EFFECT OF ADVANCING UTERINE ENVIRONMENT WITH EXOGENOUS ESTROGEN ON PORCINE CONCEPTUS SURVIVAL

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CHAPTER I

INTRODUCTION

Reproductive efficiency of the sow herd is the most economically important aspect in commercial swine production. Effective management of nutrition and environment for the sow before parturition combined with early weaning can increase the number of pigs raised by a sow each year. However, a great deal of reproductive potential is lost during pregnancy. Most of the loss in litter size occurs during gestation by factors of which a manager has little control.

Piglet losses occur with varying frequencies over the course of pregnancy. Investigators have found that the highest frequency of embryonic loss occurs very early in pregnancy, usually before day 25. Early embryonic mortality, determined by comparing the number of corpora lutea to the number of embryos present, can range from 20-45% (see review Flint et al. 1982). This is not surprising considering the complexity of crucial events, such as fertilization, embryo spacing, maternal recognition of pregnancy, blastocyst elongation and implantation (attachment), which occur during this time.

Embryonic losses after fertilization can occur through

chromosomal abnormalities and polyspermy. These factors can account for 8-12% of the embryonic death after day 10 post-coitum (McFeely, 1967; Day and Polge, 1968). The incidence of embryonic death identified by chromosomal abnormalities before day 9, appears to be very low (Bounters, Bonte, and Vandeplassche, 1974). The majority of early embryonic mortality is possibly due to factors involved with embryo-uterine and/or embryo-embryo interactions between the days of 10 to 25 post coitum.

Maternal recognition of pregnancy refers to the critical time when viable embryos must provide a biological signal to the maternal system to maintain the functional lifespan of the corpora lutea. In the pig, the signal appears to be released by the blastocyst on approximately day 11 of pregnancy (Flint et al. 1982). This embryonic signal may act locally on the uterine endometrium to prevent the endocrine release of prostaglandin F_{2a} that would cause luteolysis (Bazer and Thatcher, 1977). Estrogen is reported to be the embryonic signal produced by the pig blastocyst (Perry, Heap, and Amoroso, 1973). Blastocyst release of estrogen stimulates uterine secretion as indicated by the sequestering of calcium, prostaglandin F_{2a} and protein within the uterine lumen (Geisert et al 1982).

By days 10 to 12 of pregnancy, blastocyst development can vary as much as 48 to 72 hours between individual embryos within a litter (Anderson 1978; Geisert et al. 1982a). Since estrogen is produced at a certain stage of

blastocyst development (Geisert et al. 1982a, Bazer et al. 1982), it is possible that the older embryos will stimulate uterine changes to profit their further development, possibly creating an asynchronous uterine environment for the younger embryos.

Embryo transfer studies have demonstrated that porcine embryos must be less than 48 hours asynchronous with an advanced uterus to be capable of surviving to later stages of development. Survival rates of transferred embryos actually increase if the embryos are 48 hours ahead of the recipient (Anderson 1978; Polge 1982).

By using exogenous estrogen, the maternal recognition signal of an embryo can be mimicked to alter uterine secretions or induce embryonic mortality in pregnant gilts if given before day 11. Pope and First (1985) found that administering exogenous estrogen on days 9 and 10 caused complete embryonic death by day 30 of gestation. Morgan et al. (1987) reported that by using the same treatment schedule, the blastocysts were fragmented by day 16 of gestation gilts treated with estrogen.

This thesis will attempt to determine the possible uterine changes which result in early embryonic death after exogenous estrogen treatment on day 9 and 10. We will study the effects on embryonic survival and uterine changes during days 12 to 18 of pregnancy.

The following review of literature presents the current knowledge of factors affecting early embryonic mortality in

the gilt. This review will focus on the blastocyst and uterine endometrial development, and how they may affect early embryonic survival.

CHAPTER II

LITERATURE REVIEW

Estrous Cycle

In swine, the estrous cycle usually ranges from 18-22 days with behavioral estrus lasting 24-72 hours. Ovulation usually occurs 38-42 hrs after the onset of estrus and can occur over 4-6 hours (du Mesnil du Buisson et al. 1970).

An inverse relationship between follicular stimulating hormone (FSH) and estrogen seems to exist. During days 16-20 of the estrous cycle, FSH decreases as estrogen increases. After ovulation when estrogen is low, there is evidence of a small surge of FSH. The highest FSH levels occur approximately 1-5 hrs after the spike of luteinizing hormone (LH) ranging from 11.3 to 18.5 ng/ml (van de Wiel, 1981). During the remainder of the estrous cycle, FSH release remains near 7 ng/ml (Rayford et al. 1974).

Peripheral plasma levels of LH peak during estrus, reaching a concentration of 6 ng/ml. This preovulatory LH surge peaks on the first day of estrus (day 0) about 12 hr after the start of its initial rise (Parvizi et al. 1976). It is thought that the LH surge is actually triggered by a sustained increase of estrogen during proestrus. After the ovulatory surge, LH concentrations return quickly to basal

levels (<1 ng/ml) for the remainder of the estrous cycle (van de Wiel, 1981).

Follicular growth is initiated during the luteal phase as the number of small antral follicles (2-5 mm) increases. Between days 14-16, follicles destined to ovulate will increase to 8-11 mm in size (Anderson, 1987). During this period, follicular recruitment can occur as demonstrated by the ability of the ovary to compensate for any follicles damaged by electrocautery (Clark et al. 1979) or lost by unilateral ovariectomy (Clark et al. 1982; Coleman and Dailey, 1979). After day 16, the ovary can not replace follicles damaged or lost by these methods nor will it respond to a superovulation and still ovulate by day 21 (Phillippo, 1968; Hunter, 1972).

Follicular steroidogenesis in the gilt (Haney and Schomberg, 1981; Evans et al. 1981) follows the two cell theory originally proposed by Falck in 1959 for the rat. Granulosa cells lack the 17a-hydroxylase enzyme necessary to convert progesterone or pregnenolone to androgens (Bjersing and Caisteosen 1967). Thecal cells compensate for the granulosa cell deficiency by synthesizing androgens, mainly androstenedione from progesterone or pregnenolone (Evans et al. 1981). Androgens produced by thecal cells are transferred to granulosa cells where androstenedione is converted to testosterone and then aromatized to estrogen. The theca interna of the gilt can produce significant amounts of estrogen which is a property unique to the pig.

However, considerably more estrogen is produced by the granulosa cells when they are co-cultured with the theca interna. In vitro, thecal cells do not respond to FSH stimulation with increased androgen production. However, thecal cells will respond to LH stimulation in vitro, which results in an increased production of androgen as follicular growth continues (Evans et al. 1981). A general model for theca and granulosa cell interaction in the bovine preovulatory follicle has been proposed by Hansel and Fortune (1978). They suggest that the high estrogen concentration in the follicle inhibits progesterone production. However, estrogen causes an increase of pregnenolone available to the thecal interna for the production of androgen. The decrease in intrafollicular estrogen concentrations after the LH surge probably enhances the conversion of pregnenolone to progesterone.

Peripheral plasma estrogen concentrations begin to increase on approximately day 15-18 of the estrous cycle as progesterone concentration declines. Peak values for estrogen of 38 pg/ml appear two days before estrus which reflects the rapid growth of the graafian follicles (Guthrie et al. 1972). After reaching its peak, estrogen drops steadily to approximately 25 pg/ml by estrus and remains low (<20 pg/ml) throughout the luteal phase (Hansel and Eckternkamp, 1972).

The length of the estrous cycle is determined by the lifespan of functional corpora lutea (CL). After ovulation,

the granulosa and theca cells within the porcine follicle differentiate into large and small luteal cells of the corpus luteum (Corner, 1920). These different cell types have not been extensively studied in the gilt, but have been characterized in the ewe (Fritz et al. 1982). Large luteal cells in the ewe contain few receptors for luteinizing hormone, but have many receptors for prostaglandin E, prostaglandin F_{2a} and estrogen. Large luteal cells secrete 80% of the progesterone (P4) and make up 30% of the total CL volume. Small luteal cells have many LH receptors yet few receptors for prostaglandin and estrogen. Although small luteal cell make up the majority of CL volume and respond to LH simulation in vitro, they only secrete about 20% of the total progesterone produced by the CL. The number of large luteal cells peak at approximately day 12 in the estrous cycle of the ewe (Schwall et al. 1986). Ali and Hansen (1984) propose that as the CL ages, the small luteal cells become large luteal cells.

The CL of the pig is independent of pituitary LH support until day 12 of the estrous cycle. Up to this day, LH receptors are tightly bound and the CL is refractory to exogenous prostaglandin F_{2a} (PGF_{2a}) or hypophysectomy. After day 12, the number of unbound LH receptors and PGF_{2a} receptors increase (Henderson and McNatty, 1975).

Peripheral progesterone concentrations in the gilt are basal (<1 ng/ml) at 2 days before estrus to 2 days after estrus. After day 2 of the estrous cycle, progesterone

levels increase rapidly to a peak concentration of approximately 33 ng/ml on days 12-14 (Hansel and Eckternkamp, 1972). On approximately day 15, an abrupt decline of progesterone concentration is observed as the CL regress.

If the gilt is not pregnant, luteal regression is initiated on approximately day 15 (Guthrie et al. 1972). Luteal regression is proposed to be caused by the synthesis and release of PGF_{2a} by the uterine endometrium (Bazer et al. 1982). Anderson et al. (1961) indicated that hysterectomy before day 12 of the cycle resulted in an extension of corpora lutea lifespan. Destruction of endometrial epithelium or congenital absence of uterine endometrium also leads to prolonged CL maintenance (Anderson et al. 1969). In vitro studies have shown that the pig endometrium produces significant amounts of PGF_{2a} during the latter stage of the estrous cycle (Patek and Watson, 1976; Guthrie and Rexroad, 1980). Frank et al. (1977) and Moeljono et al. (1977) reported that the concentration of PGF_{2a} in the utero-ovarian vein was greatest during the period of luteolysis in cyclic gilts. Plasma concentration of prostaglandin F_{2a} in the utero-ovarian vein reach peak levels (2.86 ng/ml) on days 12-14 of the estrous cycle (Gleeson et al. 1974; Moeljono, 1977; Frank, 1977).

Prostaglandin F_{2a} is capable of acting systemically in the gilt since only 20% of it is metabolized during one passage through in the lungs, unlike the ewe and cow in

which 99% is metabolized (Davis et al. 1979). The gilt rarely maintains a unilateral pregnancy, since the vacant horn will release PGF_{2a} in the systemic circulation before day 14 which causes CL regression on both ovaries. Prostaglandin F_{2a} works locally on the ipsilateral ovary, but can act systemically to cause CL regression on the contralateral ovary (Anderson et al. 1961; Spies et al. 1960).

Hickens et al. (1974) suggested that PGF_{2a} binds to the receptors on the luteal cells causing the cell membrane to undergo a conformational change which decreases the LH binding capacity of luteal cells. Prostaglandin F_{2a} may also decrease the blood flow to the CL through vasoconstriction, thus causing their regression through restriction of nutrients to the cells (Jason et al. 1975). Binding of prostaglandin F_{2a} to its receptor uncouples cAMP production in the luteal cells, thus shutting down progesterone synthesis. Henderson and McNatty (1975) have proposed that prostaglandin F_{2a} activates lysosomal enzymes causing morphological regression of the luteal cells.

Pregnancy

The hormone profiles characteristic of pregnancy in the gilt are essentially identical as during the estrous cycle until day 14. At this time, the circulating hormone concentrations reflect the presence of embryos as the maternal system prepares for continued embryonic development during the 114 day gestation. This review will cover hormone

concentrations in the peripheral maternal system during early pregnancy. For hormone changes and concentrations during the last two trimesters of pregnancy, see the review by Bazer and First (1983).

Following ovulation, pituitary support is essential after day 12 through term. Du Mesnil du Buisson and Denamur (1969) found that pregnancy was terminated if the gilt is hypophysectomized at day 70. Kraeling and Davis (1974) reported a similar result when gilts were hypophysectomized at day 80-90. Therefore, these results suggest baseline LH support is needed throughout gestation.

Plasma progesterone concentrations peak at approximately 30-40 ng/ml by day 12-14 of pregnancy and then gradually decrease to 10-25 ng/ml by day 25 of pregnancy. This concentration of progesterone will be maintained until approximately day 100 of pregnancy. At this point, progesterone concentrations decline to approximately 9 ng/ml by day 112 and abruptly decrease to basal levels (<1 ng/ml) by one day after parturition (Guthrie et al. 1974; Robertson and King, 1974; Knight et al.1977).

Production of progesterone by the CL is vital to the maintenance of pregnancy in the pig. Abrupt abortion occurs if CL are removed through luteectomy or lysed by exogenous prostaglandin F_{2a} (Belt et al. 1971; Diehl and Day, 1974). However, if CL are carefully removed sequentially throughout early pregnancy, pregnancy will be maintained as the remaining CL increase their production of progesterone to

compensate (Thomford et al. 1984). Pregnancy can be maintained with as few as one CL.

Plasma prostaglandin F_{2a} concentrations in the utero-ovarian vein do not change significantly between days 12-24 of gestation with concentrations averaging .52 ng/ml. In contrast, nonpregnant gilts had increased concentrations between days 13-17 (1.37 ng/ml) which decreased by day 18 to .68 ng/ml (Moeljono et al. 1977; Bazer et al. 1982). The PGF_{2a} metabolite, (13,14 dihydro 15 keto-prostaglandin F_{2a}), is < 5 ng/ml in peripheral plasma during the 6 days before parturition afterwhich it increases abruptly to > 15 ng/ml on the day of delivery (Nara and First 1981).

Estrogen in peripheral plasma is present in both conjugated and unconjugated forms. However, estrone sulfate is the major estrogen in the peripheral circulation during pregnancy. Estrogen is produced by the pig conceptus, conjugated by the endometrium and released into the maternal circulation. Conjugated estrogens increase in a triphasic pattern throughout gestation (Knight et al. 1977; Stoner et al. 1981; Bazer et al. 1982). Concentration of estrone sulfate is detectable as a transient peak at day 12. Levels increase again from day 16 (60 pg/ml) to a peak level at day 30 (3 ng/ml) followed by a decrease by day 46 (35 pg/ml). Estrone sulfate concentrations then increase at day 60 until parturition, reaching concentrations of 3 ng/ml (Robertson and King,1974). Estradiol-17B is present in utero-ovarian vein plasma during days 12-21 of gestation with average

values around 28.2 pg/ml (Moeljono et al. 1977). Concentrations begin to increase between days 70-80 (3.8 to 14.8 pg/ml) and peak on the day of parturition at a concentration of approximately 130 pg/ml (Robertson and King, 1974; Knight et al. 1977).

Embryo and Placental Development

Fertilization rate in swine is usually quite high (95%) (Polge, 1982) barring any deformity or subclinical infection. Through natural mating, the boar will deposit 4-8 billion total sperm into the uterus (Rodolfo, 1934a; McKenzie, Miller and Bauguess, 1938; Polge, 1956). After a 2-3 hour capacitation period within the uterus (Hunter and Dziuk, 1968), there is about 100 sperm cells found at any one time between the utero-tubal junction and the ampulla (Hunter, 1982). The boar's sperm is viable in the female's reproductive tract for approximately 24 hours, but ova have a limited functional lifespan of about 6-12 hours.

If fertilization occurs normally, pig embryos will usually enter the tip of the uterine horn about 48 hours after ovulation (day 4 post initiation of estrus). Oviductal transport is under ovarian hormone control. Estrogen will cause the retention of eggs in the oviduct for up to 21 days (see review Dziuk, 1985), whereas progesterone will accelerate ova transport through the oviduct (Day and Polge, 1968). Uterine entry may take up to 24 hours before all embryos are transported into the uterine lumen (Oxenreider

and Day, 1965).

The pig embryo enters the tip of the uterine horn at the four cell stage. The four cell stage is particularly long in the pig compared to other early developmental stages (Hunter, 1974; Polge, 1982). After fertilization, the first cell cleavage occurs around 16-18 hours and the second at approximately 30 hours, however, the four cell stage can still be found 60-70 hours after fertilization. Polge (1982) reported that several proteins not found in one or two cell embryos are produced in vivo during the four cell stage. These proteins may be vital since porcine embryos become "blocked" at the four cell stage during in vitro culture (Polge and Frederick, 1968; Rudell and Vincent, 1968). By day 5, porcine embryos have reached the morula stage containing 16-32 cells and undergo the process of compaction. Compaction serves to form tight junctions between blastomeres and create a permeability seal to allow for a directional movement of fluid during blastocyst formation. Formation of outside and inside cells resulting from compaction also allows for cell differentiation of the inner cell mass by cellular position as described by McLaren, (1969). The process of compaction is dependent on the presence of calcium and calcium binding proteins. Culture of mouse embryos in calcium-free media will prevent compaction, but not cellular division (Ducibella and Anderson, 1979).

Niemann and Elsaesser (1986), reported that the

development of compacted morulae to cavitated blastocysts in vitro depends on the presence of estradiol-17B on day 5. The importance of estrogen is less clear as the blastocysts continue to expand within the zona pellucida (Niemann and Elsaesser, 1987). The specific uptake of the radiolabelled estradiol-17B indicates that early pig blastocysts contain specific binding sites for estradiol-17B, whereas the uptake of progesterone was nonspecific (Niemann and Elsaesser, 1984). Estrogen may provide a significant signal for continued embryonic development.

Blastocysts hatch from the zona pellucida during days 6-7 of gestation (Hunter, 1974). From day 8-10, hatched blastocysts remain spherical in shape increasing in diameter at a rate of approximately .25 mm/hr until they reach 10 mm in diameter (Geisert et al. 1982b). The increase in diameter at this stage appears to be the result of cellular hyperplasia. Once the blastocysts reach a 10 mm diameter, between day 11-12, they rapidly elongate at a rate of 30 to 45 mm/hr. Spherical blastocysts transform into tubular forms and rapidly become filamentous within a few hours. The tubular form is very transient, lasting only about 4 hours (Geisert et al. 1982b). Several reports have confirmed the presence of spherical, tubular and filamentous forms within the same uterine horn (Anderson, 1978; Geisert et al. 1982b). The rapid elongation phase on day 12 is not due to hyperplasia, but is the result of cellular remodeling as indicated by no change in DNA content or mitotic index

(Geisert et al. 1982b). Geisert et al. (1982b) examined the changes of trophectoderm and endoderm during elongation. They proposed that elongation starts with the movement of trophoblastic and endoderm cells toward the embryonic pole, thus forming the elongation zone. Junctional complexes of trophectoderm cells are changed allowing for a redistribution of cells toward the end of the tubular blastocyst. The endoderm maintains junctional complexes within the layer, but contacts the trophectoderm only through filopodia. This model allows a decrease in blastocysts diameter and a rapid increase in length.

Work by King and Ackerley (1985) indicated that mesoderm was forming beneath the embryonic disk by day 12 before elongating to the filamentous form. Mesoderm continued to spread from the embryonic disc and by 16 lined the yolk sac and chorion. Using immunocytochemical staining with IgG-gold complex, they found that trophectodermal cells stained weakly for estrone on day 10, which is the period when aromatase activity is first detected (Gadsby, Heap and Burton, 1980). On days 12, 14 and 16, the trophectoderm and yolk sac endoderm showed an intense reaction to antibodies to estradiol-17B. No staining was present in any cells that they identified as mesoderm. One theory suggests that the appearance of the mesoderm on day 12 may be the signal for the blastocyst to start its rapid elongation phase and that the estrogen produced at this time may be a product of the mesodermal outgrowth.

As elongation takes place, mesoderm is first identifiable as it expands from the inner cell mass to lie between the extraembryonic ectoderm (trophectoderm) and endoderm (King and Ackerly, 1985). As the mesoderm becomes more discretly organized, it splits to form two layers. One layer of mesoderm will become closely associated with the endoderm, thus forming the yolk sac. This layer is very important in the development of blood islands and blood vessels. The other layer of mesoderm that is closely associated with the trophectoderm (trophoblast), forming a portion of the chorion. A ridge of chorion will fold over the embryo, fuse together to form the amnion, later separating from the chorion. This stage in development usually occurs during days 13-17 of pregnancy.

Beginning of areolae development is first observed on the chorion by day 17 (Crombie, 1972). Areolae are specialized structures spread over the chorion positioned over uterine glands that take up nutrients. Through nonreceptor mediated pinocytosis, areolae absorb histrotroph and transfer the nutrients to an extensive network of fetal capillaries (Chen et al. 1975; Reneger et al. 1982). By the fourth week of gestation, the areolae are well developed (Perry, 1981), showing extensive folding to increase their surface area and absorptive capacity.

The pig embryo relies on the yolk sac and the transport of nutrients across the chorio-vitelline placenta until day 18. By day 20 the yolk sac will regress and become vestigial

as the allantois develops and expands to form the chorio-allantoic placenta which takes over nutrient transport (see review Perry, 1981).

The allantois, composed of an inner endoderm and outer mesoderm layer, develops as an outpouching of the hindgut on day 16 of gestation. Between days 18 and 30, the allantois expands to fill the exocoel. The allantois is important for the transport and storage of nutrients for the developing fetus. Fusion with the chorion forming the chorio-allantoic placenta has usually occurred by day 30. Mesoderm between the chorion and allantois will form the extra-embryonic blood vessels of the placenta, except for the very tips of the chorion where the allantois fails to fuse. Placental development is complete by days 60-70 of pregnancy (Bazer and First, 1983).

Blastocyst Synthetic Ability

After hatching, developing blastocysts are synthetically active in secreting steroids, polypeptides and prostaglandins. These embryonic products have been characterized but their functions for the most part have only been theorized.

Steriods

The production of estrogens by pig blastocysts has been extensively studied. Stone et al. (1986) found that spherical blastocysts collected between days 4-8 after

mating were able to produce a number of steroids including pregnenolone, progesterone, androstenedione, testosterone, estrone and estradiol from a media providing pregnenolone sulfate as a substrate. Many investigators have reported aromatase activity in preimplantation blastocysts between day 12-14 (Perry et al. 1973; Perry et al. 1976; Flint et al. 1979; Gadsby et al. 1980). Geisert et al. (1982a) correlated the types and levels of estrogens with the developmental stage of the blastocysts. They found that estrone, estradiol, and their sulphated forms were greatest in concentration in uterine flushings containing tubular and filamentous blastocysts. These results supported the estrogen concentrations found in the uterine flushings of day 12-18 pregnant gilts by Zavy et al. (1980).

All the enzymes necessary for estrogen production de novo are present in the blastocyst during days 16-20 (Heap et al. 1981). Elongated blastocysts are able to produce sufficient estrogen to reflect a rise of estrogen concentration found in the peripheral plasma levels on day 25 to 30. (Heap et al. 1981a).

Proteins

Polypeptide production by blastocysts has been characterized by Godkin et al. (1982). Blastocysts collected on day 10.5 to 18 of pregnancy were cultured with [^{3}H]-leucine to characterize polypeptides synthesized in vitro. The majority of the peptides released by blastocysts

from day 10.5 to 12 consisted of low molecular weight (20 to 25 daltons) acidic proteins (pI 5.6 to 6.2).

By day 13-16, the polypeptides released in to the media by filamentous blastocysts shifted to a more basic polypeptide of a higher molecular weight (40-50 daltons). The acidic proteins were still present but were less intense compared to the release of the basic proteins (Godkin et al. 1982). At day 18, basic proteins were still present, but lower in proportion. New polypeptides, corresponding to fetal serum proteins, appeared to be produced by the embryo and/or yolk sac (50-70 daltons) as trophectodermal protein production decreased.

Fazleabas et al. (1983) reported that the blastocyst synthesizes and releases a protease between days 10 and 16 of pregnancy. Blastocyst production of a plasminogen activator is first detected on day 10. Plasminogen activator works on a serum zymogen called plasminogen which is converted to plasmin. Plasmin acts to break down fibrin, which is a normal component of basement membranes and extracellular matrix (Werb et al. 1980). This polypeptide may even activate other proteases including collagenase (Werb and Aggelar, 1978).

Fazleabas et al. (1983) found that the release of plasminogen activator from conceptuses cultured <u>in vitro</u> is biphasic. The highest amounts are secreted between day 10-12 when rapid blastocyst elongation and cellular remodelling are taking place. The second phase occurs between days 14-16

when there is an rapid increase in cellular hyperplasia and expansion of the allantoic membrane. The function of plasminogen activator in the implantation of rodents may function to allow invasion into the uterine stroma. However, attachment in swine is non-invasive, thus plasminogen activator may be a by-product of cellular reorganization or may help the embryo loosen tight junctional complexes so that cellular remodeling can take place during elongation (Fazleabas et al. 1983).

Prostaglandins

Cultured spherical blastocysts (days 4-8) are able to release mainly prostaglandin E_2 and H_2, an endoperoxide precursor to many different prostaglandin and prostaglandin-like products (Stone et al. 1986). This corresponds to the prostaglandin E_2 receptors that are found in the endometrium of the pig during days 5, 10, and 15 of early pregnancy (Kennedy et al. 1986) Prostaglandins of the E series are proposed to increase blood flow, and activate adenylate cyclase in the uterus. Thus, PGE could enhance the synthetic ability of the endometrium for later histotroph release.

The production of prostaglandin F_{2a} from conceptuses during days 10-18 has been demonstrated in several experiments by comparing the increased concentration in the uterine flushings of a pregnant gilt as compared to nonpregnant or pseudopregnant gilts (Zavy et al. 1980;

Geisert et al. 1982a). Guthrie and Lewis (1986) found that embryonal membranes cultured in vitro on days 13, 16, and 19 were able to produce more PGF_{2a} than endometrium of the same time period on a concentration per gram of tissue basis. Lewis and Waterman (1983) reported that blastocysts cultured in vitro 16 days after estrus produced mainly PGE_2, and some PGFM and PGF_{2a} from arachidonic acid. Endometrium from the same period produced PGFM and PGE_2 from arachidonic acid, but only in small amounts.

The rate-limiting enzyme, phospholipase A_2, has been found in increased amounts in the blastocyst tissue during the period of elongation (Davis et al. 1983). Yet, prostaglandin F production may not be essential to the elongation process. Geisert et al. (1986) treated gilts with indomethacin, a prostaglandin synthetase blocker, and reported that the blastocysts were able to elongate similar to controls. However, Kraeling et al. (1985), found a loss of pregnancy after giving indomethacin from days 10 to 25 of pregnancy. So, perhaps prostaglandin has a role in attachment and placentation of the swine conceptus.

Embryonic Migration

Embryonic migration is important for embryonic survival to term in pigs as it is in other polytoccous species (i.e. rabbit, rat, mouse). Pig embryos will usually be near the tip of the uterine horn until about day 6 when they start to progress though the horns during days 7 and 8. As

early as day 9, embryos can be found on the opposite side from which they ovulated (Dhindsa et al. 1967). By day 12 of pregnancy, embryonic migration is essentially over as rapid trophoblastic elongation begins (Polge and Dziuk, 1970).

During uterine migration, embryos are able to move past each other and become randomly mixed between the uterine horns (Dziuk et al. 1964). The number of embryos present does not influence the rate of their migration nor their ability to become equally spaced (Dziuk, Polge and Rowson, 1964). The embryos are able to obtain an equidistant spacing from each other, probably by influencing uterine motility through some local factor. Pope et al. (1982b) indicated that estrogen and histamine are necessary for normal embryonic migration. Embryonic production of prostaglandins and uterine calcium may also influence the uterine myometrium locally to cause peristaltic and anti-peristaltic contractions for embryonic movement (Pope et al. 1982a).

The pig fetus depends on histotrophic nutrition provided by the endometrium of the uterus. The surface area of the placenta is important for the amount of nutrients that can be absorbed. Since the surface area of the placenta is directly correlated with the fetal weight and pig placentas will not overlap, the amount of uterine space will determine the surface area that the placentas can occupy. Uterine space is not a restrictive factor in embryonic survival prior to day 30. The uterus will grow locally and lengthen before day 30 as a direct effect of the conceptuses

presence (Knight et al. 1977). The conceptus influences during early pregnancy may be through the production of estrogen. Pope and First (1985) treated pregnant and nonpregnant gilts with estradiol valerate on day 12 and 13 and demonstrated an increased uterine length due to the estradiol treatment when examined on day 15. The filamentous blastocyst will occupy approximately 10 cm of uterine space and will cause an additional 10 cm of growth in the uterine horn. Thus, the conceptus generally needs approximately 20 cm of uterine space for development (Dziuk, 1985).

During the period of embryonic migration (days 6-12), critical morphological developments are occurring in the embryo. These crucial stages include morula compaction, blastocyst formation, hatching and elongation. Many of the factors influencing embryonic migration may also influence their development.

Placentation

Placentation in the pig is classified as epitheliochorial which means that the placental barrier between fetal capillaries and maternal capillaries include three maternal and three fetal layers. This type of placentation was considered the least efficient in the transport of nutrients by Grosser (1909).

Attachment of the pig blastocysts to the maternal uterine surface epithelium is a gradual process. King et al. (1982) has separated placentation in the pig into three

overlapping phases termed apposition, adhesion and attachment. Apposition which occurs on day 11-18, is considered to be the close positioning of the trophoblast with the uterine surface epithelium, but without any cell connections between the two layers. Adhesion is the beginning of tenuous cell contact between the trophoblast and the endometrial epithelium occurring during days 12-20. Attachment is the interdigitation of microvilli between the trophoblast and the endometrial surface epithelium occurring from day 16 to 24.

Embryonic migration essentially stops after elongation on days 12-13 (Dziuk, 1985). The uterine epithelium changes at this time from low columnar with a flat apical surface to a layer of cells having a dome-like apical surface with some protruding proliferations (Geisert et al. 1982b; Dantzer, 1985). The trophoblast will roughly match the domed endometrial surfaces by changing its apical surface to concave "caps" (Dantzer, 1985). These complementary surfaces serve to immobilize the blastocyst so that more intimate cell contacts can be established, however the trophoblast and the uterine epithelium are still separated by different amounts of uterine histotroph (Dantzer, 1985).

The trophoblast and the endometrial epithelium both display a glycocalyx on their apical surface (Dantzer, 1985). The maternal epithelium has a thicker, more defined glycocalyx that will thin as close apposition and attachment takes place. The trophoblast displays a more delicate

type of glycocalyx. Dantzer (1985) suggests that this glycocalyx may function in cell to cell recognition. Prior investigations using rabbits, (Anderson and Hoffman, 1984), rats, (Hewitt et al. 1979) and mice (Chavez and Anderson, 1985), which show a similar type of glycocalyx on the endometrial surface, found an alteration in the morphology of this glycocalyx just prior to the time of blastocyst implantation. Anderson et al. (1986) found that there is a change in the protein and saccharide make up of the apical surface of the endometrium of the rabbit prior to implantation that is regulated by progesterone. This apical surface was further modified by blastocysts at the sites of implantation. The glycocalyx is proposed to be made up of sialic acid which plays a protective role in masking antigenic or recognition sites during development in the mouse (Shutelsky et al. 1974). Removal of the sialic acid prior to the time of implantation (Anderson and Hoffman, 1984), possibly provides the blastocysts access to galactose-containing receptors (Anderson et al. 1986). Chavez et al. (1984) reported the incorporation of D-galactose in the mural trophectoderm of preimplantation mouse embryos. They propose that the blastocyst surface receptors for D-galactose may interact with the terminal residues of this sugar in the glycocalyx of the endometrium promoting adhesion. Even though the rodent species display a different type of implantation than the pig, the presence of the glycocalyx on the endometrium of both species may have a

similar function.

As close apposition occurs, the trophoblast displays only a few microvilli and the endometrial domes tend to become smoother with a disappearance of the epithelial protuberances (Geisert et al. 1982b; Dantzer, 1985). Dantzer (1985) found no evidence of junctional complexes between the trophoblast and the maternal epithelium as reported in King's review (1982). The trophoblast extends cytoplasmic branches between the epithelial domes into the pools of histotroph to increase the uptake of the histotroph. These trophoblastic "fingers" never penetrate further than the junctional complex of the endometrial epithelium. (Dantzer, 1985).

The interdigitation characteristic of the epitheliochorial type of placentation found in the pig was examined at days 15 and 16 by Dantzer (1985), which is two days earlier than reported by King et al. 1982. Trophoblast-uterine epithelial interdigitation starts at the apical domes of the maternal epithelium and the chorionic caps. This cell to cell contact starts at the area of the embryonic disk and proceeds towards the ends of the chorion with time. Thus, different phases of attachment can be observed throughout the placentation of each embryo (Dantzer, 1985).

Complete attachment by interdigitation is not accomplished until day 30 of pregnancy. However, even after interdigitation is completed, the chorioallantoic membranes

will stimulate formation of secondary ridges or rugae in the endometrium which will enlarge to increase surface area as pregnancy continues (Friess et al. 1980; Dantzer, 1984).

Maternal Recognition of Pregnancy

Maternal recognition of pregnancy is a functional relationship between the uterus, the embryo and the CL. Viable embryos maintain the CL and cue a response of the uterine endometrium to provide nutrients. A theory for maternal recognition in swine has been proposed by Bazer and Thatcher in 1977. Basically, they propose a change in the direction of secretion of PGF_{2a}, the uterine luteolysin, from an endocrine direction (i.e. toward the uterine vasculature) to an exocrine direction (i.e. toward the uterine lumen). Sequestering of PGF_{2a} into the uterine lumen of pregnant gilts is thought to be caused by the local action of estrogen produced by the elongating blastocysts around day 12 of pregnancy.

Endometrial explants of nonpregnant gilts at the late luteal stage cultured <u>in vitro</u> have a higher production of PGF_{2a} compared to pregnant gilts (Watson and Patek, 1979; Guthrie and Lewis, 1986). Yet, evidence for the sequestering of PGF_{2a} in pregnant gilts is substantial. Comparison of utero-ovarian vein plasma concentrations of PGF_{2a} between pregnant and nonpregnant gilts shows a significant decrease in levels found in pregnant gilts during days 12-17 (Moeljono et al. 1977; Frank et al. 1977).

During this same time period, the concentration of PGF_{2a} found in the uterine flushings of pregnant and nonpregnant gilts displays a dramatic increase of PGF_{2a} in the pregnant animals (Zavy et al. 1980).

This sequestering of PGF_{2a} into the uterine lumen during pregnancy prevents CL regression but doesn't protect it from the effects of exogenous PGF_{2a}. Exogenous prostaglandin F_{2a} causes luteolysis during pseudopregnancy (Kraeling et al. 1975) or pregnancy (Diehl and Day, 1974). Lung and endometrial conversion of PGF to its 13,14 dihydro 15 keto-prostaglandin F_{2a} metabolite (PGFM) is not significantly different from any day of the estrous cycle or pregnancy (Guthrie and Lewis, 1986). Thus, the maintenance of the CL is not due to an increased metabolism of PGF_{2a} or a change in sensitivity to PGF_{2a}, but the absence or low concentration of PGF_{2a} seen by the CL. There is also an increased uterine blood flow during this time (days 12-13) which may have a diluting effect on the concentration of PGF_{2a} which reaches the CL (Ford and Christenson, 1979; Keys et al. 1986).

In pregnant gilts, the developing blastocysts secrete large amounts of estrogen at the time of elongation. Zavy et al. (1980) and Geisert et al. (1982a) found a temporal relationship between the elongation of blastocyst and the rise in estrogen, PGE and PGF in the uterine flushings. Estrogen produced by the blastocyst appears to exert its effect locally. This embryonic signal is not transported

through the systemic circulation, since the pig will not maintain a unilateral pregnancy (Anderson et al. 1966; Christianson and Day, 1971). Thus, the embryo's signal from one uterine horn will not protect the CL on the contralateral horn from regression. The embryonic signal must also be quantitative since at least two embryos are necessary in each horn to provide enough of a signal for the PGF to remain in an exocrine direction throughout the uterus (Polge et al. 1966).

If a nonpregnant gilt is given exogenous estrogen to mimic the blastocyst release on days 11-15, pseudopregnancy is induced and the CL are maintained for an average of 92 days or longer (Frank et al. 1978; Bazer et al. 1982). Pseudopregnant gilts have low utero-ovarian plasma concentrations of PGF_{2a} comparable to that of pregnant gilts (Frank et al. 1977). They also have a higher concentration of PGF_{2a} in uterine flushings as compared to nonpregnant gilts on day 15-18 (Frank et al. 1978) but intermediate to levels found in pregnant animals (Zavy et al. 1980). This provides evidence that some of the prostaglandins found in the uterine flushings are of blastocyst origin (Lewis and Waterman, 1982; Guthrie and Lewis, 1986).

One theory suggests that estrogen is able to maintain the basement membrane of the endometrial epithelium so that PGF_{2a} is held in the uterine lumen (Bazer and Thatcher, 1977). Ryan and Woesner (1971) found that estrogen is a potent inhibitor of uterine collagenase activity which is

high in the involuting rat uterus on days 1-3 post-partum (Montfort and Perez-Tamayo, 1975). Further support of this theory was demonstrated by Ogra et al. (1974) in which prostaglandin F_{2a} was found in the epithelial mucosa of oviductal tissue in women during the preovulatory phase of the menstrual cycle. Yet after ovulation, PGF_{2a} was localized in the lamina propria. Another product of the uterine endometrium, uteroferrin, has been found to have a similar pathway to either the uterine lumen or the epithelial stroma. Between day 9-13 of the estrous cycle, uteroferrin is found in the uterine lumen, but by day 14, uteroferrin was found to be localized in the uterine stroma (Chen et al. 1975). The presence of blastocysts seem to keep uteroferrin secreted into the uterine lumen since it is never found in the epithelial stroma in pregnant gilts (Chen et al. 1975).

The movement of PGF_{2a} in an exocrine direction may be related to histotroph release to the uterine lumen by the same estrogen signal. Geisert et al. (1982a) reported a synchronous release of secretory vesicles from the endometrial epithelium during the estrogen release by tubular blastocysts. Estrogen treatment on day 11 of estrous will cause a rise in the calcium concentration in the uterine lumen (Geisert et al. 1982c). This calcium release may facilitate the secretory vesicle release containing histotroph, perhaps similar to the way calcium triggers the vesicle release of neurotransmitter in a nerve synapse

(Rubin and Laychock, 1978). Calcium may also trigger the enzyme, phospholipase A_2, to cause the release of arachidonic acid from the cell membrane as is found in the adrenal cortex (Rubin and Laychock, 1978). Arachidonic acid can be a substrate for prostaglandin production in both the epithelium (Guthrie and Lewis, 1986) and the trophectoderm (Lewis and Waterman, 1982). These membrane changes may facilitate exocytosis by the epithelium and endocytosis by the embryo.

Uterine Environment

The uterus provides an environment for embryonic development. Yet, the uterus is not always a place suitable for embryonic life. If the embryos are placed in the uterus too early after estrus or too late, they will not survive. Uterine and embryonic development, and the signals produced by both, must be a highly synchronized and delicately balanced proceeding, if normal pregnancy is to be achieved.

Since placentation in swine is of the non-invasive epitheliochorial type, porcine embryos must depend upon the uterus to provide a high level of nutrients throughout pregnancy. These nutrients termed histotroph or uterine milk contain a complex mixture of proteins, ions, carbohydrates and steroids.

The uterus requires progesterone to induce the synthesis and transport of the proteins and steroids characteristic of histotroph. The protein profiles

characteristic of early pregnancy in the gilt was first identified through two dimensional gel electrophoresis by Basha et al. (1980). Two proteins, uteroferrin and plasminogen inhibitor, have been selectively identified and their functions examined.

Uteroferrin

One protein found to be synthesized and released by the uterine endometrium is uteroferrin. Uteroferrin is a glycoprotein of 35,000 molecular weight with a pI of 9.7 (see review Roberts and Bazer, 1980). It has a distinctly purple color when oxidized, and a pink color when reduced (Schlosnagle et al. 1976). Although earlier reports indicated that uteroferrin carried only one molecule of iron (Fe^{+2}) (Schlosnagle et al. 1974); a more recent report suggests that uteroferrin actually carries two molecules of ferric iron (Raub et al. 1985). The glycoprotein has acid phosphatase activity which increases several fold when reduced with mild agents such as 2-mercaptoethanol or ascorbate (Schlosnagle et al. 1974). The acid phosphatase activity provides a convenient marker for detection of uteroferrin, since 95% of the acid phosphatase activity in the pig uterus is provided by uteroferrin (Basha et al. 1979).

Recent findings of Baumbach et al. (1986) has indicated another possible form of uteroferrin may exist. It has a higher molecular weight (80,000 daltons), a stable pink color that does not change with oxidation and has similar

chemical characteristics as the lower molecular weight purple uteroferrin after reduction. These authors concluded that the pink form of uteroferrin is a heterodimer of activated purple uteroferrin and another protein chain of unknown function.

Chaichimansour et al. (1985) reported that the acid phosphatase activity in uterine flushings did not change between pregnant and nonpregnant gilts from day 0-18. However, examination of epithelium for histochemical staining of uteroferrin from nonpregnant gilts during days 9-13 has indicated uteroferrin was present in the lumen of the uterine glands. But by day 14, uteroferrin is localized in the endometrial stroma surrounding the basement membrane of the uterine glands. It is thought that uteroferrin may be picked up by the uterine vasculature, transported to the liver and spleen and degraded (Chen et al. 1975). In pregnant gilts, uteroferrin is always found within the uterine epithelial cells, lumen of the uterine glands and placental areolae (Chen et al. 1975).

Uteroferrin can be induced in ovariectomized gilts under a progesterone or progesterone-estradiol treatment, but not under estradiol treatment alone (Basha et al. 1979). There is no significant increase of acid phosphatase activity in uterine flushings after estradiol treatment on day 11 compared to controls, even though this estrogen treatment will induce pseudopregnancy. However, Geisert et al. (1982c) using two dimensional gel electrophoresis

determined uteroferrin was released 12 hours earlier by estrogen treatment on day 11. The exogenous estradiol that induces pseudopregnancy or the estrogen from the elongating blastocysts seems to maintain the release of uteroferrin into the lumen of the glands instead of into the uterine stroma as found in non-pregnant controls. Estrogen may influence uteroferrin's release by working synergistically with progesterone.

Uteroferrin is thought to carry iron from the maternal system to the conceptus throughout pregnancy for use in fetal erythropoiesis. The fetus stores uteroferrin in the allantoic fluid starting by day 35 (Bazer et al. 1975). Renengar et al. (1982) proposed a pathway for uteroferrin transport from the uterine glands to the fetus according to their observations during mid to late pregnancy. Uteroferrin is secreted by uterine glands and picked up through the placental areolae by non-receptor mediated pinocytosis. It is then transferred to the fetal capillaries and carried to the fetal liver for use in erythropoiesis. If uteroferrin is not picked up by the fetal liver, it is partially filtered by the kidney and stored in the allantoic fluid.

The action of uteroferrin during early pregnancy before actual erythropoiesis is initiated in the embryo may be involved in placental development. Chen and Bazer (1973) passively immunized gilts against uteroferrin and found that placenta lengths and weights were significantly reduced. Conversely, when Knight (1974) supplemented the maternal

system with exogenous progesterone, an increase in uterine secretory activity and a corresponding increase in placental length and weight occurred.

Plasminogen Inhibitor

The uterine endometrium secretes a group of low molecular weight, basic protease inhibitors in response to progesterone stimulation. Fazleabas et al. (1982) purified and characterized a plasmin/trypsin inhibitor, also known as the plasminogen inhibitor for its known action against plasminogen activator. This inhibitor is specifically secreted by the uterine surface epithelium and by the upper glandular epithelium (Fazleabas et al. 1985).

In pregnant gilts, plasminogen inhibitor is found in low amounts on day 6 but will reach its highest concentration by day 12 and then decline (Chaichimansour et al. 1985). This time corresponds with the release of estrogen from the blastocyst (Bazer et al. 1982), the time of rapid cellular remodelling during embryonic elongation (Geisert et al. 1982b) and the time of highest concentration of plasminogen activator release by the porcine blastocysts (Fazleabas et al. 1982).

The plasminogen inhibitor concentration found in cyclic gilts can be increased during days 12-14, if these gilts are given an injection of estradiol valerate on day 11 (Fazleabas et al. 1983). This suggests that estrogen may enhance plasminogen inhibitor production or induce a release

of plasminogen inhibitor such as that seen by Geisert et al. (1982a) with secretory vesicle release after estradiol treatment.

Plasminogen inhibitor may protect the uterine epithelium from the invasive action of the blastocysts. Histochemical staining has demonstrated that plasminogen inhibitor coats the trophectoderm and the uterine surface epithelium. The trophoblast also becomes coated with the plasminogen inhibitor through endocytosis (Fazleabas et al. 1982). The amount of plasminogen inhibitor produced by the endometrium is more than enough to neutralize the plasminogen activator produced by the blastocysts (Mullins et al. 1979). Plasminogen inhibitor may also protect macromolecules present in the uterine lumen, such as uteroferrin, from enzymatic digestion before they can be absorbed by the chorion (Fazleabas et al. 1982).

Prostaglandins, Carbohydrates, Steroids

Uterine histotroph contains many other factors whose identities are known but functions are less clear. Prostaglandins F and E are sequestered within the uterine lumen during pregnancy (Bazer and Thatcher, 1977; Zavy et al. 1980). They are produced by the uterine endometrium under progesterone influence at mid to late luteal phase (Patek and Watson,1976) and also by the developing blastocysts (Zavy et al. 1980; Lewis and Waterman, 1983). The presence of the prostaglandins in the uterine lumen

suggests they may provide some support to embryonic development beyond that of just saving the CL from lysis. Maul-Walker et al. (1977) discovered that conceptus membranes were able to metabolize PGF_{2a} to PFGM thereby inactivating its biological activity. However, Kennedy (1980) and Kraeling et al. (1985) suggest that PGF may function in implantation.

Zavy et al. (1982) reported that the content of glucose and fructose in uterine flushings of pregnant gilts increases as pregnancy proceeds. Fructose may be an important storage form of glucose, since it does not freely diffuse through plasma membranes like glucose (Huggett et al. 1961), can be sequestered in the fetal membranes and/or reproductive tract and can be easily converted into glucose by the blastocyst (Goodwin, 1956). Zavy et al. (1982) indicated that the amount of glucose was correlated with the amount of estrone found in the uterine flushings. Estrone produced by the pig blastocysts at this time (Zavy et al. 1980; Stoner et al. 1981) may increase glucose transport to the uterine lumen as has been reported for rats (Roskoski and Steiner, 1967).

Zavy et al. (1982) also examined ascorbic acid concentrations in the uterine flushing. Concentrations of ascorbic acid increased during pregnancy and may be produced from glucose. Ascorbic acid has many known functions such as involvement in collagen synthesis, iron absorption and metabolism (Buhi, 1980) and serves as an antioxidant for

vitamins A, E, and B-complex.

Murray et al. (1980) and Moffatt et al. (1980) identified the presence of riboflavin in the uterine flushing collected during day 6-8 in the estrous cyc or pregnancy. The function of riboflavin in the pig is known, but in the chicken embryo, it can influence survival Also in the mouse, termination of pregnancy can be caused y treatment with antiserum to the riboflavin carrier p tein (Natraj et al. 1987). In tissues, riboflavin combine with phosphoric acid to form the coenzymes, flavin mononu eotide and flavin adenine dinucleotide, which are important s hydrogen carriers in the oxidative systems in the mitochondria. These coenzymes are essential for the oxidation of glucose and fatty acids and for cellula growth since ultimately they cause the phosphorylation of A to ATP.

Besides secreting histotroph, the uterine endom rium also has the ability to metabolize steroids of mater l and fetal origin, particularly progesterone and estrogen (Magness et al. 1986; Dwyer and Robertson, 1980). Ma ess et al. (1986) compared the levels of progesterone in th systemic and utero-ovarian vein plasma of nonpregnan pregnant and hysterectomized gilts. They indicated t ovarian production of progesterone in pregnant gilts actually increased from days 14 to 18 as compared to nonpregnant gilts. Yet the systemic levels of proges rone in pregnant gilts showed a decrease in levels on day 13-17

and then remained constant, whereas in the hysterectomized gilts, progesterone levels remained steady throughout the sampling period (to day 21). Comparing the differences between hysterectomized and pregnant gilts shows an active uptake and/or utilization of progesterone by the uterus that is influenced by the conceptuses. These results support the work by Knight et al. (1977) who showed a positive arterial venous difference with progesterone by the uterus.

Zavy et al. (1980) examined the uterine flushings of pregnant and nonpregnant gilts to determine the concentrations of steroids. He found a higher concentration of progesterone in the uterine lumen in nonpregnant gilts than in pregnant gilts. This difference supports the concept that conceptuses exert an influence over the metabolism of progesterone during pregnancy.

It has been demonstrated that progesterone can be metabolized by the endometrium to androgens, estrogens and conjugated estrogens (Dueben et al. 1977; Dueben et al. 1979). However, these results are not supported by the report of Fischer et al. (1985). These authors found no evidence that the endometrium from pseudopregnant gilts will convert tritiated progesterone into precursors that can be aromatized to estrogens. However, the endometrium from the pregnant gilts did produce some estrone and estradiol <u>in vitro</u>. An enzyme present in the endometrium, 5a reductase, will metabolize progesterone into inactive metabolites that are non-aromatizable and thus will rapidly deplete the pool

of progesterone (Fischer et al. 1985). These reduced metabolites of progesterone were also identified by Henricks and Tindal (1971).

Progesterone activates enzyme systems in the endometrium. Meyers et al. (1983) demonstrated the induction of estrogen sulfotransferase activity in ovariectomized pigs under long term progesterone treatment or in gilts primed with estrogen and then given progesterone. The priming estrogen is thought to increase the number of progesterone receptors which will in turn increase the enzymatic activity. Dwyer and Robertson (1980) studied the activity of the estrogen sulphatase and sulphotransferase systems in the endometrium of the pregnant sow and ewe. These opposing enzyme systems are found in inverse levels throughout pregnancy. In the sow, the sulphotransferase activity peaks by day 30 and then slowly declines, whereas the sulphatase activity starts to rise around day 80 of pregnancy. The sulphotransferase enzyme is thought to deactivate the estrogens produced by the conceptuses during pregnancy, before releasing them to the maternal system (Heap and Perry, 1974).

Embryonic Mortality

The percent of embryonic loss after fertilization is fairly high (35-45%) in the pig (Pope and First, 1985) with the majority occurring before day 25 (see review Flint et al. 1982). During this time, the embryo goes through many

crucial changes in its development such as migration, maternal recognition of pregnancy, elongation and placentation. Different theories have been proposed to explain the causes of embryonic mortality. Studies have suggested that embryo mortality can result from an inadequate concentration of progesterone during the early pregnancy (Sammelwitz et al. 1956; Haines et al. 1958), a limited uterine capacity (Hammond, 1921) and that the uterus secretes some critical biochemical substrate that can be limited in quantity (Bazer et al. 1969; Bazer, 1975). The investigation into embryonic mortality reveals many details on early embryonic development, embryo-embryo interactions, and embryo-endometrium interactions.

Recent studies with the Chinese breeds of swine have revealed many answers concerning uterine capacity and genetics of reproduction. The Chinese breeds, which are known for their prolificacy, have an average litter size of 16 to 17 piglets (Gianola et al. 1982). This is a noticeable difference from the breeds common to the U.S. and Europe that average a litter size of only 9 to 10 piglets. Bazer et al. (1987) have compared the Chinese Meishan breed to the Large White, a European breed commonly known for its maternal traits. They found that the ovulation rate of the Large White was actually higher than that of the Meishan. They also reported that the Large White breed had longer uterine horns and a greater endometrial surface area when measured at day 30 of pregnancy.

A basic difference found between the Meishan and the Large White was in the blastocysts. The Meishan blastocysts developed faster. Meishan blastocysts were spherical on day 8 and 10, tubular by day 11 and reached filamentous by day 12. These developments are approximately 24 hours earlier than that seen in the Large White. In addition, the variability in blastocyst development was low compared to blastocysts recovered from the Large White gilts.

When examining differences in conceptus characteristics, total protein, allantoic glucose, fructose, placental weight, embryonic weight and crown-rump length were measured. The only difference found was a higher placenta weight of conceptuses in the Large White. The researchers suggest that the placenta of the Meishan may be more efficient since it must function in a smaller space with less surface area.

The uterine flushings of the Meishan gilts showed an increased concentration of total protein, fructose, glucose, PGF, PGE and acylaminopeptidase. These researchers conclude that the conceptus must give either a stronger signal or that the uterine epithelium must have increased secretory ability per unit area. This proposal has been suggested in the Finn sheep by using latex casting. The caruncles of the Finn sheep shows many times the vascularity than the normal single bearing breeds (Barron, Bazer and Zavy, personal communication).

Goldbard and Warner (1982) reported on a gene that

controls early conceptus development in mice in which they labeled the preimplantation embryonic development (PED) gene. Since the Meishan breed displays a rapid early conceptus development, the possibility of a porcine PED gene exists. However, many other factors, such as ovulation time and/or uterine environment, probably play an important role in the subsequent litter size in the Meishan.

The development of embryos within a litter varies. This variation may be due to the length of ovulation which can take up to 6 hours (Burger,1952) and the time of entry into the uterus which can span up to 24 hours (Oxenreider and Day, 1965). Embryos of different developmental states, spherical to filamentous, have been found in the same uterine horn (Anderson, 1978; Geisert et al. 1982a). Thus, a 24 hour difference of development can normally exist. With the rapid development required of embryos during elongation, this variation in development may be detrimental.

Through embryo transfer, it has been shown that the porcine embryo can tolerate a 24 hour asynchrony with its uterine environment (Polge, 1982). Pope et al. (1982) used this information to study the possible interaction between "old versus young" blastocyst development. They transferred day 7 blastocysts and day 5 blastocysts into a day 6 recipient. The older blastocysts showed a definite increase in survival over that of the younger blastocysts when measured at day 60 (Pope et al. 1982). To check the physiological soundness of the previous study, Pope et

al. 1986 used a closer synchrony of blastocyst age. They placed day 6 blastocysts into a day 7 pregnant uterus and vice a versa. They reported that the younger embryos, whether they were of the original litter or those transferred, did not survive as well or develop as rapidly when competing with the day 7 embryos. It seems that the older blastocysts are able to wait for the uterus easier than it is for the younger blastocysts to catch up (Pope et al. 1986).

The steroidogenic activity of the blastocysts to release estrogen increases when it reaches a 10 mm spherical stage on about day 11.5 (Geisert et al. 1982a). This stage corresponds with the period of maternal recognition of pregnancy (Dhindsa and Dziuk, 1968) and only shortly precedes the rapid elongation phase (Geisert et al. 1982a,b). During rapid transition to the filamentous form, the blastocyst is able to signal the uterine endometrium to change the environment to be more suitable for its advanced embryonic development (Geisert et al. 1982b,c). It seems logical that those blastocysts that reach this developmental stage first would signal the uterus to suit their needs leaving any other underdeveloped blastocyst in a very different and perhaps even hostile environment.

To determine what sort of uterine changes are brought about due to the blastocyst release of estrogen, Geisert et al. (1982b) examined the glandular epithelia through transmission microscopy and found a concerted secretory

vesicle release in pregnant gilts carrying tubular and filamentous blastocysts. There was also an increased concentration in prostaglandins F and E, protein, calcium, and estrogens found in uterine flushings. Previous studies have also shown the estrogen appears to be responsible for the increased concentrations of prostaglandins found in the uterine lumen pregnant or pseudopregnant gilts on day 15 (Frank et al. 1978; Zavy et al. 1980). Geisert et al. (1982c) examined the endometrial changes resulting from giving estradiol valerate to cyclic gilts on day 11. The uterine flushings were analyzed at 1, 4, 12 or 24 hours post injection. They found an increase in the amount of total calcium, protein, prostaglandins E and F at 12 and 24 hours similar to those found during days 11 and 12 of pregnancy.

Knowing that an increased prostaglandin concentration is present at the time of blastocyst elongation, Geisert et al. (1986) examined the role of prostaglandins during this time. They blocked the production of prostaglandin by giving pregnant gilts indomethacin or banamine. These inhibitors decreased the amount of acid phosphatase activity and total protein released. However, the inhibitors did not stop blastocysts from rapidly elongating. Kraeling et al. (1985) also treated gilts with indomethacin during the days 10-25. They found that the embryos did not survive this prolonged indomethacin treatment. Therefore, prostaglandins or possibly the proteins that Geisert et al. (1986) found suppressed may have a role in placentation and attachment.

Dantzer (1985) examined the epithelium-chorion interaction during early attachment. He found evidence of a glycocalyx on the trophoblast and the endometrium. The glycocalyx on the endometrial side became thinner as apposition and attachment takes place. These glycocalyces are suggested to function in cell to cell recognition. One theory under investigation suggests that after the estrogen signal is given by the porcine blastocysts, this glycocalyx may be altered in someway which may allow attachment of the trophoblast for only a limited period. Those blastocysts not yet ready for attachment may forfeit survival.

Studies done by Morgan et al. (1987) examined the development of blastocyst and endometrium if the estrogen signal was provided before the blastocyst would normally release it in vivo. This experiment was aimed at mimicking the signals given by older embryos to the uterine endometrium and the resulting affect on younger embryos. They treated pregnant gilts with estradiol valerate on day 9 or days 9 and 10. They reported normal blastocyst elongation and appearance at day 11 and 12, yet by day 16, the conceptuses were fragmenting and degenerating. The uterine environment was changed by day 12 as determined by analysis of the uterine flushings which found increased concentrations of protein, acid phosphatase, and estradiol, but a decreased concentration of calcium and prostaglandin F_{2a}. On day 16, the uterine flushings had no differences in calcium, protein, and PGF_{2a}, yet had a lower acid

phosphatase activity. These results supported the earlier findings of Geisert et al. (1982) of uterine release due to estrogen administration.

The estradiol valerate itself is not embryocidal as shown by Pope et al. (1985). They demonstrated that gilts injected with estradiol valerate on day 12 and 13 had normal embryonic development. Yet, gilts injected on days 9 and 10 showed a complete loss of embryos when examined by day 30.

The present study plans to investigate more closely this period of embryonic death. Changes in the uterine environment associated with the embryonic degeneration and proteins synthesized by the blastocysts and the endometrium in vitro will be examined. Results from this study may provide some insight as to the mechanisms involved with early embryonic mortality in the gilt.

CHAPTER III

EFFECT OF ADVANCING UTERINE ENVIRONMENT WITH EXOGENOUS OESTROGEN ON PORCINE CONCEPTUS SURVIVAL

Introduction

Porcine blastocysts synthesize and release oestrogens (Perry et al. 1973, 1976; Stone and Seamark, 1985, Fischer et al. 1985) during the period (Day 11-12) of rapid trophoblastic elongation (Geisert et al. 1982a). Development from a spherical to filamentous morphology occurs coincidently to the period of maternal recognition of pregnancy in the pig (Perry et al. 1973; Flint et al. 1979). Since administration of exogenous oestradiol to cycling gilts from days 11-15 will induce pseudopregnancy (Gardner et al. 1963; Frank et al. 1978), conceptus oestrogen release during this period has been proposed to be the signal provided for maintenance of pregnancy (see review Bazer et al. 1984).

Through the analysis of uterine flushings of cycling, pregnant and pseudopregnant gilts, it has been demonstrated that the biochemical composition of the uterine environment changes after oestrogen stimulation through either production by the blastocyst or by exogenous administration (Geisert et al. 1982a,b; Morgan et al. 1987). It appears

that this uterine secretory response cannot be induced by oestrogen prior to the 10th day of the oestrus cycle (Geisert et al. 1987). The responsiveness of the uterus is therefore in synchrony with normal blastocyst development and oestrogen release. Although the uterus appears to be refractory to oestrogen until at least day 10, oestrogen administration prior to this time results in pregnancy failure. Pope et al. (1986) reported that pregnant gilts given exogenous oestradiol valerate on day 9 and 10 had total embryonic loss on day 30. In contrast, gilts administered exogenous oestrogen on day 12 and 13, when the conceptuses normally produce oestrogen, were unaffected. Using a similar oestrogen treatment regime, Morgan et al. (1987) reported that administering exogenous oestrogen resulted in conceptus degeneration by day 16. However, examination of blastocysts on day 11 and 12 indicated that development and elongation was not affected. These authors indicated that alteration in the biochemical composition of the uterine environment a premature estrogen stimulation, may have provided an uterine environment which was unfavorable to blastocyst attachment and survival.

This study was designed to more clearly define the period of embryonic death after oestrogen administration on day 9 and 10, and to determine the uterine secretory changes which coincide with conceptus degeneration.

Materials and Methods

Experiment 1

Five gilts were utilized in an initial study to determine if progesterone production from the CL was maintained after oestrogen administration on day 9 and 10 of pregnancy. Gilts were checked for oestrus activity twice daily (0700h and 1800 h) with intact boars and bred naturally upon standing heat (day 0 of pregnancy), 12 and 24 hours later. On days 9 and 10, gilts received either 5 mg oestradiol valerate i.m. (n=3) or sesame oil (n=2). Blood samples were taken via jugular venipuncture on days 12, 14, 16, 18, and 20 of pregnancy. Gilts were hysterectomized on day 20 of pregnancy and uteri were flushed to confirm the presence or absence of conceptuses. Plasma samples were frozen and stored at - 20^{0} C until analyzed for concentration of progesterone by radioimmunoassay.

Experiment 2

Sixteen cyclic crossbred gilts of similar age (8-9 months), weight (100-130 kg) and genetic background were checked for oestrus behavior twice daily (0700 h and 1800 h) in the presence of intact boars and bred naturally at the onset of oestrus,(designated day 0), and 12 and 24 hours later. After breeding, gilts were randomly assigned (n=8) to receive one of the following treatments: Control, intramuscular injection (.25 ml) of vehicle (sesame oil) on

Day 9 and 10 of pregnancy or Treatment, intramuscular injection of 5 mg oestradiol valerate [Menaval 20 by R.J. Legere and Co., Scottsdale, AZ, USA] on Day 9 and 10 of pregnancy. Gilts were randomly assigned within treatment group to be unilaterally hysterectomized on either day 12 and 14 or day 16 and 18.

Gilts were unilaterally hysterectomized after induction of anaesthesia with a 5% solution of thiopentone sodium (Abbott Laboratories, Chicago, IL, U.S.A.) and maintained on a closed circuit system of halothane (2-5% Fluothane: Aveco Co., Fort Dodge, IA, U.S.A.) and oxygen (1.0 l/min).

After exposure by midline laparotomy, one randomly selected uterine horn and its ipsilateral ovary were surgically removed, placed on ice and transported to a sterile laminar flow hood. The incision site was then closed in a routine manner. The uterine horn was trimmed free of mesometrium and flushed with 20 ml of sterile physiological (.9%) saline. Conceptuses recovered from the uterine flushing were removed, weighed and cultured in 15 ml of modified Eagle's minimum essential media (MEM). Endometrial explants were dissected from underlying myometrium, diced and cultured (approximately 500 mg) in 15 ml of MEM for 24 hours at 37^{o} C under a controlled environment. The cultures were rocked slowly during incubation to expose the tissue to media and gas alternatively. The culture box was charged every 12 hours with a gaseous atmosphere consisting of 5% CO_2, 45% N_2, and 50% O_2. Conceptus tissue and endometrial

explants were cultured in modified Eagle's minimum essential medium (MEM) prepared as previously described by Basha et al. (1979) with L-leucine reduced to one-tenth normal concentration. Media was filter sterilized, and stored at 4° C. An antibiotic and antimyotic solution (penicillan 10000U/ml and streptomycin 10mg/ml) (Sigma, St. Louis, MO. USA) was added prior to culture. One hundred microCuries of tritiated leucine (L-leucine 4,5-^{3}H, specific activity 58.4 Ci/mmol, New England Nuclear, Boston, Mass.USA) was added to individual culture dishes as a tracer for de novo protein synthesis of conceptus and endometrial tissues. At the end of the culture period, media was separated from the tissue and centrifuged at 27,000 xg for 20 minutes at 4° C. The supernatant stored at -84° C until analyzed for ^{3}H-leucine incorporation into polypeptides.

Uterine flushings were centrifuged at 12,000 xg for 15 minutes at 4° C and the supernatant stored at -84° C until analysis for protein, acid phosphatase activity, fructose, prostaglandin F_{2a} and calcium.

Analysis of Uterine Flushings

Detailed descriptions for the analysis of calcium, protein, acid phosphatase activity, and prostaglandin F have been previously described in our laboratory by Morgan et al. 1987. The intra- and interassay coefficient of variation for the PGF assays were 15% and 27.5% respectively. Sensitivity of the assay with respect to the standard curve was 25 pg.

Concentrations of progesterone in plasma were determined by radioimmunoassay as described previously by Geisert et al. (1987). Recovery of labeled tracer after hexane extraction was 89%. The minimum sensitivity of the assay was 25 pg/ml. Intra- and interassay coefficients of variation were 1.01% and 10.03% respectively.

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed (300 ug total protein loaded) on the uterine flushings for acidic and basic proteins as described by Morgan et al. 1987.

The radiolabelled proteins in the culture media of the conceptuses and endometrium were dialyzed (3500 molecular weight cutoff dialysis membrane) against several volume changes of 1.0 mM Tris-HCl buffer, lyophilized and reconstituted for analysis by 2D-PAGE as described by Morgan et al. 1987. Approximately, 200,000 DPM were loaded onto each gel from endometrial cultures; 100,000 DPM to 200,000 DPM were loaded from blastocyst cultures. Differences in the amount of radioactivity loaded from conceptus cultures were adjusted by varying the exposure time of the gel. Following staining with Coomassie Blue, gels were impregnated with sodium salicylate (Bonner and Lashey, 1974; Chamberlain, 1979), dried onto filter paper and overlayed with X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y., USA). Fluorographs from conceptus derived gels were developed after a 10 week (200,000 DPM) or 20 week (100,000 DPM) period. Fluorographs from endometrial gels were developed

after a 10 week exposure period.

Fructose concentration in uterine flushings was analyzed according to the procedures described by Roe (1934). Sample sizes ranged from 8 ml on day 12 to 1 ml on day 18. All samples were adjusted to a final volume of 8 ml with distilled water. Samples were deproteinized with 1.0 ml 10% $ZnSO_4$ and 1.0 ml .5N NaOH prior to assay. Following deproteinization, samples were vortexed and centrifuged at 3,500 rpm for 20 min. Two ml of the supernatant was pipetted into new tubes and 2.0 mls of resorcinol (1 mg/ml in 95% ethanol) was added, followed by 6.0 ml of 30% HCl. All tubes were vortexed and incubated in a 80° C water bath for 8 minutes. After incubation, samples were cooled to 20° C in a water bath. A standard curve consisting of 0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 mg fructose/ml was included in each assay. Concentration of fructose in samples was measured spectrophotometrically using a Beckman DB spectrometer at 490 nm within an hour of cooling.

Statistical Analysis

Experiment 1. Plasma progesterone concentration were analyzed by least squares analysis of variance comparing the differences between treatments (oestradiol vs control) by specifying pig within treatment as the error term.

Experiment 2. Data from measured parameters of the uterine flushings were analyzed by least squares analysis of variance. The model included effects of period (12-14 vs

16-18), treatment (oestradiol vs control), trt*period, pig(trt*period), day(period) (12, 14, 16, and 18) and trt* day interactions.

Results

Experiment 1

Plasma progesterone concentrations were similar between control and treated animals from days 12 to 20 (Fig. 1). Oestrogen administration on day 9 and 10 of pregnancy maintained CL function comparable to pregnant controls. Uteri flushed on day 20 of pregnancy revealed normal conceptus tissue from both control gilts, however, conceptus tissue was not present in any of the three oestrogen treated gilts despite the presence of functional CL.

Experiment 2

Both control and oestrogen treated gilts had viable, filamentous conceptuses present on day 12 and 14 of pregnancy (Table 1). However, on day 16 conceptuses recovered from oestrogen treated gilts were either absent (2/4) or severely fragmented (1/4). One oestrogen treated gilt contained conceptuses which were similar in development to those recovered from control gilts. On day 18 of pregnancy, conceptus tissue was evident only as cellular debris with the exception of the presence of normal tissue in the gilt which had normal embryos on day 16.

Total content of protein, calcium, fructose, acid

phosphatase activity, and acid phosphatase specific activity recovered in uterine flushings are presented in Table 2. The oestrogen treated gilt which maintained normal conceptus tissue, also maintained concentration of measured parameters similar to control values. Due to the low number of samples analyzed in the study, the concentration of fructose from this one gilt concealed treatment differences shown by the remaining treated animals, thus values from this gilt were omitted from analysis. Total protein content in uterine flushing was not different between groups. Concentration of protein was several times greater than levels previously reported by Geisert et al. (1982a) and Morgan et al. (1987). This difference may have resulted from a difference in the time of flushing, removal time of the horn or a more vigorous stripping of the saline through the uterine horn. Analysis of 2D-PAGE gels indicated an increase in serum contamination of flushings. A treatment x day effect ($P<.05$) was detected for uterine content of calcium, while only a day effect was indicated for acid phosphatase activity and specific acid phosphatase specific activity. Calcium was elevated on day 12 in control gilts followed by a decline on day 14, then increased on day 16 and 18. In contrast, calcium remained similar across days in oestrogen treated gilts. Total fructose content in oestrogen treated gilts was higher than the controls on day 12 and 14, whereas levels on day 16 and 18 were lower in treated animals, resulting in a treatment x period effect ($P<.005$).

Prostaglandin F_{2a} levels were similar between control and treated animals on days 12 and 14 (Fig. 2), but continued to increase in control gilts on day 16 and 18 resulting in a treatment x day effect ($P<.05$).

Incorporation of tritiated leucine by the conceptus tissue (total DPM/mg tissue cultured) into polypeptides revealed an increase from day 12 (4422) to 14 (8981), then revealed a decreasing trend on day 16 (7495) and day 18 (5793), resulting in a day effect ($P<.07$). A similar trend was found by the oestrogen treated conceptus tissue from day 12 (4319) and 14 (6037), however, a valid test could not be made on day 16 and 18 since conceptus tissue was absent. Analysis of fluorographs developed from conceptus in vitro cultures indicated no differences between treatments up to day 14 of pregnancy. Representative fluorographs of acidic (Fig. 3) and basic (Fig. 4) polypeptides from conceptus tissue obtained from control gilts for each day are provided. Across days, alteration in qualitative and quantitative polypeptide synthesis and release were apparent as embryonic development continued. On day 12, the main polypeptide secreted was a lower molecular weight acidic polypeptide (M_r 22,000; pI=6.2), which persisted until day 18 in diminishing quantities. By day 14, a basic polypeptide appeared (M_r 40,000; pI=9.0-7.9) which remained prominent until day 18. By day 18, there was an increase in the amount of high molecular weight polypeptides as the yolk sac developed and fetal serum proteins began to be secreted. The

oestrogen treated gilt who maintained pregnancy on day 16 and 18 had similar acidic and basic polypeptides profiles as the controls.

Endometrial incorporation of radiolabelled leucine indicated no differences between days or treatment. Analysis of 2D-PAGE gels from the uterine flushings revealed no differences in the proteins present in the control or treated gilts. Analysis of fluorographs developed from the endometrial cultures indicate that the acidic polypeptides were affected by oestrogen treatment as early as day 12 (Figs. 5 and 6). Across all days observed, a band of 3 distinct polypeptides with a molecular weight of approximately 30,000 daltons and a pI of 9.0-7.9 and an acidic polypeptide with a molecular weight of approximately 100,000 daltons and an acidic pI of 5.0-3.5 was attenuated or absent in the oestrogen treated gilts in which conceptuses degenerated on days 16 and 18. Analysis of basic proteins revealed no differences between treatments or days as uteroferrin appeared characteristically at a molecular weight of 35,000 daltons and the plasminogen inhibitors were present at approximately 14,000 daltons (Figs. 7 and 8). The oestrogen treated gilt that maintained pregnancy on days 16 and 18 had endometrial and conceptus polypeptide profiles similar to control gilts during this period.

Discussion

Peripheral plasma concentrations of progesterone were

not altered due to exogenous oestradiol treatment on day 9 and 10 of pregnancy. Geisert et al. (1987) indicated that administering oestrogen on day 9.5 of the oestrous cycle prolonged the interoestrous interval. Thus, embryonic mortality after oestradiol treatment was not caused by an insufficient concentration of progesterone.

Pope et al. (1986) reported that gilts administered exogenous oestrogen on day 9 and 10 of pregnancy experienced embryonic loss by day 30. Morgan et al. (1987) indicated that pregnant gilts treated with exogenous oestrogen on day 9 and 10, had viable filamentous blastocysts at day 11 and 12. But conceptuses were severely fragmented when recovered at day 16 of pregnancy. Results of the present study indicate viable filamentous conceptuses were recovered on day 12 and 14 of pregnancy after exogenous oestrogen on day 9 and 10. However, conceptuses were degenerating at day 16, and absent or only detectable through cellular debris at day 18. Viability of the blastocysts until day 14 was supported by their polypeptide synthesis as indicated from the comparison of fluorographs from treated and control gilts.

Godkin et al. (1982) previously described the progression of <u>in vitro</u> conceptus polypeptide synthesis and secretion from day 10 to 18. These authors indicated a major low molecular weight acidic protein was synthesized on day 10.5 followed by secretion of a major basic proteins from day 13-16. Conceptus tissue secreted a large proportion of high molecular weight acidic polypeptide on day 18-25.

Analysis of fluorographs developed from cultured conceptus tissue in our study revealed a similar progression in conceptus polypeptide secretion, however, in contrast to Godkin et al.(1982), the low molecular weight acidic proteins remained until at least day 18 of pregnancy. The difference between our study and that of Godkin may be caused by the length of fluorograph exposure (10 weeks versus 7 days).

Previous reports by Geisert et al. (1982c; 1987) indicated that after exposure of the uterine endometrium to oestrogen, either through conceptus secretion or exogenous administration, there is a triggering of an increase in calcium, protein, and acid phosphatase concentration into the uterine lumen. Morgan et al. (1987) reported that oestrogen administration on day 9 and 10 of pregnancy advanced the release of calcium, protein and acid phosphatase recovered in uterine flushings. The analysis of uterine flushings in the present study revealed a similar alteration in calcium, however, although acid phosphatase activity tended to be lower in treated gilts, the difference was not significant.

Uterine prostaglandin F_{2a} and fructose content mirrored embryonic viability since levels failed to increase compared to control gilts on day 16 and 18. These result support the evidence presented by Frank et al. (1978) that blastocysts begin to produce significant amounts of PGF_{2a} on day 12 of pregnancy and Zavy et al. (1982) who reported that the

conceptus tissue in the gilt is the primary source of fructose production probably through glucose conversion.

The qualitative analysis of polypeptides in uterine flushings through 2D-PAGE did not show any consistent differences between days or treatments. This does not support the previous study of Morgan et al. (1987) which indicated a that group of acidic (M_r 40,000; pI=5.4) polypeptides appeared with greater intensity in oestradiol treated animals. Serum contamination in the uterine flushings may have prevented the detection of sensitive changes in this study. Fluorographs produced from 2D-PAGE analysis of endometrial cultures indicated that the acidic polypeptide of Morgan et al. (1987) did not incorporate leucine suggesting that the polypeptide is possibly of serum origin. However, at day 12, fluorographs from endometrial cultures obtained from oestradiol treated gilts demonstrated definite changes which persisted through day 18 of pregnancy. An attenuation or loss of a high molecular weight acidic polypeptide and a band of three basic polypeptides, was evident in oestradiol treated gilts. These polypeptides did not stain with Coomassie blue and therefore were undetected on gels before fluorography. The alteration of these polypeptides may be the result of the lack of insufficient endometrial stimulation by the conceptus and/or modification of gene expression by the advanced oestrogen stimulation. Lack of conceptus stimulation may be questioned however, since these polypeptides were absent as early as

day 12, when viable blastocysts were present. Studies utilizing cycling gilts are needed to clarify the latter possibility. The biological function of these proteins in embryonic development and/or attachment is unknown.

The demonstrated effects of exogenous oestrogen on the uterine environment and blastocyst development may illustrate a similar action of zearalenone, a mycotoxin from moldy grains which has oestrogenic activity. Long and Diekman (1986) reported that gilts fed zearalenone from days 7-10, experienced total embryonic failure by day 30. In contrast, feeding zearalenone during days 2-6 or 11-15 had no effect on pregnancy maintenance when observed on day 30.

The critical period of embryonic survival indicated by this study is between 14 and 16 of pregnancy. This period coincides with the initiation of conceptus attachment to the endometrial surface epithelium (Dantzer, 1985). Perhaps the exogenous oestrogen administered on day 9 and 10 causes surface changes on either the endometrial surface or the trophoblastic surface which interferes with normal apposition and attachment. The role of the endometrial polypeptides in which <u>in vitro</u> synthesis was altered after oestrogen treatment may provide a possible factor which is involved in attachment and survival of embryos in the pig.

TABLE 1

PROPORTION OF GILTS WITH NORMAL EMBRYOS FOLLOWING OESTRADIOL VALERATE OR VEHICLE TREATMENT ON DAY 9 AND 10 OF PREGNANCY

DAY	12	14	16	18
VEHICLE	4/4	4/4	4/4	4/4
OESTRADIOL VALERATE	4/4	4/4	2/4	1/4

TABLE 2

LEAST SQUARE MEANS FOR TOTAL PROTEIN, ACID PHOSPHATASE, SPECIFIC ACTIVITY, CALCIUM AND FRUCTOSE IN UTERINE FLUSHINGS FROM GILTS TREATED WITH OESTRADIOL VALERATE OR VEHICLE ON DAY 9 AND 10 OF PREGNANCY

Treatment	Day of Hysterectomy	Total Protein (mg)	Acid* Phosphatase (umol Pi/min)	Specific Activity* (umol Pi/min/ mg protein)	Calcium+ (mg)	Fructose** (mg)
VEHICLE	12	134.2	104.0	.726	.717	.274
	14	197.1	686.5	3.378	.212	1.917
	16	179.3	278.1	1.665	.472	5.549
	18	244.3	181.7	.728	.802	9.695
OESTRADIOL[a] VALERATE	12	165.8	193.0	1.287	.454	2.953
	14	184.6	537.1	2.818	.398	3.171
	16	179.5	91.7	.536	.395	2.062
	18	150.8	79.3	.532	.237	.726
overall s.e.m.		±12.8	±95.0	±.517	±.104	±.73

* Day(period) effect (P<.02)
** Treatment x Period (P<.01)
\+ Treatment x Day(period) (P<.05)
[a] One oestrogen treated gilt was deleted; n=3

Figure 1. Peripheral concentration of progesterone in plasma of control (solid line) and oestrogen treated (dashed line) gilts in experiment 1.

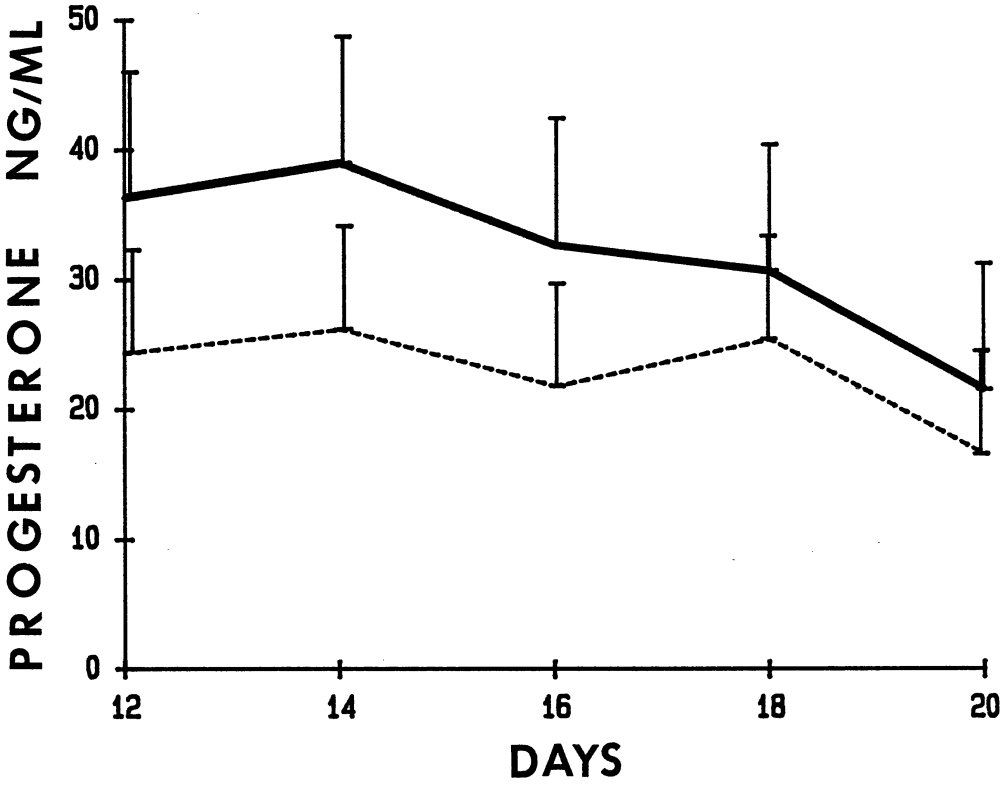

PROGESTERONE NG/ML
50
40
30
20
10
0
12
14
16
18
20
DAYS

Figure 2. Total content of prostaglandin F in the uterine flushings of control (hatched bars) and oestrogen treated gilts (clear bars) from days 12 to 18 of pregnancy. Analysis indicated a treatment x day effect ($P < .05$).

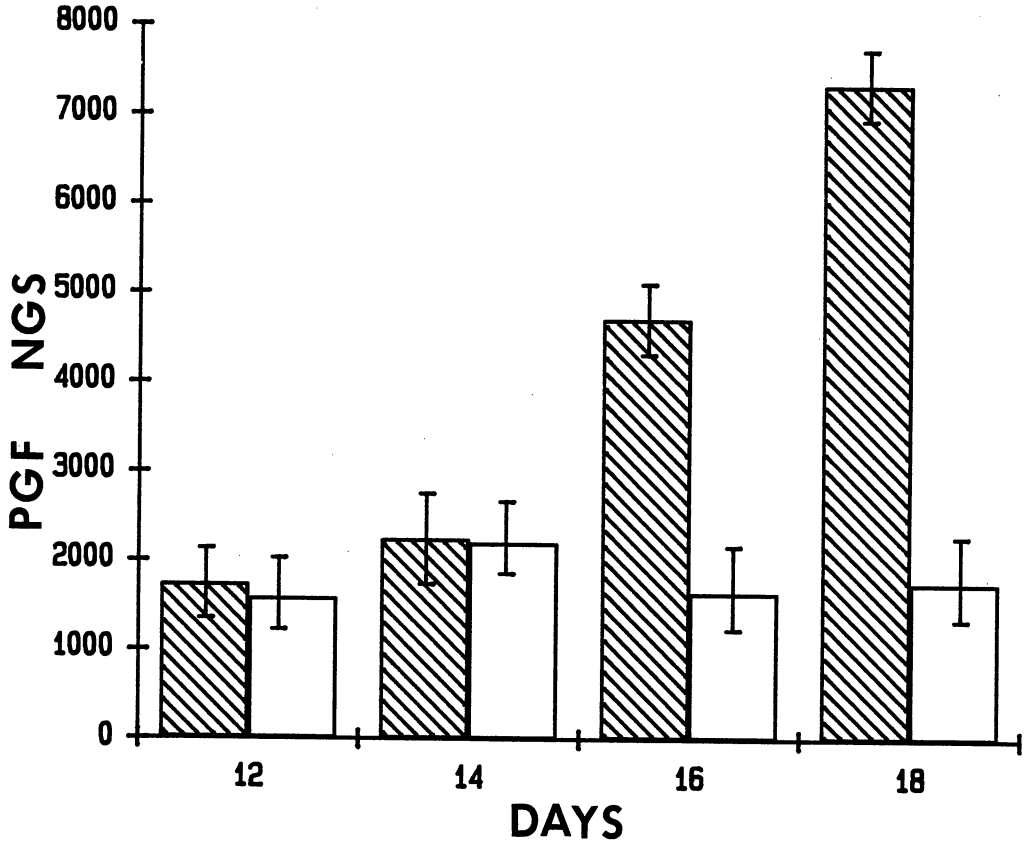

8000
7000
6000
5000
4000
3000
2000
1000
0
PGF NGS
12
14
16
18
DAYS

Figure 3. Fluorographs from 2D-PAGE analysis of acidic polypeptides secreted *in vitro* by conceptuses from control (C) gilts on day 12, 14, 16, and 18 of pregnancy.

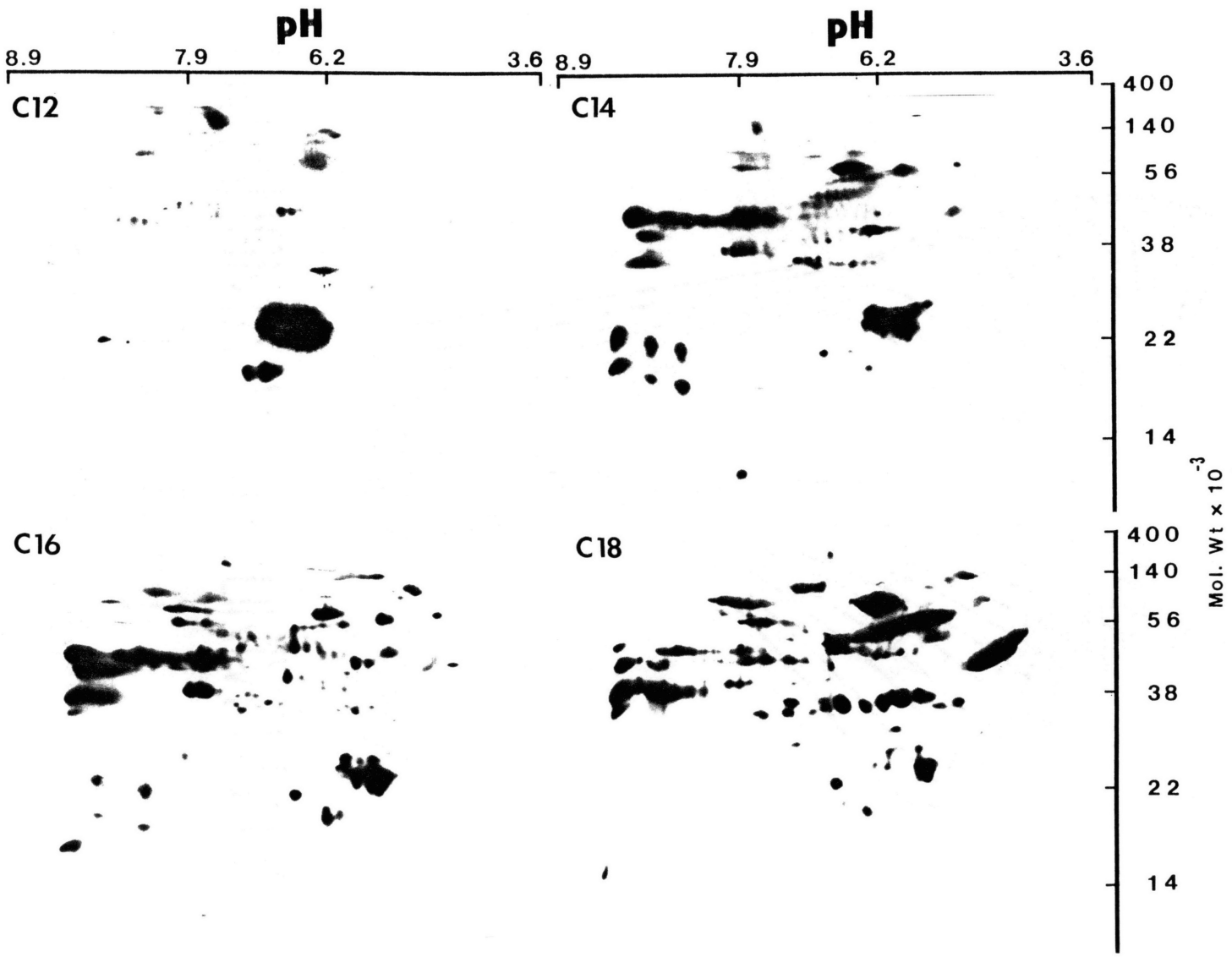

pH
8.9
7.9
6.2
3.6
C12
C14
C16
C18
400
140
56
38
22
14
Mol. Wt x 10^-3

Figure 4. Fluorographs from 2D-PAGE analysis of basic polypeptides secreted *in vitro* by conceptuses from control (C) gilts on days 12, 14, 16, and 18 of pregnancy.

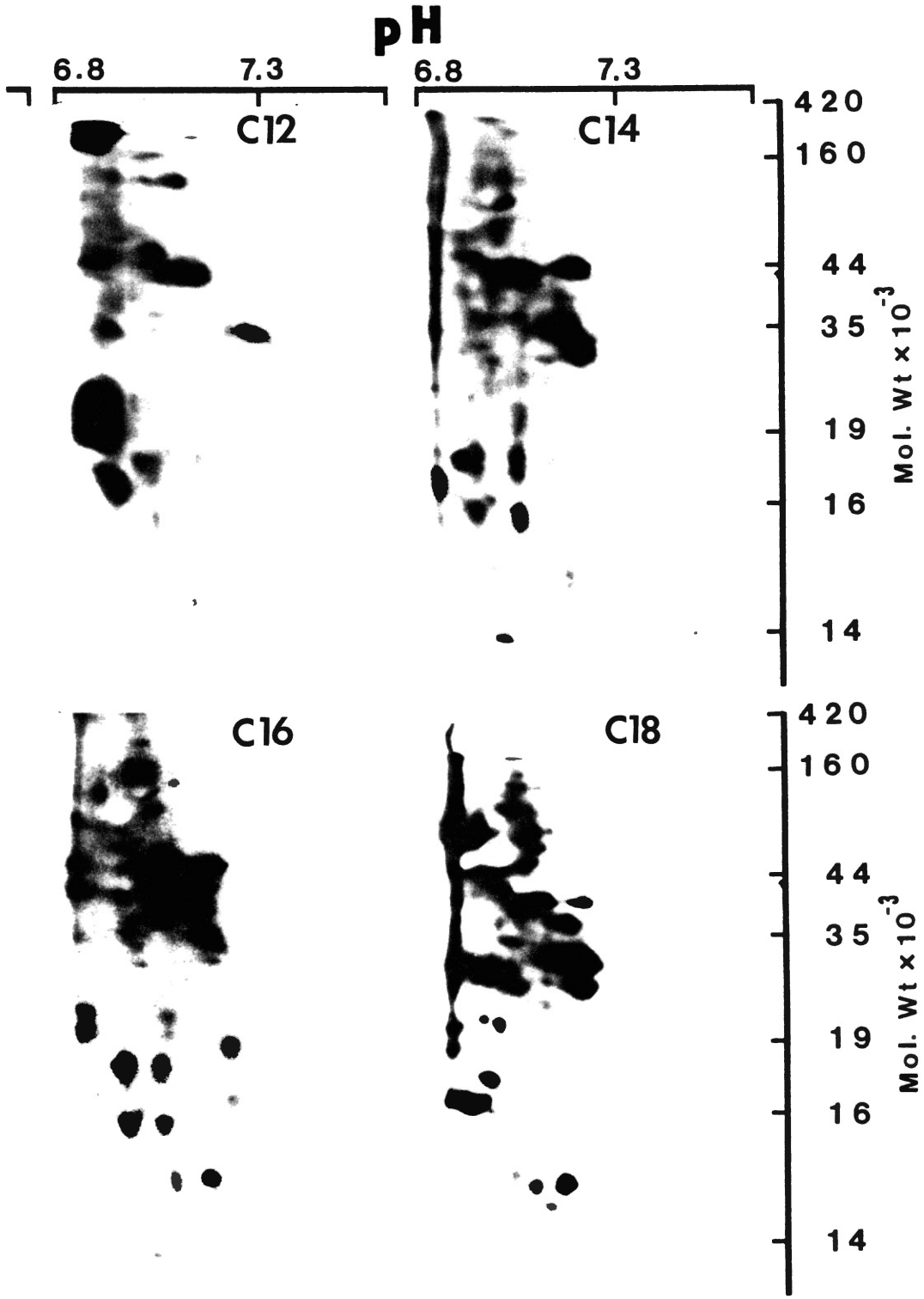

pH
6.8
7.3
6.8
7.3
C12
C14
C16
C18
420
160
44
35
19
16
14
Mol. Wt x10-3
420
160
44
35
19
16
14
Mol. Wt x10-3

Figure 5. Fluorographs from 2D-PAGE analysis of acidic polypeptides secreted in vitro by endometrial explants from control (C) and oestrogen treated (T) gilts on days 12 and 14 of pregnancy. Polypeptides designated by the open arrow (⇨) and the solid arrow (➡) are absent or their production modified in the oestrogen treated (T) gilts.

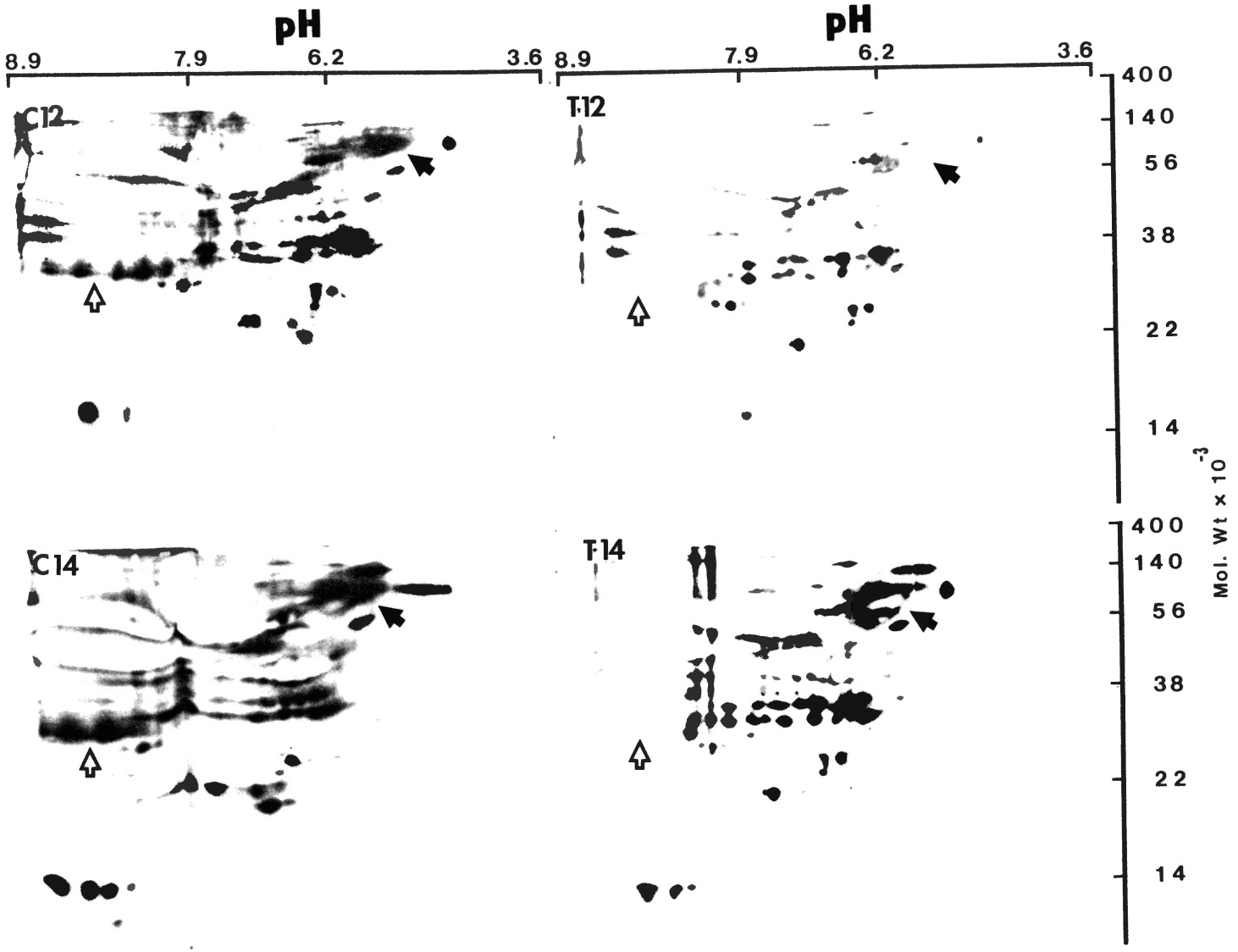

pH
8.9
7.9
6.2
3.6
C12
T12
C14
T14
400
140
56
38
22
14
Mol. Wt x 10^-3

Figure 6. Fluorographs from 2D-PAGE analysis of acidic polypeptides secreted in vitro by endometrial explants from control (C) and oestrogen treated (T) gilts on days 16 and 18 of pregnancy. Polypeptides designated by the open arrow (⇨) and the solid arrow (➡) are absent or their production modified in the oestrogen treated (T) gilts.

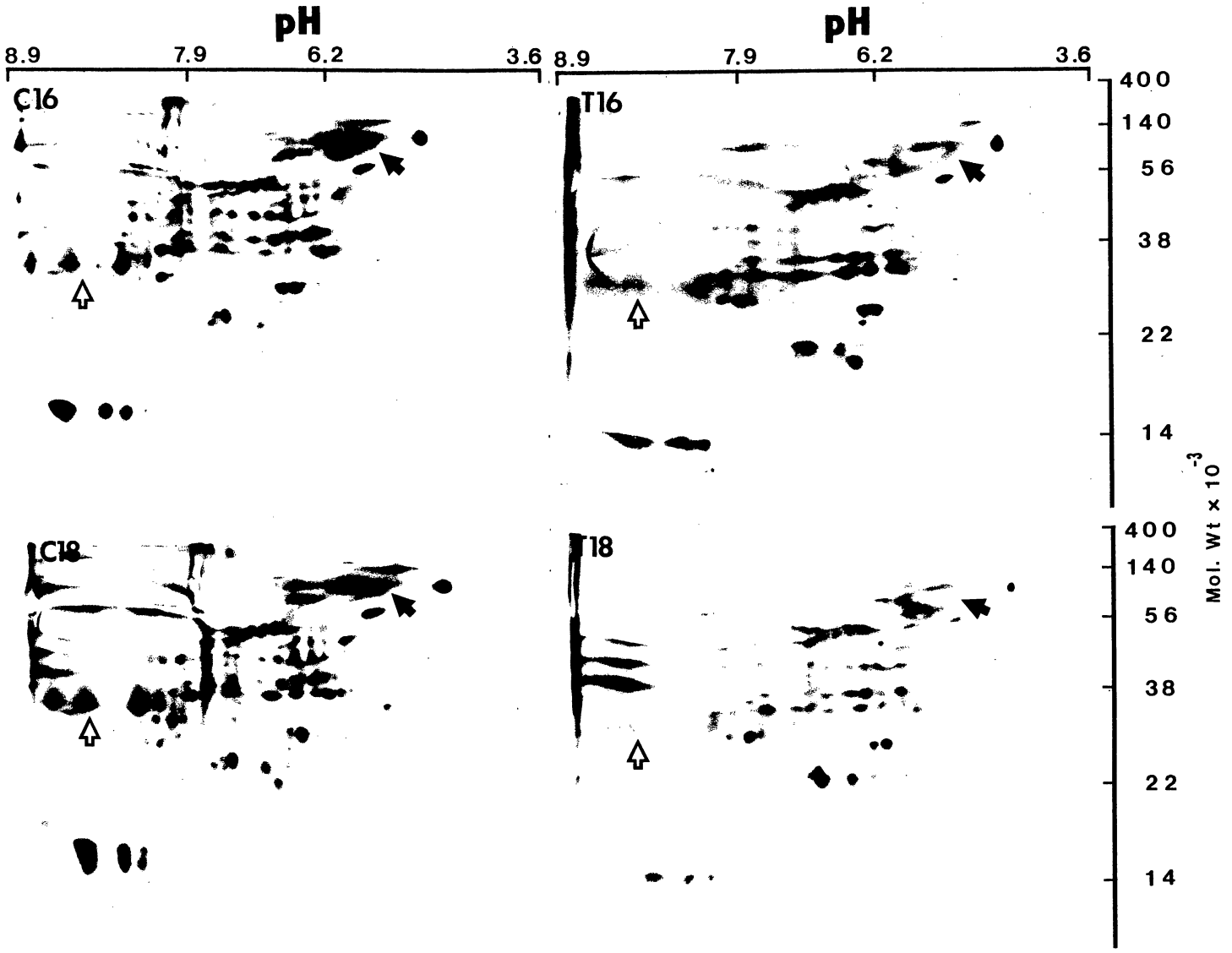

pH
8.9
7.9
6.2
3.6
C16
T16
C18
T18
400
140
56
38
22
14
Mol. Wt x 10^-3

Figure 7. Fluorographs from 2D-PAGE analysis of basic polypeptides secreted *in vitro* by endometrial explants from control (C) and oestrogen treated (T) gilts on days 12 and 14 of pregnancy. The open arrow (⇨) designates the position of uteroferrin.

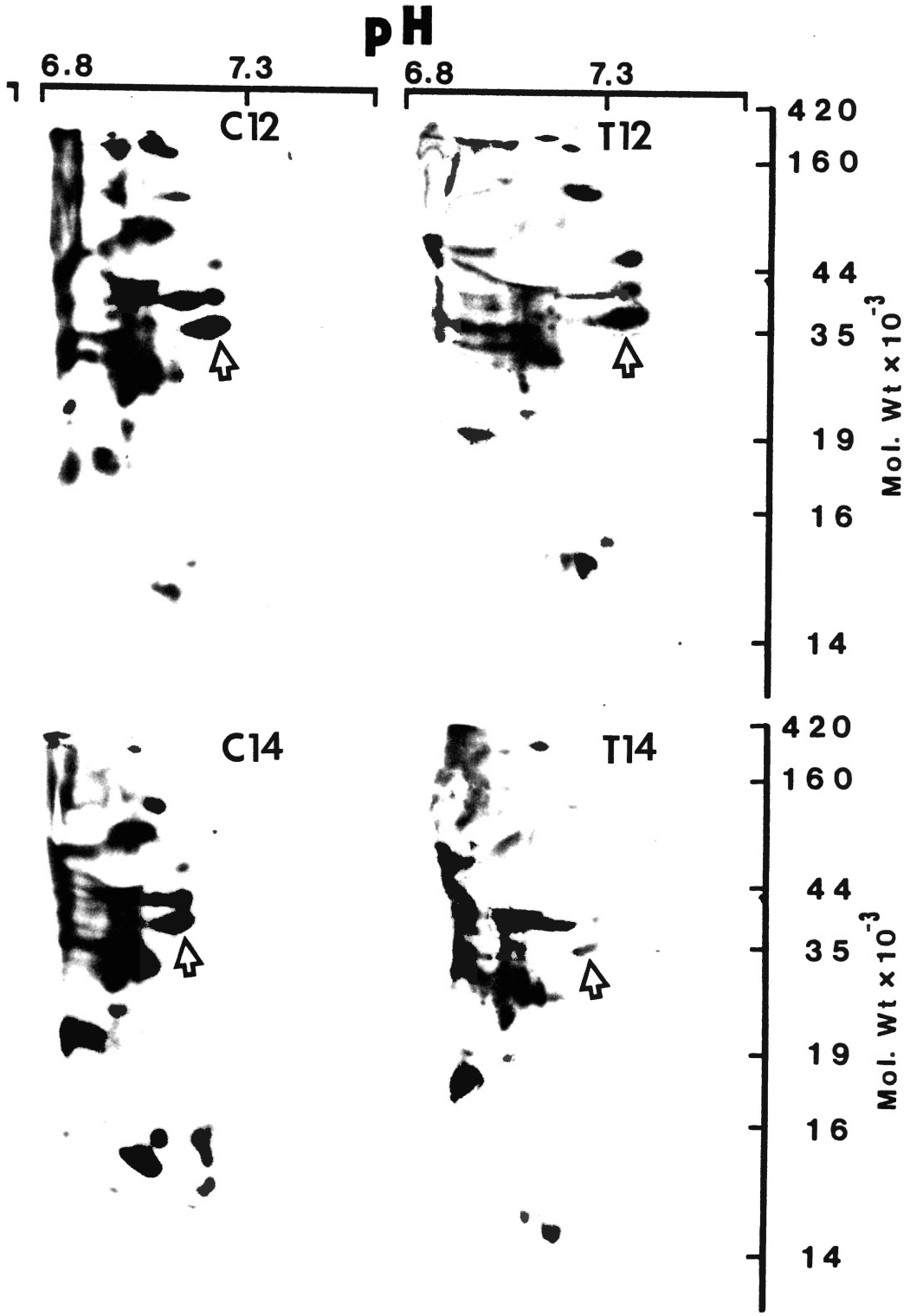
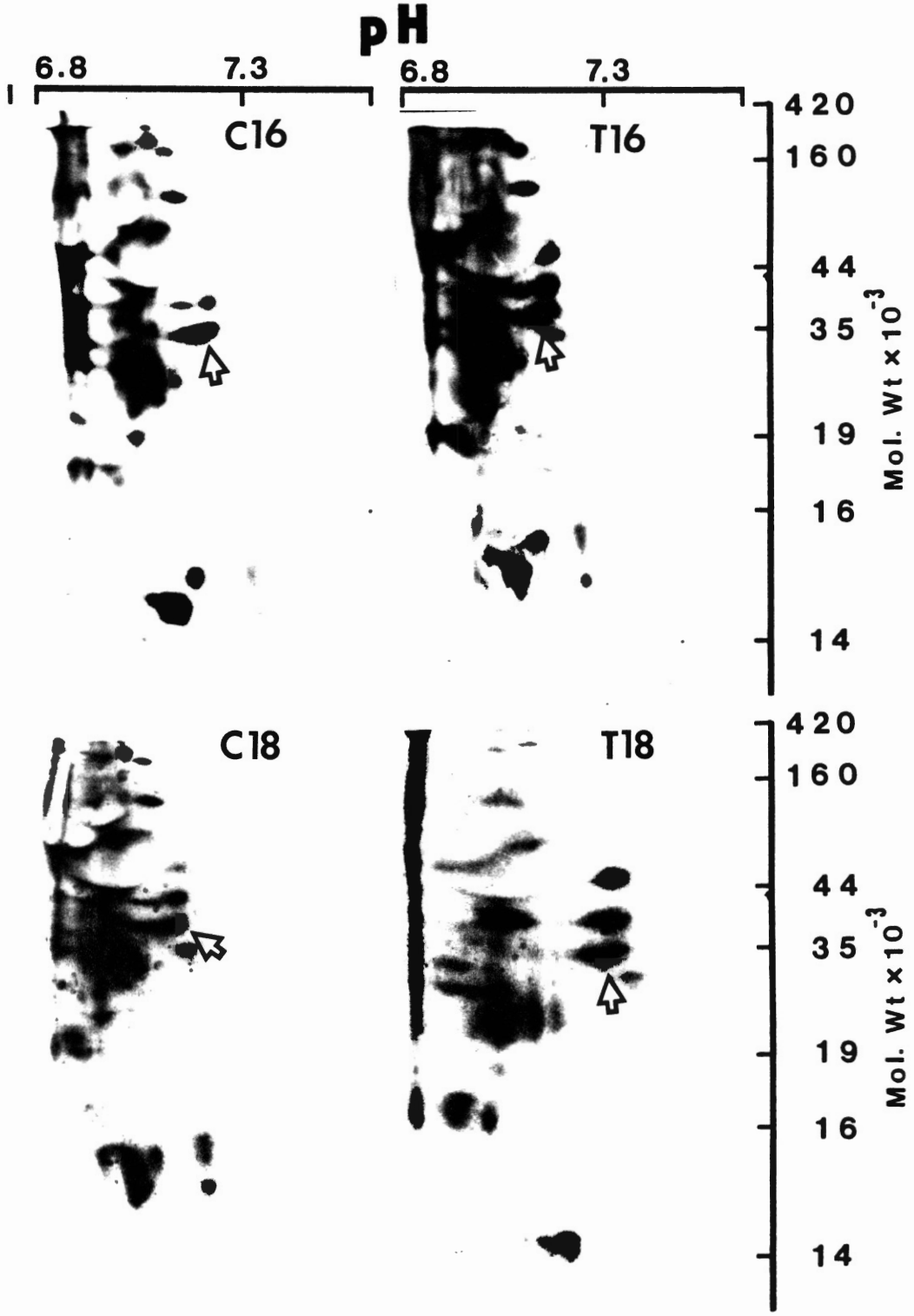

pH
6.8
7.3
6.8
7.3
C12
T12
C14
T14
420
160
44
35
19
16
14
Mol. Wt x10-3
420
160
44
35
19
16
14
Mol. Wt x10-3

Figure 8. Fluorographs from 2D-PAGE analysis of basic polypeptides secreted *in vitro* by endometrial explants from control (C) and oestrogen treated (T) gilts on days 16 and 18 of pregnancy. The open arrow (⇨) designates the position of uteroferrin.


pH
6.8
7.3
6.8
7.3
C16
T16
C18
T18
420
160
44
35
19
16
14
Mol. Wt x 10^-3

CHAPTER IV

GENERAL DISCUSSION

There is considerable variation in the degree of morphological development of porcine embryos within a litter prior to attachment (Anderson et al. 1978). This may reflect variable gene control of early embryonic development or may alternatively be the result of a prolonged ovulation span in the pig. This variation in embryonic development may possibly be explained on the basis that early embryonic mortality in swine may be a consequence of estrogen secreted by blastocysts during elongation which stimulates changes in the uterine environment so that any morphologically undeveloped blastocysts can no longer survive.

Pope et al. (1982) reported that after transferring day 5 and day 7 embryos into nonpregnant day 6 uteri, conceptuses surviving until day 60 were mainly from the older day 7 embryos. Even when day 6 embryos were placed in day 7 pregnant uteri, a higher proportion of older embryos survived. It is proposed that the more advanced blastocysts were probably able to undergo elongation first, which may in turn have changed the uterine environment to suit their needs. Those blastocysts not ready for this environmental change were not able to survive.

Geisert et al. (1987) have determined that intra-uterine changes in calcium, protein and acid phosphatase secretion can be induced by exogenous estrogen as early as day 10 of the cycle and that these same changes occur shortly after the onset of blastocyst oestrogen secretion (day 11) in pregnant gilts (Geisert et al. 1982c).

Calcium is required in such a myriad of biochemical reactions, that calcium may, in fact, serve several functions simultaneously. Calcium stimulates the release of secretory vesicles from several types of tissues (Rubin and Laychock, 1978; Rasmussen and Barrett, 1984). Geisert et al. (1982c) reported the synchronous release of secretory vesicles from the endometrial epithelium following estrogen stimulation. Coinciding with this release is a significant increase in intra-uterine calcium into the uterine lumen. Calcium has been proposed to activate the enzyme, phospholipase A2, a membrane bound enzyme that regulates the initial step in prostaglandin synthesis (Hirata and Axelrod, 1980). Calcium has also been associated with the methylation of phospholipids within the plasmalemma, thereby altering membrane fluidity (Hirata and Axelrod, 1980). Calcium is required as the trigger to many enzyme cascade systems within the cell and for the production or induction of many different kinds of proteins such as enzymes, receptors, and secreted proteins. Yet, the surge of calcium into the uterine lumen <u>per se</u> is not essential for blastocyst elongation, as viable filamentous blastocysts have been

recovered when calcium was low or undetectable (Morgan et al. 1987; present study).

The specific function for the increased release of protein at the time of estrogen exposure is unknown. It may act as a hormonal signal to the elongating blastocyst or may be a source of nutrition to the rapidly growing blastocysts before attachment. Perhaps it reflects endometrial changes as the epithelium prepares for attachment and placentation. The protein concentrations in the present study were 2-3 times greater than those previously reported by Geisert et al (1982) or Morgan et al. (1987). This increase may have been due to serum or cytoplasmic contamination during transport to the lab and/or flushing. If so, the contamination may have altered detection of differences between estrogen treated and control gilts in the analysis of acid phosphatase or gel electrophoresis of uterine flushings. Acid phosphatase activity is a convenient assay for uteroferrin concentration in uterine flushings since 95% of the acid phosphatase activity in the uterine lumen is due to uteroferrin (Basha et al. 1979). Uteroferrin has been proposed to function in placental development during early pregnancy (Chen and Bazer, 1973) and to transport iron to the developing embryo (Renegar et al 1982). Geisert et al. (1987) and Morgan et al. (1987) reported that acid phosphatase activity increased after exogenous estrogen on days 11 and 12, but were lower than controls by day 16. In the present study, acid phosphatase activity was lower in

treated animals than in the controls on all days except day 12. Acid phosphatase activity did not reveal a treatment effect in this study, since the concentrations of each treatment followed a similar trend.

Fructose was measured as another parameter of the uterine environment. Zavy et al. (1982) analyzed the fructose content in uterine flushings from cyclic and early pregnant gilts. These authors reported an increase in concentration on day 14 which continued until day 20 of pregnancy. In the present study, there was an increase in fructose concentration on day 12 and 14 of pregnancy in treated gilts followed by dramatic decline on day 16 and 18. In contrast, the pattern of release in control animals, which expressed low levels on day 12 and 14, was followed by increasing levels on day 16 and 18. Since the assay requires a large amount of sample, only a limited number of flushings were analyzed. However, statistically a treatment by period effect was detected. Fructose in early pregnancy is proposed to be an energy storage for the embryo, since it can be sequestered within the uterus and embryonic fluids and is also easily transformed into glucose.

Prostaglandin F_{2a} production in this study mirrored the periods of embryonic viability. Prostaglandin F_{2a} concentration were similar between control and estrogen treated gilts on days 12 and 14 when viable filamentous conceptuses were present. However, in estrogen treated gilts on days 16 and 18, when conceptuses was absent or degenerating,

prostaglandin levels failed to rise. Prostaglandin F_{2a} levels in the uterine flushings of pregnant gilts are produced mainly by conceptus tissues, even though, after day 14, the endometrium contributes to the uterine luminal concentration (Frank et al. 1978; Guthrie and Lewis, 1986).

The uterine environment appears to be advanced by exogenous estrogen given on days 9 and 10 of pregnancy. The consequences of these changes may be reflected in blastocyst survival. Pope et al. (1986) reported that gilts administered exogenous estrogen on days 9 and 10 of pregnancy experienced complete embryonic loss by day 30 of pregnancy. However, the same estrogen treatment given to gilts at days 12 and 13 of pregnancy, when the uterus is normally exposed to estrogen, resulted normal embryonic development when observed on day 30. Morgan et al. (1987) reported viable elongated blastocysts recovered on day 11 and 12 of pregnancy after early exogenous estrogen treatment. However, embryos were fragmented and degenerating by day 16 of pregnancy. In the present study, elongated conceptuses were recovered at day 12 and 14 of pregnancy. However, by day 16, conceptuses were similarly fragmented and by day 18, conceptuses were totally absent or detectable only through the presence of cellular debris. Analysis of fluorographs developed from blastocysts cultured in vitro revealed similar acidic and basic polypeptide profiles until day 14 which supports the conclusion of embryonic viability until this time.

The one estrogen treated gilt who retained pregnancy on day 16 and 18 may have had a uterine environment that was more advanced at the time of treatment. This gilt did not respond to treatment since fluorographs were characteristic of those found in the corresponding control animals.

The critical period of embryonic survival as indicated in this study is between day 14 and 16. Events during this period in normal embryonic development include the second phase of estrogen production for maternal recognition of pregnancy (Zavy et al. 1980; Geisert et al.1982), the production of prostaglandins from the blastocysts (Lewis and Waterman, 1983; Guthrie and Lewis, 1986), and the beginning of attachment (Dantzer, 1985). Dantzer (1985) using electron microscopy, found that the trophoblast and the endometrial epithelium have a glycocalyx on their surfaces. These glycocalyces subsequently thin as apposition occurs. In the rodent species, the glycocalyx interaction is proposed to function in cell to cell recognition, and as an anchoring of the trophoblast so that the interdigitation of microvilli can develop (Anderson and Hoffman, 1984; Hewitt et al. 1987; Chevez and Anderson, 1985). This glycocalyx has been characterized to have stage specific proteins, and sialic acid. (Anderson et al. 1986; Shutelsky et al. 1974). As the glycocalyx thins, receptors may be exposed for a limited period of time providing for attachment (Anderson et al. 1986). Even though implantation in rodents is different than in swine, the function of the glycocalyx may be similar. It

is possible that estrogen produced by elongating blastocysts could be the signal for the synthesis of receptors on the trophoblast glycocalyx, or could be the agent for unmasking such receptors to allow for apposition and attachment to take place. If exogenous estrogen is given early on day 9 and 10 as in this study, perhaps the endometrial receptors are ready for trophoblastic attachment before the corresponding receptor on the blastocysts. If attachment can only occur over a limited period of time in the pig, it is possible that the endometrial receptors maybe absent by the time the conceptuses of treated animals were ready to attach.

Changes in endometrial epithelia due to exogenous estrogen were detected through differences in secreted proteins in vitro. Fluorographs developed from cultured endometrial explants, reveal a loss or modification of a high molecular weight acidic polypeptide and a band of basic polypeptides as early as day 12 after estrogen treatment. The function of these secreted proteins and the mechanism which prevents or modifys their production is unknown. The polypeptides did not stain with Coomassie blue, indicating that their presence in the uterine flushings may have been previously undetected. These polypeptides do not appear to be induced by the blastocyst since they were absent by day 12, when viable blastocysts were present. These polypeptides may act as a hormonal signal to the conceptus. Possibly, gene expression for the synthesis of these polypeptides

could be inhibited.

In conclusion, this study supports previous work indicating changes in the uterine environment as analyzed through uterine flushings due to exogenous estrogen (Geisert et al. 1982; 1987; Morgan et al. 1987). It would appear that blastocysts survive the advanced release of calcium, acid phosphatase, protein, and prostaglandin F, but may be unable to properly attach to the endometrium. The critical period of blastocyst survival occurs between day 14 and 16 of pregnancy, when intimate attachment of trophoblast and endometrial epithelia is necessary. Exogenous estrogen changed the polypeptides secreted in vitro from the endometrium and these may play a possible role in embryonic attachment and development

Future studies may include a biochemical characterization of the glycocalyx present on both the trophoblast and the endometrial epithelium and their changes during apposition. If this glycocalyx could be blocked by antibodies, its affect on attachment could be studied. Perhaps an assay for sialic acid would be able to determine if the glycocalyx was being degraded or shed after estrogen from the blastocysts or estrogen administration.

The isolation of the polypeptides missing in the endometrial fluorographs from treated gilts may determine if these proteins are essential in blastocyst development. Perhaps a more sensitive staining technique, such as silver staining, would be able to detect these polypeptides on the

gels without fluorography.

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VITA

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