this gene called TDY (testis determining factor from the Y-chromosome) has now been shown to be essential for induction of testis formation.

Several studies have shown sex differences in various morphological variables regarding regions in the central nervous system that control reproductive processes (Gorski, 1985; Kolata, 1979; Raisman and Field, 1971; Toran-Allerand, 1976). Moreover, Sakuma and Pfaff (1981) have shown sex differences in the pattern of connectivity between various limbic regions that are related to reproductive functions. The role of sex steroids in directing or organizing brain sexual dimorphism has been well established (Beyer and Feder, 1987; Christensen and Gorski; 1978; Ellendorf et al., 1979; MacLusky and Naftolin, 1981). Several observations indicate that there is a period early in life which is species dependent (prenatal or neonatal), termed the critical period, when the neural regions that control reproduction are maximally susceptible to steroids (Erhardt and Meyer-Bahlburg, 1981;

Kolata, 1979; MacLusky and Naftolin, 1981). Testosterone treatment during the critical period prevents the occurrence of regular ovulatory cycles in rats (Barraclough, 1961), guinea pigs (Brown-Grant and Sherwood, 1971), hamsters (Paup et al., 1972), and sheep (Short, 1974). Prenatal androgen treatment masculinized the external genitalia of female hamsters (Nucci and Beach, 1971). Exposure to exogenous testosterone between days 40 to 50 of fetal life caused masculinization of female external genitalia in sheep (Clarke et al., 1976). The brain centers that control estrus and ovulation however, are not affected until some time between days 70 to 80 of gestation (Clarke et al., 1977). The pig fetus seems to be well protected against the masculinizing effect of testosterone administered to the mother. It required three treatments (2 days interval) of 25 mg testosterone propionate/kg body wt. given to day 30 pregnant sows in order to produce masculinization of female fetuses (Elsasser and Parvizi, 1979). This observation might be explained by the insufficient amount of testosterone that the fetus received. A study in guinea-pigs (Vreeburg et al., 1981) showed that after a 2 hour infusion of tritiated testosterone into 36-day pregnant mothers, tritiated androstenedione and testosterone could hardly be detected in the fetal plasma. In another study, Despres et al. (1984) showed that under physiological conditions, only a minute portion of maternal testosterone enters the fetus via the guinea-pig placenta. Elsasser and Parvizi (1979) demonstrated that the stimulatory feedback mechanism could be affected by testosterone treatment when given between days 30 to 70 of fetal life, suggesting that sexual differentiation in pigs takes place around

that period of time. In the rhesus monkey however, exposure to exogenous testosterone in utero did not affect the function of the stimulatory feedback mechanism (Steiner et al., 1976).

It has been well documented that estrogen plays an important role in mediating the sexual differentiation effect of testosterone. Christensen and Gorski (1978), showed that testosterone or estradiol when implanted in rats into specific loci at a specific time caused similar effects in terms of masculinization of gonadotropin release and behavior. Studies with rats (McDonald and Doughty, 1972) and hamsters (Etgen and Whalen, 1979) have shown that the effect of testosterone treatment can be blocked by the administration of estrogen antagonist. Also, Naftolin et al., (1975) have shown that the developing brain itself has a high aromatase activity that converts androgens to estrogens. The question now would be: How is the fetal female protected against masculinization? In rats and mice, the mechanism by which this protection is achieved is well established. The developing yolk sac and fetal liver synthesize estrogen binding proteins (feto-neonatal estrogen binding protein and α-fetoprotein) which effectively sequester much of the estrogen present in the fetal and neonatal circulations (Plapinger et al., 1973; Plapinger and McEwen 1975; Raynaud et al., 1971). The situation in humans seems to be different where only a small proportion of circulating α-fetoprotein binds estrogen (Uriel et al., 1975). Plapinger and his co-workers (1977) failed to demonstrate the existence of a fetal protein in guinea pigs similar to that of the fetal rat.

Fetal testicular androgen is critical for male differentiation of external and internal genitalia and the central nervous system. In pigs the critical period of sexual differentiation (days 40 to 60) is simultaneous with maximal testicular testosterone secretion (Colenbrander et al., 1978). Ford et al. (1980) found greater plasma testosterone concentrations in the umbilical artery of male fetuses than those obtained from female fetuses at all stages sampled from day 30 to day 112 indicating active testosterone secretion from the fetal testis of the pig. Raeside et al. (1991) reported that the fetal porcine testis is capable of aromatizing testosterone to estrogen at the different stages examined (day 32 to 114). In hamsters, Vomachka and Lisk, (1986) also found that plasma levels of androgen are higher in male than female fetuses on day 14 to 15 of gestation. A number of studies have looked at the influence of prenatal exposure to progesterone on later behavior. The data from those studies support the hypothesis that progesterone can act as a testosterone antagonist. Concomitant treatment of testosterone propionate (TP) and progesterone to adult female guinea pigs prevented the expected masculinization behavior that is usually caused by TP itself (Diamond and Young, 1963). Comparable inhibitory effects of progesterone on male behavior have been observed in rabbits (Ericson et al., 1964) mice (Erpino and Chappelle, 1971). Neonatal treatment of rats with progesterone inhibited the masculine sexual behavior of treated animals compared to controls (Cagnoni et al., 1965; Kincl et al., 1965; Diamond et al., 1973). More fetal progesterone has been reported in developing female rhesus monkeys compared to developing males

(Hagemenas and Kittinger, 1972; Resko, 1975). Resko believes that the protection effect of progesterone against testosterone applies to monkeys too.

A naturally occurring cause of phenotypic variation in females of litter bearing mammals is the in utero proximity of a female/male fetus to fetuses of the same or opposite sex. This important source of intrasexual variation within the same litter was revealed when Clemens et al. (1978) found that the anogenital distance (measure for morphological masculinization) of newborn female rats was greater when they had been located in the uterus between two males rather than between two females. This phenomenon was referred to later as the intrauterine position (vom Saal, 1981). This phenomenon apparently is not due to the position itself but rather to the interamniotic diffusion of steroid hormones between adjacent fetuses and the variation in the hormonal environment relative to the proximity of an individual fetus to the members of the same or opposite sex (Clemens et al., 1974; Tobet et al., 1982; vom Saal, 1981). The intrauterine position of a female mouse fetus has been correlated with concentrations of testosterone in amniotic fluid and fetal plasma on day 17 of pregnancy. Specifically, females located between 2 males (2M) have greater concentrations of testosterone than do female fetuses not located next to a male fetus (0M, vom Saal and Bronson, 1980). vom Saal et al. (1983) reported that in mice, higher concentrations of estradiol in the amniotic fluid of males developing between two females (2F) compared with males developing between two males (0F). As adults, 2F males had smaller seminal vesicles, were more

sexually active but were less aggressive than 0F males. Clemens et al. (1978) also reported that female rats located near a male in utero showed increased frequencies of male-like behavior as adults and virilization of genital morphology. A study by Kinsely et al. (1986) has shown that female mice located in utero between 2 females exhibited higher levels of locomotor activity in adulthood than did females located between two males. Rines and vom Saal (1984) reported that the sensitivity to testosterone treatment effect on behavior increases during aging in 0M females . Recently, Nonneman et al. (1992) reported that 5α-reductase activity (5α-reductase converts testosterone to dihydrotestosterone) was 60% greater in both seminal vesicles and prostate of male mice located between two males than in males located between two females.

Based on evidence to date, intrauterine position appears to have less effect on reproductive traits in adult swine compared to mice. Rhode Parfet et al. (1990) reported that intrauterine position affected growth of 0F males in a limit fed situation, apparently due to greater aggressiveness, but had no effect on postnatal growth rate in swine fed ad libitum. In the same study and based upon single semen samples collected at 220 days of age, semen characteristics were not markedly different between 0F and 2F males. Recently, Wise and Christenson (1992), reported no intrauterine position effect in swine on placental weight or fetal testosterone concentration.

The intrauterine position hypothesis has been challenged by another school of thought. Meisel and Ward (1981) ascribed the hormonal influence in utero to the circulation of blood within the uterus

rather than to an interamniotic diffusion. This hypothesis was based on the observation that among female rats that had developed in utero next to one male, the anogenital distance was found to be greater in those females with the male located caudally than those with the male located cephalically. The uterine blood flow in rats is unidirectional from the cervical to the ovarian end of each horn (Del Campo and Ginther, 1972), suggesting that steroid hormones secreted by fetal males may be carried by uterine vasculature to female fetuses located farther downstream. Further evidence to support this hypothesis in rats has been reported by Richmond and Sachs (1984). Vomachka and Lisk (1986) reported that in hamsters female siblings that are located caudally suppress amniotic fluid androgen and enhance estradiol concentrations of male siblings at day 14 of gestation. Gandelman (1986) concluded that both intrauterine position and the presence of a male fetus in the caudal portion of the uterus are necessary conditions for prenatal masculinization of the guinea pig.

SUMMARY

Limited uterine space and placental insufficiency are main reasons for fetal death in swine. It has been firmly established that the porcine placenta produces a changing milieu of steroid hormones during gestation and that, through local control of various intrauterine events, these steroids are key regulators of conceptus development and prenatal

survival. The effect of limited uterine space on placental steroidogenesis is not established.

Steroids clearly play a crucial role in sexual differentiation. Studies with rodents have indicated that a naturally occurring cause of intrasexual phenotypic variation within the same litter is the intrauterine proximity of a female/male fetus to fetuses of the same or opposite sex. This phenomenon is referred to as intrauterine position.Evidence to date suggests that intrauterine position has less of an effect on reproductive traits in swine compared to rodents. However, in utero effects of intrauterine position have yet to be estabished.

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CHAPTER III

STATEMENT OF THE PROBLEM

Numerous lines of evidence suggest that intrauterine crowding is a major reason for prenatal, and to a certain extent some postnatal death losses in swine. There is a mounting evidence that steroid hormones produced and metabolized by the feto-placental unit and the maternal endometrium play an essential role in this process. In order to alleviate the problem of prenatal death in pigs, a better understanding of placental and endometrial steroidogenic activities throughout gestation and their effect on conceptus development under normal and intrauterine crowded conditions is essential. Interactions that may exist between the sex of the fetus and intrauterine position during development under both normal and intrauterine crowded conditions also need to be established.

The following studies were conducted in the Reproductive Physiology laboratory of the Animal and Poultry Sciences Department at Virginia Polytechnic Institute and State University.

Chapter IV

THE EFFECT OF UTERINE SPACE ON CONCEPTUS DEVELOPMENT AND *IN VITRO* RELEASE OF PROGESTERONE AND ESTRONE BY REGIONS OF THE PORCINE PLACENTA THROUGHOUT GESTATION

ABSTRACT

A total of 36 intact and unilaterally hysterectomized-ovariectomized (UHOX) gilts were used to study conceptus development and steroidogenic capability of three regions of the porcine placenta under normal and crowded intrauterine conditions. Gilts were hysterectomized on either d 30, 50, 70, or 90 of gestation. Placentas were combined according to the sex of the fetus associated with the placental unit (except at d 30 when all were combined). Placentas were cut into three regions: middle, inner, and polar. Placental tissues were incubated, and release of progesterone (P_4) and estrone (E_1) into the incubation medium was determined. Fetal survival rate was greater ($P< .01$, .05, and .001 at d 50, 70, and 90, respectively) from intact versus UHOX gilts. Placental length and weight, fetal length and weight, and allantoic fluid volume were greater in intact compared to UHOX gilts. Less P_4 was present in the medium where the polar region was incubated compared to the other regions at d 50, 70, and 90. Uterine status affected ($P< .005$) P_4 release only at d 90. Sex of the fetus did not affect placental P_4 release. Region of the placenta affected E_1 release at d 30 ($P< .01$) and d 50 ($P< .06$). There was no uterine status effect on E_1

release. Sex of the fetus affected ($P < .001$) E_1 release only at d 50. Collectively, the results from this experiment indicated a) a differential release of P_4 and E_1 by the different regions of the placenta at certain days of gestation, and b) no compensatory increase in steroidogenic activity of the pig placenta when the total placental mass was reduced.

Key Words: Pigs, Placenta, Steroids, Conceptus

INTRODUCTION

It is well established that uterine capacity is a major factor limiting litter size in swine (Dziuk, 1968; Longenecker and Day; 1968; Fenton et al.; 1970, Webel and Dziuk; 1974; Wu et al., 1987; Wu et al., 1989). Knight et al. (1977), using unilaterally hysterectomized-ovariectomized gilts (UHOX), a treatment that induces intrauterine crowding, reported a decrease in fetal survival and fetal growth in UHOX compared to intact gilts after d 35 of gestation. Measurements of placental size were significantly smaller in UHOX compared to intact gilts at all stages of gestation studied (d 20 to 100). Their report suggested that placental insufficiency was the primary cause of increased prenatal mortality and decreased fetal growth in UHOX gilts.

Previous reports (Brambel, 1933; Knight et al., 1977) indicated that placental areolae, sites of macromolecular nutrient transport, appear initially in greatest concentration in the central section of the placenta around d 30 of gestation. Areolae then develop toward the polar sections so that by d 50 no significant difference in numerical distribution exists among the different sections of the placenta. Knight et al. (1977), however, reported that the number of areolae was significantly less in placentas collected from UHOX gilts compared to those collected from intact gilts at all days of gestation studied (d 35 to 100). Dalton and Knight (1983) demonstrated that exogenous application of progesterone (P_4) and estrone (E_1) had beneficial effects on areolae formation.

Previous studies from our laboratory (Knight and Kukoly, 1990; Knight and Jeantet, 1991) have demonstrated that the porcine placenta is capable of producing P_4 and E_1 throughout gestation. Placental estrogens play an important role in placental expansion (Knight et al., 1977), water and electrolyte movement (Goldstein et al, 1980), cell permeability (Szego and Sloan, 1961), and uterine blood flow (Ford and Christenson, 1979). However, little information exists concerning the effect of uterine space on placental steroidogenesis and its relationship to conceptus development.

The present study was designed to investigate the steroidogenic activity of three regions of the pig placenta throughout gestation. Specifically, we investigated possible differences in placental P_4 and E_1 release associated with the region of the placenta and sex of the fetus under normal (i.e., uncrowded) and intrauterine crowded conditions on d 30, 50, 70, and 90 of gestation.

MATERIALS and METHODS

Animals

Thirty-six gilts of similar size, age, and genetic background were used for this experiment. Nineteen gilts were unilaterally hysterectomized-ovariectomized between days 7 to 11 of their second estrous cycle. The UHOX model was utilized to create intrauterine crowding. The justification for this model is reviewed by Christenson et al. (1987). The uteri in the remaining 17 gilts were left intact. All gilts

were bred at their third detected estrus and assigned randomly for hysterectomy (HYSTX) at either d 30, 50, 70, or 90 of gestation.

Sample Collection

Surgical anesthesia was induced with a 5% solution of sodium thiopental and maintained with halothane utilizing O_2 and NO_2 as carriers. The reproductive tract was exposed via midventral laparotomy. The reproductive tract was then removed and processed as described by Knight et al. (1977). The corpora lutea (CL) were counted. The uterus was dissected free of connective tissue and cut along the mesometrial border. Each conceptus was exposed intact. After the aspiration of uncontaminated samples of allantoic and amniotic fluids, the chorioallantois and the amnion were then cut, the respective fluids were collected, and the fluid volumes were measured. Fetal fluid samples were stored at -20° C for subsequent steroid hormone analyses. Measurements of conceptus development (placental length and weight, fetal crown-rump length, and fetal weight) were taken following HYSTX. Placentas were combined according to the sex of the fetus associated with the placental unit (except at d 30 when sex could not be visually determined), and all placentas were divided into three regions. The portion of the placenta overlying the fetus was identified as the "middle" region. The remainder of the placenta was divided equally and identified as "interior", i.e., the part adjacent to the middle region and "polar", i.e., the portion adjacent to the necrotic tips. All tissues were kept In Medium 199 (M199, Gibco Laboratories, Grand Island, NY)

supplemented with 10 ml of Antibiotic-Antimycotic Solution (Gibco) at 37 ° C until prepared for incubation.

Tissue Incubation

Incubations were performed as described by Knight and Kukoly (1990) with some modifications. Briefly, the allantochorionic portion of the placenta was separated from the amnion, minced into 2 mm^3 pieces using razor blades, and kept in M199. Wet samples were collected from the three regions randomly, and duplicate 300 mg samples were incubated in tubes containing 3 ml of M199 in a Dubnoff shaker at 37° C in an atmosphere of 95% air and 5% CO_2 for either 0, 1.5, or 3.0 h. Samples were transferred to an ice bath at the end of each incubation period. The samples were centrifuged, and the medium was decanted and stored at -20° C for subsequent P_4 and E_1 analyses. The concentration of P_4 or E_1 present in the media will be referred to as the concentration of hormone released. We should acknowledge, however, that the method of incubation used in this study does not differentiate between the release of hormone present at the initiation of incubation and hormone produced by *de novo* synthesis and released during incubation. Tissue precipitates were also stored at -20° C for subsequent protein and DNA analyses. The duplicate tissue and media samples were combined for the subsequent assays. Initial analyses revealed no significant difference in the concentration of P_4 and E_1 present after 1.5, or 3.0 h incubation periods. Therefore, only the amounts of E_1 or P_4 present during the 1.5 h incubation period are reported. Least squares

means of P_4 and E_1 concentration released after 0 and 3.0 h of incubation are presented in Appendix Figures 1-4.

Tissue protein and DNA Determination

Tissue protein and DNA contents were determined to correct for possible sampling and weighing errors. Four milliliters of .9% saline were added to the tissue samples after thawing and the samples were sonicated with 2 10-s pulses using a Virtis sonicator (The Virtis Company, Gardiner, NY). Protein was determined using the bicinchoninic acid assay as described by Smith et al. (1985). Commercial assay standards and kits (Pierce Chemical Company, Rockford, IL) were used. Duplicate 10 µl samples of the homogenate in a microtiter plate were used, and protein was determined by measuring the absorbance at 540 nm using an ELISA plate reader (Titertek, Multiskan MCC/340, ICN Biomedicals, Huntsville, AL).

The DNA was determined using a dye-binding assay as described by Labarca and Paigen (1980). Duplicate 100 µl samples of the homogenate were assayed. Samples were diluted with 1,800 µl of phosphate-buffered saline solution, and 100 µl of working dye solution (Hoechst 33258, Sigma Chemical Co., St. Louis, Mo) was then added. Samples were vortexed for 1 min and fluorescence was measured with a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Samples were compared with calf thymus DNA (Sigma Chemical Co., St. Louis Mo) that was used as a standard.

Assays for Progesterone and Estrone

Progesterone concentrations in the media were quantified using a solid-phase radioimmunoassay kit (Diagnostics Products Corp., Los Angeles, CA). Estrone was determined using a double extraction procedure described by Hattersley et al. (1980) with a slight modification. Duplicate 200 µl samples were double extracted using 2 ml of diethyl ether at each extraction. A specific antibody (generously donated by R. B. Staigmiller, Miles City, MT) was used in the determination of E_1 (Appendix Table 1). Inter- and intra- assay coefficients of variation were as follows: progesterone, 8.5 and 7.2% and estrone, 15.2 and 11.6%, respectively.

Statistical Analyses

The data were analyzed using the General Linear Models procedures of SAS (SAS, 1988). Number of CL, number of live or dead fetuses and percent fetal survival were analyzed separately by day of gestation using one-way analysis of variance with treatment (Intact vs UHOX) as the main effect.

Conceptus variables (placental length and weight, fetal length and weight, and fetal fluid volumes) were analyzed with day of gestation and treatment as main effects. Gilt nested within day of gestation and treatment was used as the error term for the main effects and their interaction.

Analyses for the regression of P_4 and E_1 concentrations on tissue protein or DNA content did not reveal significant relationships of

hormone concentrations and protein or DNA contents. Differences in P_4 and E_1 in the media were analyzed separately by day of gestation with treatment, gilt within treatment, region of placenta, and fetal sex as main effects and also included the interactions among the main effects. Gilt within treatment was used as the error term for treatment effect. All other effects were tested using the residual mean square. Day 30 data were analyzed without sex included in the model.

When F-tests from analyses of variance were significant, means for day of gestation and region of the placenta were compared using Duncan's multiple range comparison.

RESULTS

Litter Variables

The data for litter variables are summarized in Table1. Mean number of ovulations was not significantly different between intact and UHOX gilts at any day of gestation studied except for d 90 where it was greater ($P< .01$) in intact compared to UHOX gilts. Mean number of live fetuses was greater ($P< .05$, .01, and .001 at d 50, 70 and 90, respectively) in intact versus UHOX gilts. No significant uterine status effect was detected for total number of dead fetuses. Survival rate (number of live fetuses/number of CL) was greater ($P< .01$, .05, and .001 at days 50, 70, and 90 of gestation, respectively) in intact compared to UHOX gilts. No treatment effect on survival rate was detected on d 30 of gestation.

Conceptus Variables

The data for the conceptus variables are presented in Table 2. As expected, day of gestation affected (P< .001) all conceptus variables measured. Placental length and weight and amniotic fluid volume changed modestly after d 70 of gestation, while fetal length and weight continued to increase throughout gestation. A reduction in allantoic fluid volume was detected on d 90 of gestation. Placental length and weight, fetal length and weight, and allantoic fluid volume were greater (P< .05, and .001, .05, .001, respectively) in intact compared to UHOX gilts. No effect of status on amniotic fluid volume was detected. The interaction of day of gestation with uterine status was not significant for any of the conceptus variables except for fetal length (P< .05). The data for uterine horn length, number of fetuses per horn, and uterine space per fetus are presented in Appendix Table 2.

Table 1. Effect of uterine status on litter variables at days 30, 50, 70, and 90 of gestation[1]

	Day of Gestation							
	30		50		70		90	
	Intact	UHOX	Intact	UHOX	Intact	UHOX	Intact	UHOX
No. of gilts	4	4	5	5	4	5	4	5
No. of CL	14.0[a] (.6)	13.7[a] (.6)	13.2[a] (.9)	15.0[a] (.9)	16.0[a] (1.1)	13.0[a] (1.1)	14.7[a] (.5)	12.2[b] (.5)
Live fetuses	12.2[a] (1.1)	9.0[a] (1.1)	10.6[a] (1.0)	7.0[b] (1.0)	11.3[a] (1.2)	5.4[b] (1.0)	11.7[a] (.4)	5.2[b] (.4)
Dead fetuses	1.5[a](.4)	1.3[a] (.4)	0.8[a] (.5)	2.0[a] (.5)	1.25[a] (.8)	2.0[a] (.7)	0.8[a] (.5)	1.6[a] (.5)
Survival rate (%)	87.7[a](7.5)	65.2[a] (7.5)	80.3[a] (7.6)	48.0[b] (7.6)	73.0[a] (11.0)	42.7[b] (10.2)	79.6[a] (4.0)	43.4[b] (3.8)

[1]Litter variables are expressed as least square means; standard error of the mean is in parentheses.
[a,b] Means in each row at each stage of gestation with different superscripts differ (P< .05).

Table 2. Effect of uterine status on conceptus variables at days 30, 50, 70, and 90 of gestation[1]

	Day of Gestation[2]							
	30		50		70		90	
	Intact	UHOX	Intact	UHOX	Intact	UHOX	Intact	UHOX
No. of conceptuses	49	36	53	35	45	27	47	26
Placental length[3], cm	55.0 (2.2)	48.8 (2.8)	72.8 (2.2)	60.1 (2.8)	78.8 (2.4)	83.6 (3.5)	83.0 (2.3)	67.1 (3.1)
Placental wt[3], g	32.6 (6.7)	23.5 (8.3)	142.0 (6.6)	122.6 (8.2)	216.2 (7.0)	219.7 (10.5)	212.7 (6.9)	172.4 (9.3)
Fetal length[3], cm	2.9 (.2)	2.5 (.3)	9.5 (.2)	8.8 (.3)	16.8 (.2)	15.7 (.3)	22.9 (.2)	19.6 (.3)
Fetal wt[3], g	1.8 (7.4)	1.6 (9.1)	50.4 (7.3)	42.8 (9.1)	236.0 (7.8)	215.0 (11.0)	623.1 (7.5)	476.3 (10.3)
Allantoic fluid volume[3], ml	230.9 (17.2)	171.6 (21.2)	258.9 (17.0)	185.7 (21.3)	239.4 (18.0)	137.5 (26.8)	77.3 (17.5)	45.1 (23.8)
Amniotic fluid volume, ml	1.5 (6.5)	1.7 (8.1)	48.9 (6.4)	56.2 (8.0)	171.5 (6.8)	187.9 (10.2)	166.4 (6.7)	180.7 (9.1)

[1]Conceptus variables are expressed as least square means; standard error of the mean is in parentheses.
[2]Conceptus variables differed with day of gestation (P< .001).
[3]Placental length, placental weight, fetal length, fetal weight, and allantoic fluid volume differ (P< .05) as function of uterine status.

Placental Progesterone Production

Least squares mean concentrations of P_4 released into the incubation medium following 1.5 h of incubation of the three regions of the placenta are presented in Figure 1. Neither uterine status nor region of placenta affected P_4 release at d 30 of gestation. The interaction of uterine status and region of the placenta, however, affected ($P< .05$) P_4 release. Region of the placenta influenced P_4 release ($P< .001$, .002, and .001 at d 50, 70, and 90 of gestation, respectively). The polar region released less P_4 than the middle and inner regions. Sex of the fetus did not affect placental release of P_4 at any of the days of gestation. Uterine status did not affect P_4 release except at d 90 ($P< .005$), when more P_4 was released from placentas of intact compared to UHOX gilts.

Placental Estrone Production

Least squares means of E_1 concentration released into the incubation medium after 1.5 h of incubation of the three regions of the placenta are presented in Figure 2. Region of placenta influenced placental E_1 release at d 30 ($P< .01$) and 50 ($P< .06$) of gestation. More E_1 was released by the polar region compared to the middle region, but no difference was detected between polar and inner regions or between inner and middle regions at d 30 of gestation. In contrast, at d 50 of gestation more E_1 was released by the middle region compared to the amount released by the polar region, but no difference was detected between the middle and inner regions or between the inner and polar regions. No effect of uterine space on E_1 release was detected at any day

of gestation. Sex of the fetus was not a significant effect at d 70 and 90, but it had an effect ($P < .001$) at d 50 of gestation when more E_1 was released by placentas of male fetuses compared to the amount released by placentas of female fetuses.

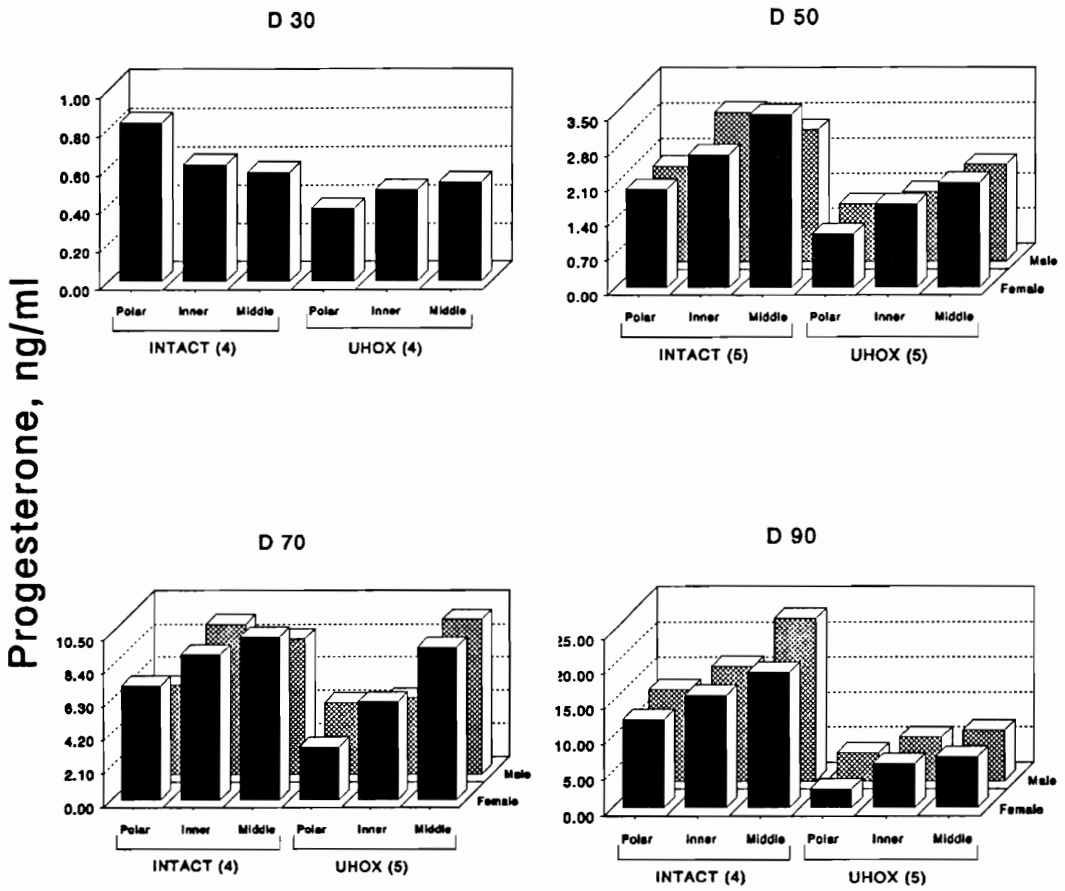

Figure 1. Progesterone release (ng/ml) after 1.5 h of incubation of three regions (polar, inner, middle) of placental tissue from male or female fetuses collected from either intact or unilaterally hysterectomized-ovariectomized gilts (n) at d 30, 50, 70, or 90 of gestation. (Sex cannot be visually determined at d 30). Progesterone release differed as a function of a) region ($P< .01$) at d 50, 70, and 90 and b) uterine status ($P< .005$) at d 90. Error mean squares are .019, .41, 11.33, and 11.73 for d 30, 50, 70, and 90, respectively.

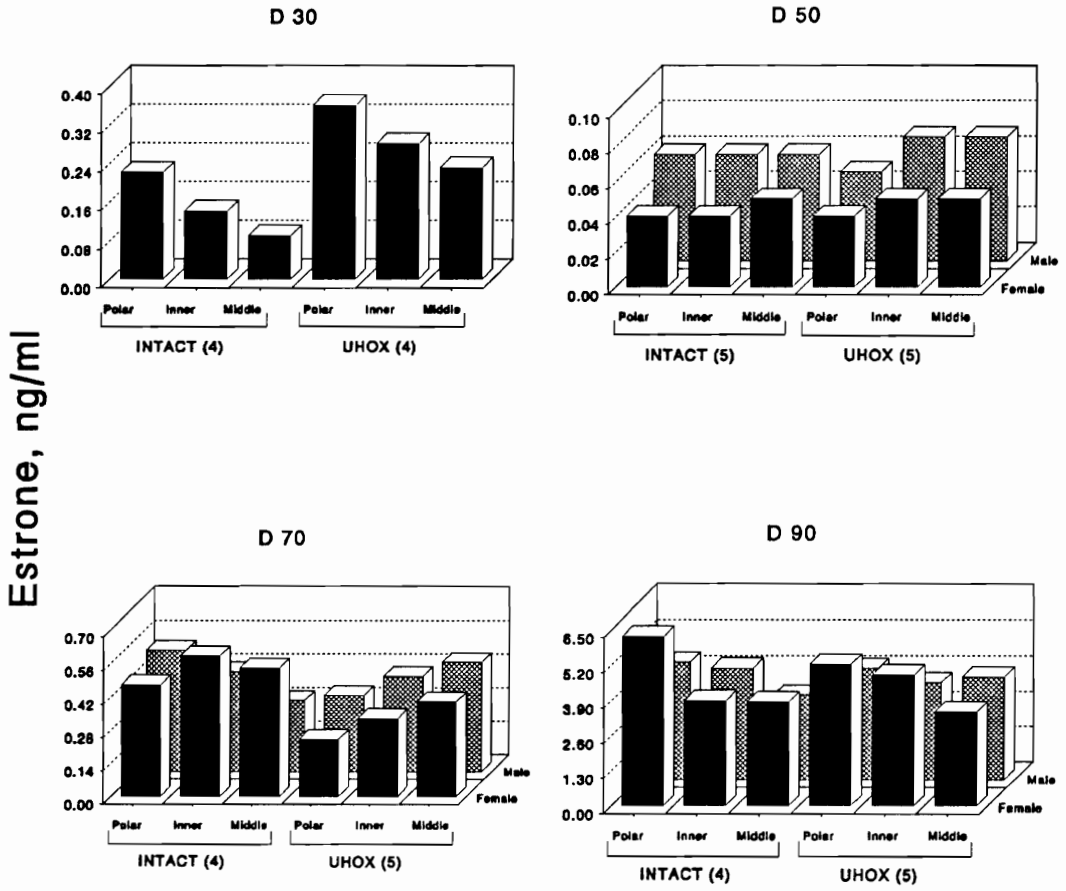

Figure 2. Estrone release (ng/ml) after 1.5 h of incubation of three regions (polar, inner, middle) of placental tissue from male or female fetuses collected from either intact or unilaterally hysterectomized-ovariectomized gilts (n) at d 30, 50, 70, or 90 of gestation.(Sex cannot be visually determined at d 30). Estrone release changed as a function of a) region at d 30 ($P < .01$) and 50 ($P < .06$) and b) sex ($P < .001$) at d 50. Error mean squares are .006, .0001, .043, and 3.65 for d30, 50, 70, and 90, respectively.

DISCUSSION

In this study, uterine space affected survival rate after d 30 of gestation. This is in agreement with the results of Knight et al. (1977) who used the UHOX model to induce intrauterine crowding. Wu et al. (1989), employing a different model to induce crowding, reported that minor crowding of conceptuses caused fetal death by d 50 of gestation, while severe crowding caused fetal death as early as d 25 of gestation.

Furthermore, results from this experiment indicate that all conceptus variables except amniotic fluid were greater in intact compared to UHOX gilts. Knight et al. (1977) reported a significant uterine space effect on all conceptus variables including amniotic fluid. The amniotic fluid has been shown to be less dynamic than allantoic fluid (Bazer et al., 1979). In agreement with previous reports (Pomeroy, 1960; Knight et al., 1977), our data indicate that the fetus continues to grow as gestation progresses, while placental growth changes relatively little from d 70 through 90 of gestation. Vallet and Christenson (1993) suggested that the disparity in growth between the fetus and the placenta (this disparity increases under intrauterine crowding conditions), is the main reason for fetal death earlier in pregnancy.

The quantities of P_4 and E_1 measured in the culture medium after 3.0 h of incubation did not differ significantly from those measured at 1.5 h of incubation. The availability of the precursor, pregnenolone (P_5), may be a limiting factor in the production of both steroids. A previous study in our laboratory (Knight and Jeantet, 1991) indicated that P_5

supplementation enhanced P_4 and E_1 production by placental tissues in vitro.

Data from the present study indicate that there is a differential release of P_4 by the three regions of the pig placenta at all days of gestation except d 30. The polar region released the least amount compared to the middle and inner regions. Previous studies have shown (Brambel, 1933; Knight et al., 1977) a diminution in the density and size of areolae moving from the middle to the polar regions of the placenta. Availability of uterine space, however, did not seem to change the capability of the different placental tissues to release P_4 until d 90 of gestation, when the three regions of the placenta collected from UHOX gilts had reduced P_4 release compared to those collected from intact gilts. This may indicate a delayed effect of intrauterine crowding on placental P_4 production. This, however, does not explain the significant decrease in fetal survival rate in UHOX compared to intact gilts as of d 50 of gestation. Fetal sex did not seem to contribute significantly to P_4 release under either uterine condition. Studies in the rhesus monkey (Hagemenas and Kittinger, 1974) have shown that placentas associated with female fetuses produced more P_4 than placentas associated with male fetuses in vitro. However, there was no significant difference in P_4 production by placentas associated with female or male fetuses when P_5 was added as a substrate.

Estrone was released in greater quantities by the polar region compared to the two other regions at d 30 of gestation. At d 50 of gestation, a shift took place in regions that released the maximum

amount of E_1; the middle and the inner regions released the most compared with the polar region. No effect of region of placenta was detected at later stages of gestation. Fetal sex on the other hand, influenced E_1 release only at d 50 of gestation when placentas associated with male fetuses produced more E_1 than placentas associated with female fetuses. Studies in humans (Frandsen and Stakemann, 1961, 1963) have shown that androgens of fetal origin are important precursors for estriol production by the human placenta. Colenbrander et al. (1978) found that maximal testosterone secretion in the fetal pig takes place between d 40-60 of gestation. In this experiment, uterine space did not have a significant effect on the amount of E_1 produced by the three regions of the placenta. The difference among the three regions in P_4 or E_1 release could be attributed to either the availability of more precursors at specific regions at a particular stage of gestation or a difference in the enzymatic complex.

Under our experimental conditions, we did not detect a significant change in the steroidogenic activity of the whole placenta and(or) the different regions of the placenta under intrauterine crowding conditions except for a decrease in P_4 release at d 90 of gestation by all placental regions. Results of this study show an increased steroidogenic activity, in terms of E_1 release, by placentas associated with male fetuses only at d 50 of gestation. Collectively, our data indicate that there is no compensatory increase in steroidogenic activity of the pig placenta per unit of tissue when total placental mass is reduced.

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Chapter V

THE EFFECT OF INTRAUTERINE POSITION ON CONCEPTUS DEVELOPMENT, PLACENTAL AND ENDOMETRIAL RELEASE OF PROGESTERONE AND ESTRONE *IN VITRO*, AND FETAL FLUID CONCENTRATION OF STEROID HORMONES THROUGHOUT GESTATION IN SWINE

ABSTRACT

Intact (n=20) and unilaterally hysterectomized-ovariectomized (UHOX) gilts (n=25) were used to study the effect of fetal intrauterine position on conceptus development, concentrations of endogenous progesterone (P_4) and estrone (E_1) in porcine placenta, and endometrium and steroid hormone concentrations in amniotic and allantoic fluids throughout gestation. Gilts were hysterectomized at either d 40, 60, 80, or 100 of gestation. Placental tissues were combined, and endometrial tissues were combined based upon the intrauterine position of the associated fetus. Placental and endometrial tissues were incubated, and release of P_4 and E_1 was determined. Uterine status (intact or UHOX) did not have a significant effect on the variables measured. Intrauterine position had a significant effect on fetal and placental weights ($P< .02$ and .01, respectively) only at d 40 of gestation. Female fetuses bordered in utero by two males, and their associated placentas were lighter in weight than those from other intrauterine positions. No significant effect of intrauterine position was detected on placental and endometrial P_4 release. At d 100 of gestation, placentas associated with fetuses bordered in utero by fetuses of the same sex released more E_1 compared

to placentas associated with fetuses bordered by fetuses of the opposite sex ($P < .01$). Estrone release by the endometrium was not affected by intrauterine position. Only trace amounts of testosterone and dehydroepiandrosterone sulfate were measured in the fetal fluids at all days of gestation. Intrauterine position had no effect on P_4, E_1, or androstenedione concentrations in fetal fluids. Results of this study indicate a limited effect of intrauterine position on conceptus development and on placental and endometrial steroidogenic activity in swine.

Key Words: Swine, Intrauterine Position, Placenta, Endometrium

INTRODUCTION

Sexual differentiation in mammals is described by Jost (1972) as a sequential process that begins at fertilization. Fetal testicular androgen secretion is critical for male differentiation of external and internal genitalia and the central nervous system (for review, see George and Wilson, 1988). In pigs, the critical period of sexual differentiation (d 40 to 60) corresponds with maximum fetal testicular secretion (Colenbrander et al., 1978; Ford et al., 1980).

A naturally occurring cause of phenotypic variation in litter bearing mammals is the in utero proximity of a fetus of the same or opposite sex, which is referred to as the intrauterine position phenomenon (vom Saal, 1981). This phenomenon has been identified as an important source of within sex variation relative to a wide array of reproductive, behavioral, and morphological traits measured during adult life in polytocous laboratory species (for review, see vom Saal, 1989). The intrauterine position effect apparently is not due to the position itself but rather to the movement of steroid hormones between fetuses and the variation in the hormonal environment relative to the proximity of an individual fetus to the members of the same or opposite sex. The intrauterine position effect in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity (vom Saal and Bronson, 1980; vom Saal et al., 1983).

Based on evidence to date, intrauterine position seems to have less of an effect on reproductive traits in adult swine compared to mice.

Rhode Parfet et al. (1990) reported that semen characteristics of boars at 220 d of age were not markedly affected by fetal position in utero. They also reported that males surrounded by two males in utero had a better growth rate in a limitfed situation, apparently due to greater aggressiveness, but that there was no effect on postnatal growth rate in swine consuming feed ad libitum. Recently, Wise and Christenson (1992) reported no intrauterine position effect in swine on placental weight or fetal testosterone at d 70 of gestation, but an effect on fetal weight was detected at d 104 of gestation. Fetuses surrounded with fetuses of the opposite sex had lower weights than fetuses surrounded by fetuses of the same sex.

Evidence indicates that intrauterine crowding results in poor fetal development and fetal death. Recently, Chen and Dziuk (1993) reported that when embryos were restricted to an initial uterine length of ≤ 20 cm, which is similar to embryos under intrauterine crowded conditions, male fetuses weighed less than their female litter mates at d 41 of gestation.

The objectives of this study were to determine the effect of intrauterine position at d 40, 60, 80, and 100 of gestation under normal and intrauterine crowded conditions on 1) conceptus development , 2) placental and endometrial progesterone (P_4) and estrone (E_1) production in vitro, and 3) steroid hormone concentrations in amniotic and allantoic fluid of pigs.

MATERIALS AND METHODS

Animals

Forty-five gilts of similar size, age, and genetic background were used for this experiment. Twenty-five gilts were unilaterally hysterectomized-ovariectomized (UHOX) between days 7 to 11 of their second estrous cycle. The UHOX model was used to create intrauterine crowding. The justification for this model was reviewed by Christenson et al. (1987). The remaining 20 gilts were left uterine-intact. All gilts were bred at their third detected estrus and randomly assigned for hysterectomy at either d 40, 60, 80, or 100 of gestation.

Sample Collection

Surgical procedures and sample collections were performed as described in Chapter IV. Placentas were combined according to intrauterine position of the fetus associated with the placental unit. Intrauterine position was noted according to the following criteria: 1) male between 2 females (2F); 2) male between two males (0F); 3) female between 2 females (0M); 4) female between 2 males (2M); and 5) males or females located adjacent to one fetus of the same or opposite sex at either end of the uterus (cranial or caudal) or in the middle of the uterus (1F, 1M). Endometrial tissue samples were stripped from the uterus and combined corresponding to intrauterine position. All tissues were processed as described in Chapter IV.

Tissue Incubation

The incubation was performed as described in Chapter IV. Preliminary analyses revealed no significant increase in the release of P_4 or E_1 in the medium beyond the 1.5 h incubation period. Therefore, only the 1.5 h values are reported. Least squares means of P_4 and E_1 released from placental and endometrial tissues at 0, and 3.0 h are presented in Appendix Figures 5-12.

Tissue Protein and DNA Determination

Tissue protein and DNA were determined as described in Chapter IV.

Assays for Steroid Hormones

Progesterone concentrations in the incubation media, and allantoic and amniotic fluids were determined using a solid-phase radioimmunoassay kit (Diagnostics Products Corp., Los Angeles, CA). Estrone in the media was determined using a double extraction procedure as described in Chapter IV. Inter- and intra-assay coefficient of variation were as follows: progesterone, 8.5 and 7.2%, and estrone, 17.6 and 10.3%, respectively. Total E_1 (E_1 plus E_1SO_4) rather than E_1 was determined in the allantoic and amniotic fluids using a modification of the assay described by Eley et al. (1981). Briefly, 100 µl sample of either fluid was incubated at 37° C with 5 µl of sulfatase enzyme type H-2 from Helix pomatia (Sigma Chemical Co., St. Louis, MO) for 12 h. After the incubation period, E_1 extracted as described previously. Inter- and intra-assay coefficients of variation were 15.7 and 9.5%, respectively.

Testosterone, and dehydroepiandrosterone sulfate (DHEAS) in the allantoic and amniotic fluids and androstenedione in the allantoic fluid were determined using solid-phase radioimmunoassay kits (Diagnostics Products Corp., Los Angeles, CA).

Statistical Analyses

The data were analyzed using the General Linear Models procedures of SAS (SAS, 1988). Initial analyses revealed no effect of uterine status on any of the variables studied. Therefore, uterine status was deleted from the general model. Also, initial analyses revealed no significant effect of intrauterine position on the variables studied. So, data collected from fetuses located between one male and(or) one female were deleted from the analyses.

Analyses for the regression of P_4 and E_1 concentrations on tissue (placenta or endometrium) protein or DNA content did not indicate a significant relationship of hormone concentration and protein or DNA content. Therefore, data were analyzed by day of gestation with gilt and intrauterine position as the main effects. Differences in P_4 and E_1 in the media and steroid hormone concentrations in the fetal fluids were analyzed using the model described above.

When F-tests for the intrauterine position effect from the analyses of variance was significant, means were compared using Duncan's multiple range comparison.

RESULTS

Conceptus Variables

Least squares means for the conceptus variables are presented in Table 1. Intrauterine position had a significant effect on placental ($P < .01$) and fetal weight ($P < .02$) but not on allantoic fluid volume at d 40 of gestation. The 2M fetuses and their associated placentas were lighter in weight compared with those from other fetal intrauterine positions at d 40. No significant effect of intrauterine position was detected for any of the conceptus variables at d 60, 80, or 100 of gestation.

Placental Progesterone and Estrone Production

Least squares mean concentrations of P_4 and E_1 in the incubation medium following 1.5 h of incubation of placentas associated with the four fetal intrauterine positions are presented in Figures 1 and 2, respectively. Intrauterine position did not significantly affect P_4 release at any of the days of gestation studied. The release of E_1 was significantly affected ($P < .01$) by intrauterine position only at d 100 of gestation where placentas associated with 0M and 0F fetuses released more E_1 compared to placentas associated with 2M and 2F fetuses.

Table 1. Effect of intrauterine position on conceptus variables at days 40, 60, 80, and 100 of gestation[1]

Day of Gestation	Intrauterine position[2]	No. of conceptuses	Placental weight[3], g	Fetal weight[3], g	Allantoic fluid volume, ml
40	0F	10	72.1 (5.8)	12.4 (.4)	106.2 (13.1)
40	0M	5	85.9 (9.0)	11.4 (.7)	126.7 (20.3)
40	2F	5	78.8 (8.4)	12.1 (.6)	84.3 (18.9)
40	2M	5	44.3 (8.3)*	9.6 (.6)*	75.4 (18.7)
60	0F	16	215.4 (19.4)	120.2 (4.5)	316.5 (56.1)
60	0M	4	180.0 (44.6)	104.5 (10.4)	168.0 (129.4)
60	2F	6	177.7 (41.0)	120.1 (9.5)	258.7 (118.9)
60	2M	6	214.5 (31.0)	112.3 (7.2)	323.8 (89.9)
80	0F	8	210.6 (29.0)	458.9 (36.0)	91.8 (45.7)
80	0M	6	245.8 (43.8)	387.4 (54.5)	30.5 (69.1)
80	2F	2	123.5 (48.9)	313.3 (60.8)	65.7 (77.1)
80	2M	9	152.8 (25.0)	351.6 (31.1)	146.1 (39.5)
100	0F	4	209.6 (42.1)	728.2 (126.8)	98.2 (22.8)
100	0M	7	231.8 (22.0)	817.5 (66.2)	73.2 (11.9)
100	2F	7	227.4 (20.0)	808.4 (60.0)	55.0 (10.8)
100	2M	7	248.1 (18.9)	888.2 (56.8)	47.5 (10.2)

[1]Conceptus variables are expressed as least squares means; standard error of the mean is in parentheses.

[2]0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses, respectively.

[3]Placental and fetal weights differ ($P < .01$ and $P < .02$, respectively) with intrauterine position at d 40 of gestation.

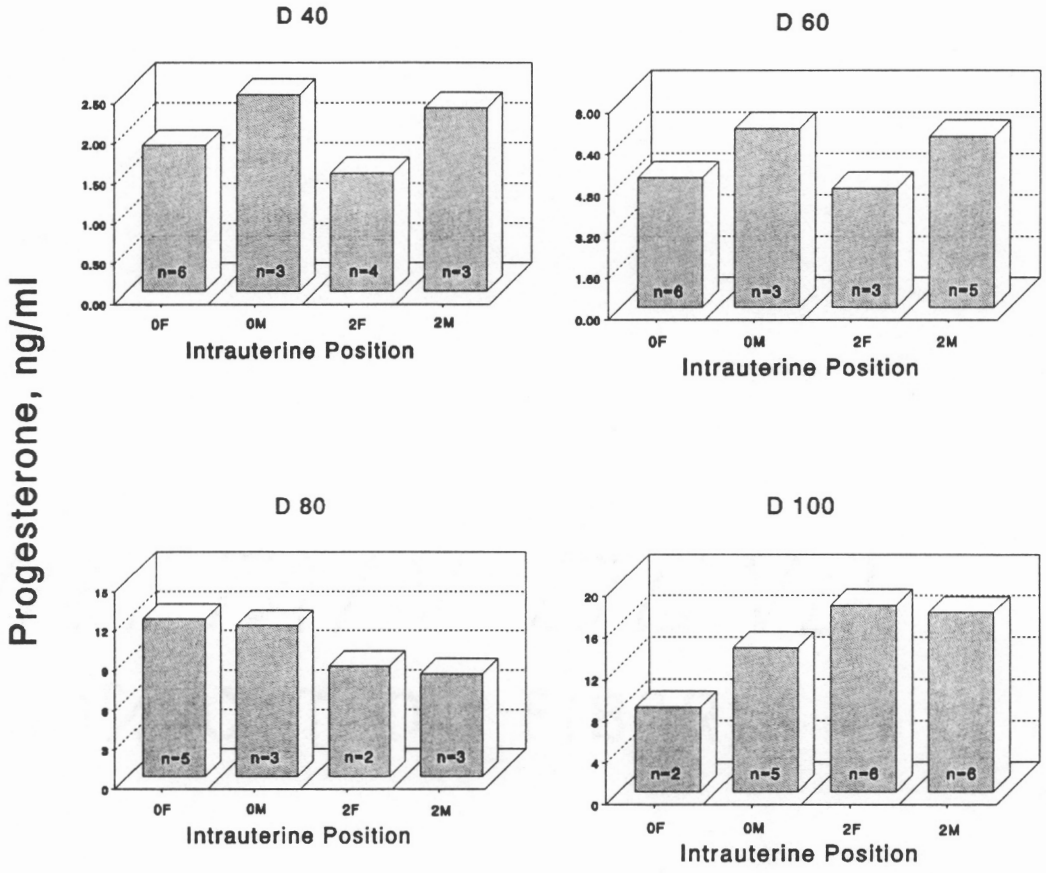

Figure 1. Progesterone release (ng/ml) after 1.5 h of incubation of placentas associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are 2.635, 10.445, 9.812, and 5.831, for d 40, 60, 80, and 100, respectively.

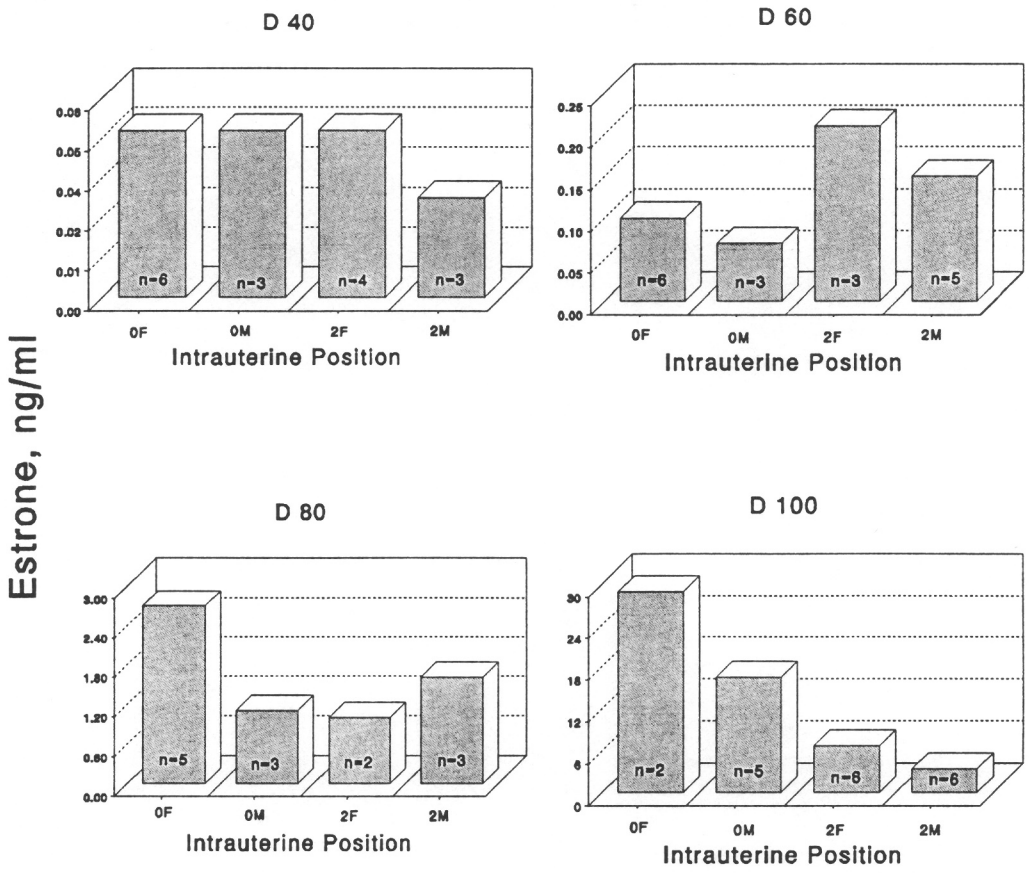

Figure 2. Estrone release (ng/ml) after 1.5 of h incubation of placentas associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .00004, .0018, .6812, and 4.8938, for d 40, 60, 80, and 100, respectively. Estrone release differs ($P < .01$) as function of intrauterine position at d 100 of gestation.

Endometrial Progesterone and Estrone Production

Least squares means of P_4 and E_1 accumulated after 1.5 h of incubation of endometrial tissue adjacent to the four intrauterine fetal

positions are presented in Figures 3 and 4, respectively. Intrauterine position did not affect P_4 or E_1 release by endometrial tissues at any of the days of gestation studied.

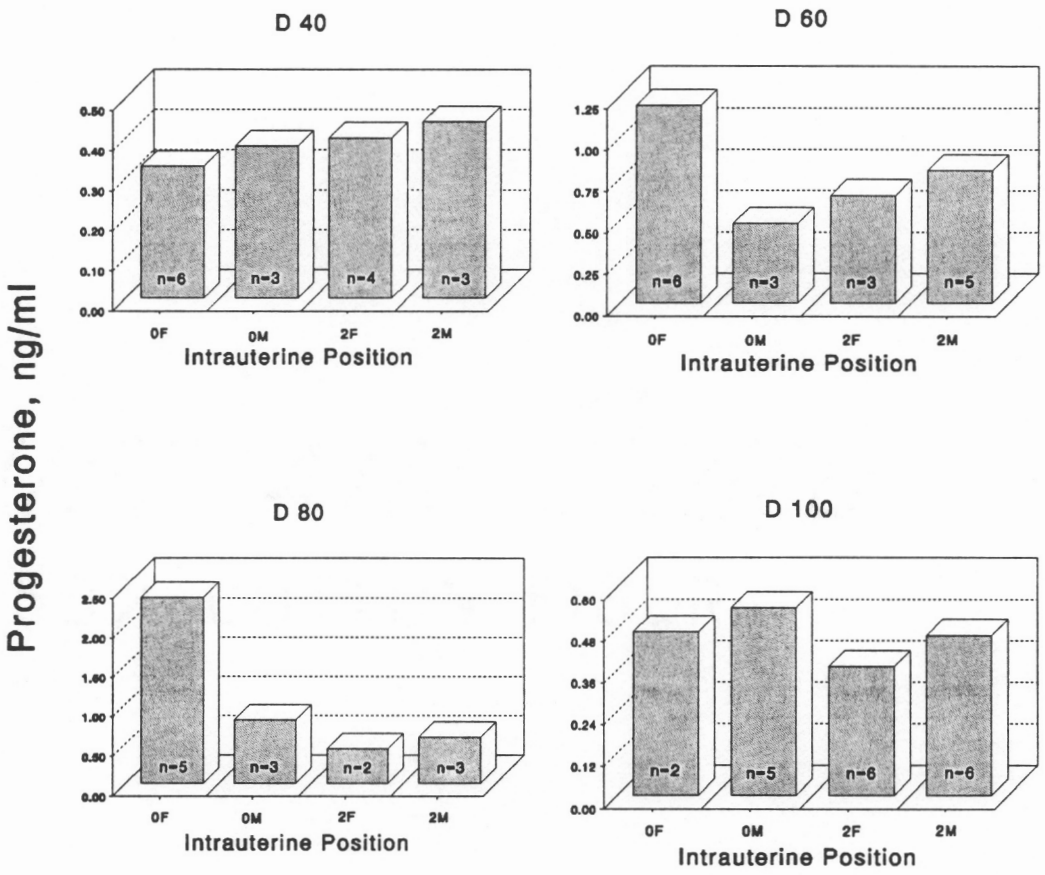

Figure 3. Progesterone release (ng/ml) after 1.5 h of incubation of endometrial tissues associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .003, .119, 2.993, and .015, for d 40, 60, 80, and 100, respectively.

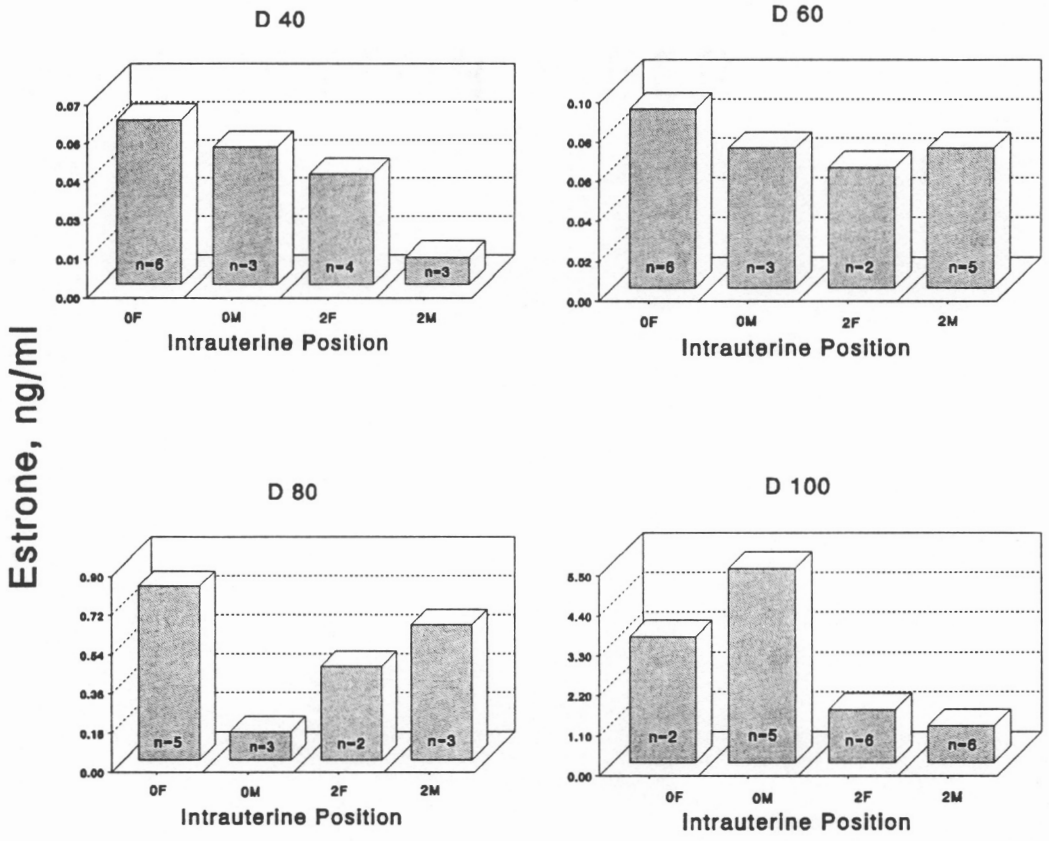

Figure 4. Estrone release (ng/ml) after 1.5 h of incubation of endometrial tissues associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .0002, .0005, .1659, and 3.9638, for d 40, 60, 80, and 100, respectively.

Steroid Concentrations in Allanotic Fluid

Negligible amounts of testosterone and DHEAS were detected in allantoic fluid at all days of gestation studied. Intrauterine position did not affect P_4, E_1 or androstenedione concentrations in the allantoic fluid

at any of the days of gestation studied (Table 2). Least square means for P_4, E_1, and androstenedione concentrations in allantoic fluids at d 50, 70, and 90 of gestation are presented in Appendix Table 3.

Table 2. Effect of intrauterine position on progesterone, estrone, and androstenedione concentrations in allantoic fluid at days 40, 60, 80, and 100 of gestation[1]

Day of Gestation	Intrauterine position[2]	Progesterone[3], ng/ml (N)	Estrone[4], ng/ml (N)	Androstene-dione[5], ng/ml (N)
40	0F	3.1 (6)	40.1 (6)	.5 (6)
40	0M	2.5 (4)	25.7 (4)	.4 (4)
40	2F	NE (4)[6]	NE (4)	.1 (4)
40	2M	1.5 (3)	34.6 (3)	.4 (3)
60	0F	3.1 (7)	1.2 (4)	.4 (7)
60	0M	1.9 (3)	.67 (3)	.2 (3)
60	2F	NE (3)	.45 (3)	.2 (3)
60	2M	.5 (5)	1.2 (5)	.2 (5)
80	0F	6.0 (5)	148.0 (5)	2.1 (5)
80	0M	5.0 (3)	152.4 (3)	1.2 (3)
80	2F	6.4 (2)	195.8 (2)	2.3 (2)
80	2M	5.8 (5)	31.2 (5)	1.6 (5)
100	0F	8.2 (2)	873.7 (2)	4.6 (2)
100	0M	7.0 (4)	1404.3 (4)	2.8 (4)
100	2F	5.9 (5)	1480.2 (5)	3.6 (5)
100	2M	10.0 (3)	3686.9 (4)	5.2 (4)

[1]Steroid concentrations are expressed as least squares means; number of observations is in parentheses.
[2]0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses, respectively.
[3]Error mean squares are 2.12, 1.14, 1.43, and 8.50 at d 40, 60, 80, and 100, respectively.
[4]Error mean squares are 4200.27, .19, 8426.04, and 1555836.4 at d 40, 60, 80, and 100, respectively.
[5]Error mean squares are .28, .01, .25 and, .88 at d 40, 60, 80, and 100 respectively.
[6]Non estimable

Steroid Concentrations in Amniotic Fluids

Negligible amounts of testosterone and DHEAS were detected in the amniotic fluid at all days of gestation studied. Progesterone and E_1 in the amniotic fluid was not affected by intrauterine position at any of the days of gestation studied (Table 3). Least square means for P_4 and E_1 concentrations in amniotic fluid at d 50, 70, and 90 of gestation are presented in Appendix Table 4.

Table 3. Effect of intrauterine position on progesterone and estrone concentrations in amniotic fluid at days 40, 60, 80, and 100 of gestation[1]

Day of Gestation	Intrauterine position[2]	No.	Progesterone[3], ng/ml	Estrone[4], ng/ml
40	0F	6	.7	.4
40	0M	3	1.3	.7
40	2F	4	.8	.6
40	2M	4	.8	1.0
60	0F	7	.9	2.4
60	0M	3	.7	1.0
60	2F	3	.5	1.3
60	2M	5	.4	1.2
80	0F	5	.7	72.3
80	0M	3	.2	35.2
80	2F	2	.5	132.9
80	2M	6	.6	14.5
100	0F	2	2.5	6.7
100	0M	5	3.0	116.0
100	2F	6	NE	35.0
100	2M	6	1.5	21.9

[1]Steroid concentrations are expressed as least squares means.
[2]0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses, respectively.
[3]Error mean squares are .157, .269, .089, and 8.448 at d 40, 60, 80, and 100, respectively.
[4]Error mean squares are .269, 1.343, 8603.96, and 4480.36 at d 40, 60, 80, and 100, respectively.
[5]Non estimable.

DISCUSSION

Results of this study indicate that intrauterine position has little effect on conceptus development, intrauterine steroidogenesis, and steroid contents in fetal fluids in swine. Studies with rodents (Clemens et al., 1978; vom Saal and Bronson, 1980; vom Saal, 1981; vom Saal et al., 1983; vom Saal, 1989) have shown considerable variability in morphological, behavioral, and reproductive characteristics among adult animals that are partially traced to intrauterine position during prenatal development.

In this study, intrauterine position affected fetal and placental weights only at d 40 of gestation. Female fetuses located between 2 males had lower fetal and placental weights compared to fetuses from other positions. Recently, Wise and Christenson (1992) reported that at d 104 of gestation, a fetus surrounded in utero by fetuses of the opposite sex was lighter in weight than a fetus surrounded by fetuses of the same sex. No difference, however, was detected at d 70 of gestation. Rhode Parfet et al. (1990) did not find any difference in birth weights (d 112 of gestation) associated with pigs from different uterine positions.

Recently, Chen and Dziuk (1993) used a different method than ours to restrict space per embryo reported that under restricted uterine space conditions males were lighter than females at d 41 of gestation. In our study however, uterine space had no significant effect on fetal weight when the data was analyzed with either fetal sex or intrauterine position as a main effect.

The quantities of P_4 and E_1 measured in the incubation medium after 3.0 h of incubation did not differ from those measured at 1.5 h of incubation. The availability of the precursor, pregnenolone (P_5), may be a limiting factor in the production of both steroids. Previous studies (Hagemenas and Kittinger, 1974; Knight and Jeantet, 1991) indicated that P_5 supplementation enhanced steroid production in vitro. Therefore, P_4 and E_1 concentrations in the media reported in this study reflect the endogenous steroid concentrations in the placenta rather than *de novo* biosynthesis.

Data from the present study indicate no intrauterine position effect on the amount of P_4 released by the porcine placenta and endometrium or on P_4 concentration in allantoic and amniotic fluids at any of the days of gestation studied. Hagemenas and Kittinger (1974) reported that the endogenous P_4 concentrations in placental tissues obtained from rhesus monkeys during the last trimester of pregnancy were greater in genotypically female compared to male placentas. However, when P_5 was added as a substrate, no difference in P_4 production was detected between genotypically female and male placentas. In our study however, we were not able to detect an effect of fetal sex on either placental P_4 production or P_4 concentration in fetal fluids when the data were analyzed with fetal sex rather than intrauterine position as the main effect. Similar observations relative the concentrations of P_4 in the fetal fluids has been reported for mice (vom Saal and Bronson, 1980), where no intrauterine position effect was detected on P_4 concentration in amniotic fluids. Knight and Kukoly (1990) also reported no differences in

P_4 concentrations between male and female fetuses in either amniotic or allantoic fluid of porcine. Several studies in rats (Cagnoni et al., 1965; Kincl and Magueo, 1965), mice (Erpino and Chappelle, 1971), and rhesus monkeys (Resko, 1975) indicated a role for P_4 in sexual differentiation by antagonizing the action of androgens.

An intrauterine position effect on the release of E_1 by the porcine placenta was detected only at d 100 of gestation. Placentas associated with fetuses surrounded in utero by fetuses of the same sex (0M and 0F) released more E_1 compared to placentas associated with fetuses surrounded in utero by fetuses of the opposite sex (2M and 2F). The same trend was reported by Wise and Christenson (1992) for fetal weight at d 104 of gestation. They suggested that 2M and 2F fetuses may be at developmental disadvantage and may have a greater chance of being developmentally behind litter mates at birth (i.e., a runt pig) and hence be a prime candidate for neonatal death. In our study, no intrauterine position effect on fetal weight was detected at d 100 of gestation, hence we cannot draw a relationship between placental E_1 production and fetal weight.

No effect of intrauterine position on E_1 release by the endometrium was noted. Previous studies in our laboratory (Faillace, 1989; Knight and Jeantet, 1991) indicated that the endometrium has considerably less steroidogenic activity than the placenta and that E_1 production by the endometrium remains constant throughout gestation.

Testosterone and DHEAS were undetectable in amniotic and allantoic fluids. This is in contrast to reports in mice (vom Saal and

Bronson, 1980; vom Saal et al., 1983) where appreciable amounts of testosterone were measured in the amniotic fluid that varied by intrauterine position. Elsasser and Parvizi (1979) were unable to masculinize female pig fetuses born to mothers where testosterone concentrations were 50 times greater (testosterone propionate treatment) than that of controls, indicating either decrease of permeability of the uterus to testosterone or increased aromatization of testosterone by the placenta. Knight and Kukoly (1990) and Knight and Jeantet (1991) reported a lack of measurable quantities of testosterone in the medium following incubation of porcine placental tissues. In the fetal pig, however, testicular testosterone concentrations are increased (Raeside and Sigman, 1975), and testosterone secretion by testicular tissue in vitro is enhanced (Stewart and Raeside, 1976) between 35 and 40 days post coitum. Also, Colenbrander et al. (1978) reported measurable amounts of testosterone in fetal pig serum as early as d 40 of gestation. Based on the aforementioned observations, the findings of our study indicate that either a limited amount of testosterone is excreted from the fetus to the fetal fluids, or testosterone is rapidly metabolized to estrogens.

Androstenedione, however, was detected in allantoic fluid as early as d 40 of gestation. Investigations in rats (Sridaran et al., 1983; Warshaw et al., 1986) indicated that placental androstenedione secretion exceeded that of testosterone. Studies with humans (Bloch and Benirschke, 1959) have shown that adrenal tissue slices collected from fetuses at different stages of pregnancy are capable of synthesizing

androstenedione in vitro. These observations indicate that the placenta and(or) the fetal adrenals are the main sources of androgen in the allantoic fluid. No effect of intrauterine position on androstenedione concentrations, however, was detected at any of the days of gestation.

Intrauterine position did not affect the total amount of E_1 in fetal fluids at any day of gestation. However, it should be mentioned that considerable variability in the amount of total E_1 in fetal fluids was detected , especially late in gestation.

In contrast to studies with rodents that have related intrauterine position to a wide array of morphological, behavioral, and reproductive characteristics, results of this study indicate a limited effect of intrauterine position in swine. However, reduced release of E_1 by placentas associated with fetuses surrounded by fetuses of the opposite sex indicates that intrauterine position may influence placental E_1 production or release.

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APPENDIX

LIST OF ABREVIATIONS

Corpus luteum	CL
Hysterectomy	HYSTX
Unilateral hysterectomy-ovariectomy	UHOX
Dehydroepiandrosterone sulfate	DHEAS
Estrone	E_1
Estrone Sulfate	E_1SO_4
Estradiol 17-β	E_2
Pregnenolone	P_5
Progesterone	P_4
Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$
Prostaglandin E_2	PGE_2
Luteinizing hormone	LH
Human chorionic gonadotropin	hCG
Male between two females	2F
Male between two males	0F
Female between two females	0M
Female between two males	2M

Table1. Crossreactivities of some common steroids with antibodies from described assays[a]

	Steroid antibody				
Steroid	P_4	E_1	A	DHEA-SO_4	T
Aldosterone			ND[b]	0.03	ND
Androstenediol	ND		0.02		0.2
Androstenedione		< 0.1	100	0.12	0.5
Corticosterone	0.4		0.00		0.00
Cortisol	ND		0.02	0.01	0.00
Cortisone		0.01	0.05		0.00
11-Deoxycorticosterone	1.7				
DHEA		< 0.1	4.3	0.08	0.00
DHEA sulfate				100	0.00
20α-Dihydroprogesterone	2.0	0.1			
17α-Estradiol					
17β-Estradiol	ND	< 0.1		0.03	0.02
Estriol				0.03	
Estrone		100	0.75	0.01	0.01
17α-Hydroxyprogesterone	0.3	0.1			
Pregnenolone	ND	< 0.1			
Progesterone	100		0.19	0.01	ND
Testosterone	ND		0.20	0.10	100

[a] Suazo, 1989

[b]ND = Not detectable.

Table 2. Effect of uterine status and day of gestation on uterine horn length, number of live fetuses per horn, and uterine uterine space per fetus[1]

	Day of Gestation[2]							
	30		50		70		90	
	Intact	UHOX	Intact	UHOX	Intact	UHOX	Intact	UHOX
No. of gilts	4	4	5	5	4	5	4	5
Uterine horn length,cm	233.2(10.4)	244.0(14.7)	240.2(9.3)	243.0(13.1)	280.0(10.4)	285.2(13.2)	294.6(10.4)	260.6(13.2)
No of live fetuses per uterine horn	6.1(.4)	9.0(.6)	5.3(.4)	7.0(.5)	5.6(.4)	5.4(.5)	5.9(.4)	5.2(.5)
Uterine space per fetus, cm^3	38.6(5.7)	28.8(8.1)	51.2(5.1)	36.6(7.2)	50.4(5.7)	67.3(7.2)	51.8(5.7)	50.3(7.2)

[1]Variables are expressed as least square means; standard error of the mean is in parentheses.
[2]Variables differed with day of gestation ($P < .05$).
[3]Uterine space per fetus at the time of hysterectomy based upon uterine length divided by the number of live fetuses. In reality, space occupied earlier in gestation by a fetus that subsequently died will not be invaded and utilized by surviving fetuses.

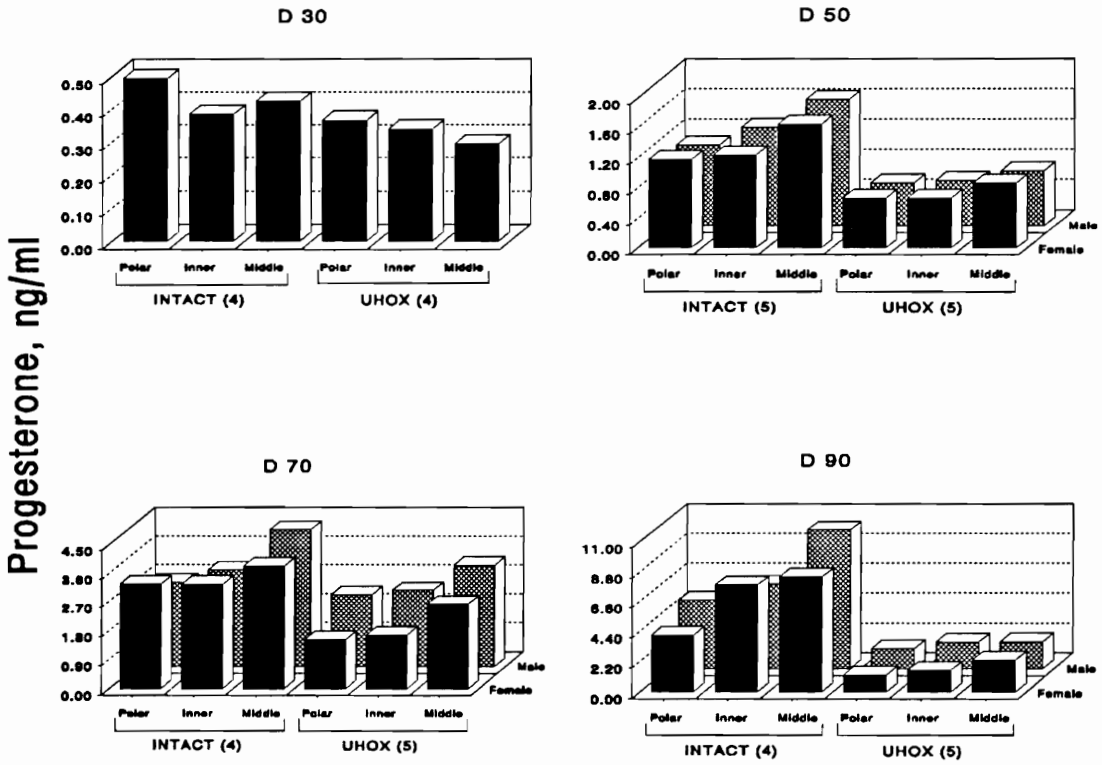

Figure 1. Progesterone release (ng/ml) after 0 h of incubation of three regions (polar, inner, middle) of placental tissue from male or female fetuses collected from either intact or unilaterally hysterectomized-ovariectomized gilts (n) at d 30, 50, 70, or 90 of gestation. (Sex cannot be visually determined at d 30). Progesterone release differed as a function of a) region ($P < .01$) at d 50, 70, and 90 and b) uterine status ($P < .005$) at d 90. Error mean squares are .101, 2.71, 19.65, and 65.57 for d 30, 50, 70, and 90, respectively.

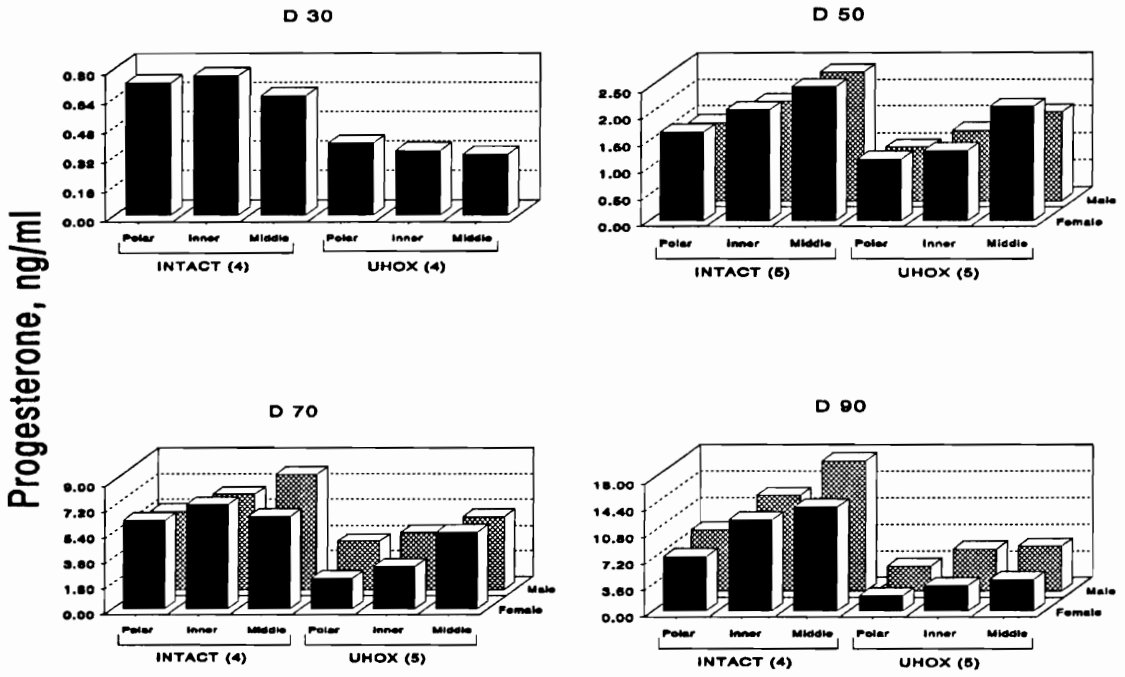

Figure 2. Progesterone release (ng/ml) after 3 h of incubation of three regions (polar, inner, middle) of placental tissue from male or female fetuses collected from either intact or unilaterally hysterectomized-ovariectomized gilts (n) at d 30, 50, 70, or 90 of gestation. (Sex cannot be visually determined at d 30). Progesterone release differed as a function of a) region (P< .01) at d 50, and 90 and b) uterine status (P< .005) at d 30, and 90. Error mean squares are .46, 105.3, 1233.0, and 2373.2 for d 30, 50, 70, and 90, respectively.

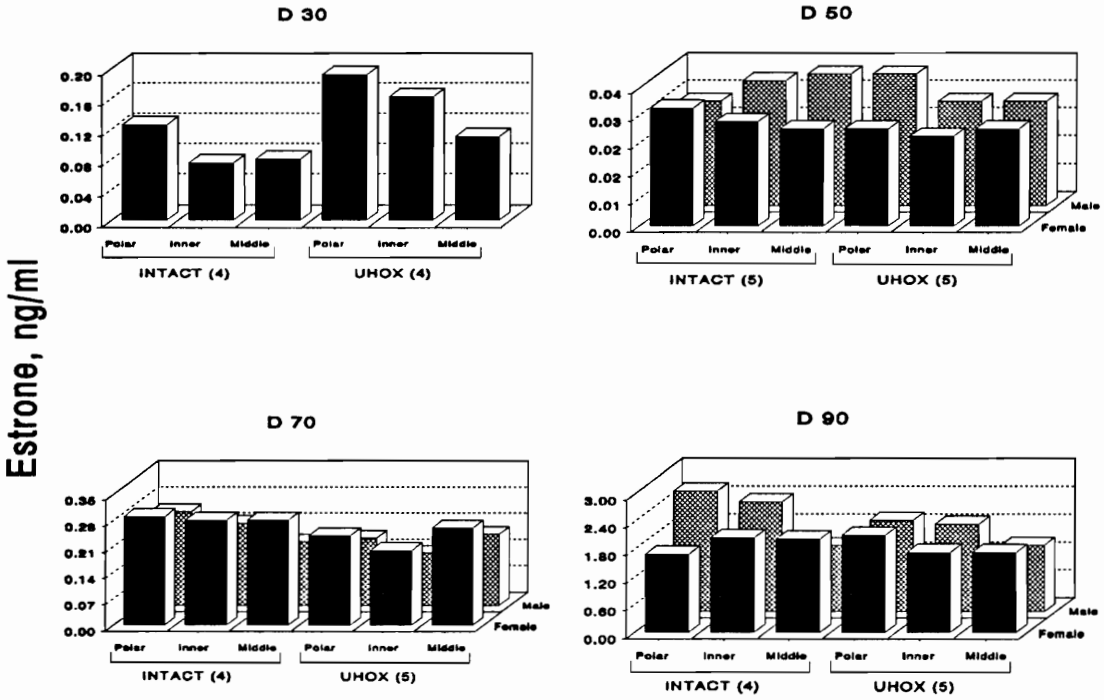

Figure 3. Estrone release (ng/ml) after 0 h of incubation of three regions (polar, inner, middle) of placental tissue from male or female fetuses collected from either intact or unilaterally hysterectomized ovariectomized gilts (n) at d 30, 50, 70, or 90 of gestation.(Sex cannot be visually determined at d 30). Estrone release changed as a function of region at d 50, and 70 (P< .001). Error mean squares are .034, .003, .163, and 21.84 for d30, 50, 70, and 90, respectively.

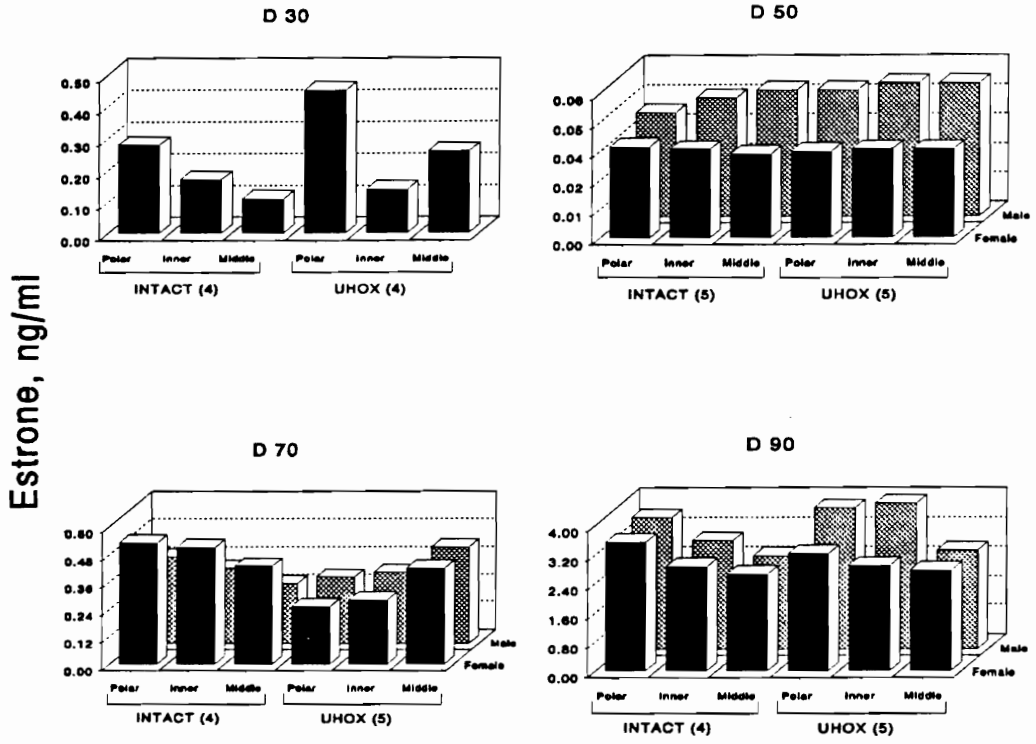

Figure 4. Estrone release (ng/ml) after 3 h of incubation of three regions (polar, inner, middle) of placental tissue from male or female fetuses collected from either intact or unilaterally hysterectomized ovariectomized gilts (n) at d 30, 50, 70, or 90 of gestation.(Sex cannot be visually determined at d 30). Estrone release changed as a function of a) region at d 30 (P< .001), and b) sex at d 50 (P< .001). Error mean squares are .113, .035, 8.517, and 410.11 for d30, 50, 70, and 90, respectively.

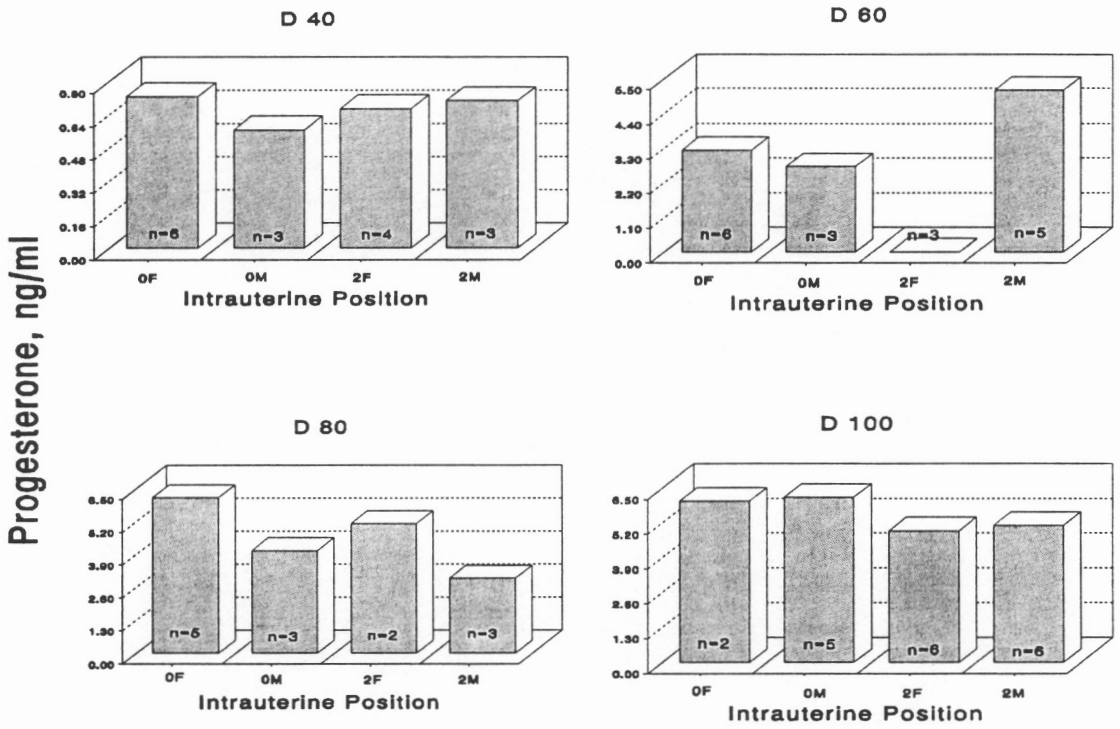

Figure 5. Progesterone release (ng/ml) after 0 h of incubation of placentas associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .11, 200.01, 13.19, and 14.66, for d 40, 60, 80, and 100, respectively.

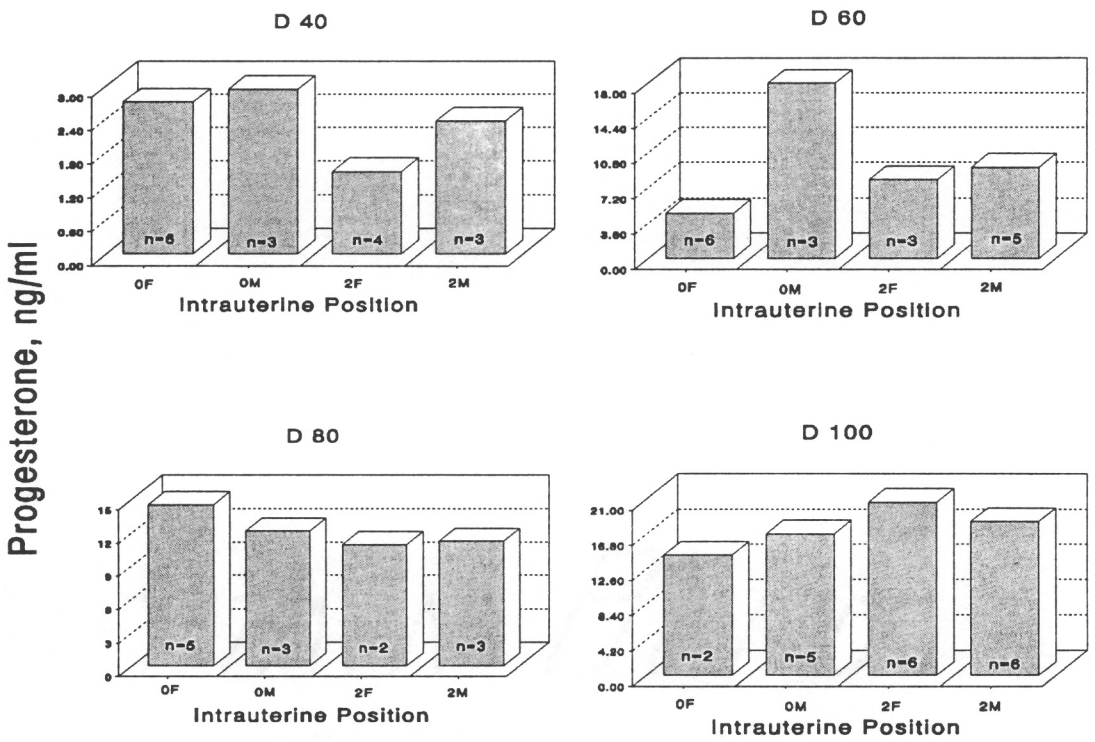

Figure 6. Progesterone release (ng/ml) after 3 h of incubation of placentas associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are 6.56, 275.71, 33.89, and 75.95, for d 40, 60, 80, and 100, respectively.

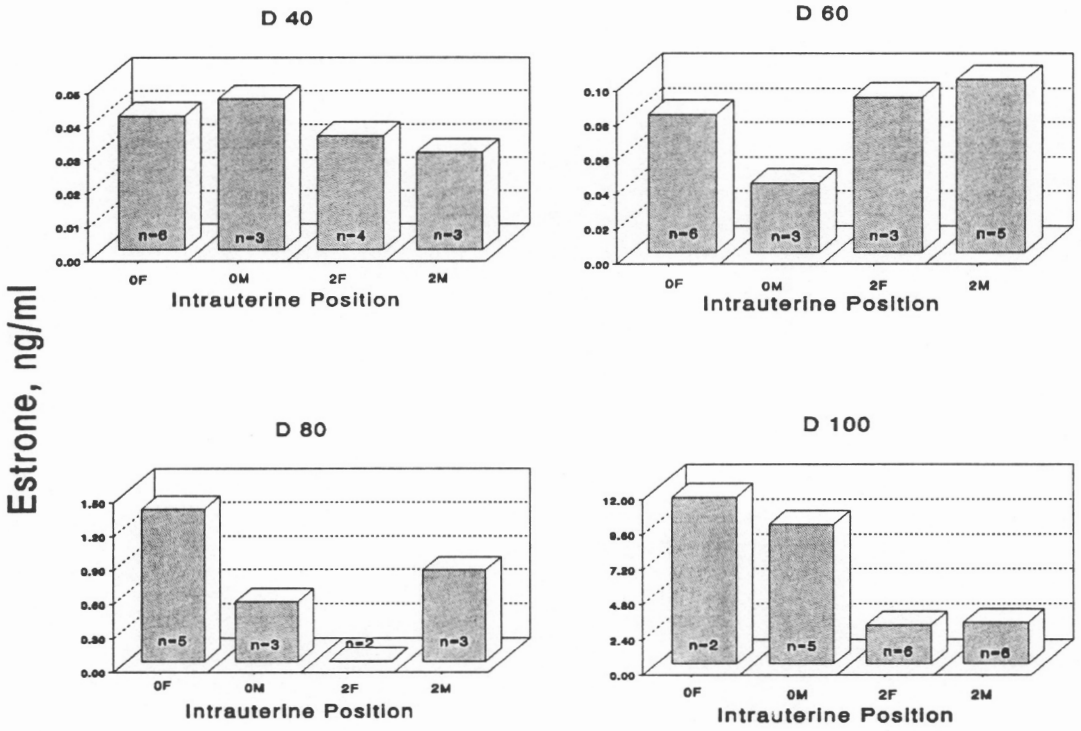

Figure 7. Estrone release (ng/ml) after 0 h of incubation of placentas associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .0005, .006, .678, and 12.35, for d 40, 60, 80, and 100, respectively. Estrone release differs as a function of intrauterine position at d 100 ($P < .01$).

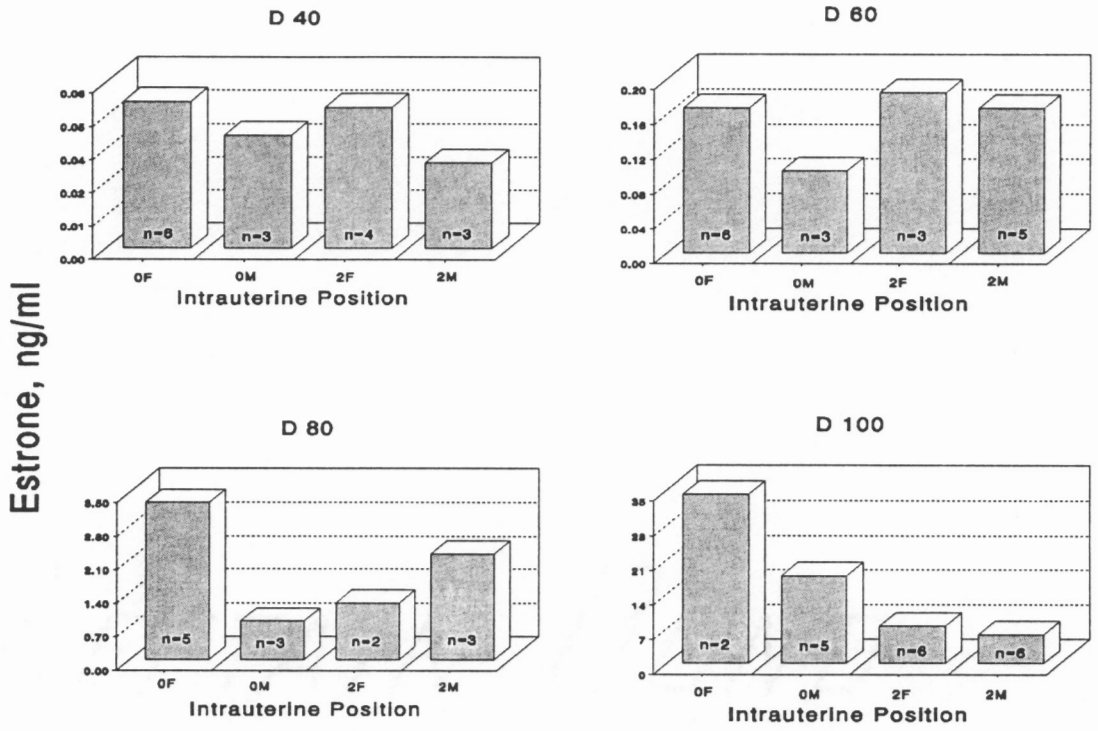

Figure 8. Estrone release (ng/ml) after 3 h of incubation of placentas associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .0003, .017, 2.097, and 77.086, for d 40, 60, 80, and 100, respectively. Estrone release differs as a function of intrauterine position at d 100 ($P < .01$).

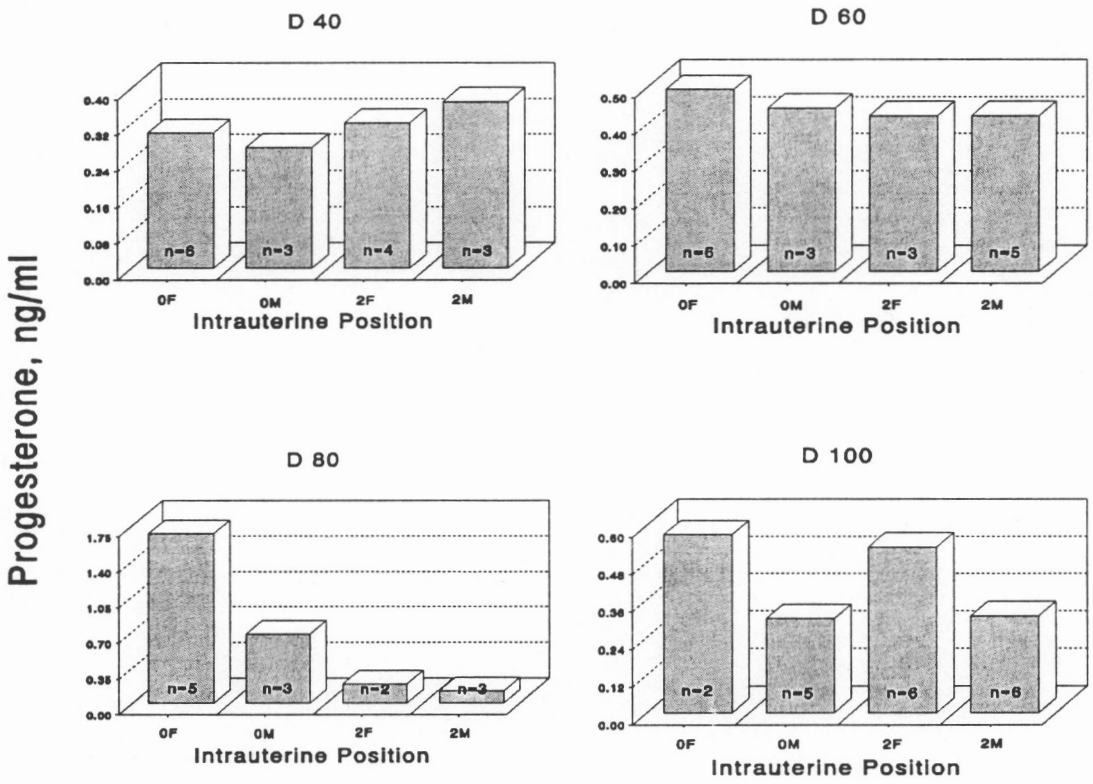

Figure 9. Progesterone release (ng/ml) after 0 h of incubation of endometrial tissues associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .108, .039, 6.784, and .037, for d 40, 60, 80, and 100, respectively.

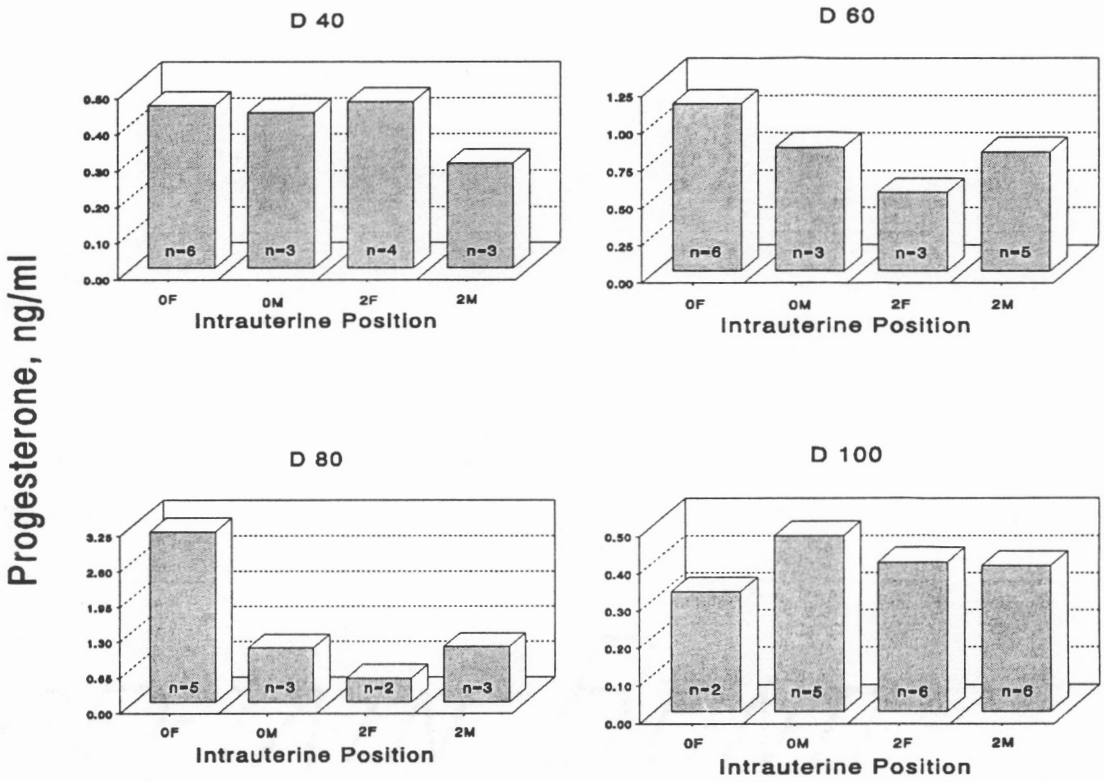

Figure 10. Progesterone release (ng/ml) after 3.0 h of incubation of endometrial tissues associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .04, .74, 15.84, and .01, for d 40, 60, 80, and 100, respectively.

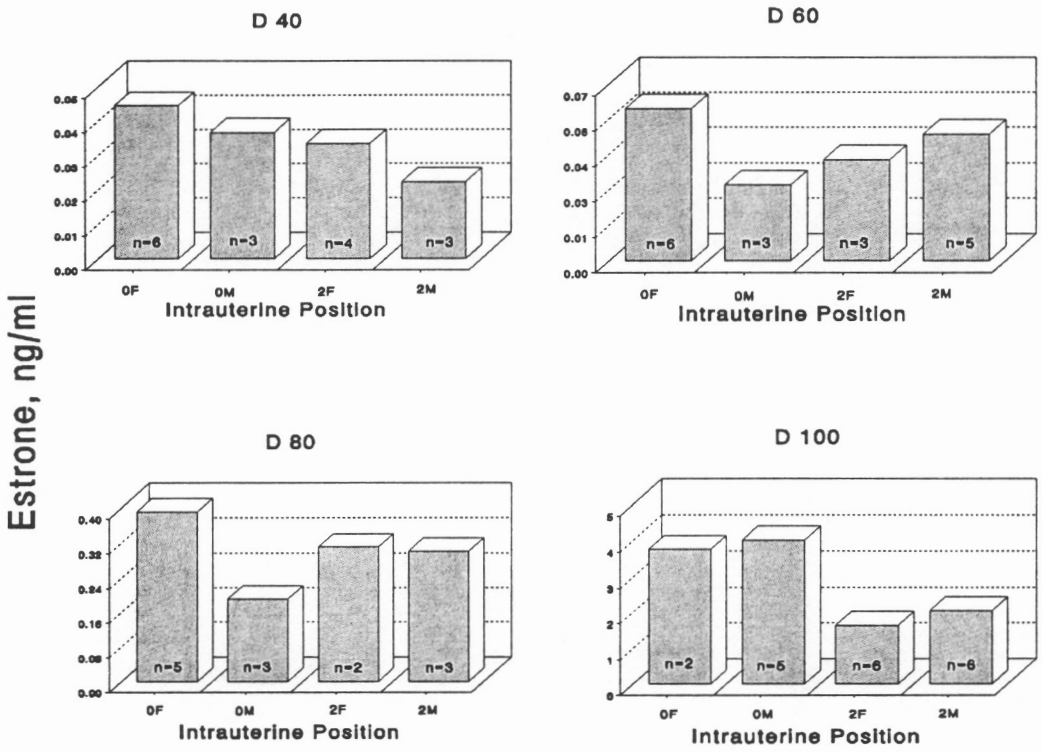

Figure 11. Estrone release (ng/ml) after 0 h of incubation of endometrial tissues associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .0004, .0003, .078, and 5.01, for d 40, 60, 80, and 100, respectively.

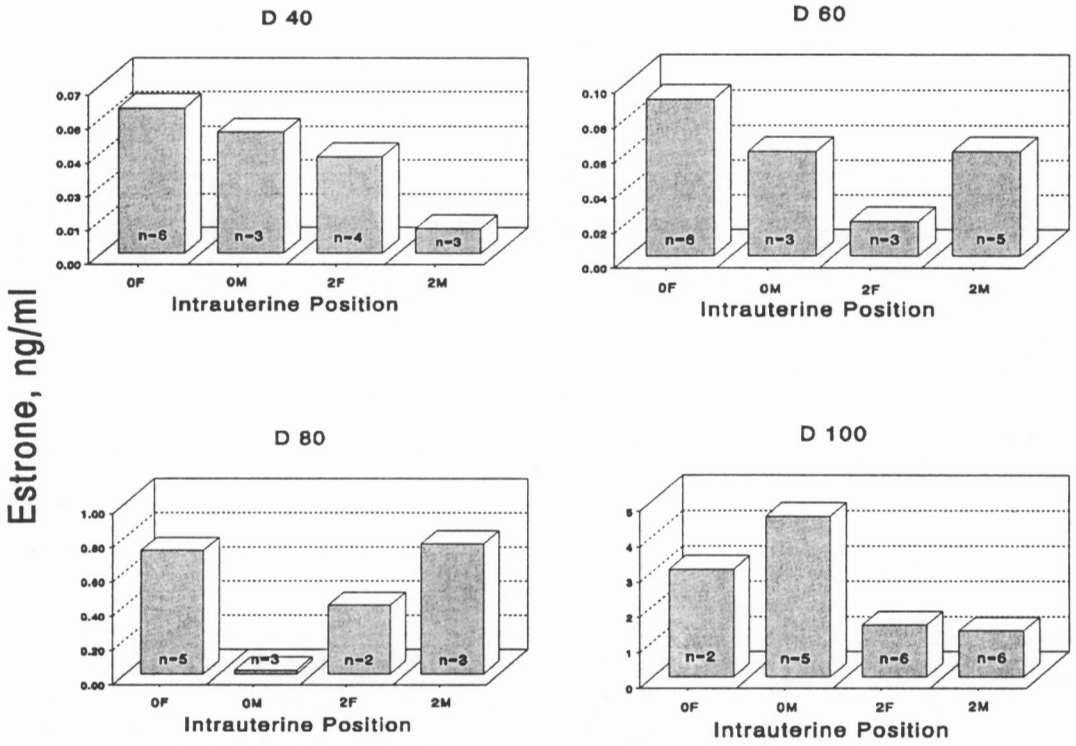

Figure 12. Estrone release (ng/ml) after 3 h of incubation of endometrial tissues associated with fetuses of different intrauterine positions at d 40, 60, 80, or 100 of gestation. 0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses respectively. Error mean squares are .0006, .0107, .4509, and 7.3975, for d 40, 60, 80, and 100, respectively. Estrone release differs as a ffunction of intrauterine position at d 40 (P< .05).

Table 3. Effect of intrauterine position on progesterone, estrone, and androstenedione concentrations in allantoic fluid at days 50, 70, and 90 of gestation[1]

Day of Gestation	Intrauterine position[2]	Progesterone[3], ng/ml (N)	Estrone[4], ng/ml (N)	Androstene-dione[5], ng/ml (N)
50	0F	.8 (5)	.1 (5)	.1 (5)
50	0M	2.0 (2)	.7 (2)	.1 (2)
50	2F	1.0 (6)	.8 (6)	.2 (6)
50	2M	1.6 (5)	1.5 (5)	.2 (5)
70	0F	5.0 (5)	41.5 (5)	.5 (5)
70	0M	5.7 (1)	41.8 (1)	.5 (1)
70	2F	NE (4)[6]	NE (4)	.2 (4)
70	2M	.4 (5)	58.2 (5)	.5 (5)
90	0F	18.4 (3)	316.9 (2)	1.8 (3)
90	0M	3.3 (2)	223.1 (2)	2.0 (2)
90	2F	.1 (4)	58.5 (4)	5.2 (5)
90	2M	28.8 (3)	584.0 (3)	5.8 (2)

[1]Steroid concentrations are expressed as least squares means; number of observations is in parentheses.
[2]0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses, respectively.
[3]Error mean squares are .549, 9.792, and 931.909 at d 50, 70, and 90, respectively.
[4]Error mean squares are 4.233, 7034.16, and 708046.52 at d 50, 70, and 90, respectively.
[5]Error mean squares are .026, .100, and, 4.178 at d 50, 70, and 90 respectively.
[6]Non-estimable

Table 4. Effect of intrauterine position on progesterone and estrone concentrations in amniotic fluid at days 50, 70, and 90 of gestation[1]

Day of Gestation	Intrauterine position[2]	No.	Progesterone[3], ng/ml	Estrone[4], ng/ml
50	0F	6	.4	.2
50	0M	2	1.0	1.0
50	2F	6	.3	.9
50	2M	5	.5	1.0
70	0F	5	.5	.5
70	0M	1	.9	2.7
70	2F	4	.2	4.7
70	2M	5	.6	1.3
90	0F	3	.9	48.5
90	0M	2	.8	31.3
90	2F	5	.5	11.9
90	2M	3	.7	21.2

[1]Steroid concentrations are expressed as least squares means.
[2]0F and 2F represent male fetuses bordered in utero by 2 male or 2 female fetuses, respectively. 0M and 2M represent female fetuses bordered in utero by 2 female or 2 male fetuses, respectively.
[3]Error mean squares are .122, .128, and .047 at d 50, 70, and 90, respectively.
[4]Error mean squares are 1.104, 11.602, and 172.263 at d 50, 70, and 90, respectively.

VITA

Charbel G. Tarraf was born the son of Gergi and Marie Tarraf in Hadeth-Beirut, Lebanon on March 26, 1959. Charbel graduated from the Lebanese University in 1980 majoring in Natural Sciences. In 1981 he enrolled in the Faculty of Agricultural and Food Sciences of the American University of Beirut (A.U.B.) where he graduated in 1984 with a diploma of "Ingénieur Agricole" and a Master of Science in Animal Science. Charbel worked as an instructor of Animal Science at A.U.B. until 1987. In April of 1987, Charbel was awarded a scholarship from the "Deutschen Akademischen Austauschdienst" for study and training in Germany at the University of Göttingen and the Institute for Animal Breeding and Animal behavior of the Federal Research Center for Agriculture in Mariensee. In 1990, Charbel started his Ph.D. program at Virginia Polytechnic Institute and State University.

Charbel married Hoda Nehme on March 21, 1987. They have three daughters, Zeina, Reema and Samar.

Charbel is a member of the American Society of Animal Science and the Society of the Study of Reproduction.