AN ABSTRACT OF THE THESIS OF

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Experiments were conducted to investigate regulation of the mink uterus by estradiol-17β (E_2) and prolactin (PRL). In Exp. 1, anestrous mink were injected sc daily with 50 ug E_2 or vehicle for 2 days. In Exp. 2, mink were injected sc daily with 2 mg bromocriptine or vehicle for 14 days beginning July 3. On day 14, both groups were divided into two subgroups and injected sc with 100 ug E_2 or vehicle. In Exp. 3, mink were assigned to one of two treatments or to a control group. Groups 1 and 2 were implanted (July 1) with 10 mg silastic melatonin (MLT) implants. On Oct. 25, group (Grp) 1 received 100 ug E_2 (sc) while Grp. 2 and the nonimplanted controls (Grp. 3) were similarly injected with vehicle. Mink in Exp. 1, 2 and 3 were sacrificed 24 h after E_2 treatment. In Exp. 4, mink in two groups were mated with vasectomized males on Mar. 7-9 (Grp. 1) and

Mar. 17-18 (Grp. 2) to induce pseudopregnancy. Each group was sacrificed 10 days after mating. Sera collected prior to sacrifice (Exp. 2-4) were analyzed for PRL, E_2 and progesterone (P_4). Aliquots of uterine tissue (Exp. 1-4) were used to quantitate concentrations of estrogen (ER) and P_4 receptor (PR), and *in vitro* glucose oxidation, protein and DNA synthesis (Exp. 1, 2 and 4 only).

Estradiol (Exp. 1-3) significantly increased uterine weight, concentrations of ER and PR, glucose oxidation and protein and DNA synthesis. Bromocriptine significantly suppressed serum levels of PRL, increased levels of E_2 and concentrations of PR but had no effect on levels of P_4, uterine weight, glucose oxidation, protein and DNA synthesis or levels of ER. Melatonin (Exp. 3) reduced serum concentrations of PRL ($P<0.01$), increased levels of E_2 ($P<0.01$) but had no effect on levels of P_4. Both MLT and MLT+E_2 increased uterine weight ($P<0.001$) and concentrations of ER and PR ($P<0.01$). In Exp. 4, concentrations of PRL and PR were significantly greater after the vernal equinox (March 21). Glucose oxidation was greater after the equinox ($P<0.05$) but not protein and DNA synthesis or levels of E_2 and P_4. These data indicate that the previously reported inability of E_2 and P_4 to induce implantation in mink is not due to uterine insensitivity to steroid stimulation and suggest that PRL regulates uterine function in this species.

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Endocrine Regulation of Uterine Physiology in Mink

INTRODUCTION

Mink have an annual reproductive cycle during which mating occurs only during a brief 4-5 week period in February and March. Like many other mammals in the family mustelidae, a unique feature of their gestation is the occurrence of an embryonic diapause during which blastocyst development is temporarily arrested and implantation is delayed. In mink ovulation is induced by mating and females will remate during the preimplantation period. Although more than 10 ova are often ovulated and fertilized during the breeding season, the average dark mink litter is only 4-6 kits per female. This difference between number of ova fertilized and number of kits born is believed to be a result of embryo mortality during diapause. Diapause related embryo mortality represents a substantial pitfall to successful rearing of mink and other species that possess this trait. Procedures that would induce early embryo reactivation and thereby reduce the preimplantation period would have potential for decreasing embryo loss and therefore improve the efficency of rearing species with delayed implantation.

In mink, embryonic diapause appears to be controlled by changes in daily photoperiod and shortly after the vernal equinox (March 21) blastocyst development resumes and embryos implant into the endometrium. Serum concentrations of prolactin increase just prior to implantation and this is associated with a concomitant stimulation of luteal steroidogenesis and an increase in serum concentrations of

progesterone. Moreover, treatments that increase serum concentrations of prolactin during embryonic diapause advance the date of embryo implantation (nidation).

Progestational stimulation of the uterus is required to permit embryo development, implantation and maintenance of pregnancy. However, treatment of mink with progestins alone or in combination with estrogen have been unsuccessful in advancing the date of nidation. Therefore, it is likely that prolactin either acts directly or through the stimulation of an additional endocrine factor to regulate uterine function during pregnancy in this species.

Prolactin receptors have been identified in the uterus of anestrous mink, suggesting that this peptide hormone directly regulates uterine physiology, but the nature of the uterine response remains unknown. The central goal of this thesis research was to investigate the relationship between prolactin, estrogen and progesterone in regulating uterine function in mink during anestrus and the breeding season. This objective was accomplished by quantifying changes in uterine metabolism and concentrations of estrogen and progesterone receptors. Additionally, influence of prolactin on serum concentrations of estrogen and progesterone was also determined. Pursuant to the overall goal, this thesis includes a review of literature involving reproductive physiology of mink, embryonic diapause and the mechanism by which prolactin, estrogen and progesterone exert their effects on target tissues.

REVIEW OF THE LITERATURE

First comprehensive description of the reproductive biology of mink was made by Hansson (1947). Although mink are not a traditional laboratory animal, their value as a furbearer and their relatively unique reproductive cycle (and delayed implantation) has prompted substantial research on this species. This research has resulted in a wealth of practical information concerning the reproductive endocrinology (Sundqvist et al., 1988) and physiology (Sundqvist et al., 1989) of mink.

Delayed Implantation and Reproduction in Mammals

Fries (1880) first described embryonic diapause in the European badger and this trait has since been shown to occur in at least 65 mammalian species representing 17 families (Wimstat, 1975; Renfree, 1976). Among these species, two different forms of delayed implantation, facultative and obligate, are generally distinguished. Facultative or lactation-induced embryonic diapause occurs in species that have more than one reproductive cycle each year and has been observed in marsupials, insectivors and rodents (Sadleir, 1969; Wimstat, 1975). Facultative (lactational) type delay of implantation, as the name suggests is characterized by retarded development of embryos if conception takes place during the lactation of a previous reproductive cycle. Because lactational delay of implantation is not reported to occur in carnivores it will not be discussed further (for a review see: Sadleir, 1969; Wimstat, 1975; Renfree, 1976). In contrast to species with facultative diapause,

species with an obligate or seasonal diapause have an annual reproductive cycle and embryo reactivation takes place during a specific time of year that is similar for all females in a population (Sadleir, 1969). Obligate diapause has been described in carnivores, pinnipeds, edentates, bats, a deer and a mole. Among these, obligate embryonic diapause has been most intensively studied in the carnivore family mustelidae (Sadleir, 1969; Wimstat, 1975; Mead and Wright, 1983).

Among species within the mustelidae, considerable variation exists both in occurrence of delayed implantation as well as the length of time embryos remain in a state of diapause (Wimstat, 1975; Mead and Wright, 1983). Mead and Wright (1983) categorized species of mustelids into three groups on the basis of their reproductive cycle. One category contains species that display no delay of implantation. Most thoroughly described in this regard are the domestic ferret (Mustela putorius), least weasel (Mustela nivalis) and eastern subspecies of spotted skunk (Spilogale putorius putorius). Gestation length for ferrets, least weasel and eastern spotted skunk is approximately, 44–47, 35–37 and 50–65 days, respectively (Mead, 1968a; Foresman and Mead, 1978; Mead and Wright, 1983). A second group of mustelids in this classification includes species that have a variable period of embryonic diapause resulting in gestations of variable length. Within this group, mink (Mustela vison) and the striped skunk (Mephitis mephitis) have gestations that vary greatly among individuals within each population. In mink embryonic diapause lasts from 5–55 days depending upon the date of

mating (Hansson, 1947; Enders, 1952;). Because the interval from implantation to parturition is approximately 30 days (Enders, 1952) total gestation length can vary from 39-76 days (Bowness, 1942; Hansson, 1947; Enders, 1952). A similar range of gestation (55-80 days) has been reported for the striped skunk (Wade-Smith and Richmond, 1978; Wade-Smith et al., 1980). Following Mead and Wright's (1983) classification, a third group includes species that have a prolonged embryonic diapause. Most investigated species in this group is the western spotted skunk (Spilogale putorius latifrons), which unlike the eastern subspecies has a prolonged period of diapause resulting in a total gestation length of 230-250 days (Mead, 1968b). The European badger (Meles meles), which has a 8-9 month embryonic diapause (Bonnin et al., 1978) and the fisher (Martes pennanti), American river otter (Lutra canadensis) and short-tailed weasel (Mustela ermina) are placed in this category because they have a prolonged period of diapause and gestations exceeding 9 months (Daniel, 1970a; Mead and Wright, 1983).

Numerous authors have speculated on the ecological significance of embryonic diapause and the selective pressures that might have favored its evolution (Sadleir, 1969; Mead and Wright, 1983). Generally, it is accepted that embryonic diapause is a reproductive specialization evolved independently within each taxonomic group, from a more primitive condition that consisted of a constant but short period of gestation (Sadleir, 1969; Mead and Wright, 1983). In wild populations this trait appears to be of adaptive significance in that regardless of the date of mating embryonic diapause allows for

synchronization of parturition with seasonally favorable climatic conditions (Lack, 1954). Alternative explanations for adaptive fitness of embryonic diapause have been proposed on the basis of male-male and female-female competition (Sandell, 1990).

Reproductive Cycle of Female Mink

Estrous Behavior

In the northern hemisphere, the majority of mink mate during a 4-5 week period in late winter (February-March; Hansson, 1947; Enders, 1952; Venge, 1959). Mink are induced ovulators (Enders, 1952) and because luteal function is reduced as the blastocysts enter diapause, females will often remate during the preimplantation period. Therefore, mink are often reported to be a polyestrous species. However, the number of times a female will allow mating varies among individuals and some females will mate only once during the breeding season. Martinet and Allain (1985) describe mink as a monoestrous species because it only produces one litter each year.

Onset of estrous behavior in mink varies among color mutations and the first observed matings occur in dark mink (Standard Dark) toward the end of February. Lighter color mutations (e.g., Iris and Sapphire) generally mate later but rarely do domestic mink show estrous activity past the first week of April (Enders, 1952; Venge, 1959). Mating of female mink with sterile males results in a state of pseudopregnancy and corpora lutea of pseudopregnancy are maintained until mid-April (Duby and Travis, 1972). After this time, mink that have not mated (or are pseudopregnant) become seasonally

anestrus. Seasonal anestrus in mink is generally described as the period from parturition (May) to the onset of the breeding season for the next year (February). Other species of mustelids that exhibit embryonic diapause probably display similar periods of anestrus (Mead and Wright, 1983). However, this pattern may be quite different in mustelids that exhibit no delay of implantation. One such species, the ferret, will remate and produce a second litter before entering seasonal anestrus if the first litter is lost during pregnancy (Mead and Wright, 1983).

Seasonal variation in photoperiod synchronizes the annual breeding season of mink, spotted skunk, ferret and probably other mustelids (Pearson and Enders, 1944; Duby and Travis, 1972; Mead, 1981). Although this correlation between increasing photoperiod and onset of the breeding season has been observed in mink by numerous investigators (Hansson, 1947; Hammond, 1951; Enders, 1952; Duby and Travis, 1972; Pilbeam et al., 1979; Sundqvist et al., 1989) a systematic study has not yet been conducted to determine the mechanism by which photoperiod stimulates the onset of seasonal breeding in female mink.

Neuroendocrinology of seasonal breeding has been most-intensely investigated in the ewe, rat and hamster, which have spontaneous rather than mating-induced ovulation (for review see Karsch et al., 1984; Lang et al., 1984; Karsch and Wayne, 1988; Karsch et al., 1989). According to this model natural changes in photoperiod affects pineal secretion of melatonin (see effect of photoperiod page 21). Changes in daily exposure to melatonin in turn modifies the

effect of steroid negative feedback on the pulsatile pattern of gonadotropin (follicle stimulating hormone, FSH; luteinizing hormone, LH) secretion (Karsch et al., 1987). During anestrus, LH pulses occur infrequently (every 8-12 hours in the ewe). Photoperiod-induced increase in pulsatile secretion of gonadotropins is believed to stimulate ovarian activity (folliculogenesis) at the onset of the breeding season. Systemic estradiol concentrations increase concomitant with increased follicular development and elevated estradiol concentrations stimulate estrous behavior. In the ewe, a spontaneous ovulator, secretion of estrogen decreases and luteal secretion of progesterone increases after ovulation. Progestational stimulation results in a decrease in LH pulse frequency. Luteinizing hormone pulse frequency increases again as luteal regression occurs. This creates the spontaneous estrous cyclicity observed in this species during the breeding season. A photoperiod-induced steroid negative feedback relationship with gonadotropin secretion has been described for the ferret (Sisk and Desjardins, 1986; Caroll and Baum, 1989), which is an induced rather than spontaneous ovulator. However, to date no one has attempted to measure changes in FSH in mink. As a consequence, knowledge of the mechanism of seasonal control of folliculogenesis is limited in this species.

Duby and Travis (1972) conducted an experiment where weekly photoperiod was altered such that beginning June 21st mink experienced a rate of photoperiodic decrease and then increase doubled to that of controls. This treatment resulted in an accelerated breeding season (Dec. 8-13) but it is unclear if this

effect was brought about by decreasing or increasing photoperiod. In subsequent experiments, Duby and Travis (1972) demonstrated that photoperiod in excess of 16 hours of light had a negative effect on reproduction. Female mink maintained on short photoperiod (even total darkness) from December to May were reported to become reproductively active at approximately the same time of year (but less synchronized) as females under natural photoperiod (Kirk, 1962) while increasing photoperiod after the vernal equinox initiated anestrus. While female mink are receptive to mating only during February and March, testicular development of males begins during late fall. Under experimental conditions, it has been shown that short photoperiod (8 hours light, 16 hours dark) effectively stimulates testicular activity and abrupt changes from short to long photoperiod result in testicular atrophy. These gonadal responses to photoperiod are similar to those observed in the ferret (Herbert et al., 1978). Further, Murphy et al. (1990) reported that chronic treatment of female mink with 5 mg melatonin implants did not interfere with onset of breeding behavior (effects of melatonin on implantation will be discussed below). Together, these results suggest that in mink, short photoperiods (< 12 hours daylight) may allow onset of the breeding season while long photoperiod (> 12 hours daylight) inhibits breeding behavior and initiates seasonal anestrus. It is still unclear to what extent increasing (or decreasing) photoperiod acts to synchronize breeding within a population. As previously mentioned, lengthening photoperiod stimulates embryonic implantation and this will be discussed in more detail later.

Ovarian Structure

Hansson (1947) published the first description of the mink ovary contrasting it with that of the ferret. Ovaries of mink are roughly oval or bean-shaped and enclosed by a connective tissue capsule, the ovarian bursa, except for a small slit that is covered by the fimbria of the oviduct. Like other mammals, the ovary is comprised of a connective tissue rich medulla surrounded by a cortical region containing the developing follicles. The ovarian medulla of the newborn mink is reported to consist primarily of disorganized connective tissue (Enders, 1952). The ovarian medulla of adult mink, unlike some other mammals (including ungulates) has two distinct layers. Inner most medullary layer, consists of connective tissues derived embryologically from the rete ovarii. A second medullary layer, peripheral to the first, is comprised of steroidogenic interstitial cells (Sundqvist et al., 1988). An endocrinological role for these interstitial cells is not known.

Irregularities in the smooth oval structure of the ovary are generally due to large protruding follicles or corpora lutea (Enders, 1952) and change with the reproductive condition of the female. Ovary size is also dependent on reproductive condition, being small (100-150 mg) during summer anestrus and gradually increasing to about 250-300 mg immediately prior to mating (Enders, 1952; Pilbeam et al., 1979). Because of luteal development, greatest ovarian weights (1.0-1.6 g) are attained during pregnancy just prior to parturition (Enders, 1952).

Follicular Development

At birth, primordial germ cells (oogonia) and primary oocytes are reported to be scattered throughout the ovarian cortex (Hansson, 1947). In young mink primordial follicles accumulate in the ovarian cortex by approximately 5-6 weeks of age and recognizable folliculogenesis has been reported in kit mink sacrificed in October (Enders, 1952). Folliculogenesis continues at a low level from this time until the onset of the breeding season. A detailed description of follicular development has been presented by Hansson (1947).

In mink, the prepubertal ovary is slightly smaller but otherwise resembles that of anestrous adults (Hansson, 1947; Enders, 1952). Follicular growth can be stimulated in anestrous mink with injection of 100 IU of pregnant mares serum gonadotropin (PMSG; Hammond, 1952). Under natural conditions during anestrus, small groups of primary follicles increase in size to approximately 0.3-0.4 mm (Enders, 1952) after which growth stops and the follicles become atretic. Some follicular development continues throughout the year even during pregnancy, but during the short annual breeding season, primary follicles grow beyond stages observed during anestrus reaching approximately 1.0 mm in diameter. Graafian follicles that have reached a diameter $<$ 0.7mm appear to be representive of proestrus (Enders, 1952). Number of developing follicles is greater during the breeding season than anestrus and folliculogenesis appears to increase as the breeding season progresses (Hansson, 1947; Enders, 1952).

Ovulation

In mink, like the ferret and rabbit, ovulation is induced by mating and follicles that mature in overlapping waves, regress unless the female is stimulated to ovulate. Enders (1952) reported that ovulation can be induced in mink by both the struggling of mating as well as physical stimulation of the vagina and cervix. While copulation in mink is normally prolonged (often lasting more than an hour), it has also been established that only brief exposure to a male (that may not involve seizing the female or intromission) can induce ovulation (Enders, 1952; Adams, 1973; 1981). Ovulation can also be induced artificially by interperitoneal injections of human chorionic gonadotropin (hCG; Hammond, 1952) or intramuscular injections of gonadotropin-releasing hormone (Murphy, 1976; Murphy, 1978).

In mink, spontaneous ovulation may occasionally occur. Corpora lutea are reported to have formed in unmated mink, removed from the vicinity of males for several months before the onset of the breeding season (Moller, 1974). However, interpretation of these results is difficult because of the possibility that ovulation could be induced by the stress of human handling (Daniel, 1971).

Reports of the period between coitus and ovulation vary. Hansson (1947) estimated the period from mating to ovulation to be 36-37 hours. Enders (1952) reestimated this period as 42-52 hours and Venge (1959) reported 33-66 hours. Injection of hCG results in ovulation 35-40 hours after treatment (Hammond, 1952; Chang, 1968).

As in other mammals, the ovum of preovulatory follicles is surrounded by a layer of granulosa cells, the corona radiata and attached to the follicular wall by a stalk of granulosa cells, the cumulus oophorus. Enders (1952) reported that the cumulus oophorus and corona radiata reach their greatest development just prior to ovulation (Enders, 1952). As in other mammals, following ovulation the ovulated follicles form corpora lutea, but in mink, European badger and western spotted skunk, luteal function is retarded during embryonic diapause.

Pilbeam et al. (1979) reported that vulval swelling increased steadly during the breeding season. Anestrous mink have lower plasma levels of estradiol and demonstrate no similar vulval stimulation (Pilbeam et al., 1979). Therefore, it is likely that estrogen production increases during the breeding season, along with the maturation of each wave of developing follicles (Pilbeam et al., 1979). Systemic concentrations of estrogen may be responsible for changes in the receptivity of females during different periods in the reproductive season. During the breeding season, four or more waves of follicles mature at approximately 8-day intervals (Enders, 1952). The interval between mating and the period of greatest receptivity is approximately 7-10 days (Sundqvist et al., 1989) and coincides with the maximum number of mature follicles (Hansson, 1947).

It is standard practice for mink producers to begin mating their female mink in early March and repeat mating each female at approximately 7-10 days after its first successful mating (Sundqvist et al., 1989). Therefore, female mink are often mated two to three

times resulting in an average of 19.2 follicles being ovulated during the breeding season (Adams, 1973). This practice results in both superfetation and superfecundation as a result of multiple ovulation and fertilization (Hansson, 1947; Enders, 1952; Shackelford, 1952; Enders and Given, 1977). When superfetation occurs, most of the offspring in a given litter originate from ovulations induced during the second (or subsequent) matings (Johansson and Venge, 1951; Shackelford, 1952). Johansson and Venge (1951) reported that mink mated 6 days apart produced litters with an average of 14% of the kits arising from the first and 86% from the second mating. This difference in the number of kits produced from the first and second matings is believed to be due to mortality of unimplanted embryos at the time of the second mating (Adams, 1981).

Preimplantation Embryonic Development

At ovulation, the ovum of mink is surrounded by both the acellular zona pellucida as well as the granulosa cells of the corona radiata and cumulus oophorus. Hansson (1947) reported that ova surrounded by granulosa cells can be collected from the upper oviduct, but Enders (1952) indicated that the ovum is free of the corona radiata at the time of fertilization, and suggested that in earlier reports, rough handling during necropsy may have resulted in the expulsion of ova and granulosa cells from immature follicles. Regardless, fertilization occurs in the upper oviduct (and perhaps ovarian bursa) and if corona cells are present, they are shed during oviductal transport (Enders, 1952). Cleavage begins in the upper

oviduct and ovum transport down the upper half of the oviduct requires about 12 hours (Hansson, 1947). Cleavage continues but at a slower rate as the embryos pass the lower half of the oviduct, entering the uterus as blastocysts approximately 8 days after mating (Hansson, 1947).

Upon entering the uterus the blastocysts become quiescent and remain unattached during embryonic diapause. Ovulation from one pair of ovaries results in the equal distribution of embryos in both uterine horns (Enders, 1952). Migration, and spacing of the blastocysts occurs prior to embryo reactivation, and nidation. It has been postulated that in mink, estrogens produced by developing waves of follicles may be responsible for uterine migration as has been described for the rabbit and swine (Boving, 1971).

Embryonic Diapause

Each blastocyst consists of a single layer of squamous trophoblast cells that form a hollow sphere surrounding a spherical cluster of cells, the inner cell mass. It is believed that development slows and blastocysts enter diapause shortly before the 300 cell stage. Enders (1952) recovered embryos that were at approximately 300 cells from the anterior uterus of mink between 10 and 17 days post-coitus. The inner cell mass of these embryos consisted of 28 to 164 cells.

Although embryonic development does not progress beyond the blastocyst stage during diapause, embryos of the short-tailed weasel, mink, European badger, American badger and western spotted skunk

gradually increase in diameter (Deanesly, 1943; Neal and Harrsion, 1958; Wright, 1966; Canivenc and Bonnin, 1981; Mead and Rourke, 1985). Total cell numbers also increase slowly throughout the period of delayed implantation but this increase is restricted to the trophoblast in the spotted skunk and perhaps the American badger (Wright, 1966) and European badger as well (Harrison, 1963). There is no evidence of increased trophoblast cell number in embryos of mink or short-tailed weasels during diapause (Baevsky, 1963; Shelden, 1973). Despite this growth, the zona pellucida is retained until just prior to the period of implantation in all carnivores (Wright, 1963) and hatching from the zona represents one event in the reactivation of embryos after diapause. However, this is not the case in all species exhibiting delayed implantation. Hatched blastocysts can be recovered from the uterus of the Armadillo (Enders, 1966) and Roe deer (Aitken et al., 1973) during embryonic diapause.

Enders et al. (1986) investigating the ultrastructure of spotted skunk blastocysts indicated that metabolic activity is reduced during embryonic diapause. Several lines of evidence indicate that blastocysts during diapause are metabolically active but at a rate well below that of embryos after diapause. First, some DNA synthesis must occur in trophoblast cells in those species where cell number is increasing. Second, blastocyst RNA synthesis appears to occur at very low levels in the mink (Gulyas et al., 1969) and spotted skunk (Mead and Rourke, 1985). Similarly, protein is synthesized in blastocysts of the spotted skunk and mink during diapause (Gulyas et

al., 1969; Rourke and Mead, 1982). It is likely that at least part of this protein synthesis occurs as a result of RNA synthesized prior to the onset of diapause (Rourke and Mead, 1982). Gulyas and Daniel (1970) also reported that oxygen consumption of diapausing mink embryos was as high or higher than that of "activated" rabbit blastocysts.

Embryo Reactivation and Nidation

Following the period of diapause the embryo is reactivated and development resumes with the embryo hatching from the zona pellucida (Hansson, 1947). Mechanisms involved in reactivation of the embryo have not been thoroughly described, but a few days prior to implantation in mink and the spotted skunk numerous cytological and metabolic changes can be detected within the blastocysts, which are now undergoing renewed development. There is a rapid increase in cell number of both the trophoblast and inner cell mass (Mead, 1981; Mead and Rourke, 1985; Enders et al., 1986). The inner cell mass loses its spherical appearance, forming a more flattened embryonic disc and a new germ layer (endoderm) differentiates from the inner cell mass. Cells of both the trophoblast and inner cell mass develop a more extensive rough endoplasmic reticulum (Enders et al., 1986). These histological and cytological changes in the skunk and mink blastocyst are strongly correlated with a marked increase in RNA and protein synthesis (Rourke and Mead, 1982; Mead and Rourke, 1985).

Although, endocrine signals affecting embryo reactivation in mink appear to be of uterine origin, it is also possible that the

blastocyst induces changes in endometrial morphology and secretion through production of growth factors as has been suggested for the rat (Acker et al., 1989). Shortly after embryo reactivation, distinct stage-specific changes occur in the apical endometrial surface, apparently in preparation for embryo attachment (see Uterine Morphology). Stage-specific uterine changes have not been well described in mink, but in the rabbit, these include changes in glycoprotein content, resulting in a loss of negatively charged molecules on the endometrial surface (Hewitt, 1979; Anderson and Hoffman, 1984). Changes in surface negativity appear to be involved in attachment of the trophoblast to the endometrium in the rat, rabbit and ferret (Enders and Schlafke, 1972; 1979). Upon endometrial contact in the ferret there is a proliferation of trophoblastic cells concomitant with epithelial proteolysis permitting intrusion of the trophoblast into the endometrium (Enders and Schlafke, 1972).

Parturition

Duration of gestation in mink is highly variable (Bowness, 1942; Pearson and Enders, 1944; Hansson, 1947; Enders, 1952) and the date of mating is the most important factor accounting for this variability. Matings later in the breeding season tend to result in shorter gestation lengths. Bowness (1968) reported that in mink the average gestation is 52.37 days in length (based on 22,919 observations) and the range of gestation is apparently 39-76 days (Enders, 1952). Because of the relatively synchronous nature of

implantation in mink, most litters are born in early May. Enders (1952) reported that mink litters can be as large as 17 kits, but the average litter is approximately 4-5. At birth, young mink weigh 7-10 g and are blind and naked. Kits usually open their eyes at about 5 weeks and are weaned at approximately 7 weeks of age. During the first 8 weeks of life, kit mink grow rapidly and then their growth gradually slows with age until they attain adult size in early November. Mink are sexually mature by February at approximately 10 months of age and mate in synchrony with adults of the previous generation (Enders, 1952).

Endocrine Regulation of Luteal Function

Luteal Development

After ovulation the granulosa cells of the ovulated follicle proliferate filling the follicular antrum (antrum folliculi) to form an immature corpus luteum within a few hours (Hansson, 1947; Enders, 1952). Histologically, immature corpora lutea appear as a loose aggregate of luteinizing cells and there is a lack of distinct luteal vascularization. In the first week of luteal development angiogenesis occurs with an ingrowth of blood vessels into the developing luteal tissue. However, luteal development and function is arrested shortly after the onset of luteal angiogenesis at approximately the same time that the embryos reach the blastula stage of development. In the European badger this results in corpora lutea with a central avascular region (Sarker and Canivenc, 1982). In this species luteal reactivation is associated with an increase in

capillary diameter, renewed angiogenesis and vascularization of the central region of the gland (Sarker and Canivenc, 1982). Regulatory factors involved in this unique pattern of angiogenesis have yet to be characterized.

Corpora lutea of mink, spotted skunk and European badger remain as distinct but relatively inactive structures during embryonic diapause. Luteal inactivity during this period can be confirmed by histological appearance of the corpora lutea and low serum concentrations of progesterone (Hansson, 1947; Enders, 1952; Moller, 1973; Bonnin et al., 1978; Mead, 1981; Sarker and Canivenc, 1982). At this time, luteal cells are small, densely packed and stain strongly basophilic. Luteal cells during embryonic diapause in the spotted skunk appear to consist of two populations based on cell size (large and small). Small luteal cells range from 12-20 um in diameter while large cells range form 20-45 um (Sinha and Mead, 1975). During diapause small luteal cells predominate. These cells have only modest amounts of rough and smooth endoplasmic reticulum, and small rod-shaped mitochondria (Sinha and Mead, 1975).

During luteal quiescence, concomitant with delayed implantation, the uterus is characterized by an obvious lack of progestational stimulation and female mink are inclined to remate (Hansson, 1947; Enders, 1952; Mead, 1981). The inactive luteal phase persists until just prior to nidation when both corpora lutea and blastocysts are reactivated. Luteal reactivation is preceded by a dilation of vasculature supporting the corpora lutea after which there is an increase in luteal mass and significant increase in synthesis of

progesterone (Hansson, 1947; Stoufflet et al., 1989). In mink, corpora lutea of the post-implantation period are characterized by large luteal cells that are less irregular in outline and stain more acidophilic (Enders, 1962). Corpora lutea of the spotted skunk at embryonic implantation contain predominantly large luteal cells and very few small cells can be found 24 hours after nidation (Sinha and Mead, 1975). These large luteal cells contain an extensive rough and smooth endoplasmic reticulum and abundant mitochondria characteristic of steroidogenic cells. Sinha and Mead (1975) proposed that in the spotted skunk small luteal cells of the preimplantation period develop into large cells after embryo activation. If this is the case, origin of large and small luteal cells may differ among species. There is some controversy over the origin of large and small luteal cells in ungulates (Alila and Hansel, 1984; Farin et al., 1986; Schwall et al., 1986). For a further discussion of the regulation of luteal composition and function in ungulates see Appendix 2.

Effect of Photoperiod

In mustelids, a variety of physiological functions are synchronized by seasonal changes in daily photoperiod. These include winter and summer fur growth, onset of the breeding season (for both males and females) and timing of implantation in species with embryonic diapause (Duby and Travis, 1972; Allain and Rougeot, 1980; Allain et al., 1981; Martinet et al., 1981b; Rose et al., 1987). For

more information regarding some of the influences of photoperiod on winter fur growth see Appendix 1.

Manipulation of photoperiod has been demonstrated to induce embryo implantation within populations of mink, western spotted skunk and European badger. In mink and spotted skunks, embryo implantation is associated with exposure of the animal to increasing photoperiods of late winter and early spring while in the European badger it is associated with decreasing photoperiod of late autumn (Mead and Wright, 1983). Despite this obvious difference in their natural history it is apparent that seasonal changes in photoperiod influence the timing of nidation. In mink, duration of embryonic diapause may be experimentally shortened by exposing the animals to additional hours of daylight (Holcomb et al., 1962). Likewise, blinding prevents implantation in mink (Murphy and James, 1974a) and spotted skunks (Mead, 1971).

Pineal Gland and Melatonin

In mink as in many other mammalian species the pineal gland appears to mediate effects of seasonal change in photoperiod (Martinet et al., 1985; Karsch and Wayne, 1988). Although our knowledge of this mechanism is limited, it has been shown that the pineal functions as a neuroendocrine transducer, converting cyclic neuronal signals generated by the retina into cyclic hormonal changes, which in turn may regulate other physiological functions (Lang et al., 1984; Martin, 1985; Martinet et al., 1985).

The pineal is an elongate endocrine gland, positioned dorsally and medially to the posterior end of the third ventricle of the brain (Rouvet, 1982; Martin, 1985). Embryologically the pineal develops as an evagination (the epiphysis) of the diencephalon (Martin, 1985), the pinealocytes being derived from the ependymal cells lining the epithalamus. Unlike the ferret (David and Herbert, 1973) and spotted skunk, the pineal of mink remains attached to the brain by a thin stalk (Rouvet, 1982).

Changes in photoperiod initially perceived by the retina of the eyes is transmitted via optic nerves and the monosynaptic retinohypothalmic nerve tract to the suprachiasmatic nucleus (SCN) of the hypothalamus (May and Mead, 1986). The pineal gland is then innervated by a multisynaptic pathway involving the superior cervical ganglia (SCG). In this way, the pineal is an effector system of the autonomic nervous system. Increased neuronal activity within post-ganglionic fibers of the SCG during periods of darkness stimulate a release of norepinephrine from axons contacting the pineal. Norepinephrine stimulates beta adrenergic receptors on the surface of pinealocytes and induces an increase in the enzyme N-acetyltransferase. N-acetyltransferase activity is the rate limiting step in synthesis of a variety of indoleamines (Reiter, 1980) of which melatonin is secreted in greatest quantity. Consequently, melatonin is secreted in greater concentrations at night, and for a longer period during nights of winter, in species such as the mink, spotted skunk, ferret (Ravault et al., 1986) and rat (Nishino et al., 1976).

Treatment of mink or western spotted skunks (May and Mead, 1986) with melatonin during the preimplantation period results in a further delay of nidation, suggesting that the pineal plays a major role in mediating effects of light in synchronizing timing of nidation. Inhibitory effects of melatonin on implantation are consistent with the observation that pinealectomy does not prevent implantation in mink or spotted skunks (Mead, 1972).

Site of action and mechanism whereby melatonin from the pineal acts to delay implantation remains largely unknown. _In vitro_ incubation of ovarian tissue with melatonin resulted in an increase in steroidogenesis (MacPhee et al., 1975). Danforth and Lippman (1981) found melatonin to increase concentrations of estrogen receptor in human MCF-7 carcinoma cells. Melatonin has also been reported to increase uterine growth and metabolism in the hamster by increasing concentrations of estrogen receptor _in vivo_ (Danforth et al., 1983). However, denervation of the pineal in mink (Martinet et al., 1985) and administration of melatonin to mink or western spotted skunks caused a decrease in serum concentrations of prolactin and prolonged pregnancy (Martinet et al., 1983; Martinet et al., 1984; Rose et al., 1985). It has been demonstrated that prolactin is luteotropic in mink and required for promoting transition of inactive corpora lutea during diapause to an active state at the time of nidation (Allais and Martinet, 1978; Papke et al., 1980). Further, Rose et al. (1986) identified binding sites for prolactin in the ovary as well as uterus of the anestrous mink suggesting that it may directly regulate ovarian and uterine function.

Gonadotropins (LH, FSH and Prolactin)

As in other mammals, the pituitary gland of mink consists of an anterior (adenohypophysis) containing the pars distalis and pars tuberalis and a posterior lobe (neurohypophysis). It is connected to the hypothalamus by a pituitary stalk, which is comprised of nerve tracts extending to the posterior lobe and blood vessels of the hypothalamo-hypophyseal portal system (Martin, 1985). Hypothalamic neurons secrete a variety of hormones [gonadotropin-releasing hormone (GnRH), dopamine and thyroid stimulating hormone-releasing hormone (TRH)] into the portal circulation, which act to stimulate secretory activity of the anterior pituitary. The pituitary must be functional for implantation to occur in all mustelids thus far examined. Hypophysectomy of mink after the preimplantation rise in progesterone leads to an abrupt reduction in progesterone concentrations as well as degeneration of embryos (Murphy et al., 1980).

Early investigators postulated that insufficient secretion of gonadotropic hormones from the anterior pituitary resulted in reduced progesterone secretion and delayed implantation in mustelids. Pearson and Enders (1944) first implicated gonadotropins (FSH and LH) in stimulating implantation in mink, suggesting that these gonadotropins were required to maintain luteal function. This hypothesis was supported by histochemical studies indicating that secretion of gonadotropins from the anterior pituitary of the mink and European badger, was less in individuals during diapause than in individuals in the post-implantation phase of pregnancy (Mead, 1981; Sundqvist et al., 1988). Hypophysectomy, prior to implantation

abolished the periimplantation increase in blood progesterone in the spotted skunk (Mead, 1975) and mink (Murphy and Moger, 1977) and there is evidence that LH is luteotropic in mink both before and after implantation occurs. Further, Murphy et al. (1984) demonstrated that administration of monoclonal antibodies against GnRH to mink during gestation significantly reduced systemic levels of LH and progesterone.

It is noteworthy that Mead (1975) could detect no effect of hypophysectomy on a change in the histological appearance of corpora lutea of spotted skunks during embryonic diapause. This observation suggests that during this period corpora lutea of the skunk, and most likely those of the mink and European badger, remain independent of support from the pituitary gland. Delayed implantation appears to be a period of luteostasis in these species during which corpora lutea may be totally autonomous and maintained by a "luteostasin", which, if such a factor exists, is not of pituitary origin. Hysterectomy of pseudopregnant mink (Duby and Travis, 1972) and pregnant skunks (Foresman and Mead, 1974; Mead and Swannack, 1978) does not interfere with luteal activation or time that it occurs (near the vernal equinox). Thus, hormones of uterine or embryonic origin do not appear to be involved in the luteostatic effect.

Prolactin is luteotropic in mink and required for promoting transition of corpora lutea from the inactive to active state (Allais and Martinet, 1978; Papke et al., 1980). Luteal activation coincides with an increase in systemic concentrations of prolactin that occurs shortly before implantation (Murphy, 1979; Martinet et al., 1981a;

Murphy et al., 1981). Rose et al. (1986) found that unoccupied prolactin receptors in the ovaries of mink decreased as serum concentrations of prolactin increased after the vernal equinox. Prolactin appears to act on the ovary to stimulate luteal secretion of progesterone. When administered to mink during delayed implantation, bromocriptine, a dopamine agonist that inhibits prolactin secretion, suppressed the seasonal increase in serum concentrations of progesterone and proloned the preimplantation period (Martinet et al., 1981a). Administration of pimozide (which stimulates prolactin release) or exogenous prolactin caused a premature rise in serum progesterone and hastened implantation (Papke et al., 1980; Martinet et al., 1981a; Murphy et al., 1981; Murphy et al., 1983a). Prolactin can maintain progesterone secretion in pregnant hypophysectomized mink, suggesting that prolactin is the primary gonadotropin responsible for stimulating luteal activity.

Prolactin was initially reported to play a lesser role in stimulating implantation in the western spotted skunk and European badger (Mead, 1981). Administration of ovine prolactin to spotted skunks for 7 days during delayed implantation had no effect on plasma concentrations of progesterone or the length of the preimplantation period. However, this effect may depend on seasonal timing of prolactin administration because recent evidence (Berria et al., 1989) indicates that prolactin is involved in stimulating implantation in western spotted skunks. Role of gonadotropic hormones in stimulating implantation in the European badger remains to be elucidated.

In mink basal prolactin and LH concentrations increase in parallel with the spring rise in blood concentrations of progesterone, which coincides with the vernal equinox. While prolactin is obviously important for promoting luteal progesterone synthesis in mink, it is unknown whether this effect is by activation of steroidogenic enzymes, or via stimulation of increased numbers of LH receptors as was reported to occur in rats (Gibori and Richards, 1978). Prolactin is necessary to maintain normal numbers of receptors for LH in the developing corpus luteum (Richards and Williams, 1976) and this effect is consistent with its known role in maintaining the corpus luteum in rodents. There is some evidence that LH also regulates prolactin receptors in the rat. However, this relationship is not universal because prolactin does not appear to regulate luteal function in the cow (Hansel et al., 1973) or ewe (Karsch et al., 1971). Prolactin receptors have been found in porcine luteal tissue and stimulation by prolactin has been shown to increase luteal low-density lipoprotein receptors and lipoprotein induced steroidogenesis in swine and ferrets (McKibbin et al., 1984).

Prolactin Synthesis and Secretion

Mammalian prolactin is a single polypeptide chain approximately 200 amino acid residues long, with three disulfide bonds and a molecular weight of about 24 kDa (Lewis et al., 1984; Kohmoto et al., 1984; Nicoll et al., 1986a). Prolactin as well as growth hormone and placental lactogen are peptide hormones generally considered to belong to the same hormone family because of common structural and

immunological features (Nicoll et al., 1986a,b). Physiochemical studies indicate that prolactin and other hormones in the prolactin-growth hormone (PRL-GH) family are simple globular proteins with regions of similar helical structure (Nicoll et al., 1986a). Except for some specific forms of prolactin most of the hormones in the PRL-GH family are unglycosylated. The most distinctive feature of secondary structure, which identifies prolactin from other hormones in the PRL-GH family, is the presence of a prolactin specific loop at the amino terminus of prolactin that is maintained by a disulfide bridge (Nicoll et al., 1986a). However, it is unclear how this difference in secondary structure affects the biological action of the molecule. It is paradoxical that with approximately 85% sequence homology of amino acid residues between growth hormone and prolactin, prolactin is virtually inactive in binding growth hormone receptors or evoking a response as determined by growth hormone bioassays. However, growth hormone and placental lactogens bind to prolactin receptors and stimulate responses detectable by bioassays for prolactin (Nicoll et al., 1986a,b).

In mammals synthesis and secretion of prolactin from the anterior pituitary (adenohypophysis) is believed to be primarily regulated by inhibitory factors originating in the hypothalamus (del Pozo and Brownell, 1979). Major evidence supporting the concept of inhibitory regulation was derived from experiments in which the pituitary stalk was severed or the anterior pituitary was transplanted to a site distal to the hypothalamus (Leong et al., 1983).

Although a variety of hypothalamic hormones and neurotransmitters have been reported to have an effect on prolactin secretion, dopamine is believed to be the major prolactin inhibitory factor (Ben-Jonathan et al., 1977; Ben-Jonathan, 1985; Arbogast and Ben-Jonathan, 1988). Dopamine is a catecholamine secreted by tuberoinfundibular neurons of the hypothalamus impinging on portal blood vessels of the median eminence that lead to the anterior pituitary (Ben-Jonathan et al., 1977; MacLeod et al., 1980; Maurer, 1981). Regulation of prolactin synthesis has been proposed to involve a feedback loop with prolactin inhibiting dopamine release and the number of dopamine specific receptors on lactotropes (Heimen and Nira-Ben Jonathan, 1983).

Although structurally similar to other catecholamines, dopamine does not bind well to α or β adrenergic receptors and has its own distinct set of receptors. Dopamine receptors are categorized (D1 and D2) according to their anatomical distribution and ability to stimulate adenylate cyclase (Kebabian and Calne, 1979). The D1 receptors in this classification stimulate adenylate cyclase and have been found primarily in the parathyroid gland (Ben-Johnathan, 1985). The D2 receptors are found within the anterior pituitary, and either inhibit or have no effect on stimulation of adenylate cyclase.

Upon contact with pituitary lactotropes, dopamine interacts with D2 receptors on the cell surface to inhibit prolactin synthesis and secretion. Because of the lack of a relationship of D2 receptors to adenylate cyclase, the mechanism by which dopamine inhibits prolactin secretion is poorly understood. Agonists (bromocriptine, lisuride and lergotrile), which specifically bind to the D2 but not D1

receptor, are potent inhibitors of prolactin secretion (Ben-Jonathan, 1985). Nevertheless, an effect of dopamine via adenylate cyclase cannot be completely ruled out in the control of prolactin secretion (Giannattasio et al., 1981; Enjalbert and Blockaert, 1983). Taraskevich and Douglas (1978) reported that dopamine blocks entry of calcium ions into the lactotrope thus altering cellular membrane potentials. Intracellular concentrations of calcium ion also appear to be directly involved in prolactin secretion (MacLeod and Fonthan, 1970) supporting the model that dopamine regulates prolactin secretion through calcium entry. Dopamine as well as bromocriptine inhibit hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and diacylglycerol (Canonico et al., 1983). Both compounds are involved in calcium dependent stimulation of protein kinase C and it is likely that this pathway is involved in dopaminergic inhibition of prolactin secretion by lactotropes.

Circadian variation in prolactin secretion has been identified in a number of species. Dopamine antagonists (pimozide) alter the daily pattern of prolactin secretion and substantial evidence indicates that the inhibitory action of melatonin on prolactin synthesis is mediated by dopamine (Murphy, 1983b; Agu et al., 1986). It also has been suggested that the hypothalamus exerts stimulatory control of prolactin secretion (Clemens et al., 1980). Evidence supporting the concept of a prolactin releasing factor arises from the fact that in the preovulatory rat, dopamine turnover rates in neurons of the hypothalamus do not always correlate with changes in circulating levels of prolactin. However, it is not known if these

differences are due to stimulation by a prolactin releasing factor or due to lactotrope desensitization to the inhibitory action of dopamine (Ben-Jonathan, 1985).

Although it has been difficult to demonstrate a requirement for a prolactin releasing factor, a wide variety of hypothalamic peptides affect prolactin secretion. Two hormones in particular, thyroid-stimulating hormone-releasing hormone (TRH) and vasoactive intestinal peptide (VIP) have been shown to increase prolactin production *in vitro* (Martin and Tashjian, 1977; Shaar et al., 1979). Further, antisera to these prospective regulators suppresses prolactin secretion *in vivo*. Studies also indicate that antibodies prepared against oxytocin will inhibit prolactin release *in vivo*, but investigators report that *in vitro* treatment with oxytocin will not stimulate prolactin production (Frawley et al., 1985). These results make interpretation of the immunological studies difficult.

It has long been recognized that estrogens stimulate prolactin secretion through several mechanisms in a variety of species (Maurer and Gorski, 1977; Dannies, 1985). Estrogen pellets implanted in ovariectomized rats appear to stimulate an increase in the number of prolactin secreting cells of the anterior pituitary (Dannies, 1985). These cells also showed an increase in synthetic ability, having more rough endoplasmic reticulum and Golgi bodies than those from non-implanted animals. It also appears that estrogen increases prolactin storage (granules) within lactotropes and inhibits the effect of dopaminergic agents on the degradation of prolactin secretory granules (Dannies and Rudnick, 1980).

Mechanism of Prolactin Action

The mechanism by which prolactin induces its effect on its target cell remains largely enigmatic. Like other protein hormones, prolactin is believed to initiate a cell response by interacting with prolactin specific receptors in the plasma membrane of its target cells. Convincing evidence for the presence of biologically active cell surface prolactin receptors was initially presented by Shiu et al. (1973) who used antibodies prepared against purified prolactin receptor to abolish prolactin-induced responses. Presence of membrane-bound receptors suggests that an intracellular second messenger system is involved in the mechanism of prolactin action. However, to date, the nature of this second messenger system is not known.

Receptors for prolactin have been identified by binding of radiolabled prolactin in a wide variety of mammalian tissues including mammary gland, ovaries, testis, uterus, liver, kidney, adrenal, prostate, seminal vesicle, hypothalamus, choroid plexis, pancreas, and lymphoid tissues (Posner et al., 1974; Manni et al., 1987; Williams et al., 1978; Posner and Kahn, 1983). Specific binding affinity constants for the prolactin receptor range from 10^{-9} to 10^{-10} M (Shiu et al., 1973).

The exact molecular construction of the prolactin receptor is not known, and it has been suggested that more than one receptor type may be present in and among target cells (Nicoll et al., 1986a). Prolactin receptors partially purified from rabbit mammary gland have

a molecular weight of approximately 32 kDa (Kelly et al., 1983; 1985). However, prolactin specific binding molecules with smaller molecular weights also have been recovered (Shiu et al., 1973). Prolactin receptors also have been isolated from the liver of lactating mice and human placentae by treatment with detergents (Liscia and Vonderhaar, 1982; Liscia et al., 1982; Vonderhaar et al., 1985). Binding studies of the detergent purified receptor (of 37 kDa molecular mass) indicated binding affinities (K_d) similar to that of high affinity sites in membranes. Because recovery of purified receptor required a detergent, these data suggest that the prolactin receptor is an integral membrane protein that requires a lipid environment to be functional (Luscia et al., 1982).

Endocrine Regulation of Uterine function

Uterine Morphology

Because the uterus mediates composition of the environment bathing the blastocyst, it is directly involved in regulation of delayed implantation. Mink, like the ferret (Hansson, 1947) and the spotted skunk (Mead, 1981) have a bicornuate uterus. During anestrus each uterine horn is approximately 30 mm in length, thread-like (2-3 mm wide) and oval in cross section (Enders, 1952). During estrus the uterus becomes enlarged to approximately 40 mm in length and 4-5 mm in width. As in other mammals, mink uteri consist of a secretory luminal epithelium supported by a vascularized connective tissue stroma (endometrium) and layers of smooth muscle (myometrium). The

uterine lumen is characterized by five lateral folds of endometrium giving it a pentaradiate appearance in cross section.

Mink, like other carnivores have a richly glandular endometrium (Enders and Enders, 1963), the endometrial glands extending deep within the stroma. Histologically distinct changes occur in the endometrium of the mink, skunk, weasel and European badger in response to endocrine stimulation associated with embryo implantation. Cells of the endometrial glands of mink during embryonic diapause have a reduced rough and smooth endoplasmic reticulum, few secretory granules and a high glycogen content (Schlafke et al., 1981). These features are described as absorptive rather than secretory and change dramatically immediately prior to implantation. At implantation endometrial cells increase in size becoming more columnar and there is a concomitant increase in both smooth and rough endoplasmic reticulum indicative of a secretory epithelium (Schlafke et al., 1981). Murphy and James (1974b) reported that in mink there is a significant decrease in endometrial glycogen and a concomitant increase in luminal mucopolysaccharide. It is likely that this shift in mucopolysaccharide synthesis is indicative of changes in the overall secretory activity of the uterus prior to nidation. In carnivores, embryonic implantation involves adhesion of the trophoblast to the endometrium, and then penetration of the endometrium (Enders, 1976), and it is possible that changes in mucopolysaccharide synthesis are involved in this process (Murphy and James, 1974b). Implantation-associated synthesis of endometrial glycoproteins has been reported to occur in rodents (Hewitt, 1979)

and rabbits (Anderson et al., 1986). In rabbits, stage-specific changes in the synthesis of apical membrane glycoproteins of the endometrium appear to be involved in blastocyst adhesion (Anderson et al., 1986).

Total protein content of uterine fluid of the ferret (Daniel, 1970b) and western spotted skunk (Fazleabas et al., 1984) increase in parallel with changing blastocyst diameter. It is likely that the quantity of various constituents of the uterine fluid also undergo dramatic changes. However, changes in the concentration of constituents in uterine flushing are currently unknown and this information is difficult to obtain because flushing usually damages the endometrium resulting in contamination of uterine fluid.

Proximate factors that regulate embryonic development in mammals appear to be of uterine origin. Transplanting 'dormant' rat blastocysts into 'active' rat uteri resulted in activation of dormant blastocysts (Dickman and DeFeo, 1967). Chang (1968) obtained similar results by transferring diapausing mink blastocysts into uteri of ferrets (which do not have delayed implantation) resulting in activation and subsequent implantation of the embryos; although they degenerated 4-8 days after implantation. Further, transfer of ferret morulas into the uteri of pseudopregnant mink resulted in a prolonged inactive period of 6-25 days.

A pregnancy associated serum protein has been isolated from mink (Larson et al., 1971) but the function of this factor is unknown. Krishnan and Daniel (1967) reported that uterine flushings from the rabbit contain a stimulatory factor called blastokinin (uteroglobin).

This protein appears in the uterine fluid prior to implantation, and has been shown to stimulate blastulation of rabbit morula _in vitro_ (Krishnan and Daniel, 1967; Daniel, 1976). A similar protein has been reported to exist in the uterine fluid of mink (Daniel, 1968; Daniel and Krishnan, 1969). Further, addition of blastokinin to _in vitro_ culture of mink embryos increased cell division over cultures without blastokinin (Daniel and Krishnan, 1969).

Evidence for inhibitory factors affecting blastocyst development are also convincing. Weitlauf (1976) showed that active blastocysts can be partially inhibited by incubating them with uterine extracts from animals during embryonic diapause. Several proteases such as plasminogen activator appear to be involved in embryo attachment and implantation in rodents, primates, ungulates and carnivores (Denker, 1980). Implantation can be inhibited in mice by uterine administration of protease inhibitors (Kubo et al., 1981). Uterine flushings from diapausing spotted skunks yield an inhibitor of plasminogen activator with a molecular mass of approximately 70 kDa. Unfortunately, to date, no temporal relationship has been established between this inhibitor and implantation in this species.

Mead and Wright (1983) have suggested three possibilities for proximate control of embryonic diapause: 1) during the preimplantation period the uterine environment may lack necessary factors that promote embryo growth and development; 2) the uterus may secrete regulatory substances that inhibit embryonic development; and 3) the uterus may secrete substances endocrine in nature that stimulate blastocyst development and promote implantation.

Ovarian Factors

How ovarian hormones induce implantation in largely unknown. The most likely hypothesis is that these hormones act on the uterus rather than the embryo to induce the endometrium to modify the intrauterine environment in such a manner that a renewed rate of embryonic development can occur. Numerous studies indicate that the uterus undergoes striking cytological changes during the preimplantation period (Enders and Given, 1977; Enders, 1981; Schlafke et al., 1981).

Ovariectomy during both pre- and post-implantation gestation results in a lack of progestational support and termination of pregnancy in mink, spotted skunk, long-tailed weasel and the European badger. The corpus luteum appears to be the only ovarian component that consistently undergoes pronounced morphological and physiological changes that temporally coincide with renewed embryonic development after diapause in mustelids (Mead, 1981).

Ovariectomized mink with transplanted ectopic corpora lutea activate and promote implantation of blastocysts when supplemented with exogenous progesterone indicating that the corpus luteum is the only ovarian component needed to induce implantation in these species (Foresman and Mead, 1978; Murphy et al., 1983). There is an increase in luteal diameter and serum concentrations of progesterone increase prior to implantation in all species of mustelid carnivores examined (Blatchley and Donovan, 1968; Duby and Travis, 1972; Murphy and Moger, 1977; Mead et al., 1981; Kinter and Mead, 1983; Martinet et al., 1983; Matson and Donovan, 1986). Plasma estrogen concentrations

appear to decline during preimplantation in the spotted skunk (Ravindra and Mead, 1984) European badger (Mondain-Monval et al., 1980) and during pregnancy in mink (Pilbeam et al., 1979). Estrone concentrations appear to be very low during pregnancy (Stouffet et al., 1989) and no data appear to exist on levels of estriol during pregnancy in mink or other mustelids. Attempts to induce implantation in intact or ovariectomized mustelids by administering progesterone (Hammond, 1951; Mead, 1981), other synthetic progestins such as medroxyprogesterone acetate (MPA; Murphy et al., 1982; Murphy, 1983b) or a combination of estrogens and progestins (Cochrane and Shacklford, 1962; Shelden, 1973) have failed.

It has been proposed by (Mead, 1981) that because neither progesterone nor estrogen can stimulate implantation, corpora lutea may secrete another nonsteroidal regulator that induces implantation. However, contrary to this model, Concannon et al. (1980) and Jarosz and Dukelow (1985) report that MPA will advance timing of implantation. Ability of MPA to affect gestation length appears to be dependent upon timing of treatment post-coitum (Jarosz and Dukelow, 1985). It appears that the uterus becomes more receptive to progestational stimulation as the end of embryonic diapause approaches. However, interpretation of these results is difficult because MPA has been found to persist longer in circulation than endogenous steroids. Concannon et al. (1980) found that treatment with MPA resulted in detectable levels 23 days after administration. Further, treatment with this steroid prior to nidation has been shown to interfere with parturition (Jarosz and Dukelow, 1985).

In vitro studies regarding steroid metabolizing potential of corpora lutea of western spotted skunk (Ravindra et al. 1984) and domestic ferret (Kinter and Mead, 1983) indicate that progesterone is the predominate steroid produced but that both species have enzymatic capability to aromatize androgens to estrogens. Rose et al. (1983a) showed that 5α-pregnane-3,20-dione is a major product of progesterone metabolism in the uterus, but a function for this steroid is not known. Although Mead (1981) concluded that ovarian factors other than progesterone are required to initiate implantation in the skunk, and possibly other mustelids, these factors have yet to be identified.

Mechanism of Estrogen and Progesterone Action

Current concepts on how steroid hormones initiate a response in target tissues have changed considerably since Mueller (1958) summarized early studies with a model of steroid action. These changes have promoted a number of in-depth reviews on the role of estrogen and progesterone receptors in initiating a response in target tissues (Leavitt et al., 1983; Rochefort and Westley, 1984; Gorski et al., 1986), the molecular nature of receptors (Horwitz et al., 1985; Savouret et al., 1989) and the nature of nuclear steroid response elements (Yamamoto, 1985).

Steroid Binding

Like other steroids, estrogens and progestins are lipophilic and diffuse freely through the cell membrane, cytoplasm and nuclear

envelope and are believed to exert their actions by binding to nuclear receptors (Gorski et al., 1986). Steroid binding to specific nuclear receptors is believed to induce a conformational change (transformation) of the receptor protein within the nucleus and the transformed receptor-ligand complex binds to regulatory regions of chromatin (Yamamoto, 1985). Binding of the hormone-receptor complex to chromatin of the nuclear matrix acts to increase rate of mRNA transcription for specific genes (Gorski et al., 1986).

Measurement of concentrations of steroid receptors has been used extensively to assess the responsive state of steroid target tissues. Steroid receptors exist in at least two binding states, unoccupied (not bound to the ligand) and occupied (ligand bound; Gorski et al., 1986). Unoccupied receptors are usually quantified by exposing the receptors to increasing quantities of [^{3}H]-labeled ligand in the presence or absence of excess non-labeled ligand. Occupied receptors are similarly quantified after dissociation of the ligand-receptor complex. Often, measurement of total concentrations of receptor (occupied and unoccupied combined) can be accomplished either by manipulating temperature during the exchange and binding assay, or by assaying with synthetic ligands that have greater binding affinity than the natural steroid (for a review of various techniques see: Clark and Peck 1979; Clark et al., 1988).

Localization (and to some extent quantitation) has also been accomplished by autoradiographic and immunocytochemical techniques (Brenner et al., 1974). Regardless of the technique used, these receptor sites are expected to display specific binding criteria

based on a theoretical description of receptor action. First, receptors should exist in a finite number within a target cell and tissue. This criterion is generally demonstrated by showing that receptor binding of increasing concentrations of labeled ligand follows Michaelis-Menten saturation kinetics (Clark et al., 1988). Second, receptor sites should have a ligand binding affinity sufficient to capture the ligand at physiological concentrations (10^{-10} to 10^{-9} M). Affinity constants (K_a) or their reciprocal (K_d) are calculated from saturation data by the method of Scatchard (1949). Third, hormone receptors should show higher affinity for specific ligands than for other members of the hormone family. However, hormone specificity is not absolute. _In vitro_ and _in vivo_ binding of androgens to estrogen receptors (at concentrations of 1-10 uM), and progesterone and glucocorticoids to both progesterone and glucocorticoid receptors (at approximately 1 nM concentrations) have been demonstrated (Lippman et al., 1977). This is of particular importance in attempts to quantify progesterone receptors (Walter and Clark, 1977). In addition to criteria discussed above for the biochemical description of receptors, these binding sites should also show both target tissue specificity and correlate with specific hormone-induced biological responses _in vivo_.

Early models of steroid action described steroid receptors as existing in the cytoplasm of target cells (Gorski et al., 1986). Hormone binding was reported to stimulate translocation of the hormone receptor complex into the nucleus. Evidence for the presence of cytoplasmic estrogen receptors was initially based on observations

that unoccupied receptors were present in the cytosols prepared by homogenizing target tissues such as uterus and oviduct (Jensen et al., 1969; Gorski et al., 1986). General findings were that the majority of unoccupied receptors were found in the cytosolic fraction and that injections of estradiol into rats resulted in a significant increase in concentrations of ligand-bound receptor in the nucleus. Early studies using autoradiography at low temperature (Jensen et al., 1969), and immunocytochemistry with polyclonal antibodies further supported a model that suggested that steroid receptors existed in the cytoplasm (Jensen et al., 1986).

Current interpretations indicate that both the unoccupied and ligand-bound receptor exist in the nucleus rather than cytoplasm of target cells (Gorski et al., 1986). The first convincing report suggesting that steroid receptors exist solely in the nucleus was presented by McCormack and Glasser (1980) who found that high concentrations of both unoccupied as well as occupied receptors exist in the nucleus of uterine cells grown in culture. Further evidence that both unoccupied and ligand-bound receptors exist in the nucleus came from the use of monoclonal-immunocytochemical techniques (King and Greene, 1984). Further, McClellan et al. (1984), also using immunocytochemical methods, noted that unoccupied receptors were more easily removed from the nucleus than bound receptors. Similar results have been demonstrated for the progesterone receptor. However, it should be noted that use of similar immunocytochemical techniques indicated that glucocorticoid receptors exist in both

nuclear and cytoplasmic compartments (Yamamoto, 1985; Gorski et al., 1986).

Convincing research showing an absence of cytoplasmic receptors was presented by Welshons et al. (1984) using GH_3 cells treated with cytochalasin and centrifuged in an Percoll gradient to form nuclear free cytoplasmic vesicles (cytoplasts). Enucleation by this technique produced cytoplasts that were metabolically active, but contained no nuclei. Exchange and binding assays of cytoplasts and cells treated in similar fashion but containing nuclei showed that only cells that had nuclei had estrogen receptors. Little or no receptors were found to exist in nuclear free cytoplasts (Welshons et al., 1984) and similar results have been demonstrated for the progesterone receptor (Welshons et al., 1985; Gorski et al., 1986).

Enucleation experiments of Welshons et al. (1984), and the immunocytochemical studies of King and Greene (1984) also indicated that no receptors were localized in the periphery of cells as reported by Szego and Pietras (1984). Likewise, immunocytochemical evidence indicates that binding does not occur on the nuclear envelope. Estrogen binding was shown to occur only within the nuclear matrix. Many nuclear proteins do not leave the nucleus even when the nuclear envelope is disrupted (Gorski et al., 1986). Therefore, these investigators (Gorski et al., 1986) concluded that nuclear receptor proteins are bound or become bound to components of the nuclear matrix.

Estrogen and Progesterone Receptor Structure

Although structural and biochemical characteristics of estrogen and progesterone receptors are not yet fully understood substantial information has been obtained on the molecular nature of these binding proteins (Leavitt et al., 1983; Rochefort and Westley, 1984; Horwitz et al., 1985; Yamamoto, 1985; Gorski et al., 1986; Savouret et al., 1989). In accordance with current molecular descriptions steroid receptors are believed to be linear proteinaceous molecules that may be comprised of multimeric units (Puca et al., 1979; Yamamoto, 1985).

Estrogen receptors isolated from the cytosol of target tissues such as the uterus and oviduct of the rat demonstrate a sedimentation coefficient of 8S when centrifuged in a sucrose density gradient. Treatment of the 8S estrogen receptor with high ionic strength buffers results in measurement of an aggregate of estrogen binding molecules consisting of 4S units. A variety of sedimentation gradients can be obtained by manipulating the protein concentrations in the sedimentation gradient, or by adding heparin (Stancel et al., 1973). Subunits (4S) with molecular weights ranging from 65-70 kDa have been isolated and until recently it was assumed that the 8S form of the estrogen receptor was a dimer made up of the two 4S subunits each with ligand binding capacity. However, isolation of 90 kDa (4-5S subunits) that lack the ability to bind steroids suggests that the 8S complex consists of at least two subunits, one in which steroid binding is absent (Joab et al., 1984). Non-steroidal binding sites have been described for glucocorticoid (Yamamoto, 1985) and

progesterone receptors (Tai and Faber, 1985).

Progesterone receptors are reported to have sucrose gradient sedimentation values ranging from 3.5 to 8S. Schrader et al. (1972) reported that progesterone receptor of the chick oviduct exists as a 6S dimer consisting of two 4S subunits. This subunit relationship is similar to that reported for progesterone receptors of the rabbit and guinea pig uterus (Milgrom et al., 1970; Savouret et al., 1989). The two 4S subunits have been shown to have molecular masses of approximately 80 kDa and 108 kDa. Because of endogenous proteases each 4S subunit is susceptible to proteolysis (Savouret et al., 1989), which in turn generates two fragments. It has been shown that one of these fragments (approximately 43 kDa) contains both the progesterone and DNA binding site, while the other (23 kDa) lacks both DNA and steroid affinity. Dissociation of progesterone receptor subunits is of particular importance in validating binding assays. Chen et al. (1981a,b) reported that the progesterone receptor is more stable in buffers containing sodium molybdate, glycerol and protease inhibitors.

As described above, binding of a steroid to its receptor results in a transformational change in the receptor protein that changes its affinity for nuclear binding sites. It has been proposed that estrogen binding converts the estrogen receptor subunits from 4 to 5S (Jensen et al., 1986). This was initially described to occur in the cytosol but probably takes place in the nucleus as pointed out previously. Regardless of the location, it appears that the chromatin binding affinity of the estrogen-receptor complex increases

during transformation. It has been proposed that formation of the 5S complex represents dimerization of two 4S subunits (Notides and Nielson, 1974), which contain nuclear binding sites and have a slower dissociation rate for estrogen binding. Notides et al. (1981) reported that estradiol modulates the equilibrium between the low and high affinity states (4 and 5S, respectively) and that this results in positive cooperativity of binding. However, this is in contrast to the findings of others (Sakai and Gorski, 1984) who have shown that estradiol binding is noncooperative at physiological concentrations and may be a result of techniques used in the cytosolic isolation of the receptor (Gorski et al., 1986).

Unlike the estrogen receptor, transformation of the progesterone receptor is reported to involve dissociation of receptor subunits. Progesterone receptors are believed to exist as a 6 to 8S dimer that upon binding dissociates into 4S subunits. One subunit is then believed to bind to DNA, while the other may bind to non-DNA chromatin (Savouret et al., 1989). It has been suggested that dissociation of progesterone is responsible for the prevalence of the receptor being lost from the nucleus and degraded during receptor assay conditions.

Specificity of Chromatin Binding

Several investigators have shown that binding of estrogen and progesterone receptors to chromatin of the nuclear matrix is a saturable phenomenon. This suggests that specific receptor sites exist on the chromatin. However, others report that specific nuclear

sites do not exist and that saturation kinetics represent measurement artifacts. Nuclear acidic proteins that may represent chromatin acceptor sites for the progesterone receptor have been studied extensively in the avian oviduct (Spelsberg et al., 1971; 1976; Spelsberg, 1976; Kon and Spelsberg, 1982). Although it is unclear if these proteins actually represent acceptor sites (Littlefield and Spelsberg, 1985) it does appear that transformation of the receptor results in binding of the steroid-receptor complex to DNA. Steroid receptors recognize different nucleotide sequences that represent regulatory elements of target DNA. These receptor binding regions, collectively termed response elements, reside in multiple copies within or near regulated genes (Stewart et al., 1988). Regulatory sequences, which have strong binding affinity for progesterone receptor have been identified 150-200 base pairs upstream from the ovalbumin gene of the chicken oviduct. Extensive nuclease footprinting experiments have been conducted indicating that a control sequence of bases (TATATAT) exist at this site. Similar binding sequences have been described for both glucocorticoid and progesterone receptor complexes associated with the promoter of the mouse mammary tumor virus (Yamamoto, 1985). Much of our understanding of steroid action arises from research on the mouse mammary tumor virus (MMTV), which has proven useful in studying steroid regulation of gene expression (Payvar et al., 1981; Hager et al., 1984).

Evidence for hormone regulation of the MMTV gene was first demonstrated in cells derived from murine mammary carcinomas. Mouse

mammary tumor virus expression has also been demonstrated in transfected non-murine cells, which are free of endogenous MMTV genomes (Cato and Weinman, 1988). Use of transient expression assays, has shown that progestins, dihydrotestosterone and glucocorticoids induced expression in chimeric proviral genes while estradiol had no inductive effect (Otten et al., 1988; Wu and Pfahl, 1988). Localization of the glucocorticoid receptor binding region was established using a MMTV proviral clone containing the oncogene from Harvey murine sarcoma virus. Further, removal of bases between -350 and -105 (0 = initiation of proviral transcription) from the viral genome resulted in a gradual loss of hormone inducibility (Ciardiello et al., 1988). Use of exonuclease protection techniques supported these findings and further demonstrated multiple binding regions within the LTR (Chalepakis et al., 1988). Common to these binding regions was a core sequence (TGTTCT). Overall, this suggests that activated receptor binds at a largely nonspecific DNA sequence (Payvar et al., 1981; Yamamoto, 1985; Wasner et al., 1988). Binding specificity may therefore be due to a regulatory, non-binding property of the receptor complex.

The intact glucocorticoid receptor is a protein of approximately 94 kDa. Comparing the binding of intact receptor with protease cleavage products of purified receptor preparations has indicated that DNA binding activity resides in a relatively small region of the receptor. Presence of a larger 50-55 kDa domain, which lacks inherent binding ability, greatly enhances binding affinity of the DNA binding domain. A third separate region of the receptor appears

to be involved in hormone binding. Several structural motifs have been described for DNA binding proteins (Struhl, 1989). One form contains nine repeating units of 30 amino acid residues, including two cysteine residues, and 7-11 Zn^{+2} molecules, which stabilize the protein to form finger-like structural projections (Struhl, 1989). It is believed that these structural "zinc fingers" interact to bind with the DNA. Binding of the hormone receptor complex may induce changes in chromatin structure that allow initiation of RNA polymerase II transcription and viral expression.

Physiological Responses to Ovarian Steroids

Estrogen and progesterone stimulate a variety of biosynthetic events in their target cells. These events have been classically described as early and late responses. Naturally, they are dependent upon hormone binding to a receptor and association of the hormone-receptor complex with DNA. Further, the primary sequences of activated genes are important, such that in different target cells different responses can be anticipated. Because uterotropic responses to estrogen have been most extensively described, these will be described as a model for steroid action.

Early uterine responses to estrogen in mice include hyperemia, histamine mobilization, water imbibition, increased glucose metabolism, lipid biosynthesis, biosynthesis of induced proteins, and increased activity of RNA polymerase II (Spanzani and Szego, 1958; Spaziani, 1963). Late responses constitute a continuation of early responses. This is particularly true of biosynthetic responses

including mRNA and DNA synthesis, cellular hypertrophy and hyperplasia and increased synthesis of histones (Gorski, 1964; Glasser et al., 1972; Anderson et al., 1975).

Of particular interest is the effect of estrogens and progestins on concentrations of estrogen and progesterone receptors. In estrogen target tissues like the uterus and mammary gland, basal estrogen receptor concentrations are maintained constitutively at constant low levels. Stimulation of the these receptors results in an increase in the concentration of newly synthesized estrogen receptors (Anderson et al., 1975). Similarly, in estrogen and progesterone target tissues, treatment with estrogen stimulates an increase in the concentration of progesterone receptors. Because of low progesterone receptor concentration, progesterone treatment of non-estrogenized uteri will not result in the production of a progestational or secretory endometrium (Reynolds, 1951). However, pretreatment with estrogen results in a significant increase in progesterone receptors and the secretory response. It has been shown that in the rat estrogen and progesterone receptor concentrations increase within 24 hours of estradiol injection. Further this response can be blocked by both cycloheximide, and actinomycin D, indicating that both mRNA and protein synthesis are involved in the up-regulation of estrogen and progesterone receptors (Anderson et al., 1975; Kassis and Gorski, 1986).

While estradiol has a stimulatory effect on concentrations of both the estrogen and progesterone receptor, progesterone in the absence of estrogen inhibits recruitment of both estrogen and

progesterone receptors (Milgrom et al., 1973; Freifield et al., 1974; Hsueh et al., 1975; 1976). This ability of progesterone is often described as being antagonistic to estrogen. However, progesterone does not affect the ability of estrogen to bind to its receptor, but rather acts to stimulate differentiation in the target cell, which appears to ultimately render it less responsive to both estrogen and progesterone stimulation (Lerner, 1964).

Effects of Prolactin on Steroid Action

While estrogen has been observed to stimulate prolactin secretion in a variety of species, influence of this protein hormone on steroid synthesis and action are less well documented. In rat granulosa cells, prolactin has an inhibitory effect on aromatase activity, resulting in a decrease in estradiol synthesis *in vitro* (Wang et al., 1980). Inhibition of ovarian aromatase also occurs *in vivo* (Tasi-Morris et al., 1983) and may be involved in lactation-induced diapause in rats and mice. Prolactin receptors have previously been detected in the ovaries of mink (Rose et al., 1986) suggesting that prolactin regulates ovarian function. Pilbeam et al. (1979) reported a decrease in serum estradiol concentrations concomitant with periimplantation increase in serum concentration of prolactin (Rose et al., 1986). Increased concentrations of prolactin are also associated with the post-implantation rise in progesterone (Murphy et al., 1981) and treatment with ovine prolactin will reverse melatonin-induced inhibition of luteal function and implantation.

Occurrence of prolactin receptors in the uterus is in agreement with results suggesting that prolactin may act directly on the uterus of a number of species (Joseph and Mubako, 1975; Rose et al., 1983b; Daniel et al., 1984; Chilton and Daniel, 1985; Chilton and Daniel, 1987). In the ovariectomized rabbit, this effect of prolactin appears to occur through modulation of progesterone receptor activity (Daniel et al., 1984) and inhibition of progesterone-induced down-regulation of the progesterone receptor. In rabbits, one effect of progesterone on the uterus is the stimulation of uteroglobin synthesis. Similarly, prolactin stimulates the production of uteroglobin in this species. However, growth hormone does not stimulate uteroglobin synthesis or progesterone receptor concentrations in this species (Chilton and Daniel, 1987).

The ability of prolactin to stimulate uterine progesterone receptors may be a highly specific response. Saiduddin and Zassenhaus (1977) were unable to demonstrate an effect of prolactin on concentrations of estrogen receptors in ovariectomized rats. Similar results were presented by Sartor et al. (1983) who showed that prolactin does not induce uterine estrogen receptors in young mice.

STATEMENT OF THE PROBLEM

Embryonic mortality associated with delayed implantation represents a major pitfall to husbandry programs involving species with embryonic diapause. In the mink industry, multiple mating practices are currently employed to reduce losses due to reproductive failure. Investigation of the physiological mechanisms involved in regulating implantation in this species may lead to the development of techniques that will reduce the period of embryonic diapause and embryonic wastage.

Implantation can be induced by treatments that increase serum levels of prolactin (Murphy et al., 1981; Murphy, 1983a). Prolactin is luteotropic in mink and stimulate progesterone synthesis. Although progesterone is required for implantation and maintenance of pregnancy in mink, all attempts to induce implantation in these species with exogenous progesterone or estrogen alone or in combination have failed (Cochrane and Shackleford, 1962; Holcomb, 1967; Mead, 1981). Because receptors for prolactin have been found in the uteri of anestrous mink, it is likely that prolactin has a direct effect in regulating uterine physiology of this species as has been described for the rabbit (Daniel et al., 1984; Chilton and Daniel, 1985; Chilton and Daniel, 1987). Research, as described herein, was conducted to elucidate the relationship between prolactin, estrogen and progesterone in regulating uterine physiology in mink.

ANIMAL CARE AND HOUSING

Standard dark mink were selected at random from the herd at the Oregon State University Experimental Fur Farm during the course of the following four experiments. Mink were kept individually in galvanized wire cages approximately 265.2 cm x 600 cm x 375 cm (high) separated by solid polyvinyl chloride dividers and each cage was equipped with a cylindrical wire-mesh nest box. Cages were housed in open-sided sheds at the Experimental Fur Farm, and the animals were maintained under natural changes of photoperiod and temperature. Animals were fed once daily (in the afternoon) a quantity of a standard ranch mink diet (see appendix I) adequate to appease appetite without wastage and water was supplied *ad libitum*.

Anesthesia for vasectomies (Exp. 4) was induced with Vetalar (Ketamine HCl; 100 mg/ml; Parke-Davis, Morris Plains, NJ; dosage delivered to effect, 0.7-1.0 ml) and surgical procedures were performed by the researcher under aseptic condtions. All blood samples were collected from animals under anesthesia (with 0.4 ml Vetalar) by cardiac puncture immediately prior to euthanasia.

EXPERIMENT 1

ESTROGEN STIMULATION OF UTERINE METABOLISM, AND NUCLEAR CONCENTRATIONS OF ESTROGEN AND PROGESTERONE RECEPTORS IN MINK DURING WINTER ANESTRUS

Introduction

Although delayed implantation in mink was first described in detail by Hansson (1947), endocrine control of this phenomenon is only partially understood. During embryonic diapause, corpora lutea formed after ovulation remain small, relatively inactive and systemic concentrations of progesterone are low compared with those during post-implantation pregnancy (Moller, 1973; Pilbeam et al., 1979; Stoufflet et al., 1989). Lengthening photoperiod during spring stimulates an increase in hypophyseal secretion of prolactin, which acts to induce luteal reactivation and stimulate progesterone production (Papke et al., 1980; Murphy et al., 1981; Rose et al., 1986). Ovarian steroids (estradiol and progesterone) are believed to act on the uterine lining (endometrium) to promote embryo reactivation and implantation (Pilbeam et al., 1979; Concannon et al., 1980). However, treatment with exogenous progesterone alone or in combination with estrogen does not advance the date of nidation (Hammond, 1951; Cochrane and Shackelford, 1962; Murphy et al., 1982). Contrary to these findings, Concannon et al. (1980) and Jarosz and

Dukelow (1985) reported that the synthetic progestin, medroxyprogesterone acetate (MPA), will advance timing of implantation. Ability of MPA to affect duration of gestation, and litter size appears to be dependent upon timing of treatment post-coitum (Jarosz and Dukelow, 1985). The uterus may become more receptive to progestational stimulation as the end of natural embryonic diapause approaches.

Apparent nonresponsiveness of the uterus to endogenous progesterone and estrogen during diapause could in part be attributed to insufficient concentrations of estradiol and progesterone receptors. In the rat, exposure to estrogen appears to be required to create a progestin responsive uterus (Reynolds, 1951). In this model, estrogen stimulation results in an increase in concentrations of newly synthesized estrogen and progesterone receptor (Anderson et al., 1973; 1975; Feil et al., 1975). Concentrations of rat uterine estrogen and progesterone receptor increase within 24 hours (h) of estradiol injection (Stormshak et al., 1976). Despite these well documented effects of estrogens, concentrations of estrogen and progesterone receptors have not been previously measured in the mink. Measurement of estrogen and progesterone receptors should be a useful approach for assessing the responsiveness of the mink uterus to steroid stimulation.

In addition to receptor regulation, estrogens stimulate a variety of biosynthetic events in target tissues. These include protein and DNA synthesis, which result in cellular hypertrophy and hyperplasia (Gorski, 1964; Glasser et al., 1972; Anderson et al.,

1975; Stormshak et al., 1976). Biosynthetic responses of the mink uterus to estrogen treatment have not previously been documented and may also indicate changes in relative responsiveness to steroid stimulation.

The objective of this experiment was to determine basal concentrations of uterine estrogen and progesterone receptors in nulliparous mink during winter (immediately prior to the breeding season) and to evaluate effects of exogenous estradiol on concentrations of these receptors. A second goal was to determine the effect of estradiol on uterine glucose metabolism, protein synthesis and cell division in 'proestrous' mink prior to the onset of the breeding season.

Materials and Methods

During December 55 nulliparous dark mink were assigned to a treatment (n = 20) or a control group (n = 35). Treatment consisted of a daily injection of 50 ug estradiol-17β (sc) for two consecutive days. Control mink were similarly injected with vehicle (1 ml corn oil). Mink were necropsied 24 h after the last injection and uteri were collected, dissected free of connective tissue and fat and weighed.

Uterine horns from 20 mink (10 treated and 10 control) were cut into longitudinal strips and pooled within each group (2 uteri per pool). Replicate aliquots of pooled tissue (n = 5) were used to quantitate _in vitro_ incorporation of $[^3H]$thymidine into DNA,

[^{14}C]leucine into protein and oxidation of [^{14}C]glucose to $^{14}CO_2$ (described below).

Uteri from 35 mink (15 treated and 20 control) were cut into strips, pooled within each treatment group (3 treated uteri per pool and 4 control uteri per pool). Replicate aliquots of pooled tissue were used to quantify changes in the number of uterine estradiol and progesterone receptors. For comparative purposes, estrogen and progesterone binding were also quantified in diaphragm tissue collected from estradiol-treated and control mink (n = 3 each) at necropsy.

Radioreceptor Assays

Nuclear estrogen and progesterone receptors were quantified by exchange and binding assays following procedures previously described (Koligian, and Stormshak, 1977; West and Brenner, 1985; West et al., 1986) and modified as indicated. Optimal estrogen and progesterone receptor assay conditions were determined on uteri collected from 32 additional anestrous adult mink injected with 50 ug estradiol for 2 days prior to sacrifice.

For estrogen receptor analysis, uterine tissue from separate pools (4 uteri/pool; n = 4) was homogenized on ice in 10 mM Tris-HCl, 1.5 mM EDTA (TE) and centrifuged at 800 x g to recover the nuclear pellet. Nuclear pellets were washed with TE buffer three times, recentrifuged (800 x g) after each wash and resuspended. A saturating concentration of [^{3}H]estradiol was determined by incubating aliquots containing 50 mg equivalents of resuspended

nuclear pellet with increasing concentrations of [6,7,-^{3}H(N)]estradiol (60 Ci/mmol;NEN) in the presence or absence of 100-fold excess unlabeled diethylstilbestrol (Sigma; 30° C for 30 min). Incubation was stopped by cooling the aliquots of nuclear suspension to 4° C. After incubation the aliquots were centrifuged, washed four times with cold TE buffer (centrifuging at 800 x g after each wash) and extracted (12 h) with 2 ml cold absolute ethanol. Extracted samples were centrifuged (1000 x g) and extractants were decanted into scintillation vials containing scintillation fluid (10 ml; 0.7% 2,5-diphenyloxazole, 0.05% p-Bis[2-(5-phenyloxazolyl)]-benzene, in toluene: Triton X-100, 2:1 vol/vol). Bound [^{3}H]estradiol was measured by liquid scintillation spectrometry (LSC) of extractant. Quench determination and correction of LSC was accomplished by preparation of a external-standard quench correction curve following the procedure of Horrocks (1969). Sample counting efficiency for ^{3}H in samples prepared above was $37 \pm 1\%$. After extraction, each nuclear pellet was precipitated with 2 ml of 0.5 M perchloric acid (PCA), hydrolysed at 90° C and total DNA determined by the procedure of Burton (1955).

For progesterone receptor analysis, pooled uterine tissue (4 uteri/pool; n = 4) was homogenized on ice in 10 mM Tris-HCl, 1.0 mM EDTA, 12 mM monothioglycerol, 5 mM sodium molybdate, 30% v/v glycerol (TEMG) and centrifuged at 1000 x g to recover the nuclear pellet. The nuclear pellet was washed as described above and resuspended in TEMG buffer. A saturating concentration of [17α-methyl-^{3}H]promegestone (R5020; 87 Ci/mmol;NEN) was determined by incubating

aliquots containing 50 mg equivalents of the nuclear pellet resuspended in TEMG buffer containing 10 nM dexamethasone (Sigma) and increasing concentrations of [^{3}H]R5020 (at 4° C for 16 h) in the presence or absence of 100-fold excess unlabled R5020 (NEN:NLP 004). After incubation the aliquots were centrifuged, washed four times with cold TEMG buffer (centrifuging at 1000 x g after each wash) and extracted (16 h) with 2 ml cold absolute ethanol. Bound [^{3}H]R5020 was measured by LSC of extractant and DNA content of the remaining pellet was determined as described above.

A concentration equal to 2 x 10^{-8} M [^{3}H]estradiol in incubation buffer was found to saturate estrogen receptors in 50 mg equivalents of the nuclear resuspension (Figure 1). Subsequent estrogen receptor assays of tissue were conducted (in duplicate) using this saturating concentration of 2 x 10^{-8} M [^{3}H]estradiol. Scatchard analysis for specific binding of [^{3}H]estradiol revealed linear plots (Figure 1) and a K_d of 5.45 nM (r^2 = 0.96).

A concentration of 1 x 10^{-8} M [^{3}H]R5020 was found to saturate the progesterone receptors in estradiol-treated mink and further progesterone receptor assays were conducted (in duplicate) using this concentration of ligand. Scatchard plots of specific binding of [^{3}H]R5020 were linear and indicated a binding K_d of 2.43 nM (r^2 = 0.94, Figure 2).

Determination of Glucose Oxidation, DNA and Protein Synthesis

Glucose oxidation, DNA and protein synthesis were measured as described by Stormshak et al. (1976). Briefly, weighed aliquots of

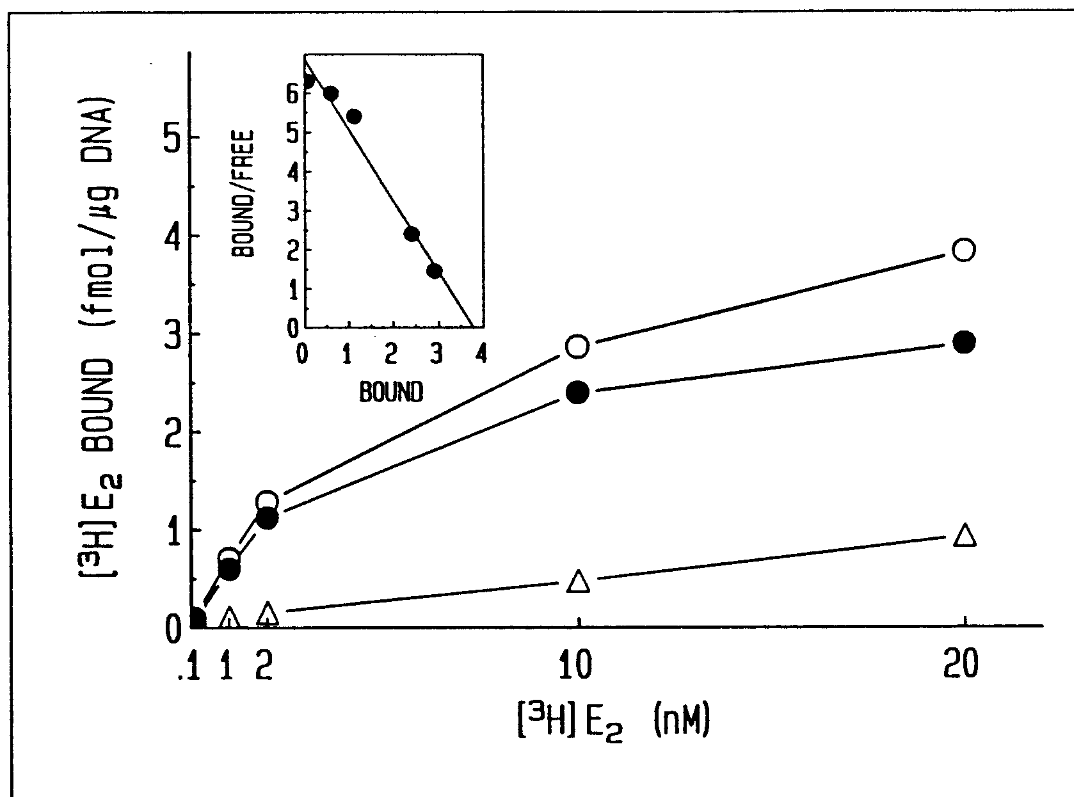

Figure 1. Saturation analysis of nuclear estrogen receptor in uteri of mink injected with 50 ug estradiol-17β (E_2) for 2 days prior to sacrifice. Aliquots of nuclear pellet (n = 4) were incubated with each concentration of $[^3H]E_2$ alone (Total bound; ○——○) or with a 100-fold excess unlabeled diethylstilbestrol (Nonspecifically bound; △——△). Specific binding (●——●) was determined as the difference between total and nonspecific binding. Inset: Scatchard analysis of specifically-bound $[^3H]E_2$, K_d = 5.45 nM (r^2 = 0.96).

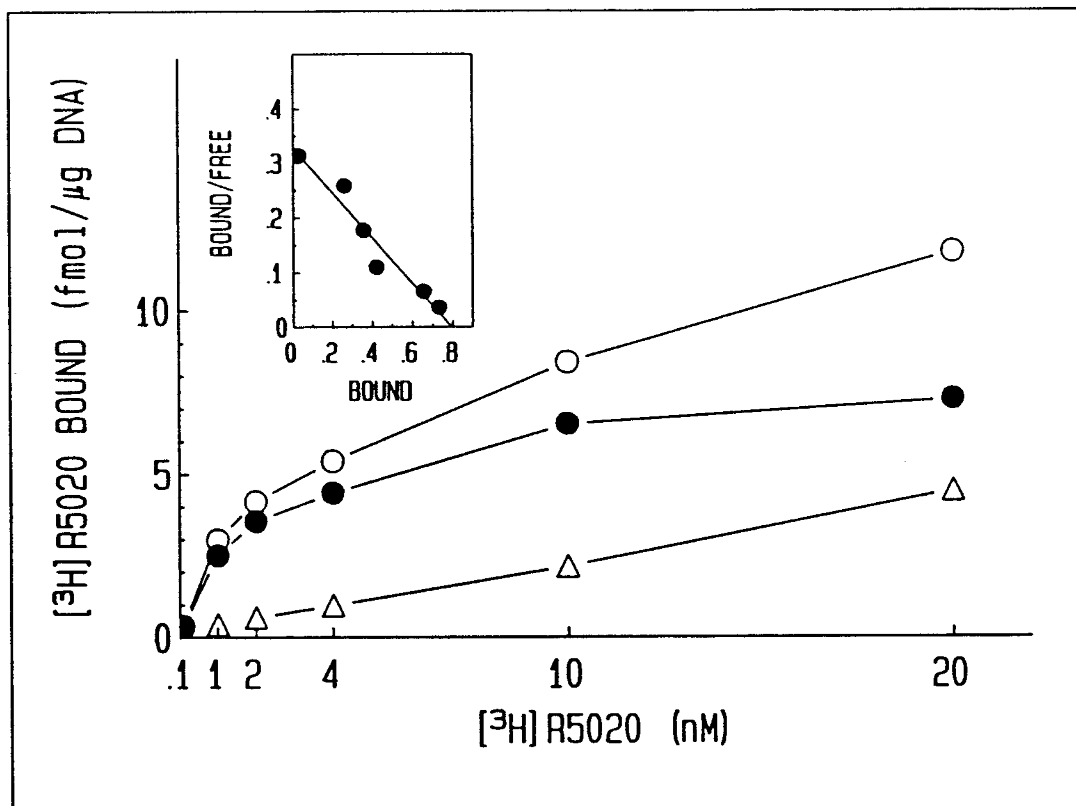

Figure 2. Saturation analysis of nuclear progesterone receptor in uteri of mink injected with 50 ug estradiol-17β for 2 days prior to sacrifice. Aliquots of nuclear pellet (n = 4) were incubated with each concentration of [^{3}H]promegestone (R5020) alone (Total bound; ○——○) or plus 100-fold excess of unlabeled R5020 (Nonspecifically bound; △——△). Specific binding (●——●) represents the difference between total and nonspecific binding. Inset: Scatchard analysis of specifically-bound ligand, K_d = 2.43 nM (r^2 = 0.94).

uterine tissue from control and treated mink were incubated in sealed flasks containing 2 ml Minimal Essential Medium (MEM alpha; Sigma) and 0.5 uCi/ml of D-[$U^{14}C$]glucose (250 mCi/mmol;NEN) or 0.5 uCi/ml of L-[$U^{14}C$]leucine (308 mCi/mmol; NEN) and 1 uCi/ml of [methyl-^{3}H]thymidine (20 Ci/mmol; NEN). Incubation was for 1 h at 37°C under an atmosphere of 95% O_2-5% CO_2. Incubation was terminated in flasks containing [^{14}C]glucose by adding 1 ml 2N H_2SO_4 and $^{14}CO_2$ was trapped for 2 h on filter paper saturated with hyamine hydroxide (NEF-921; NEN). Replicate flasks were similarly treated at the beginning of incubation to serve as unincubated controls. [^{14}C]glucose oxidation to $^{14}CO_2$ was measured by LSC of the filter paper and expressed as disintegrations per minute (dpm) per mg uterine tissue. Counting efficiency for ^{14}C in samples prepared as described above was 97±1%.

Incubations of uterine tissue in the presence of [^{14}C]leucine and [^{3}H]thymidine were terminated by rinsing with cold medium and homogenizing in 2 ml 0.5 M PCA. Replicate incubation flasks were similarly treated at the beginning of incubation to serve as unincubated controls. Homogenates were centrifuged at 1000 x g to precipitate protein and DNA. Precipitated tissue pellets were washed three times with 0.5 M PCA and centrifuged (800 x g) after each wash. The pellet was resuspended in 2 ml 0.5 M PCA and duplicate aliquots (0.5 ml) were recentrifuged and the pellet dissolved in 1 ml NCS Tissue Solubilizer (Amersham) and incorporation of [^{3}H]thymidine and [^{14}C]leucine in the solublized pellet was measured by dual-label LSC. Counting efficiency for ^{14}C and ^{3}H in samples prepared for dual label

counting was 92±2% and 31±2.1%, respectively. Replicate aliquots of homogenate were hydrolysed in 0.5 M PCA and total DNA determined by the procedure of Burton (1955).

Statistical Analysis

Analysis of data on uterine weight, concentrations of estrogen and progesterone receptor and *in vitro* incorporation of [^{14}C]leucine into protein and [^{3}H]thymidine into DNA and [^{14}C]glucose oxidation was accomplished by Student's t-test (Snedecor and Cochran, 1980). Log transformations of data on uterine weight and glucose oxidation were performed prior to analysis because variances were heterogeneous. However, for purposes of presentation data are represented by untransformed values.

Results

Basal uterine concentrations of estrogen receptor were found to be greater than basal concentrations of progesterone receptor (Figure 3A). Binding of [^{3}H]estradiol in uterine nuclei was fivefold greater than that in negative control tissue (diaphragm; $P < 0.001$). Although far less pronounced, binding of [^{3}H]R5020 in uteri was also significantly greater than in negative control tissue ($P < 0.05$). Injection of 50 ug estradiol for 2 days significantly increased concentrations of estrogen receptor ($P < 0.01$) and induced a five fold increase in progesterone receptor ($P < 0.001$; Figure 3B). Treatment with estradiol had no significant effect on the binding of [^{3}H]estradiol or [^{3}H]R5020 to negative control tissue (Figure 3AB).

Treatment with estradiol increased uterine weight approximately 2.5-fold ($P < 0.001$; Figure 4A) and stimulated *in vitro* glucose oxidation ($P < 0.05$; Figure 4B). *In vitro* incorporation of [^{14}C]leucine into protein and [^{3}H]thymidine into DNA was three- and 2.5-fold greater in estrogen-stimulated uteri, respectively ($P < 0.01$; Figure 5AB).

Discussion

Concentrations of progesterone receptor in uteri of control animals were only slightly above that observed in the negative control tissue (diaphragm) and reflect the relative nonresponsiveness of the uterus to progesterone stimulation during this period. Both estradiol and progesterone receptor concentrations increased in the uterus following estradiol treatment indicating that the uterus is responsive to estrogen. Treatment with estradiol had no effect on binding of [^{3}H]estrogen or [^{3}H]R0520 in diaphragm tissue and it is likely that the observed low level of binding of steroids in this tissue was nonspecific in nature.

Treatment with exogenous estradiol elicited a marked increase in metabolic activity as reflected by increased glucose oxidation and protein synthesis. Estradiol-induced increase in uterine weight may have included fluid uptake. However, growth was not limited to fluid uptake and appeared to involve both an increase in cell number and increase in cellular metabolism and protein synthesis. Together, these results are supportive of the general model for estrogen action

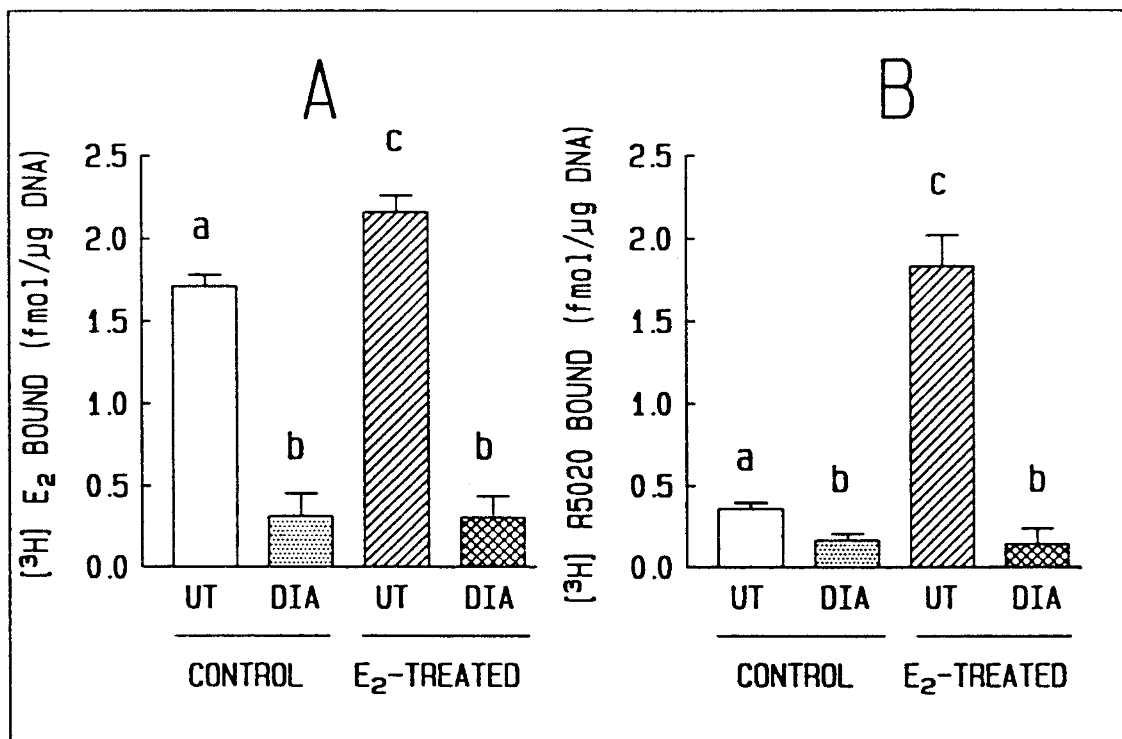

Figure 3. Nuclear concentrations (mean ±SE) of specifically bound [^{3}H]estradiol (A) and [^{3}H]R5020 (B) in uteri of nulliparous mink treated daily with 50 ug estradiol (E_2) for 2 days prior to sacrifice (n = 5). Diaphragm tissue (DIA) was collected from both treated and control mink (n = 3) to serve as a negative control. Treatment of mink with E_2 resulted in a significant increase in [^{3}H]E_2 ($P < 0.01$) and [^{3}H]R5020 binding ($P < 0.001$). Basal level (non-stimulated) of specifically bound [^{3}H]E_2 were fivefold that of negative control tissue (DIA; $P < 0.001$). Although less pronounced, basal [^{3}H]R5020 binding was greater than that of negative control tissue ($P < 0.05$). Estradiol had no effect on receptor concentrations in negative control tissue.

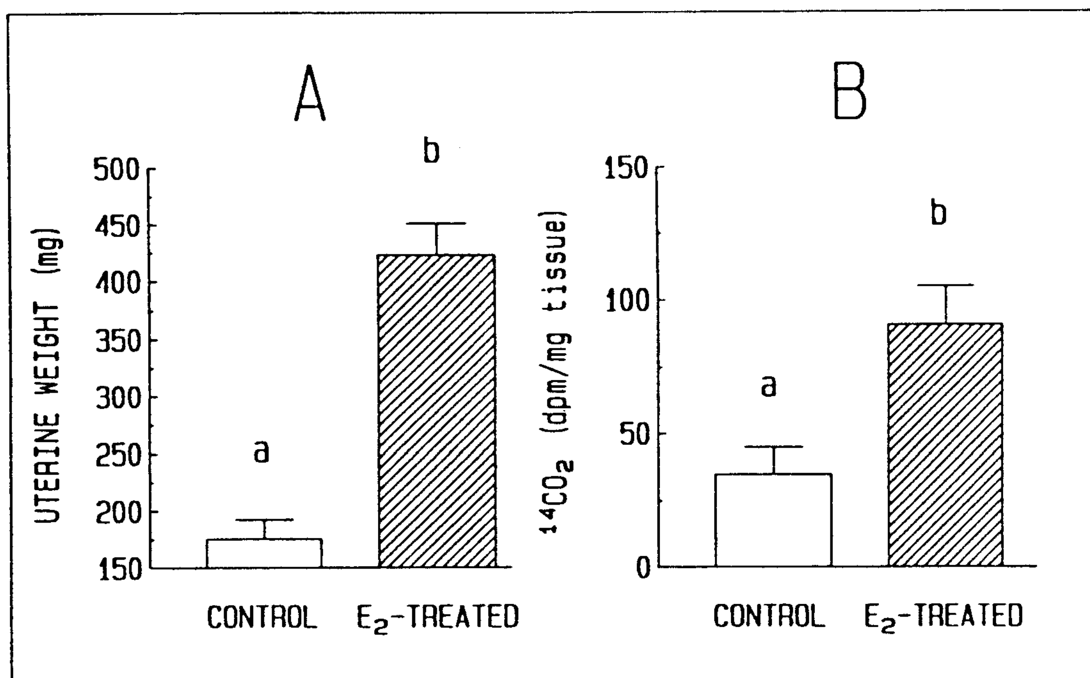

Figure 4. Mean (±SE) uterine weight (A) and <u>in vitro</u> [^{14}C] glucose oxidation (B) in controls (n = 20) and mink treated with 50 ug estradiol-17 β (E_2) (n = 15) sc for 2 days prior to sacrifice. Estradiol significantly increased uterine weight ($P < 0.001$) and oxidation of glucose ($P < 0.05$).

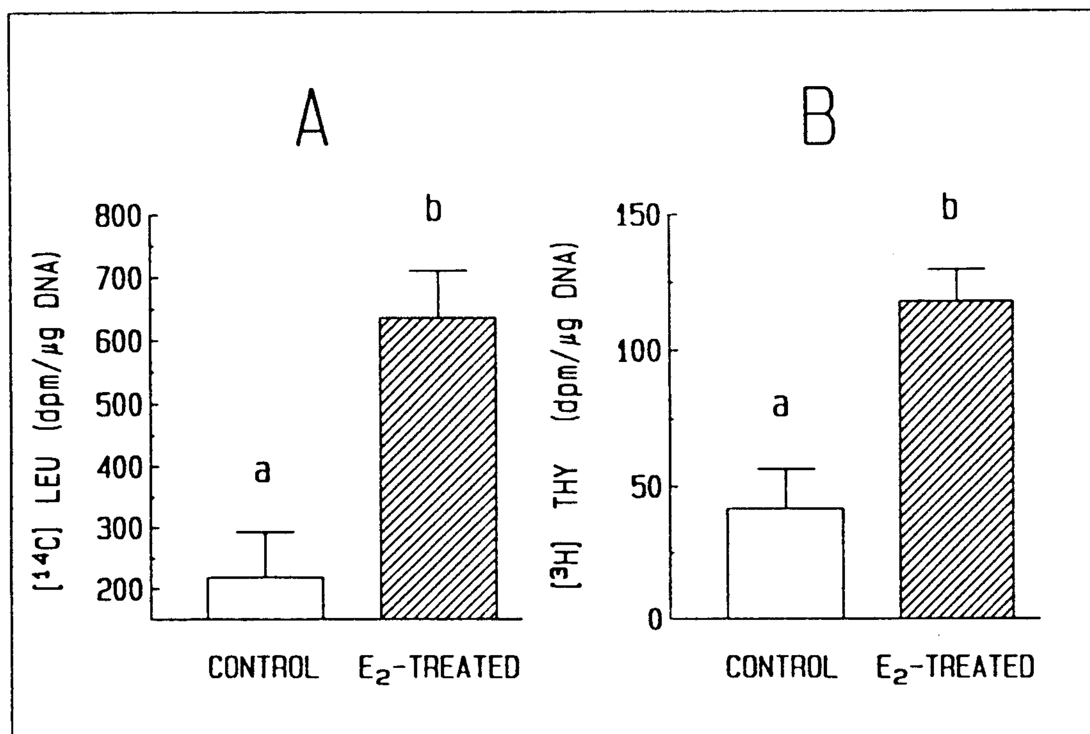

Figure 5. Mean ($\pm$SE) *in vitro* protein (A) and DNA synthesis (B) in uteri of mink injected with 50 ug estradiol (E_2) daily for 2 days. Protein synthesis was measured as the quantity (dpm) of [^{14}C]leucine incorporated per ug DNA. DNA synthesis is expressed as dpm [^{3}H]thymidine incorporated per ug DNA. Treatment with estradiol significantly increased both protein ($P < 0.01$) and DNA synthesis ($P < 0.01$).

in other species (Gorski, 1964; Anderson et al., 1975). These results do not explain lack of uterine responsiveness observed during the breeding season and it is possible that another endocrine factor is involved in regulation of uterine function during this reproductive state.

Serum concentrations of prolactin increase concomitant with implantation in mink (Martinet and Allain, 1985). Further, prolactin receptors have been identified in the uterus of the mink (Rose et al., 1983b), but it is not known how prolactin affects uterine physiology. Increases in prolactin secretion following the vernal equinox (period of implantation) could alter the ability of the uterus to respond to endogenous estrogen and progesterone. Prolactin has been implicated in the induction of progesterone receptors in the rabbit uterus (Chilton and Daniel, 1987).

Summary

Concentrations of [^{3}H]estradiol and [^{3}H]promegestone (R5020) needed to saturate uterine estrogen and progesterone receptors in the estrogen-primed mink have been established. Exogenous estradiol was shown to induce hyperplasia of the uterus as well as an increase in the abundance of estradiol and progesterone receptors. These results suggest that the relative nonresponsiveness of the mink uterus to steroid stimulation may not be due to insufficient estrogen receptors and implicates another endocrine regulator.

EXPERIMENT 2 & 3

UTERINE METABOLIC ACTIVITY AND STEROID RECEPTOR CONCENTRATIONS IN RESPONSE TO SUPPRESSED SECRETION OF PROLACTIN AND EXOGENOUS ESTRADIOL IN ANESTROUS MINK

Introduction

Mink are seasonal breeders that mate during late winter (February-March). One feature of gestation in mink is the occurrence of an embryonic diapause during which blastocyst development is temporarily arrested and implantation is delayed (Hansson, 1947). Diapause is under photoperiodic control and shortly after the vernal equinox (March 21) blastocyst development resumes and the embryos implant into the endometrium. Serum concentrations of prolactin increase in response to lengthening daily photoperiod after the vernal equinox. Treatments that increase serum concentrations of prolactin during diapause result in a concomitant increase in serum concentrations of progesterone and induce embryo implantation (Murphy et al., 1981). However, exogenous progesterone alone or in combination with estrogen does not advance the date at which embryos implant (Cochrane and Shackelford, 1962).

After the vernal equinox, prolactin concentrations continue to rise during pregnancy and lactation to peak during mid-summer anestrus (Martinet and Allain, 1985). High affinity binding sites for prolactin have been identified in the ovaries of mink during the

mating season (Rose et al., 1986) as well as in uteri of anestrous mink (Rose et al., 1983b). Presence of these uterine receptors suggests direct regulation of uterine function by prolactin. In rabbits, prolactin has been reported to control uterine concentrations of progesterone receptor (Chilton and Daniel, 1987). However, it is not known if prolactin acts to regulate uterine physiology in mink. Responses of uteri of anestrous mink to exogenous estradiol-17β in the face of maximal or suppressed secretion of prolactin may provide insight regarding the effects of this peptide hormone on uterine function in this species. Secretion of prolactin in mink can be suppressed by administration of melatonin or the ergot alkaloid bromocriptine (Martinet et al., 1982; Rose et al., 1985).

The objective of this research was to assess in summer anestrous mink the effects of melatonin- and bromocriptine-induced suppression of prolactin secretion and treatment with estradiol on uterine metabolic activity and concentrations of estrogen and progesterone receptors. Concomitantly, effect of treatments on systemic concentrations of prolactin, estradiol and progesterone were also measured.

Materials and Methods

Experiment 2

Beginning on July 3, 20 mink were injected daily sc with 2 mg bromocriptine mesylate (2-Bromo-α-ergocryptine; No. B-2134; Sigma Chemical Co., St. Louis, MO; in 2 ml saline) for 14 days. Controls

(n = 20) were similarly injected with vehicle only. On day 14 both bromocriptine-treated and control groups were divided into two subgroups. One subgroup was injected sc with 100 ug estradiol (Sigma No. 8875; in 2 ml corn oil; n = 10) while the other (control; n = 10) was similarly injected with vehicle. Twenty-four hours after estradiol injection, all mink were bled and euthanized. Blood samples (10 ml) were allowed to clot at room temperature then stored at 4° C for 24 h. Serum was separated by centrifugation (500 x g) for 15 min at 4° C and stored at -20 ^{o} C until assayed for prolactin, estradiol and progesterone. Uteri collected at necropsy were placed on ice, dissected free of connective tissue and fat, weighed and cut into longitudinal strips. Aliquots of pooled uterine tissue (2 uteri/pool) were used to quantitate nuclear estrogen receptor, progesterone receptor, *in vitro* incorporation of $[^{3}H]$thymidine into DNA, incorporation of $[^{14}C]$leucine into protein and oxidation of $[^{14}C]$glucose to $^{14}CO_2$ (as described in Exp 1).

Experiment 3

Treatment with bromocriptine in Exp. 2 was expected to reduce serum concentrations of prolactin but it was not known if this acute (14 day) regimen would have a maximum effect on the uterus. Previous studies have shown that prolactin secretion can be chronically suppressed by treating mink with constant-release melatonin implants (Rose et al., 1985). Because treatment with bromocriptine resulted in an increase in serum concentrations of estradiol and progesterone receptor, Exp. 3 was designed to investigate whether prolonged

prolactin suppression with melatonin would effect serum concentrations of estradiol, progesterone and uterine concentrations of estrogen and progesterone receptors. In this experiment 15 mink were assigned to one of two treatment groups or to a control group. On July 1, mink in treatment groups 1 and 2 (n = 5 each) were implanted with silastic melatonin implants (10 mg melatonin; Sigma) constructed as described by Rose et al. (1984) and inserted sc over the scapular area. On Oct. 25, mink in group 1 were injected with 100 ug estradiol (sc in 2 ml corn oil) while mink in group 2 and unimplanted controls (group 3) were injected with vehicle only. On Oct. 26 (24 h after injection) all mink were bled and sacrificed as described in Exp 2. Serum samples were analyzed for prolactin, estradiol and progesterone. At necropsy, uteri were collected and concentrations of nuclear estrogen and progesterone receptor determined as described in Exp 1.

Radioimmunoassays

Serum concentrations of prolactin were measured in duplicate by heterologous double antibody radioimmunoassay as described by Rose et al. (1986) and modified as follows. The assay employed goat antiserum to porcine prolactin (Research Products International, Mt. Prospect, IL; Lot 10117L) and rabbit anti-goat precipitating antibody (Antibodies Inc., Davis, CA; Lot 5TA90Y). Optimal concentrations for primary and precipitating antibody were 1:150,000 and 1:15, respectively. Highly-purified porcine prolactin (pPRL-I-2;AFP-5000) was iodinated by chloramine-T method (Kraeling et al., 1982) and free

^{125}I was separated from [^{125}I]prolactin by passing the iodination mixture through an anion exchange resin column (AG 2-X8, 50-100 mesh, Bio-Rad, Richmond, CA). Serum concentrations of prolactin were expressed using porcine prolactin standards (pPRL-I-2; 0.05-36 ng/tube). Assay sensitivity was 0.125 ng/assay tube (50-200 ul) and intra- and interassay coefficients of variation were 8.5 and 14%, respectively (four assays).

Progesterone was assayed in duplicate after hexane:benzene (2:1) extraction following the procedure of Koligian and Stormshak (1976). Progesterone, [1, 2, 6, 7 $^{3}H(N)$]progesterone (12 x 10^{3} dpm; 115 Ci/mmol; New England Nuclear [NEN], Boston, MA) was added to a third tube containing an aliquot of each sample to determine and correct for procedural loss due to extraction. Extraction efficiency of this procedure was 87 $\pm$ 0.7% (n = 40). Progesterone radioimmunoassay utilized a progesterone specific antibody, anti-progesterone-11-BSA. Assay sensitivity was 10 pg/assay tube (100 ul) and intra- and interassay coefficients of variation were 9.6 and 17%, respectively (3 assays).

Concentrations of estradiol in sera were determined in duplicate after diethyl ether extraction as described by Hotchkiss (1977). Procedural losses were determined and corrected for by adding 3.5 x 10^{3} dpm [2, 4, 6, 7 $^{3}H(N)$]estradiol (115 Ci/mmol; NEN) to a third tube containing an aliquot of each sample. Mean extraction efficiency was 98 $\pm$ 0.2% (n = 40). Estradiol radioimmunoassay followed methods described by Butcher (1977) using estradiol standards (0.5-200 pg/tube), [2, 4, 6, 7 $^{3}H(N)$]estradiol,

and an estradiol specific antibody (Anti estradiol-3-HSA; final concentration, 1:10,000). Sensitivity of the assay was 1 pg/assay tube (300 ul) and intra- and interassay coefficients of variation were 9.1 and 15%, respectively (4 assays).

Statistical Analysis

Analysis of data from Exp. 2 on serum concentrations of progesterone, uterine [^{3}H]thymidine incorporation and [^{14}C]glucose oxidation was accomplished by two-way analysis of variance (ANOVA). Log transformations of serum concentrations of estradiol, uterine weight, [^{14}C]leucine incorporation and concentrations of estrogen and progesterone receptor were performed prior to ANOVA (Snedecor and Cochran, 1980) because variances were heterogeneous. Data from Exp. 3 were analyzed by one-way ANOVA after log transformation. However, for purposes of presentation data from both experiments are represented by untransformed values.

Results

In Exp. 2. treatment with bromocriptine (2 mg/day for 14 days) reduced serum concentrations of prolactin ($P < 0.001$; Figure 6A) and resulted in increased concentrations of estradiol over that of controls ($P < 0.05$; Figure 6B). Injection of mink with estradiol alone had no significant effect on prolactin concentrations compared with controls, but treatment with estradiol after treatment with bromocriptine resulted in an increase in serum concentrations of prolactin compared with mink treated with bromocriptine alone

($P < 0.05$; Figure 6A). As anticipated, injection of mink with estradiol alone or after bromocriptine increased serum concentrations of estradiol over that of controls and bromocriptine-treated mink ($P < 0.001$; Figure 6B). Neither estradiol nor bromocriptine altered serum concentrations of progesterone and no corpora lutea were observed on the ovaries at necropsy. Treatment of mink with estradiol alone or bromocriptine plus estradiol increased uterine weight ($P < 0.01$) but there was no apparent effect of bromocriptine alone on uterine size. Mean ($\pm$ SE) uterine weights in control, bromocriptine, estradiol and bromocriptine plus estradiol-treated mink were 203.3 $\pm$ 15.16, 208.4 $\pm$10.1, 280.9 $\pm$ 16.9 and 289 $\pm$ 21.7 mg, respectively. Injection of estradiol alone or bromocriptine plus estradiol stimulated an increase in uterine concentrations of estrogen receptor ($P < 0.01$; Figure 7A). Uterine concentrations of estrogen receptor were not affected by treatment with bromocriptine alone. Compared with controls, uterine concentrations of progesterone receptor were greater in mink treated with bromocriptine, estradiol and bromocriptine plus estradiol (bromocriptine x estradiol interaction, $P < 0.05$; Figure 7B). Further, concentrations of progesterone receptor were greater in mink treated with bromocriptine plus estradiol than those treated with estradiol alone ($P < 0.05$).

Relative to control animals, uterine metabolic activity as reflected by glucose oxidation was increased only by treatment with estradiol and bromocriptine plus estradiol (bromocriptine x estradiol interaction; $P < 0.05$; Figure 8A). Treatment with estradiol and

bromocriptine plus estradiol markedly stimulated protein synthesis (Figure 8B) and increased cell division, as indicated by enhanced incorporation of [^{3}H]thymidine into DNA ($P < 0.01$ Figure 8C). Administration of bromocriptine alone did not alter glucose oxidation or DNA and protein synthesis.

In Exp. 3, melatonin and melatonin plus estradiol injection resulted in a reduction of serum concentrations of prolactin prior to sacrifice ($P < 0.01$; Figure 9A). Exposure to melatonin increased serum concentrations of estradiol ($P < 0.01$; Figure 9B) but had no effect on serum concentrations of progesterone. Treatment of mink with melatonin plus estradiol did increase serum concentrations of progesterone ($P < 0.05$; Figure 9C). However, progesterone levels were less than one-third the concentration expected for mink during the breeding season. Uterine weight was greater in mink treated with melatonin (mean$\pm$SE; 361$\pm$35 mg), and melatonin plus estradiol (426$\pm$27 mg) compared with controls (177$\pm$11 mg; $P < 0.001$). Treatment with melatonin and melatonin plus estradiol increased nuclear concentrations of estrogen and progesterone receptors ($P < 0.05$ Figure 10AB) compared with controls. Further, treatment with melatonin plus estradiol increased nuclear progesterone receptor compared with treatment with melatonin alone ($P < 0.05$; Figure 10B). However, there was no significant difference in concentration of nuclear estrogen receptor between melatonin-and melatonin plus estradiol-treated mink.

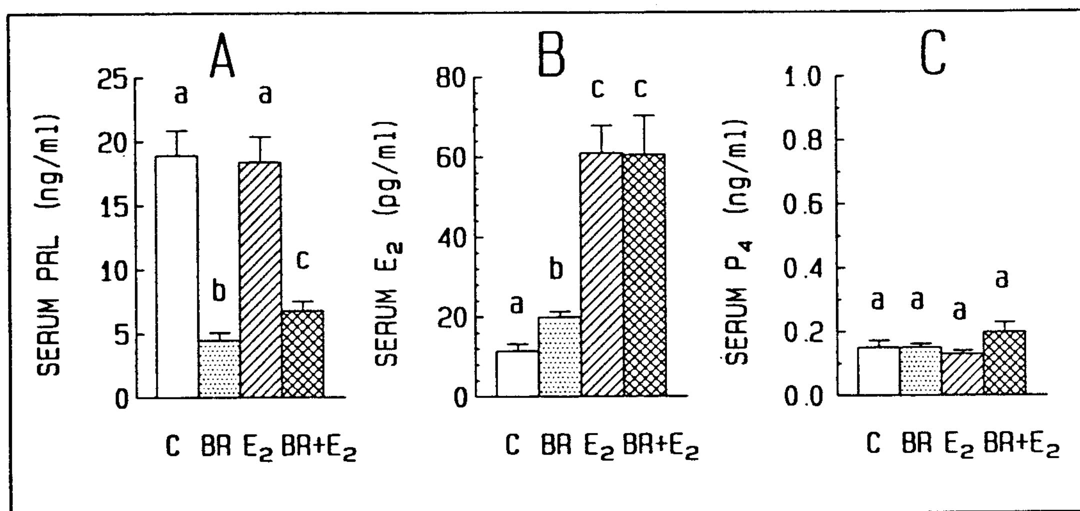

Figure 6. Serum concentrations (mean ±SE) of prolactin (PRL; A), estradiol-17β (E_2; B) and progesterone (P_4; C) in mink injected daily with 2 mg bromocriptine (BR) and(or) with 100 ug E_2 (n = 4) 24 h prior to sacrifice (means with different superscripts differ ($P < 0.05$). Treatment with BR+E_2 resulted in a an increase in serum concentrations of prolactin compared to those treated with BR alone ($P < 0.05$). Treatment with BR reduced serum concentrations of prolactin ($P < 0.001$) and increased serum concentrations of E_2 ($P < 0.05$). Injection with E_2 resulted in a significant increase in serum concentrations of E_2 ($P < 0.001$).

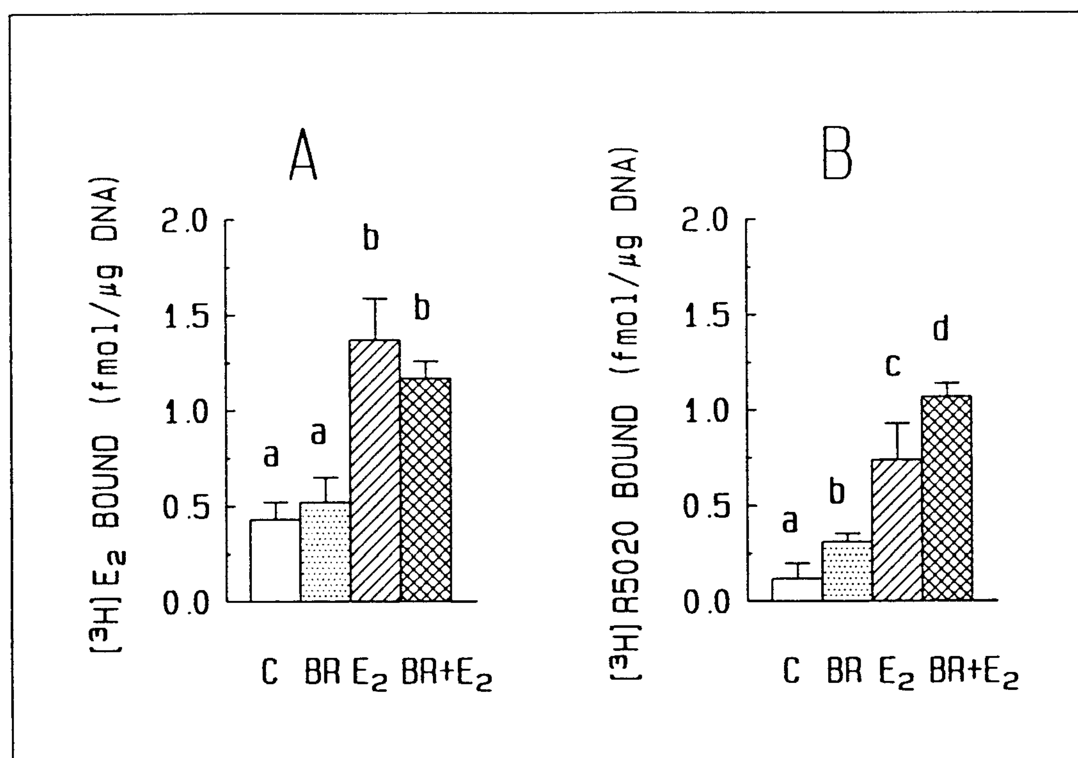

Figure 7. Nuclear concentrations (mean ±SE) of specifically bound [^{3}H]estradiol (A) and [^{3}H]R5020 (B) in uteri of mink treated daily with 2 mg bromocriptine (BR) for 14 days and(or) 100 ug estradiol-17β (E_2) 24 h prior to sacrifice. Estradiol, BR and BR plus E_2 increased nuclear concentrations of PR (BR x E_2 interaction, $P < 0.05$) but had no effect on ER concentrations. Treatment with E_2 alone and with bromocriptine significantly increased ER ($P < 0.01$). Means with different superscript letters differ ($P < 0.05$).

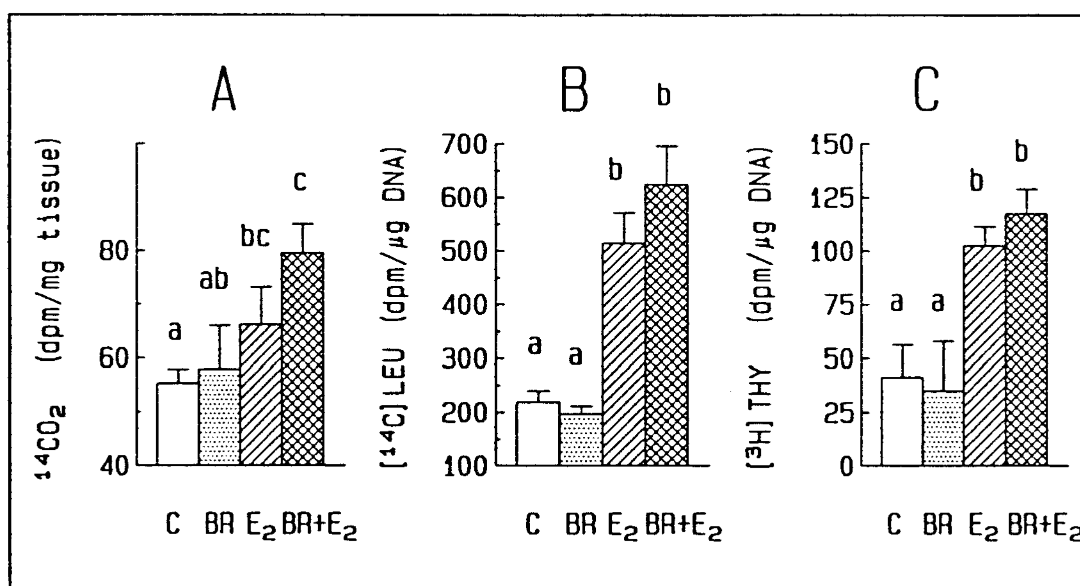

Figure 8. Mean (± SE) oxidation of [^{14}C]glucose to $^{14}CO_2$ (A) and relative <u>in vitro</u> DNA (B) and protein synthesis (C) in uteri of mink injected with 2 mg bromocriptine (BR) daily for 14 days and(or) 100 ug of estradiol-17β (E_2) 24 h before sacrifice. Estradiol but not BR increased protein and DNA synthesis <u>in vitro</u> ($P < 0.01$). Estradiol and BR plus E_2 stimulated a significant increase in glucose oxidation. ($P < 0.05$). Means with different superscript letters differ ($P < 0.05$).

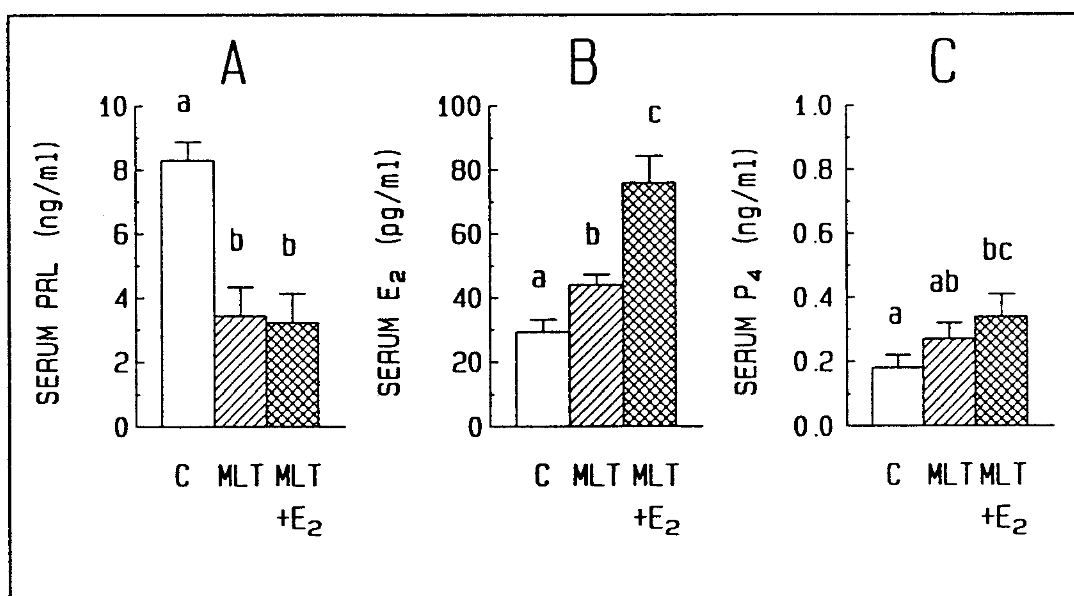

Figure 9. Serum concentrations (mean $\pm$SE) of prolactin (PRL; A), estradiol-17β (E_2; B) and P_4 (C) in mink treated with melatonin (MLT; 10 mg implants) alone or in combination with 100 ug E_2 or vehicle (sc; n = 5 each) 24 h prior to sacrifice. Treatment with MLT and MLT plus E_2 reduced serum concentrations of prolactin ($P < 0.01$) and increased serum concentrations of E_2($P < 0.01$). Treatment with melatonin plus E_2 increased serum concentrations of P_4 ($P < 0.05$). Means with different superscript letters differ ($P < 0.05$)

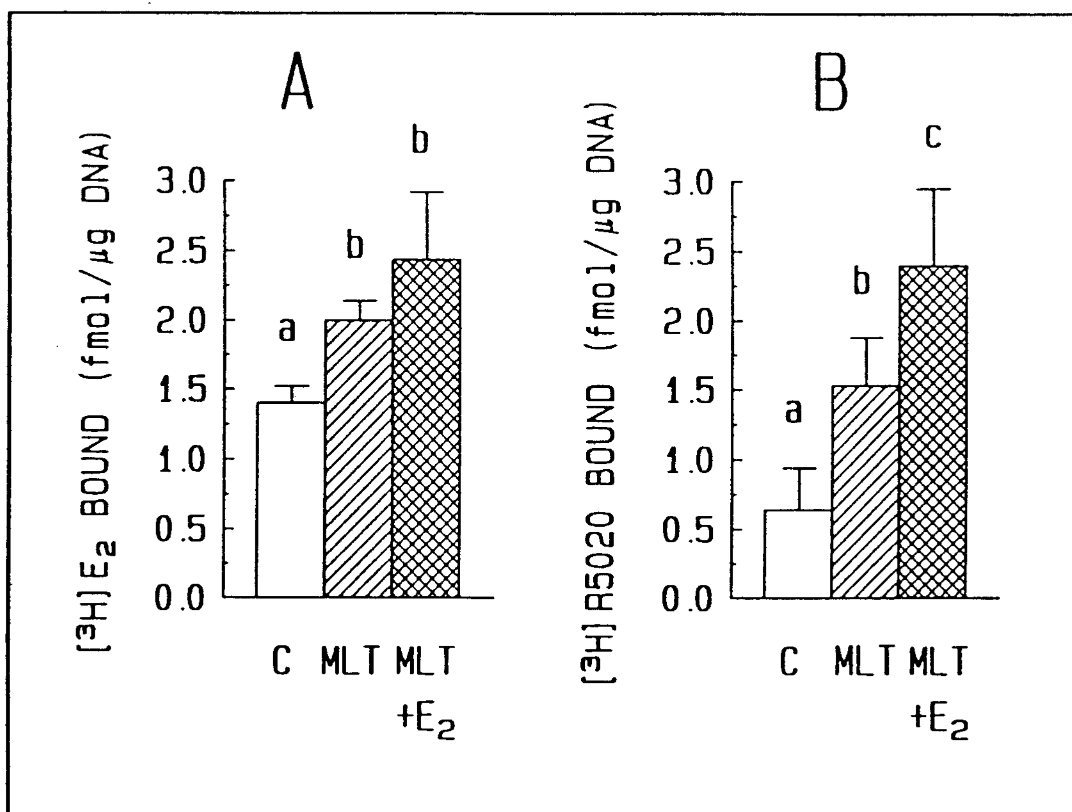

Figure 10. Nuclear concentrations (mean ±SE) of specifically bound [^{3}H]estradiol (A) and [^{3}H]R5020 (B) in uteri of mink implanted with 10 mg melatonin (MLT) alone or implanted and injected with 100 ug estradiol-17β (E_2) 24 h prior to sacrifice. Melatonin alone and MLT plus E_2 increased nuclear concentrations of estrogen and progesterone receptor ($P < 0.05$). Means with different superscript letters differ ($P < 0.05$).

Discussion

Bromocriptine and melatonin are effective inhibitors of prolactin secretion in mink (Martinet et al., 1982; Rose et al., 1985). As anticipated, injection of bromocriptine for 14 days and treatment with melatonin implants significantly suppressed serum concentrations of prolactin. Treatment with estradiol after bromocriptine stimulated an increase in serum prolactin compared with that of mink treated with bromocriptine alone. This suggests that estradiol stimulates prolactin secretion in mink as has been demonstrated to occur in the rat (Maurer and Gorski, 1977). However, this effect may in part be due to the short duration of bromocriptine exposure as this trend was not detected in Exp. 3, where melatonin suppressed serum concentrations of prolactin regardless of exposure to estradiol.

Bromocriptine and melatonin significantly increased serum concentrations of estradiol suggesting that prolactin inhibits estradiol biosynthesis. Several lines of evidence support the concept that prolactin acts at least in part to regulate estradiol concentrations in mink. Pilbeam et al. (1979) reported that serum concentrations of estrogen in mink decreased during April when concentrations of prolactin have been found to be increasing (Rose et al., 1986). Further, it appears that systemic estradiol concentrations begin to rise in late summer (Pilbeam et al., 1979) when prolactin secretion decreases in response to the shortened photoperiod (Rose et al., 1985). In rats, exogenous prolactin

inhibited *in vivo* estrogen synthesis by reducing ovarian aromatase activity (Tsai-Morris et al., 1983).

Uteri of summer anestrous mink appear to be fully responsive to estrogen stimulation and it is possible that elevated serum concentrations of estradiol induced by bromocriptine and melatonin treatment represents part of the underlying cause for the ability of these agents to increase uterine steroid receptors. Further, exposure to exogenous estradiol, resulted in an increase in the number of estrogen receptors, which is consistent with previously reported effects of this hormone in regulating uterine concentrations of estrogen and progesterone receptor (Feil et al., 1972; Anderson et al., 1973). Synergistic effects of bromocriptine and estradiol may be due to the duration of exposure because treatment with bromocriptine began 13 days prior to treatment with estradiol. Therefore, animals in the bromocriptine plus estradiol group may have been exposed to increasing estrogens for a substantially longer time than mink treated with estradiol alone. However, unlike estradiol, bromocriptine treatment had no effect on protein and DNA synthesis or concentrations of estrogen receptor. This suggests that the effect of bromocriptine is not solely through the stimulatory action of estradiol and it is therefore possible that prolactin has a specific effect in the regulation of progesterone receptors. A direct regulatory action by prolactin is implicated by the presence of prolactin receptors in the uteri of anestrous mink (Rose et al., 1983b). Prolactin regulation of steroid receptors has been reported to occur in the rat (Advis and Alvarez, 1977), and this action of

prolactin in mink may be dependent on the presence of progesterone as described for the rabbit (Chilton and Daniel, 1987). Progesterone concentrations during both Exp. 2 and 3 were low because mink were anestrus and therefore lacked corpora lutea. Treatment with melatonin plus estradiol did significantly increase serum concentrations of progesterone, but these levels although statistically significant are 10-fold less than that produced by normal luteal activity after embryonic implantation (Allais and Martinet, 1978).

Treatment of mink with melatonin during preimplantation gestation inhibits photoperiodic stimulation of prolactin and progesterone secretion, and prolongs the period of embryonic diapause. In further support of this model, Murphy et al (1990) reported that the inhibitory action of melatonin can be reversed by treatment with exogenous ovine prolactin. Because of the inhibitory action of melatonin on prolactin secretion, results of melatonin treatment presented here support the hypothesis that prolactin may also be involved in the regulation of uterine steroid receptors. Administration of melatonin to hamsters increased uterine estrogen receptor (Danforth et al., 1983), but it is unclear if this was a direct action of melatonin or one mediated by prolactin. However, Danforth et al. (1981) reported an _in vitro_ regulation of estrogen receptor activity by melatonin in human mammary carcinoma cells.

Summary

Results of this study support evidence presented in Exp 1. indicating that the uterus of anestrous (as well as nulliparous) mink is responsive to exogenous estradiol. We have presented evidence that prolactin may be involved in regulating uterine progesterone receptor. Further research is needed to elucidate the role of prolactin in augmenting estrogen and progesterone in stimulating the uterus.

EXPERIMENT 4

ASSOCIATION OF SERUM PROLACTIN, ESTROGEN AND PROGESTERONE WITH CHANGES IN UTERINE METABOLISM AND STEROID RECEPTOR CONCENTRATIONS IN MINK DURING THE BREEDING SEASON

Introduction

The hypophyseal hormone prolactin, and two ovarian steroid hormones, estradiol and progesterone, appear to play pivotal roles in controlling the duration of embryonic diapause and stimulating implantation in mink (Pilbeam et al., 1979). Estradiol (the primary estrogen) is produced by developing ovarian follicles and maximum serum concentrations occur during the mating season immediately prior to nidation. Progesterone secretion by corpora lutea, increases shortly after the vernal equinox and reaches greatest concentration approximately one week before parturition (Pilbeam et al., 1979; Stoufflet et al., 1989). Despite seasonal correlation in the secretion of these hormones, implantation cannot be induced in mink with injections of progesterone and(or) estrogen (Cochrane and Shackelford, 1962).

In experiment 1 it was shown that treatment with exogenous estradiol increased uterine concentrations of both estrogen and progesterone receptors. These data suggest that the inability of estrogen and progesterone to induce implantation is not due to a lack

of response to steroids and implicates another endocrine regulator of uterine function. Treatments that increase systemic levels of prolactin induce luteal reactivation and embryo implantation in mink (Murphy et al., 1981; 1990). Results of Exp. 2 suggest that prolactin inhibits estrogen secretion in mink and also influences concentrations of progesterone receptor. Progesterone is reported to reduce concentrations of its own receptor and in the rabbit prolactin appears to influence this down-regulation (Chilton and Daniel, 1987). However, because progesterone was at undetectable levels during these previous experiments it is difficult to extrapolate results of Exp 1, 2 and 3 to changes that occur during the breeding season. Measurement of estrogen and progesterone receptors, and comparison of relative metabolic activity during March should indicate the relative responsiveness of the uterus to changes in prolactin when progesterone is also present.

Although embryonic factors do not appear to affect luteal reactivation immediately prior to nidation, these effects cannot be ruled out in regulation of the uterus. Because mink are induced-ovulators, a condition of pseudopregnancy can be induced by mating with vasectomized males (Duby and Travis, 1972). Pseudopregnancy is reported to last through mid-April in mink (Duby and Travis, 1972) and pseudopregnant mink represent a useful model for investigating uterine function in the absence of embryonic factors.

The objective of this experiment was to compare concentrations of estrogen and progesterone receptors, relative glucose metabolism and protein and DNA synthesis in uteri of pseudopregnant mink before

and at the expected time of embryonic implantation (i.e., prior to and after the vernal equinox).

Materials and Methods

Experimental Design

Twenty four standard dark adult mink were assigned to two groups. Mink in group 1 were mated with vasectomized males from March 7 to 9 to induce a state of pseudopregnancy and then sacrificed 10 days after mating on March 17-19. Mink in group 2 were similarly mated on March 17 and 18 to induce pseudopregnancy and sacrificed on March 27 and 28 (6 and 7 days after the vernal equinox). Blood was collected immediately prior to sacrifice and serum was analyzed for prolactin, estradiol and progesterone by radioimmunoassay as described in Exp 2. At sacrifice, uteri were removed dissected free of connective tissue and fat, weighed and uteri were cut into strips. Within each treatment group uteri were pooled (2 uteri per pool) and replicate aliquots of pooled tissue (n = 6) were used to quantitate _in vitro_ incorporation of [^{3}H]thymidine into DNA, [^{14}C]leucine into protein and oxidation of [^{14}C]glucose to $^{14}CO_2$ as described in Exp. 1.

Statistical Analysis

Data on glucose oxidation, and thymidine incorporation and serum estradiol concentrations were analyzed for significance by Student's t-test. Data on serum progesterone and uterine weight, glucose oxidation, thymidine incorporation and progesterone receptor

concentrations were analyzed after log transformation (Snedecor and Cochran, 1980) because variances were found to be heterogeneous. However, data are presented as untransformed means.

Results and Discussion

Serum concentrations of prolactin were significantly greater after the equinox ($P < 0.001$); Figure 11A). Serum concentrations of progesterone and estradiol were greater in mink after than before the vernal equinox but these differences were not significant (Figure 11BC). There was also no significant change between Mar. 19 and Mar. 28 in uterine concentration of nuclear estrogen receptors (Figure 12 AB). In contrast, uterine nuclear progesterone receptors were greater after the equinox ($P < 0.05$). Likewise, uteri of mink before and after the vernal equinox did not differ in weight (Figure 13 A). Uterine glucose oxidation was increased after the equinox ($P < 0.05$; Figure 13B) but this metabolic trend was not reflected in a significant change in protein and DNA synthesis (Figure 14AB).

In mink, onset of the breeding season is generally signaled by increased reddening of the vulva of the female and their ability to induce sexual aggressiveness in the male (Pilbeam et el., 1979). These features have been previously attributed to increased estrogens during this period. Serum concentrations of estradiol were greater during the breeding season than control levels during both winter and summer anestrus. Serum concentrations of estrogen were reported to decline as concentrations of prolactin and progesterone increase as the equinox approaches (Pilbeam et al., 1979; Murphy et al., 1981;

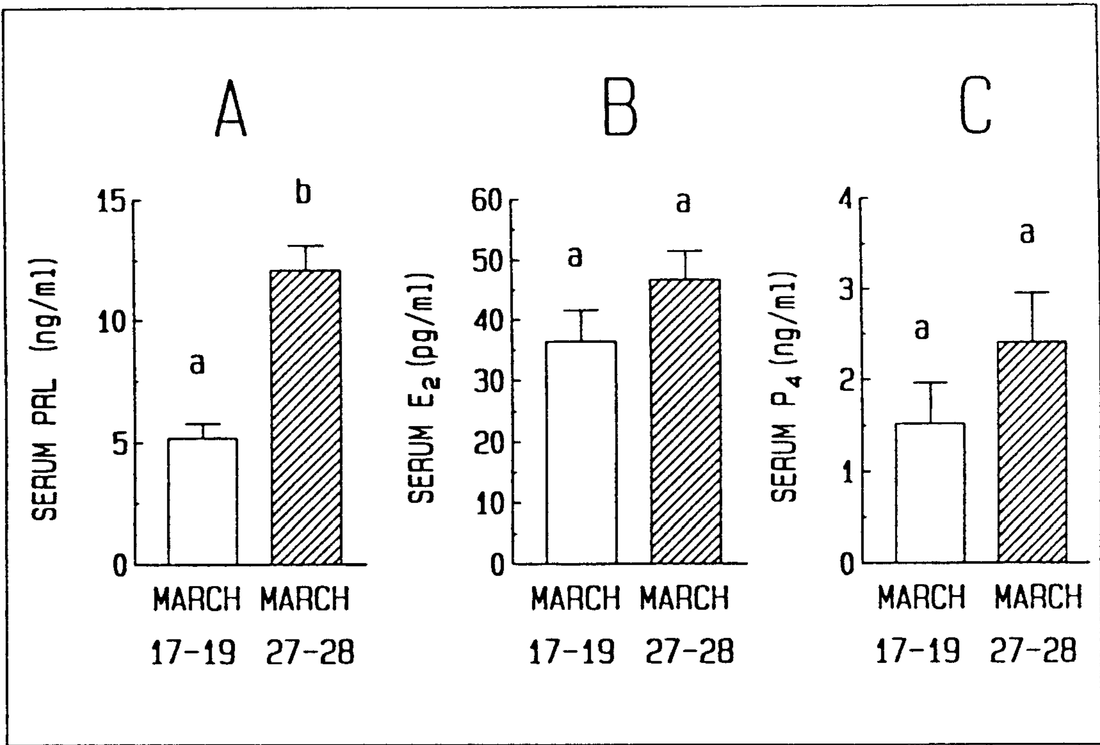

Figure 11. Mean (± SE) serum concentrations of prolactin (PRL; A) estradiol-17β (E_2; B) and progesterone (P_4;C) in pseudopregnant mink sacrificed before and after the vernal equinox (Mar. 17-19 and 27-28). Prolactin concentrations were greater after the vernal equinox (Mar. 27-28; $P < 0.001$). However, no significant change was detected in concentrations of estradiol or progesterone during this period. Means with different superscripts letters differ ($P < 0.05$).

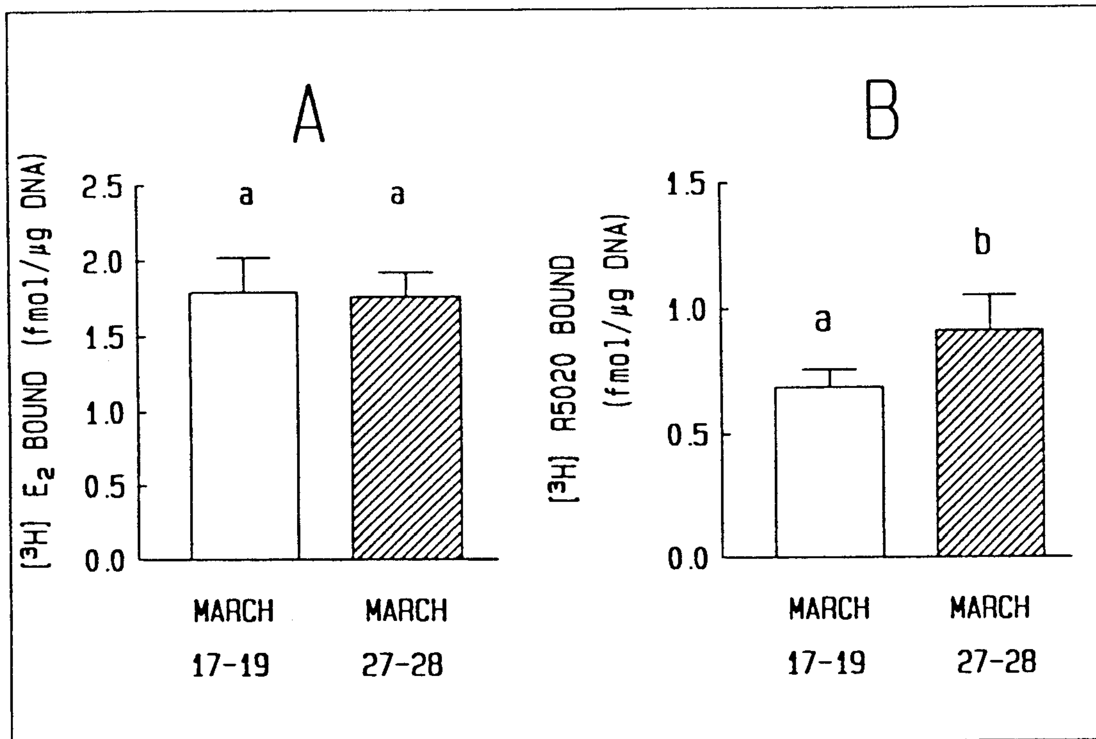

Figure 12. Nuclear concentrations (mean ±SE) of specifically bound [^{3}H]estradiol (A) and [^{3}H]R5020 (B) in uteri of pseudopregnant mink sacrificed before and after the vernal equinox (Mar. 17-19 and 27-28). Binding of [^{3}H]R5020 (estimate of progesterone receptor) was found to be greater on Mar 27-28 than on Mar. 17-19 ($P < 0.05$). No significant change was detected in concentrations of estrogen receptor during this period. Means with different superscript letters differ ($P < 0.05$).

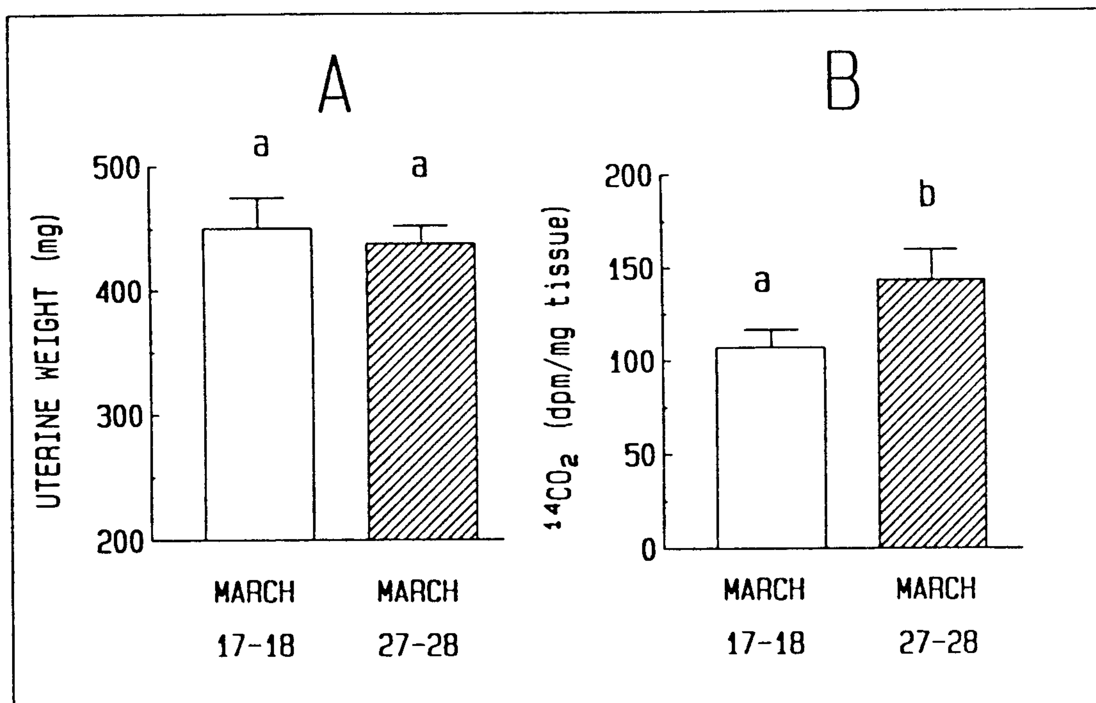

Figure 13. Mean (+SE) uterine weight (A) and _in vitro_ [^{14}C]glucose oxidation (B) in mink sacrificed before and after the vernal equinox (Mar 17-19 and 27-28). No significant change occurred in weight during this period. However, uterine glucose metabolism was greater ($P < 0.05$) after the equinox (March 27-28). Means with different superscript letters differ ($P < 0.05$).

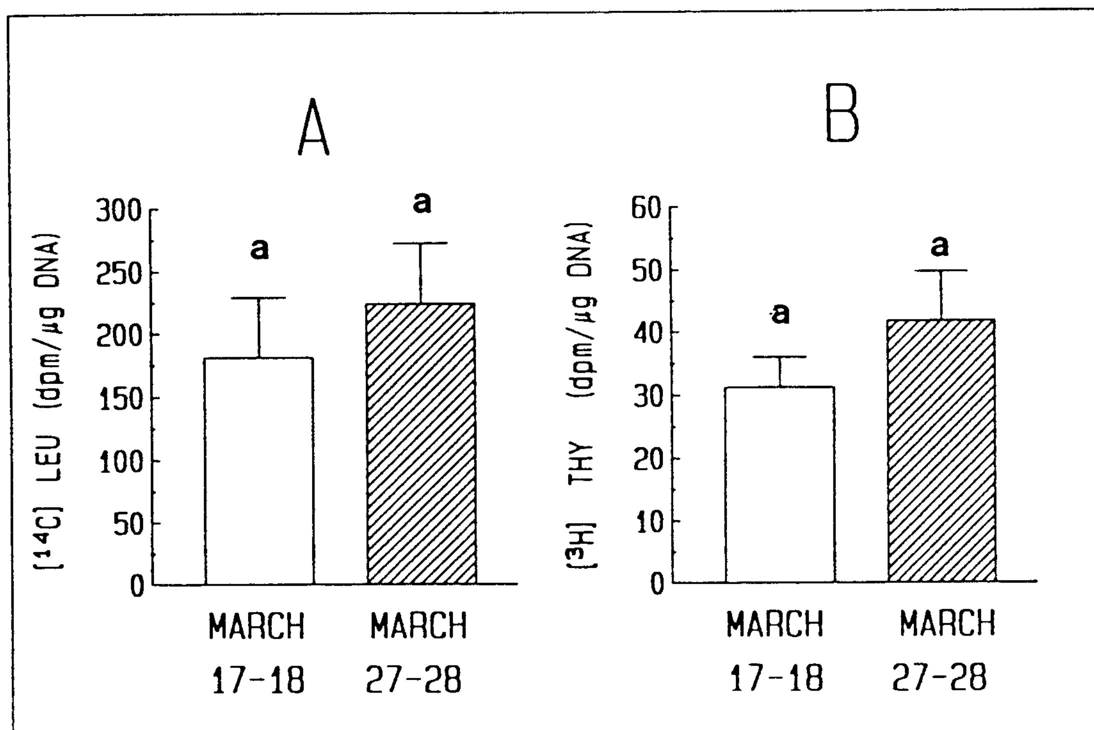

Figure 14. Mean (± SE) in vitro protein (A) and DNA synthesis (B) in uterine tissue of mink before and after the vernal equinox (Mar. 17-19, and 27-28). Protein and DNA synthesis are expressed as dpm [^{14}C]leucine and dpm [^{3}H]thymidine incorporated per ug DNA, respectively. There was no significant change in protein synthesis during the study period. Means with different superscript letters differ ($P < 0.05$).

1990). Results presented here indicate that although prolactin concentrations were increasing, estrogen concentrations had not yet decreased. Further, progesterone concentrations were lower than reported for mink during post-implantation pregnancy (Pilbeam et al., 1979; Stoufflet et al., 1989). Thus, although progesterone concentrations were greater than 10-fold those observed during anestrus it is likely that the uterus was still under only low level progestational stimulation.

While progesterone is reported to reduce concentrations of its own receptor in the rat (Fiel et al., 1972), exposure of the uterus of preparous and anestrous mink to estradiol has previously been shown to increase nuclear estradiol and progesterone receptors (Exp. 1, 2 and 3). The fact that ovarian secretion of estradiol did not differ statistically before and after the vernal equinox suggests that estrogen was at least partially responsible for increased uterine concentrations of progesterone receptor after the equinox. However, prolactin concentrations were increasing during the study period. In the rabbit prolactin has been reported to reduce progesterone-induced progesterone receptor down-regulation (Chilton and Daniel 1987).

Because uteri were apparently exposed to similar concentrations of endogenous ovarian steroids over the 9- to 10- day period, it is not surprising that no differences were detected in uterine weight, protein synthesis or cell division. However, it is evident from the present data that cell metabolism of glucose increased to become greater after than before the vernal equinox. This increase in

glucose oxidation may reflect enhanced response of the uterus to stimulation by progesterone.

Summary

Results of this study demonstrate that estrogen and progesterone receptors exist in the mink uterus in adequate concentrations for this tissue to be responsive during the breeding season. Systemic concentrations of estradiol did not decrease after the vernal equinox. It is therefore likely that estrogens were responsible for increased numbers of uterine progesterone receptors despite the presence of measureable serum concentrations of progesterone. Further research is needed to elucidate the role of these hormones in stimulating implantation in this species.

GENERAL DISCUSSION

During this research uterine metabolic activity and concentrations of estrogen and progesterone receptor have been measured during winter and summer anestrus and in pseudopregnant mink during the breeding season. Concomitantly, the effects of estradiol and prolactin on estrogen and progesterone receptor concentrations were determined. Estrogen receptors were found to be at ample concentrations to respond to exogenous stimulation both during anestrus and the breeding season. During winter and summer anestrus, estrogen stimulation resulted in an increase in uterine concentrations of estrogen and progesterone receptor as well as an increase in uterine metabolic activity.

Endogenous estrogen concentrations were found to be greater in pseudopregnant mink during the breeding season than in anestrous mink. On the basis of these data, it appears that estrogen concentrations decline either during pregnancy, or shortly thereafter and remain low during summer anestrus. Lengthening photoperiod has been reported to stimulate secretion of prolactin and consequently prolactin concentrations rise through gestation to peak during mid-summer (Martinet and Allain, 1985; Rose et al., 1986). Inhibition of prolactin with bromocriptine and melatonin resulted in increased serum concentrations of estradiol suggesting that prolactin is involved in regulation of estrogen synthesis as has been described in the rat (Tsai-Morris et al., 1983). Thus it may be hypothesized that the reported increases in systemic estrogen that occurs in autumn (Pilbeam et al., 1979) is due to increased endogenous melatonin and

the concomitant decrease in secretion of prolaction. However, it is unclear if this effect of melatonin is through suppression of prolactin (Rose et al., 1987) or via a direct effect of this indoleamine on steroidogenic cells. Regardless, the hypothesis that melatonin stimulates estradiol secretion is supported by data presented by Duby and Travis (1972), which indicate that serum concentrations of estrogen gradually increase during autumn.

Anestrous mink appear to be fully responsive to estrogen stimulation and inhibition of prolactin secretion by bromocriptine and melatonin does not interfere with this response. Exposure of the uterus of preparous and anestrous mink to estradiol increased uterine nuclear estrogen and progesterone receptors and stimulated increased metabolic activity. Further, treatments suppressing prolactin and stimulating secretion of estrogen during summer anestrus increased progesterone receptor concentrations. It is possible that bromocriptine plus estradiol treatment induced a longer exposure to increased systemic estrogens than mink treated with estradiol alone. In Exp. 4, increased uterine concentrations of progesterone receptor during the breeding season may also have been due to the fact that ovarian secretion of estradiol remained high during this study period.

Although these results suggest that prolactin affects progesterone receptors through changes in estrogen secretion, it does not rule out a direct effect of prolactin on the uterus of the mink. The fact that suppression of prolactin in Exp. 2 did not result in an increase in uterine metabolism suggests that prolactin is acting via

a mechanism independent of estrogen. These results suggest that the synergistic effects of bromocriptine and estradiol in Exp. 2 are through a direct effect of prolactin, which is specific for the progesterone receptor and may be altered by the presence of endogenous progesterone. Chilton et al. (1987) suggests that prolactin specifically affects the ability of progesterone to down-regulate its own receptor. Treatment with melatonin implants also stimulated estrogen secretion and resulted in an increase in uterine concentrations of progesterone and receptor. Unlike treatment with bromocriptine, melatonin treatment stimulated an increase in estrogen as well as progesterone receptor. This latter observation suggests action by estrogen rather than specifically prolactin.

Segregation of the regulatory effects of prolactin and estrogen on uterine progesterone receptor is needed to further elucidate the role of these hormones in affecting uterine physiology. This could be accomplished through experimentation with ovariectomized mink, or by use of antiestrogen therapy. It is also unclear whether effects of prolactin and estrogen are modulated by the presence of elevated concentrations of progesterone. In other species, progesterone acts to reduce concentrations of both estrogen and progesterone receptor. Antagonistic effects of progesterone (compared with estrogens) on levels of estrogen and progesterone receptor may be modulated by the presence of prolactin. It is possible that further experimentation with progesterone antagonists (before and after the vernal equinox) may help elucidate this complex hormonal interrelationship.

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APPENDICES

APPENDIX 1

QUALITY AND COLOR OF MELATONIN AND BROMOCRIPTINE-INDUCED WINTER FUR GROWTH IN MINK (*Mustela vison*) FED A SUPPLEMENTED DIET[1]

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ABSTRACT

An experiment was conducted to evaluate the effects of diet on fur color and quality of bromocriptine- and melatonin-induced winter pelage in mink. At weaning, 90 standard dark female kit mink were assigned randomly to one of two dietary regimens. Diet 1 (n=30) was a basal ranch mink diet, and diet 2 (n=60) was the basal diet, supplemented with liver and eggs. On 26 June 1986, mink fed each diet were assigned to treatment (n=15) and control groups (n=15). Animals receiving diet 1 were treated with 120 mg melatonin implants (silastic) while those fed diet 2 were treated with 120 mg melatonin implants, 60 mg bromocriptine pellets, or 60 mg bromocriptine pellets plus 18.6 mg melatonin implants (silastic). Control groups on each diet received no implants. Subsequently, fur growth was measured at biweekly intervals. Mink treated with 120 mg melatonin, 60 mg bromocriptine, and 60 mg bromocriptine plus 18.6 mg melatonin all molted 1 mo earlier than controls and exhibited significantly greater fur growth during the months of August and September ($P < 0.05$). Mink implanted with 120 mg melatonin, and bromocriptine plus melatonin were considered to be in fully prime pelage by mid-October and were pelted October 23. Mink receiving bromocriptine alone were not considered to be in prime pelage in October and were pelted in December with controls. Neither diet supplementation with liver and eggs, nor treatment to induce early fur growth had an effect on fur color or quality. It was also concluded that treatment of mink with bromocriptine may not completely mimic the effect of exogenous melatonin on winter fur growth.

INTRODUCTION

Autumn molt and growth of winter fur in mink are stimulated by decreasing daylength during late summer. Mink treated with implants of melatonin in June exhibit an early onset of autumn molt and produce prime winter pelage by mid- October, 6 to 8 wk earlier than normal (Allain and Rougeot, 1980; Rose et al., 1984). Providing fur color or quality are not compromised, melatonin-induced early growth of winter pelage should be of substantial economic benefit to the mink industry, from the standpoint of savings in labor and feed. However, concerns have been expressed by some producers that pelage color and pelt quality of melatonin-treated mink are not comparable to those of mink having grown prime winter pelage under natural conditions. Unfortunately, no scientifically acquired data have been reported to substantiate these concerns. Further, the presumed effects of melatonin on pelt quality may be attributed in part or wholly to the composition of the diet fed to mink during the period of melatonin-induced fur growth. Nutrition is a primary determinant of body and fur growth in kit mink and experimental substitution of protein sources has been shown to increase overall pelt size (Glem-Hansen, 1980b). Producers have indicated that improved pelt quality results when mink are fed diets supplemented with liver and eggs. However, whether supplementation of mink diets with these additional protein sources improves fur color and quality in mink has not been critically evaluated.

The precise physiological mechanism by which melatonin stimulates winter fur growth is not known. However, melatonin and

artificially short photoperiod-induced growth of winter fur coincides with the ability of these treatments to inhibit the synthesis and(or) secretion of prolactin (Martinet et al., 1983; Rose et al., 1985). Under natural photoperiod, prolactin may inhibit winter fur growth until endogenous levels decline as a consequence of shortened hours of daylight in the fall. The ergot alkaloid bromocriptine has also been shown to inhibit production of prolactin in mink (Martinet et al., 1981; 1982). Daily injections of bromocriptine mimic the effects of melatonin and induce molting and growth of winter fur without altering the length of the actively growing phase of the cycle (Rose et al., 1987). While the use of daily injections of bromocriptine is impractical for mink producers the effect of an implant of this dopaminergic agonist has yet to be tested.

The objectives of the present study were two fold: 1) To compare the effect of bromocriptine implants with melatonin implants on the stimulation of growth of prime winter pelage in mink fed a basal and liver- and egg-supplemented diet; 2) to ascertain whether these treatments would affect the market value as determined by pelage color and quality scores assigned by commercial fur graders.

MATERIALS AND METHODS

At weaning, 90 standard dark female kit mink were assigned randomly to one of two dietary regimens. Diet 1 (n=30) consisted of the standard ranch mink diet used by the Oregon State University Experimental Fur Farm, and diet 2 (n=60) consisted of the standard

mink diet supplemented with liver and eggs. Composition of both diets is presented in Table 1-1. All animals were fed once daily a quantity adequate to appease appetite without wastage and water was supplied ad libitum.

On 26 June 1986, mink in each of the two dietary groups were assigned to control (n=15) and treatment groups (n=15). Mink receiving diet 1 were treated with silastic implants containing 120 mg melatonin. Mink fed diet 2 were treated with either 120 mg melatonin implants, pellets containing 60 mg bromocriptine mesylate (Innovative Research of America, Toledo, Ohio, USA) or 60 mg bromocriptine pellets and 18.6 mg melatonin implants (silastic). Rate of release of bromocriptine from the pellets was established by the manufacturer to be 2 mg daily. Implants were inserted sc over the scapular area of each mink after anesthetizing with 0.3 ml ketamine hydrochloride administered im (Rose et al., 1987). Control groups received no implants. On August 1, prior to molting summer pelage, all animals had a 4 cm^2 patch of fur shaved from the right hip and fur length on each animal was measured to the nearest millimeter at biweekly intervals as described by Rose et al. (1984). All mink were housed in open-sided sheds and exposed to natural photoperiod.

Beginning in October, mink were examined for primeness of pelts by visual inspection of external skin pigmentation and each treatment group was pelted when it was determined that animals had fully prime pelage. As previously stated, an objective of this study was to acquire data that could be tested statistically to compare the market

quality of prime winter pelts from treated and untreated mink. Thus, pelts were processed and sent to the Seattle Fur Exchange, Seattle Washington, USA, for evaluation of color and quality by experienced commercial fur graders who had no prior knowledge of treatments or treatment groups. Color of pelage and quality of pelt were scored by the graders from 1 to 6 with a score of 1 representing the most desirable color or highest quality (see legend to Table 1-2).

No attempt was made by the investigators to quantitate the effects of treatments on pelage density because such a procedure would necessitate removal of a portion of the pelt for histological examination which would markedly reduce the value of the pelt. Further, subjective evaluation of pelage density is a criterion routinely utilized by fur graders in determining the ultimate value of pelts. Pelt length, is also a major factor in determining the final value of a pelt and this measurement was made (in mm) from the nose to the base of the tail on the dry pelts after grading.

Data on fur growth were analyzed by repeated measures analysis of variance for an experiment of factorial design. Fur color and quality were analyzed by one-way analysis of variance after square root transformation of data and pelt length was analyzed by two-way analysis of variance.

RESULTS

Mink treated with 120 mg melatonin, 60 mg bromocriptine, and 60 mg bromocriptine combined with 18.6 mg melatonin all molted summer fur and grew winter fur 1 mo earlier than controls (Fig. 1-1). These

treatments caused significantly greater fur growth during August and September than was evident in control animals ($P < 0.05$). Mink implanted with bromocriptine plus melatonin exhibited greater fur growth during August than did those treated with either bromocriptine or melatonin alone, but the differences were not significant statistically. Mink implanted with 120 mg melatonin and 60 mg bromocriptine combined with 18.6 mg melatonin were considered to be fully prime by mid-October and were pelted October 23. Mink receiving 60 mg bromocriptine alone attained full fur growth by early October, but based on the presence of dark skin pigment, the pelage was not considered fully prime and they were subsequently pelted in December as were the controls. There was no significant effect of liver-and- egg-supplemented diet on fur growth rates of either control or melatonin-treated mink (Fig. 1-2).

Pelt color and quality scores and mean length of pelts for each of the treatments are presented in Table 1-2. There was no significant difference in color, quality or size of pelts due to diet or treatment to induce early fur growth. Prime winter pelage is typically longer and more dense than summer pelage. Fur length and density are major criteria in evaluating fur quality in the mink industry. Allain and Rougeot (1980) have shown that the induction of winter fur growth with melatonin increases the number of underfur fibers per hair follicle compared with that of untreated animals in summer pelage. On this basis the prime pelage of melatonin-treated animals is indistinguishable from that of naturally primed winter pelage. Results presented here agree with those of Rose et al.

(1987) who reported that treatment with melatonin hastened the onset of winter fur growth. Treatment with melatonin, however, did not alter the time required for fur to grow to a length comparable to that of naturally primed winter pelage.

DISCUSSION

The mechanism by which melatonin regulates the fur growth cycle is not completely understood. It has been shown that melatonin implanted into mink in summer causes a reduction in serum prolactin concentrations (Rose et al., 1984) suggesting that this indoleamine acts on the hypothalamus to alter pituitary function. Similarly, mink treated with melatonin in the spring have delayed spring molt, inhibited growth of summer fur and reduced endogenous prolactin concentrations (Allain et al., 1981; Martinet et al., 1981; 1983). Treatment of mink with the dopamine agonist bromocriptine results in a similar reduction in prolactin concentrations and induction of autumn molt (Martinet et al., 1984; Rose et al., 1987). It therefore appears that the presence of high levels of prolactin permits summer fur growth while inhibiting winter fur growth. However, 60 mg bromocriptine implants alone did not induce fully prime pelts in October as did 120 mg melatonin implants or 60 mg bromocriptine pellets plus 18.6 mg melatonin implants. Serum concentration of prolactin in mink treated with bromocriptine was not determined. It is presumed that release of this agonist at a rate of 2 mg daily was effective in suppressing secretion of this pituitary hormone, otherwise, it is doubtful that any change in growth of winter fur

would have been observed. If this premise is true, these data suggest that inhibition of prolactin secretion by bromocriptine alone may not mimic the effects of exogenous melatonin to stimulate fur growth in the mink through the entire cycle culminating in prime winter pelage.

Fur represents a large component of the total body protein in mink, and it is likely that during the active fur growth period there is a higher requirement for methionine and cystine (Glem-Hansen, 1980a). The basal requirement for protein is also expected to be greater for kit mink induced to produce early winter fur while still actively growing. However, results of this study indicate that supplementing basic dietary protein sources with additional liver and eggs had no effect on color of fur induced to grow with exogenous melatonin. Mink have a large capacity to reserve essential amino acids and on low protein diets still produce high quality pelts (Glem-Hansen, 1980b). It appears that the basal diet used here (130.2 g/kg protein) was of higher quality than needed for maximum fur growth.

In summary, we have shown that hormone treatment with melatonin and bromocriptine plus melatonin will induce growth of prime winter pelage without deleterious consequences on pelt color, quality or size. Diet of treated animals did not alter the quality of fur produced. Our use of bromocriptine pellets strengthens the hypothesis that reduced secretion of prolactin may be involved in the melatonin-induced fur growth in mink. The fact that 60 mg bromocriptine implants alone initiate early fur growth in mink, but

do not produce a prime pelt, may indicate a synergistic effect of prolactin and melatonin at the level of the hair follicle.

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TABLE 1-1. DIET COMPOSITIONS[a]

	Diet 1 g/kg	Diet 2 g/kg
Ingredients		
Fish scrap,	550	450
Chicken offal,	250	150
Tripe/lung mix,	100	100
Beef liver,	0	100
Eggs[b]	0	100
Dry mix[c,d]	100	100
Analysis		
Dry matter	360.8	368.9
Protein[e]	130.2	140.3
Fat	116.5	99.8
Fiber	8.8	4.4
Ash	25.9	36.6

[a]Ingredients were obtained from the Northwest Fur Breeders Cooperative, Edmonds, Washington USA.

[b]Whole, cooked with the shell.

[c]Composed of 80% finely ground pop-cooked wheat and 20% wheat bran, with 110 IU vitamin E and 2.475 grams thiamine mononitrate/kg.

[d]10% additional water was added at feeding.

[e]N X 6.25.

TABLE 1-2. FUR QUALITY AND COLOR SCORES[a] AND PELT LENGTH[b]

Treatment[c]	Color[d]		Quality[e]		Pelt Length	
Diet 1	Mean	(s.e.)	Mean	(s.e.)	Mean	(s.e.)
Control	4.3	(0.25)	1.5	(0.16)	572	(5.7)
Melatonin, 120 mg	4.1	(0.17)	1.8	(0.35)	565	(6.7)
Diet 2						
Control	4.0	(0.24)	1.7	(0.25)	583	(5.5)
Melatonin, 120 mg	3.9	(0.32)	1.9	(0.18)	580	(10.1)
Bromocriptine, 60 mg	3.9	(0.22)	1.3	(0.25)	569	(6.0)
Bromocriptine, 60 mg + melatonin, 18.6 mg	4.5	(0.35)	1.9	(0.21)	590	(7.9)

[a] Evaluation of color and quality was made by fur graders at the Seattle Fur Exchange.

[b] Dry pelt length (mm) measured from tip of nose to base of tail.

[c] Each subgroup listed under diet 1 and 2 consisted of 15 mink.

[d] 1 = XX-Dark, 2 = X-Dark, 3 = Dark, 4 = Dark brown, 5 = Brown, 6 = Red.

[e] 1=Highest, 6=Lowest.

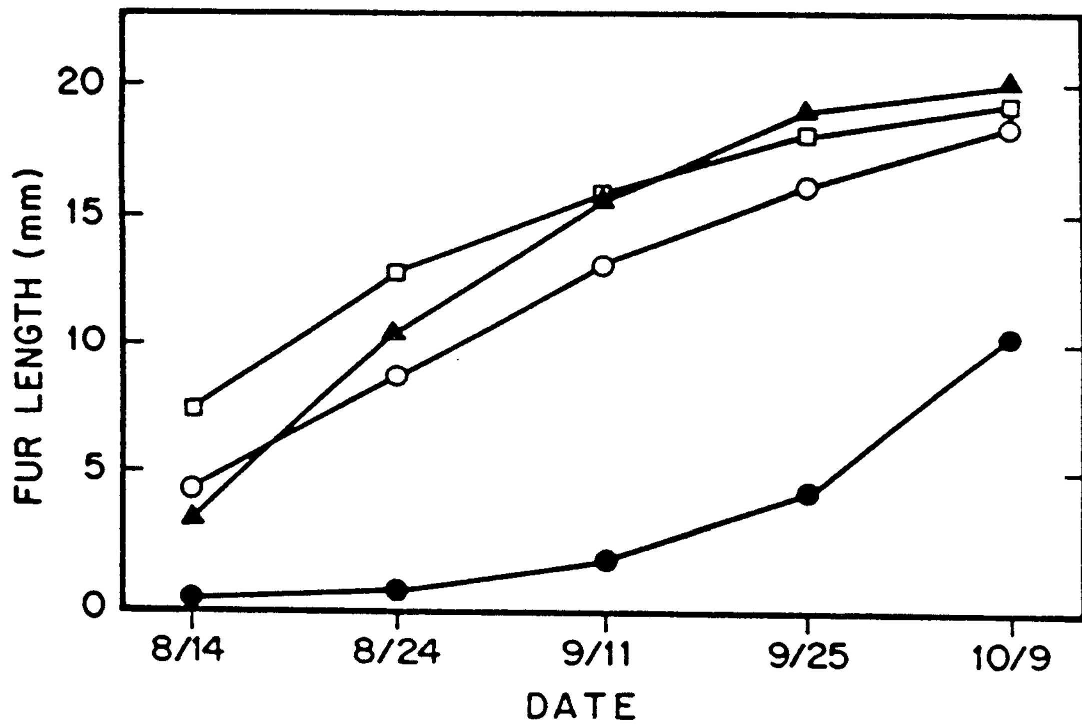

Figure 1-1. Fur growth of mink treated with 120 mg melatonin (○—○), 60 mg bromocriptine (▲—▲), 60 mg bromocriptine + 18.6 mg melatonin (□—□) and control (●—●). Common estimate of SE =3.4.

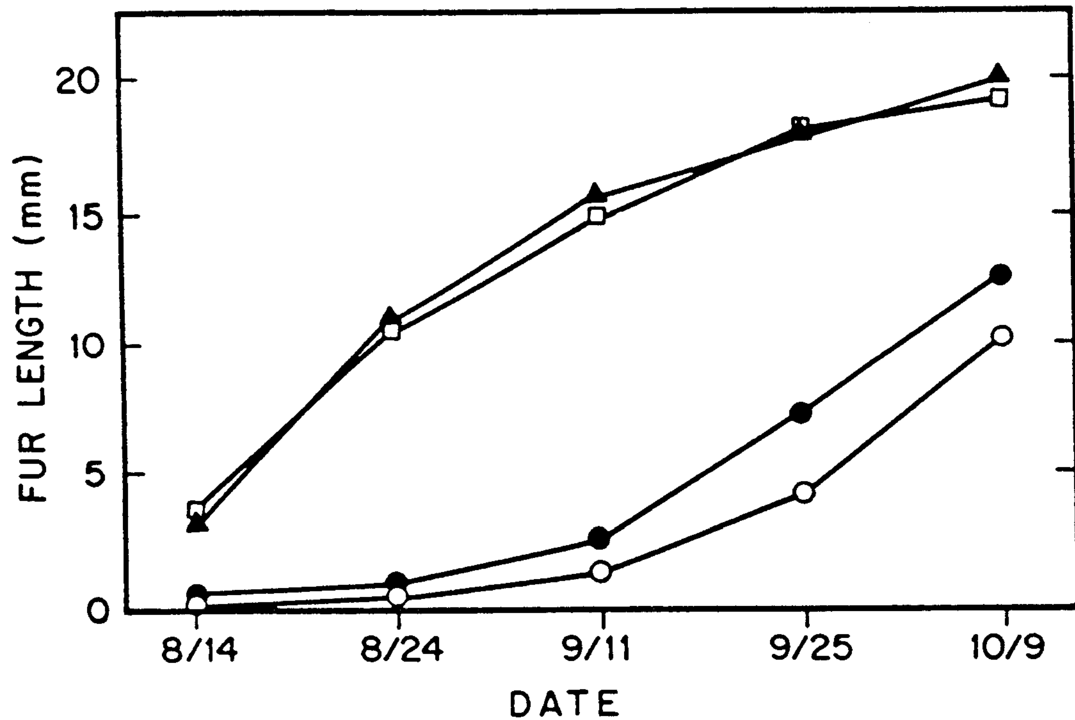

Figure 1-2. Effect of supplemented diet on fur growth in mink. Mink fed a basal diet treated with 120 mg melatonin (□—□), basal diet with no implant (●—●), diet supplemented with beef liver and eggs and treated with 120 mg melatonin (▲—▲), supplemented diet with no implant (○—○). Common estimate of SE = 3.9.

APPENDIX 2

SUPPRESSIVE ACTION OF GONADOTROPIN-RELEASING HORMONE AND LUTEINIZING HORMONE ON FUNCTION OF THE DEVELOPING OVINE CORPUS LUTEUM[1,2]

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ABSTRACT

Experiments were conducted to examine the effects of exogenous GnRH and LH on serum concentrations of progesterone (P_4) in the ewe. Ewes in Exp. 1 and 2 were laparotomized on d 2 of the cycle and ewes with corpora lutea (CL) in both ovaries were unilaterally ovariectomized. Ewes with CL in one ovary only were not ovariectomized. While anesthetized, ewes (n=5) were injected with 25 ug GnRH (Exp. 1) or 50 ng GnRH (Exp. 2) into the artery supplying the ovary bearing the CL. Control ewes (n=5 each experiment) were injected similarly with saline. In Exp. 3, six ewes were injected iv (jugular) on d 2 with 100 ug oLH (t = 0) and 50 ug oLH at 15, 30 and 45 min while six control ewes were injected similarly with saline. Jugular blood was collected from all ewes at frequent intervals after treatment for LH analysis and on alternate days of the cycle through d 10 or 11 for P_4 analysis. Treatment with 25 ug GnRH increased serum concentrations of LH at 15, 30, 45 and 60 min post-injection ($P < 0.001$) and reduced serum concentrations of P_4 on d 7 through 11 (treatment x day interaction; $P < 0.05$). Injection with 50 ng GnRH caused a slight increase in serum concentrations of LH at 15 min and had no effect on serum concentrations of P_4. Treatment of ewes with LH increased serum concentrations of LH for up to 45 min after the last injection of gonadotropin and reduced serum concentrations of P_4 on d 6 and 8 (treatment x day interaction; $P < 0.05$). Increased systemic concentrations of LH resulting from treatment of ewes with GnRH or LH early in the cycle appears to impair subsequent function of the CL. (Key Words: Corpus Luteum, Estrous Cycle, Gonadotropin

Releasing Hormone, Luteinizing Hormone, Ovine, Progesterone.)

INTRODUCTION

Administration of GnRH to heifers and cows attenuated serum concentrations of progesterone during the estrous cycle and early pregnancy without causing luteal regression (Lucy and Stevenson, 1986; Rodger and Stormshak, 1986). Altered bovine luteal function after GnRH treatment may be attributed to induced release of LH resulting in receptor down-regulation (Rodger and Stormshak, 1986). However, presence of high affinity binding sites for GnRH in ovaries of the rat (Clayton et al., 1979) and the antisteroidogenic effect of this decapeptide in hypophysectomized rats (Hsueh and Erickson, 1979) has implicated a possible direct action of GnRH on the mammalian ovary (Hsueh and Jones, 1981). Moreover, a GnRH-like ovarian hormone (GLOH), which binds to rat GnRH receptors, has been isolated from ovaries of the ewe and the cow (Aten et al., 1987). Despite the presence of this ovarian protein, binding sites for GnRH in ovarian tissues have not been demonstrated in cattle, sheep or swine (Brown and Reeves, 1983). Considering all available evidence, the mechanism by which GnRH reduces luteal steroidogenesis in domestic species remains enigmatic.

The present research was conducted to determine whether low doses of GnRH, injected into the ovarian artery of the ewe, would interfere with luteal function as reflected by a reduction in serum concentrations of progesterone. In addition, an experiment was conducted to determine whether the effect of GnRH on serum

concentrations of progesterone could be mimicked by systemic administration of LH.

MATERIALS AND METHODS

Animals and Surgical Techniques

A total of 32 mature crossbred ewes were utilized in three experiments. Ewes were checked twice daily for behavioral estrus with vasectomized rams and allowed to complete two estrous cycles (16.7 $\pm$ .2 d) before being assigned to an experiment. Ewes were fasted 24 h prior to surgery. Anesthesia for midventral laparotomy was induced by an intravenous injection of sodium thiamylal (Biotal 2.5%) and maintained by halothane-oxygen inhalation. All surgical procedures were conducted under aseptic conditions.

Experiment 1

This experiment was designed to determine whether administration of GnRH into the ovarian artery would act locally to interfere with luteal progesterone production. Ten ewes were assigned to a control or a treatment group (n = 5 each). All ewes were laparotomized on d 2 of the cycle (detected estrus = d 0 of the cycle) and animals with developing corpora lutea (CL) in both ovaries were subjected to unilateral ovariectomy. Ewes with CL in one ovary only were not ovariectomized. While anesthetized, treated ewes received a single injection of 25 ug GnRH (in 0.5 ml) into the artery supplying the ovary bearing the CL. Controls were injected similarly with an equal volume of saline. Jugular blood was collected from all ewes at 0,

15, 30, 45 and 60 min after injection for analysis of serum LH and on d 3, 5, 7, 9 and 11 of the cycle for serum progesterone analysis.

Experiment 2

Because injection of 25 ug GnRH in Exp. 1 caused LH release, Exp. 2 was designed to investigate the local effect of a lower dose of GnRH on serum concentrations of progesterone. Ten ewes were assigned to control and treatment groups (n = 5 each), laparotomized and unilaterally ovariectomized on d 2 of the cycle following the procedure described for Exp. 1. Treatment consisted of an injection of 50 ng GnRH (in 0.2 ml saline) or saline (control) into the artery supplying the ovary bearing the CL. Jugular blood was collected from all ewes at 0, 15, 30, 45 and 60 min after LH injection for LH analysis and on d 4, 6, 8 and 10 of the cycle for progesterone analysis.

Experiment 3

Experiment 3 was conducted to evaluate the effect of exogenous LH on luteal progesterone production. Twelve ewes were assigned at random to a control and treatment group (n = 6 each). On d 2 of the estrous cycle ewes were injected iv (jugular) with 100 ug oLH (NIADDK-oLH-25; t = 0) followed by 50 ug oLH at 15, 30 and 45 min. Controls were injected similarly with an equal volume of saline. Jugular blood was collected for LH analysis 15 min prior to the first injection, immediately after each injection and at 15-min intervals for 1 h after the last injection. Jugular blood collected on d 2, 4,

6, 8 and 10 of the cycle was analyzed for progesterone.

Radioimmunoassays

All blood samples (10 ml) were allowed to clot at room temperature then stored at 4° C for 24 h. Sera were separated by centrifugation (500 x g) for 15 min at 4° C and stored at -20° C until assayed.

Radioimmunoassay for LH followed the procedure of McCarthy and Swanson (1976) using highly-purified ovine LH (LER-05 6-C2) iodinated by chloramine-T method, and concentrations of LH were expressed using ovine LH standards (NIADDK-oLH-25) with assay sensitivity of 0.5 ng/assay tube. Intra- and interassay coefficients of variation were 8.4 and 14%, respectively.

Sera were assayed for progesterone in duplicate after hexane:benzene (2:1) extraction. Twelve thousand dpm 1,2,6,7 [^{3}H]-progesterone [4] were added to a third tube containing an aliquot of each sample to determine and correct for procedural loss due to extraction. Mean extraction efficiency of this procedure was 86 $\pm$ 1% (138 samples).

Progesterone RIA was performed on extracted samples following the procedure of Koligian and Stormshak (1976). An antibody (anti-progesterone-11-BSA) was utilized in the assay. Sensitivity of the assay was 10 pg/assay tube and intra- and interassay coefficients of variation were 9 and 16%, respectively.

[4] Net-381, New England Nuclear, Boston, MA.

Statistical Analysis.

Difference in the number of CL between control and treated ewes in Exp. 1 and 2 was analyzed by Student's t-test. Data on serum concentrations of progesterone and LH from all three experiments involving repeated measurements from the same animal were analyzed by split-plot analysis of variance. Differences among means were tested for significance by Fishers Protected Least Significant Difference test (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

There was no significant difference observed in the number of CL between treatment and control ewes in Exp. 1 and 2. Mean ($\pm$ SE) number of CL in treated vs control ewes in Exp. 1 and Exp. 2 were 1.8 $\pm$ 0.2 vs 2.2 $\pm$0.2 and 1.8 $\pm$0.2 vs 1.8 $\pm$ 0.2, respectively.

In Exp. 1, treatment with 25 ug GnRH increased ($P < 0.001$) serum concentrations of LH at 15, 30, 45 and 60 min and reduced serum concentrations of progesterone on d 7 through 11 of the cycle (treatment x day interaction; $P < 0.05$). Mean peak concentrations of LH in control and treated ewes were 4.9 and 19.2 ng/ml, respectively (Figure 2-1). Mean serum concentrations of progesterone in control and treated ewes on d 11 were 1.83 vs 1.53 ng/ml, respectively (Figure 2-2). In Exp. 2, treatment of ewes with 50 ng GnRH resulted in only a slight increase in serum LH at 15 min (Figure 2-3) and was without significant effect on serum concentrations of progesterone on d 4 through 10 of the cycle (Table 2-1).

Injection of ewes with LH in Exp. 3 increased serum concentrations of LH that were maximal at 15 min. Injections of LH at 15-min intervals for 45 min maintained elevated concentrations of LH for 90 min (Figure 2-4). This dose of gonadotropin, administered in an attempt to mimic the response of GnRH described in Exp. 1, suppressed serum concentrations of progesterone on d 6 and 8 of the cycle (Figure 2-5; treatment x day interaction; $P < 0.05$).

Results of these experiments indicate that exposure of the developing CL to increased concentrations of LH impairs subsequent development of the CL or the ability of luteal cells to synthesize and(or) secrete progesterone at maturity. Treatment with 25 ug GnRH, which provoked a surge-like increase of LH suppressed serum progesterone. Similar responses to GnRH occurred in cows and heifers (Lucy and Stevenson, 1986; Rodger and Stormshak, 1986). Injection of GnRH (50 ng), which did not provoke a concomitant increase in serum LH, also did not result in an antigonadotropic response. It is possible that a single injection of 50 ng GnRH may be subthreshold to a dose that will induce a direct effect on the CL. However, these data suggest that the antisteroidogenic effect of GnRH is mediated through LH in the ewe rather than directly on the ovary as has been reported to occur in the rat (Hsueh and Erickson, 1979).

Luteal development involves both an increase in the number and size of the steroidogenic luteal cells (Schwall et al., 1986). After these cells mature they can be classified morphologically into two distinct groups commonly referred to as small and large luteal cells (O'Shea et al. 1986). In the mature CL, both cell types synthesize

progesterone, but this synthesis appears to be controlled by independent factors. Large luteal cells appear to have fewer LH receptors, but possess a greater endogenous (basal) rate of progesterone synthesis than small luteal cells (Fitz et al., 1982). Further, small luteal cells have large numbers of LH receptors and respond to LH with a threefold increase in progesterone secretion.

Developing CL in the ewe may have few if any large luteal cells on d 2 of the cycle and hypophyseal support in the form of LH is required for luteal development and maintenance in this species (Kaltenbach et al., 1968). It has been proposed that the luteotropic effect of LH may be to stimulate transformation of small cells into large cells (Donaldson and Hansel, 1965). This concept is supported by differential binding of monoclonal antibodies developed against theca and granulosa-specific antigens to small and large luteal cells, respectively (Alila and Hansel, 1984). Further, repeated injection of ewes (every 6 h from d 5 to 10) with large doses of LH increased the number of large luteal cells and concomitantly decreased the number of small luteal cells without affecting serum concentrations of progesterone measured on d 10 (Farin et al., 1988).

Luteal concentrations of LH receptors decreased following administration of large quantities of LH to ewes (Suter et al., 1980) and after GnRH treatment of heifers (Rodger and Stormshak, 1986). Therefore, it appears that GnRH may act by stimulating hypersecretion of LH to induce down-regulation of LH receptors with a subsequent reduction in development of large cells as reflected by reduced serum concentrations of progesterone.

IMPLICATIONS

Our data indicate that administration of 25 ug GnRH via the ovarian artery and the resultant increase in systemic concentrations of LH, as well as injection of high doses of LH on d 2, impairs the ability of the ovine CL to produce progesterone later in the estrous cycle. Low doses of GnRH (50 ng) did not suppress serum concentrations of progesterone. From these data we conclude that the antisteroidogenic action of GnRH is mediated through release of endogenous LH, but does not exclude other possible functions of this decapeptide in the ovary.

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TABLE 2-1. MEAN (± SE) SERUM CONCENTRATION OF PROGESTERONE (NG/ML) AFTER INJECTION OF EWES WITH 50 NG GNRH (EXP. 2)

	Day of cycle			
Treatment	4	6	8	10
Control	0.36±0.01[a]	0.82±0.07[a]	1.25±0.08[a]	1.29±0.08[a]
GnRH	0.42±0.08[a]	0.89±0.1[a]	1.47±0.28[a]	1.27±0.32[a]

[a] Means in each column with the same superscripts do not differ ($P > 0.05$).

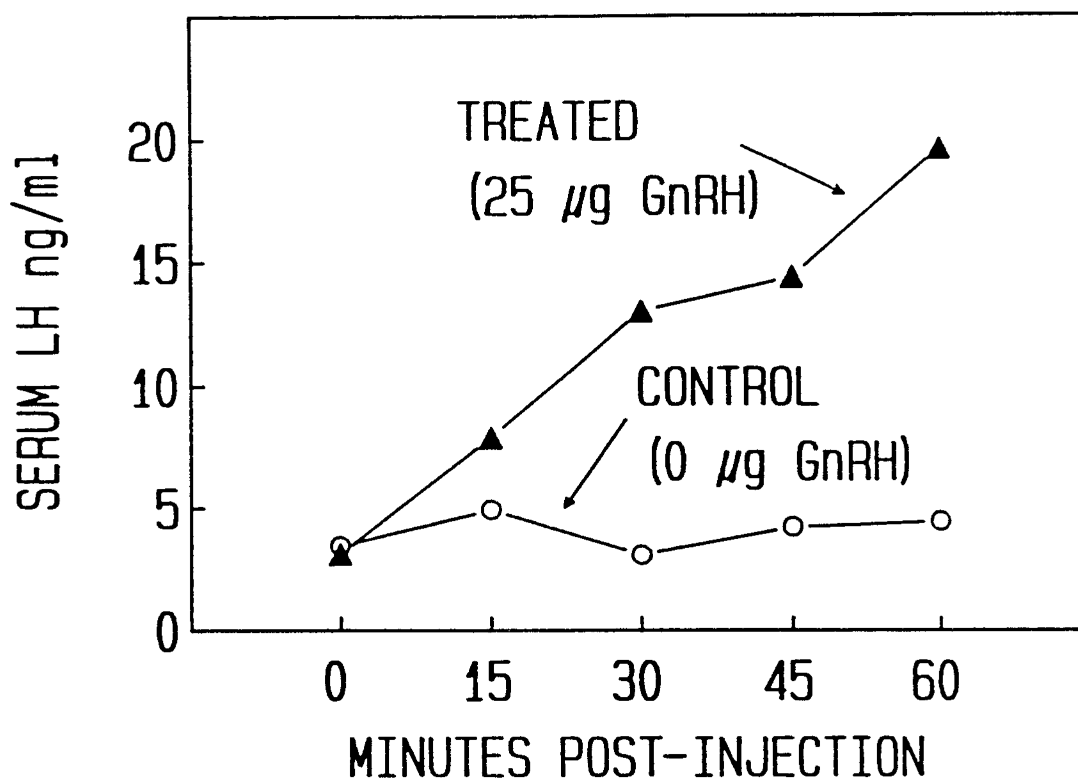

Figure 2-1. Mean serum concentrations of LH in ewes injected with 25 ug GnRH via the artery supplying the ovary bearing the corpus luteum on d 2 of an estrous cycle (pooled SE = 1.37; $P < 0.001$).

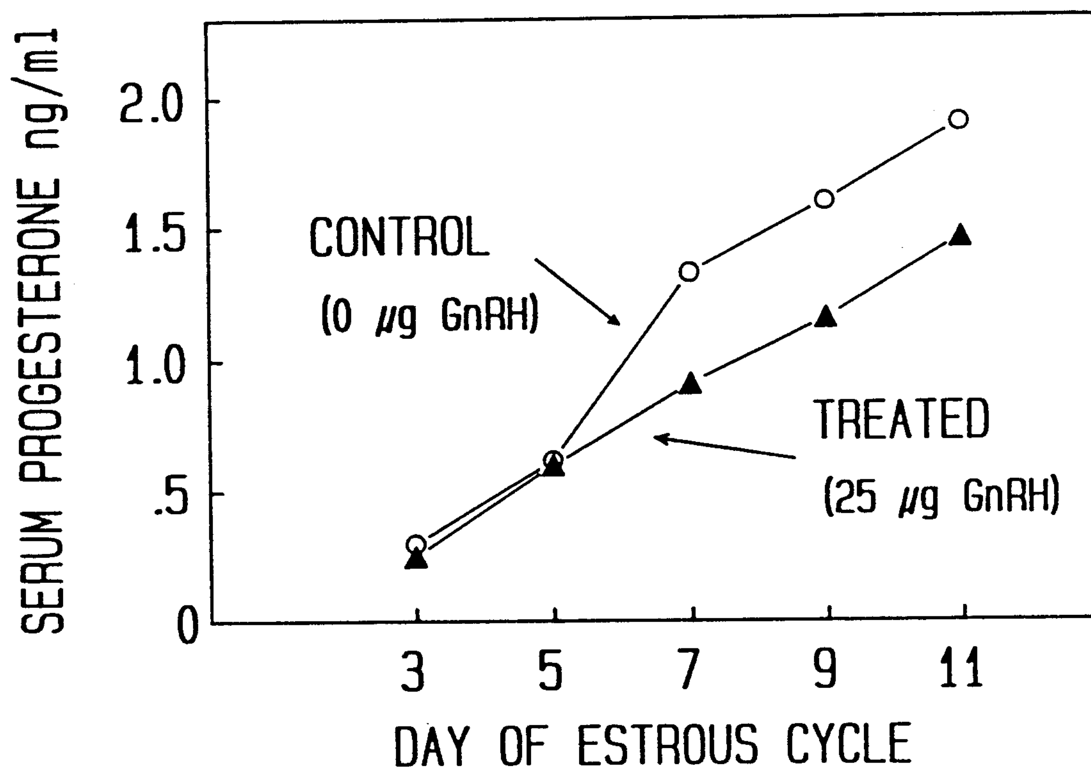

Figure 2-2. Mean serum concentrations of progesterone in ewes injected on d 2 of an estrous cycle with 25 ug GnRH via the artery supplying the ovary bearing the corpus luteum (pooled SE =0.21; treatment x day interaction; $P < 0.05$).

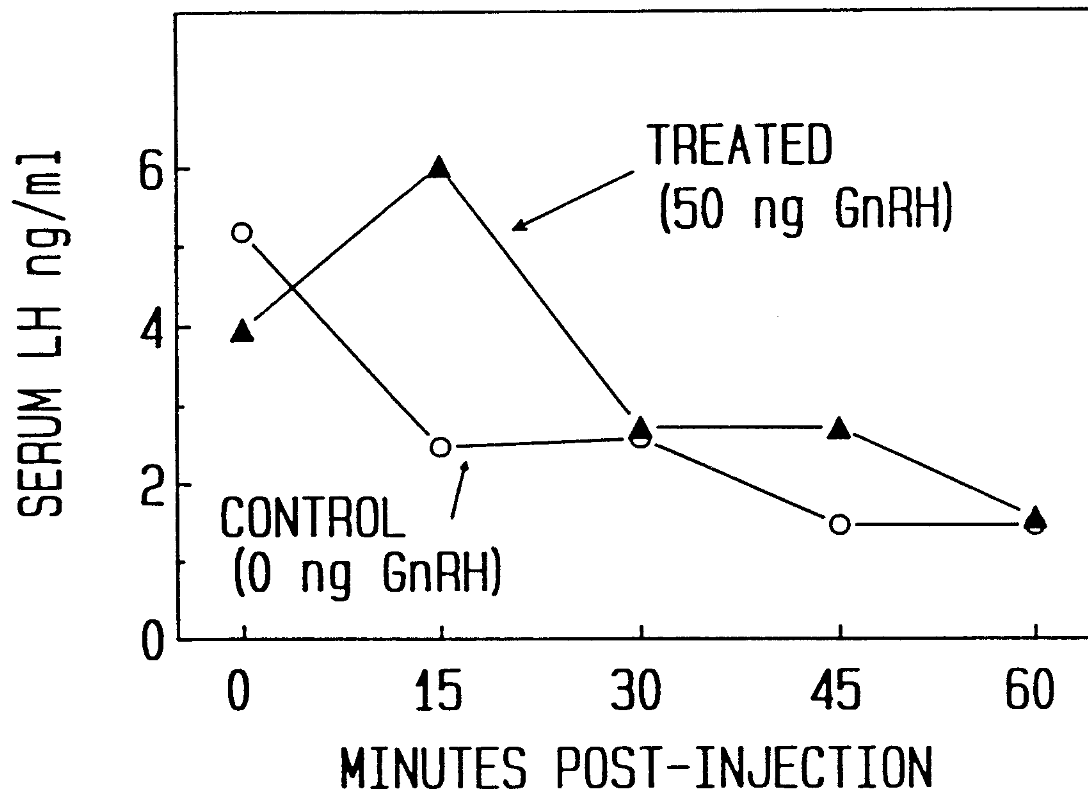

Figure 2-3. Mean serum concentrations of LH in ewes on d 2 of an estrous cycle after injection with 50 ng GnRH via the artery supplying the ovary bearing the corpus luteum (pooled SE = 1.21; $P > 0.05$).

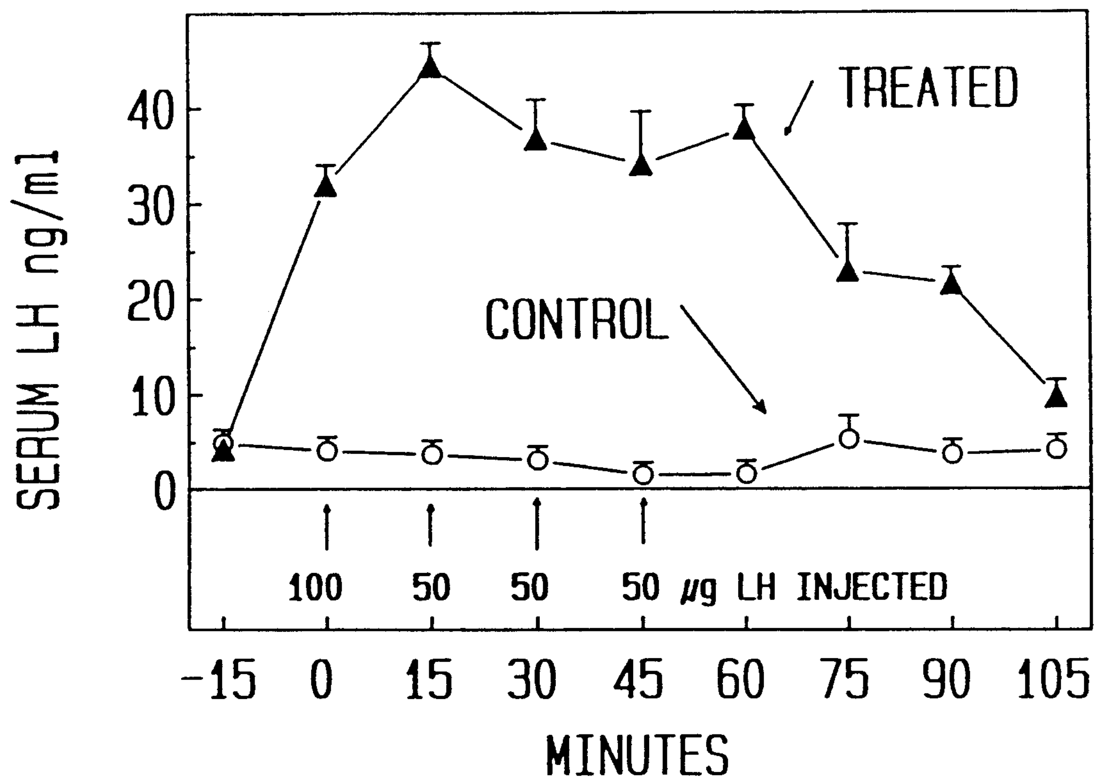

Figure 2-4. Serum concentrations of LH (mean ± SE) of ewes after iv (jugular) injection of 250 ug oLH (100 ug at t = 0, followed by 50 ug at 15, 30 and 45 min).

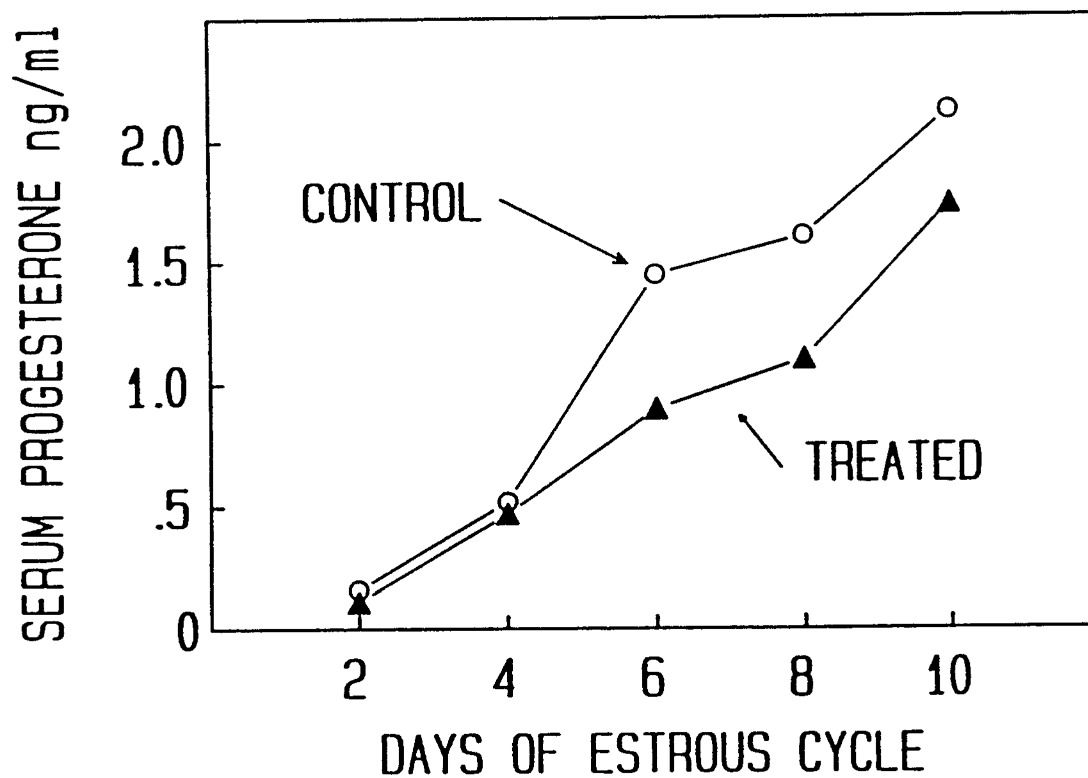

Figure 2-5. Mean serum concentrations of progesterone after treatment of ewes on d 2 of the cycle with 250 ug LH (pooled SE = 0.31; treatment x day interaction; $P < 0.05$).

APPENDIX 3

IN VIVO AND *IN VITRO* EFFECTS OF A CYCLOPROPENOID FATTY ACID ON OVINE CORPUS LUTEUM FUNCTION[1,2]

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[1] Technical paper No. 9071, Oregon Agr. Exp. Sta. Published in Endocrinology 127:3166-3171 (1990).

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ABSTRACT

Experiments were conducted to examine the effect of a cyclopropenoid fatty acid (CPFA) on progesterone (P_4) production by the ovine corpus luteum (CL) during the estrous cycle. Ewes in Exp. 1 and 2 were laparotomized on day 2 of the estrous cycle and animals with CL in both ovaries were subjected to unilateral ovariectomy. Ewes with CL in one ovary only were not ovariectomized. During surgery, ewes were injected with a mixture of fatty acids [sterculic acid, 39%; palmitic, 29%; linoleic, 12%; malvalic acid, 9%; oleic, 8%; stearic, 3%] containing 500 ug sterculic acid (SA; Exp. 1), 750 ug SA or with 750 ug oleic acid (Exp. 2) via the artery supplying the ovary bearing the CL. Control ewes were similarly injected with vehicle only (0.1 - 0.2 ml DMSO; Exp. 1 and Exp. 2, respectively). Sera from blood samples collected at 15-min intervals for 1 h after injection or once daily on alternate days of the cycle after surgery were analyzed for LH and P_4, respectively. In Exp. 3, slices of CL removed from 5 ewes on day 10 of the cycle were incubated for 90 min in medium containing 100 ng/ml SA or vehicle (10 ul DMSO). Slices were then reincubated for 90 min in medium containing 10 ng/ml oLH or saline (10 ul). Tissue and medium were analyzed for P_4. Injection of 500 ug SA suppressed serum levels of P_4 ($P<0.01$) but did not alter mean cycle length. Injection of 750 ug SA reduced serum concentrations of P_4 and shortened estrous cycle duration ($P<0.005$). Oleic acid (750 ug) or as much as 1.9 mg of a mixture of fatty acids devoid of CPFA, had no effect on cycle length or serum levels of P_4 suggesting that altered luteal function was due to type and not

quantity of fatty acid injected. Treatments had no effect on serum concentrations of LH. Preincubation with SA interfered with the ability of luteal slices to synthesize P_4 when subsequently incubated alone or with oLH ($P<0.01$). It is concluded that SA acts on the CL to impair steroidogenesis and ultimately cause luteal regression.

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) are naturally occurring plant lipids that contain a highly strained propene ring in their carbon chains. Two CPFA, the 18 carbon malvalate (2-octyl-1-cyclopropene-1-heptanoate) and 19 carbon sterculate (2-octyl-1-cyclopropene-1-octanoate) are found in seed and other tissue oils of plants in the order Malvales (Nunn, 1952; Carter, 1964; Kircher, 1964). This order includes species (kapok and cotton) that are economically important dietary items for humans and livestock (Shenstone et al., 1965).

Cyclopropenoid fatty acids are not known to have specific metabolic functions in plants but cause a variety of toxic effects in animals. These unique fatty acids have been shown to increase infertility in rats and delay sexual maturity in poultry and rats (Phelps et al., 1965), increase concentrations of saturated fatty acids in tissue lipids (Raju and Reiser 1967), increase plasma and liver cholesterol (Ferguson et al., 1976; Matlock et al., 1985) and alter membrane permeability (Doberenz et al., 1962). Cyclopropenoid fatty acids are both carcinogenic and cocarcinogenic in fish (Hendricks et al., 1980). Sterculic acid is reported to be the most

biologically active CPFA and its activity appears to be contingent upon incorporation into biomembranes, and saturation of the propene ring leads to inactivity (Pawlowski et al., 1985).

Reproductive responses after CPFA treatment are poorly documented, but may be related to altered steroidogenesis. If this hypothesis is true, resolving the mechanism by which CPFA acts on cells may provide a valuable tool in studying the control of steroid metabolism. The present research was conducted to investigate whether CPFA could alter progesterone production by the ovine corpus luteum. This tissue was selected for study because of its well-documented pattern of hormone secretion during the estrous cycle (Auletta and Flint, 1988).

MATERIALS AND METHODS

Animals and surgical techniques

Thirty six mature crossbred ewes were checked twice daily for behavioral estrus with vasectomized rams and allowed to complete one estrous cycle (16.5 $\pm$ 0.2 days) before being assigned to one of three experiments. Ewes were fasted 24 h prior to surgery. Anesthesia for midventral laparotomy was induced by an intravenous injection of sodium thiamylal (Biotal, 2.5%) and maintained by halothane-oxygen inhalation. All surgical procedures were conducted under aseptic conditions.

Cyclopropenoid fatty acid preparation

Pure preparations of CPFA are unavailable commercially and these

fatty acids are difficult to isolate and purify from natural sources (Pawlowski et al., 1981). Therefore, a mixture of CPFA [sterculic acid, 39%; palmitic, 29%; linoleic, 12%; malvalic acid, 9%; oleic, 8%; stearic, 3%] extracted from Sterculia foetida seeds was used in this study. This mixture was stored at -20 C prior to dilution with dimethyl sulfoxide (DMSO). Because sterculic acid is reported to be the most biologically active CPFA (Pawlowski et al., 1985) presentation of CPFA concentrations are based on the sterculate (SA) fraction in the extract.

Experiment 1

This experiment was exploratory in nature and designed to determine whether an injection of SA into the ovarian artery of the ewe would affect progesterone secretion by the corpus luteum. Ten ewes assigned to a control and treatment group (n = 5 each) were laparotomized on day 2 of the cycle (detected estrus = day 0) and those ewes with corpora lutea in both ovaries were subjected to unilateral ovariectomy. Ewes with corpora lutea in one ovary only were allowed to remain intact. Treatment consisted of a single injection of 500 ug SA (1.3 mg S. foetida extract, in 0.1 ml DMSO) into the artery supplying the ovary bearing the corpus luteum. Control ewes were injected similarly with vehicle (DMSO) only. Ewes were allowed to recover from surgery for 24 h and then checked twice daily for estrous behavior to monitor cycle length. Jugular blood samples collected on days 3, 5, 7, 9 and 11 of the cycle were analyzed for serum progesterone by radioimmunoassay.

Experiment 2

Because injection of 500 ug SA into the ovarian artery attenuated serum concentrations of progesterone, Exp. 2 was designed to evaluate the effect of a larger dose (750 ug) of SA and to determine whether effects of this CPFA could be mimicked by an equivalent quantity of oleic acid. This fatty acid (oleic acid) was specifically selected as a treatment because it differs from sterculate only in the absence of the propene ring in the center of its carbon chain.

In this experiment, seventeen ewes were assigned to a control or to one of two treatment groups. As in Exp. 1, ewes were laparotomized on day 2 of the cycle and those ewes with developing corpora lutea in both ovaries were subjected to unilateral ovariectomy. Treatment consisted of an injection of 750 ug SA (n = 5) or an injection of 750 ug oleic acid (cis-9-octadecenoic acid, Sigma Chemical Co. St. Louis, MO; n = 6) via the artery supplying the ovary bearing the corpus luteum. Control ewes were similarly injected with vehicle only (n = 6). Treatments that elevate serum concentrations of LH early (day 2) in the cycle, have been shown to alter serum concentrations of progesterone (Slayden and Stormshak, 1990). To determine if SA treatment could acutely affect LH secretion, jugular blood was collected at 0, 15, 30, 45, and 60 min after injection and the sera were analyzed for LH. Ewes were allowed to recover from surgery for 24 h then checked twice daily for estrous behavior to determine cycle length. One ewe from each group was relaparotomized on day 6 of the cycle and CL were removed, weighed

and fixed for cytological examination. From the remaining ewes, jugular blood collected on days 2, 4, 6, 8 and 10 of the cycle, or until animals exhibited behavioral estrus, was analyzed for progesterone.

The response observed in Exp. 1 was presumed to be due to SA in the administered extract of S. foetida seeds. To rule out the possibility that other fatty acids in this mixture were in fact responsible for the reduced function of the corpus luteum, a comparable mixture of fatty acids containing no CPFA was prepared and tested. In this corollary trial, four ewes on day 2 of the cycle were injected during laparotomy as previously described with either 1.9 mg of a mixture of fatty acids [oleic acid, 56%; palmitic, 29%; linoleic acid, 12%; stearic acid, 3%; in 0.1 ml DMSO] or with vehicle alone (n = 2 each). Ewes were checked twice daily for estrous behavior to determine cycle length and jugular blood collected on days 2, 4, 6, 8, 10, 12, 14 and 16 of the cycle was analyzed for progesterone.

Experiment 3

The objective of Exp. 3 was to determine if pretreatment with SA would affect synthesis of progesterone by luteal tissue during short-term incubation. Five ewes were laparotomized on day 10 of the cycle and corpora lutea were removed and transported to the laboratory in incubation medium (Ham's F-12) supplemented with 24 mM Hepes buffer, 100 U/ml penicillin, 100 ug/ml streptomycin, 0.25 ug/ml amphotericin, 5 ug/ml bovine insulin, 5 ug/ml human transferrin and 5 ng/ml

selenium (Sigma Co., St. Louis). Corpora lutea were dissected free of ovarian stroma, weighed and sliced (0.3 mm) with a Harvard apparatus #140 tissue slicer. Tissue slices from each ewe were washed twice with incubation medium and divided into six, 100 mg aliquots. Three aliquots of tissue were preincubated in 4 ml medium containing 10 ul DMSO while the remaining three aliquots were preincubated in the same volume of medium containing 100 ng/ml SA (in 10 ul DMSO). This concentration of SA is similar to that shown to induce a 50% inhibition of liver fatty acyl desaturase activity _in vitro_ (Raju and Reiser, 1967) while being 0.01 the concentration shown to have non-specific detergent effects (Raju and Reiser, 1972). Preincubation was for 90 min in a Dubnoff shaking incubator at 37 C under an atmosphere of 95% O_2:5% CO_2. After preincubation, medium was removed and tissue slices were washed twice with fresh medium. One aliquot of tissue having been preincubated with SA and one with vehicle alone were chosen at random to serve as unincubated controls. Six milliliters of cold (0 C) absolute ethanol were added to flasks containing these two aliquots of tissue to stop further synthesis of progesterone. The four remaining aliquots of tissue were placed into 4 ml medium containing either 10 ng/ml oLH or vehicle (10 ul saline) and reincubated for 90 min. Thus, tissues preincubated with SA and vehicle were incubated in the absence or presence of LH. Incubation was stopped as described for unincubated controls and tissue in medium plus ethanol were stored at -20 C until analyzed for progesterone.

Radioimmunoassays

All blood samples (10 ml) were allowed to clot at room temperature then stored at 4 C for 24 h. Sera were separated by centrifugation (500 x g) for 15 min at 4 C and stored at -20 C until assayed for progesterone in duplicate after hexane:benzene (2:1) extraction. Progesterone (1, 2, 6, 7 [^{3}H]-progesterone, 12 x 10^3 dpm, New England Nuclear: Net-381) was added to a third tube containing an aliquot of each sample to determine and correct for procedural loss due to extraction. Extraction efficiency of this procedure was 87 $\pm$ 0.7% with 110 samples.

Tissue samples were prepared for progesterone assay following the procedure of Koligian and Stormshak (1976). Briefly, tissue plus medium in absolute ethanol were homogenized with an additional 6 ml absolute ethanol in a Duall 24 ground glass homogenizer. Progesterone (1, 2, 6, 7, [^{3}H] progesterone, 45 x 10^3 dpm) were added to each sample prior to homogenization. Samples were filtered through Whatman #1 filter paper with an additional 10 ml absolute ethanol. Filtered samples were evaporated to dryness under vacuum at 45 C, resuspended in 4 ml phosphate buffered saline with 1% gelatin and extracted with 20 ml hexane:benzene (2:1). A one milliliter fraction of extractant was used to determine procedural loss. Extraction efficiency was 83 $\pm$ 1.4% in 30 samples. Addition of [^{3}H]-progesterone to tissue samples increased total progesterone in tissue extractant by 3.5 pg/ml which was below the limit of detection of the assay procedure.

Progesterone radioimmunoassays were performed on all extracted samples (sera and tissues) following the procedure of Koligian and Stormshak (1976) utilizing a progesterone antibody, anti-progesterone 11-BSA. Sensitivity of the assay was 10 pg/assay tube (100 ul) and intra- and interassay coefficients of variation were 8.9 and 12%, respectively.

Radioimmunoassay for LH followed the procedure of McCarthy and Swanson (1976) using highly-purified ovine LH (LER-05 6-C2) iodinated by chloramine-T method, and concentrations of LH were expressed using ovine LH standards (NIADDK-oLH-25) with assay sensitivity of 0.5 ng/assay tube. Intra- and interassay coefficients of variation were 9.1 and 13%, respectively.

Cytology

Luteal tissue samples 2-3 mm thick were fixed in 2.5% glutaraldehyde (in phosphate buffer) for 60 min, washed for 60 min and transferred to phosphate buffer for storage. Tissue was post-fixed with 1% osmium tetroxide for 1 h, washed, ethanol dehydrated, embedded in Epon-Araldite, sectioned (1 um), stained with toluidine blue and mounted. Light micrographs of tissue sections were compared on the basis of cellular and nuclear integrity.

Statistical Analysis

Data on serum concentrations of progesterone and LH were analyzed by analysis of variance for repeated measures. Differences among means were tested for significance by Fishers Protected Least

Significant Difference test (Snedecor and Cochran, 1980). Data on cycle length were analyzed by unpaired Students t-test in Exp. 1 and by one-way analysis of variance in Exp. 2. Medium and tissue concentrations of progesterone from Exp. 3 were analyzed by analysis of variance for a randomized block split-plot design.

RESULTS

Injection of 500 ug SA in Exp. 1 resulted in suppressed serum concentrations of progesterone on days 7 through 11 of the cycle (Fig. 3-1; treatment x day interaction; $P < 0.01$). Ovariectomy on day 2 had no significant influence on concentrations of progesterone among the two groups later in the estrous cycle (ovx. x treatment interaction; $P>0.09$). Serum concentrations of progesterone increased later in the cycle (day 11-13) in ewes treated with 500 ug SA, but this increase was not significant statistically compared to concentrations measured on days 3 - 7. Sterculic acid treatment did not significantly alter mean cycle length (mean $\pm$ SE; control, 16.8 $\pm$ 0.5 vs SA, 15.6 $\pm$2.5 days) but one ewe treated with SA did have a shortened cycle of 8 days duration.

Injection of 750 ug SA in Exp. 2 significantly shortened the estrous cycles compared with the duration of cycles in oleic acid-treated ewes and DMSO controls (mean$\pm$SE; control, 17$\pm$0.3; oleic acid, 17.3$\pm$0.5; SA, 9.8$\pm$2.0 days; $P<0.005$). Therefore, data on serum concentrations of progesterone of SA-treated ewes (Fig. 3-2) were not compared statistically with those of animals receiving oleic acid or vehicle only. Injection of 750 ug oleic acid had no significant

effect on estrous cycle length or serum concentrations of progesterone compared with these characteristics of control animals (Fig. 3-2). Treatment with a mixture of fatty acids (1.9 mg), which contained no CPFA, had no significant effect on serum concentrations of progesterone (Table 3-1) or on the mean cycle length.

Although _in vivo_ treatment with SA reduced luteal function, examination of light micrographs of luteal tissue collected from a limited number of ewes 4 days after treatment revealed no difference in cell integrity among ewes injected with 750 ug SA and those injected with 750 ug oleic acid or vehicle (Fig. 3-3). This indicates that the suppressive action of SA on progesterone production in the majority of ewes was apparently not due to an acute effect on cell morphology. The CL recovered from a ewe injected with oleic acid was slightly larger than that collected from the SA and vehicle injected ewes (CL weight for SA-treated, oleic acid-treated and control ewes were 267, 333.4 and 186 mg, respectively).

There was no significant acute effect of of SA treatment on serum concentrations of LH indicating that reduced luteal function _in vivo_ was not due to altered gonadotropin secretion (Table 3-2). Because shortened cycles of ewes injected with 750 ug SA were marked by behavioral estrus of normal duration it is unlikely that treatment impaired folliculogenesis. Further, SA treatment had no effect on duration of the subsequent estrous cycle (mean $\pm$ SE for SA, oleic acid, and vehicle were 15.6 ± 1.2, 16.1 ± 0.3 and 16.5 ± 0.4 days, respectively).

In Exp. 3, preincubation of luteal slices with 100 ng/ml SA, compared with those preincubated with vehicle, resulted in a suppression in progesterone production by tissue when subsequently incubated with oLH (SA x LH interaction; $P < 0.01$). Progesterone production by tissues exposed to SA was significantly less in the presence than in the absence of LH (Fig. 3-4). In contrast, luteal tissue preincubated with vehicle responded to subsequent incubation alone and to the presence of LH with significant increases in progesterone production.

DISCUSSION

Results of the present experiments indicate that *in vivo* exposure of corpora lutea to SA caused a reduction in progesterone secretion and, depending upon dose of SA administered (500 or 750 ug), complete luteolysis. The unique ability of this CPFA to attenuate luteal function can be attributed to the presence of the propene ring. The possibility that malvalic acid in the preparation administered impaired luteal function cannot be entirely excluded. However, this possibility seems remote because the content of malvalic acid in the preparation was low (9%) and this CPFA has been reported to possess limited biological activity (Pawlowski et al., 1985). Treatment of ewes with oleic acid, similar in structure to SA, but lacking the propene ring, was without affect on the corpus luteum. It is unlikely that the observed responses were due to the total concentration of fatty acid administered because 1.9 mg of a similar fatty acid mixture was without effect on luteal function. Previous evidence indicates that altered secretion of LH during

metestrus affects subsequent progesterone production in ewes (Slayden and Stormshak, 1990) and cows (Rodger and Stormshak, 1986). However, administration of a luteolytic dose of SA to ewes did not acutely alter secretion of LH suggesting that the CPFA acted directly on the corpus luteum. In support of this conclusion, preincubation of luteal tissue removed from ewes on day 10 of the cycle with SA resulted in a reduction in progesterone synthesis when tissue was subsequently incubated in the presence or absence of oLH. Collectively, these data indicate that the developing as well as the mature corpus luteum is vulnerable to CPFA. Vulnerability of the mature corpus luteum to CPFA has been confirmed by the observation that treatment of ewes on day 10 of the estrous cycle with 750 ug SA (1.09 mg S. foetida extract), but not 1.09 mg oleic acid, suppressed serum concentrations of progesterone within 12 h of treatment (Slayden et al., 1989).

Although the mechanism by which SA inhibits luteal steroidogenesis is unknown, the action of this fatty acid does not appear to be the result of acute luteal cell death (Fig. 3-3). However, it is possible that inhibition of luteal steroidogenesis by SA is a consequence of altered plasma or intracellular membrane function. Incorporation of intact SA into membrane phospholipids (Eisele et al., 1982), or SA-induced change in the ratio of endogenous fatty acids incorporated into phospholipids could alter membrane fluidity.

Decreased plasma membrane fluidity could result in reduced responsiveness of luteal cells to LH, the primary gonadotropin in the

ewe (Kaltenbach et al., 1968), by interfering with LH-induced activation of adenylate cyclase. Reduced adenylate cyclase activity has been reported to occur during luteal regression induced by the natural luteolysin prostaglandin $F_{2\alpha}$ (Fletcher et al., 1982). Results of the present _in vitro_ experiment indicate that SA can block the response of the luteal cell to LH. Further, progesterone production by SA-treated luteal tissue exposed to LH was significantly less than in its absence.

Alternatively, SA treatment may inhibit steroidogenesis directly through impairment of cholesterol utilization (Matlock, 1985). Ingestion of SA resulted in accumulation of this fatty acid in hepatic microsomal and mitochondrial membrane fractions (Nixon et al., 1977) and SA treatment inhibited hepatic microsomal mixed-function-oxidase in rabbits (Eisele et al., 1982) and fish (Perdew et al., 1988). Further, treatment of fish with SA altered hepatic microsomal concentrations of acetyl-CoA carboxylase (Perdew et al., 1988) and specific microsomal glycoproteins (Perdew et al., 1986). Therefore, it is likely that activity of microsomal enzymes involved in regulating cholesterol metabolism may be impaired by SA treatment.

Regardless of the mechanism of action, evidence indicates that SA has a marked luteolytic effect on the ovine corpus luteum inhibiting progesterone synthesis during short-term incubation and causing luteal regression _in vivo_. Because of the effect of this CPFA on luteal steroidogenesis it may prove useful as a tool in elucidating the action of endogenous luteotropins and luteolysins.

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Table 3-1. SERUM CONCENTRATIONS OF PROGESTERONE (MEAN ± SE, ng/ml) AFTER INJECTION 1.9 mg OF A MIXTURE OF FATTY ACIDS OR VEHICLE (DMSO) INTO THE OVARIAN ARTERY ON DAY 2 OF THE ESTROUS CYCLE (n = 2 each).

Treatment	Day of the Cycle[a]							
	2	4	6	8	10	12	14	16
Vehicle	0.13 ±0.04	0.28 ±0.05	0.43 ±0.04	1.24 ±0.43	1.51 ±0.30	1.60 ±0.25	1.72 ±0.28	0.28 ±0.18
Fatty acid[b]	0.14 ±0.01	0.34 ±0.13	0.76 ±0.22	1.18 ±0.18	1.27 ±0.13	1.52 ±0.06	1.82 ±0.04	0.18 ±0.05

[a] Means within each column do not differ (P>0.05)

[b] Mixture of fatty acids consisted of oleic acid, 56%; palmitic acid, 29%; linoleic acid, 12%; and stearic acid, 3%; in 0.1 ml DMSO.

Table 3-2. SERUM CONCENTRATIONS OF LH (MEAN ± SE, ng/ml) AFTER INJECTION OF STERCULIC ACID INTO THE OVARIAN ARTERY ON DAY 2 OF THE ESTROUS CYCLE*.

	Minutes post-injection				
Treatment	0	15	30	45	60
Vehicle	2.6 ± 0.5	2.9 ± 0.3	3.5 ± 0.2	5.1 ± 1.8	3.9 ± 1.0
Oleic acid	3.2 ± 0.9	2.7 ± 0.2	2.9 ± 1.0	2.5 ± 0.3	3.8 ± 1.1
Sterculic acid	2.6 ± 0.3	2.8 ± 0.9	3.6 ± 0.5	2.4 ± 1.2	3.6 ± 0.9

* Means within each column do not differ ($P>0.05$)

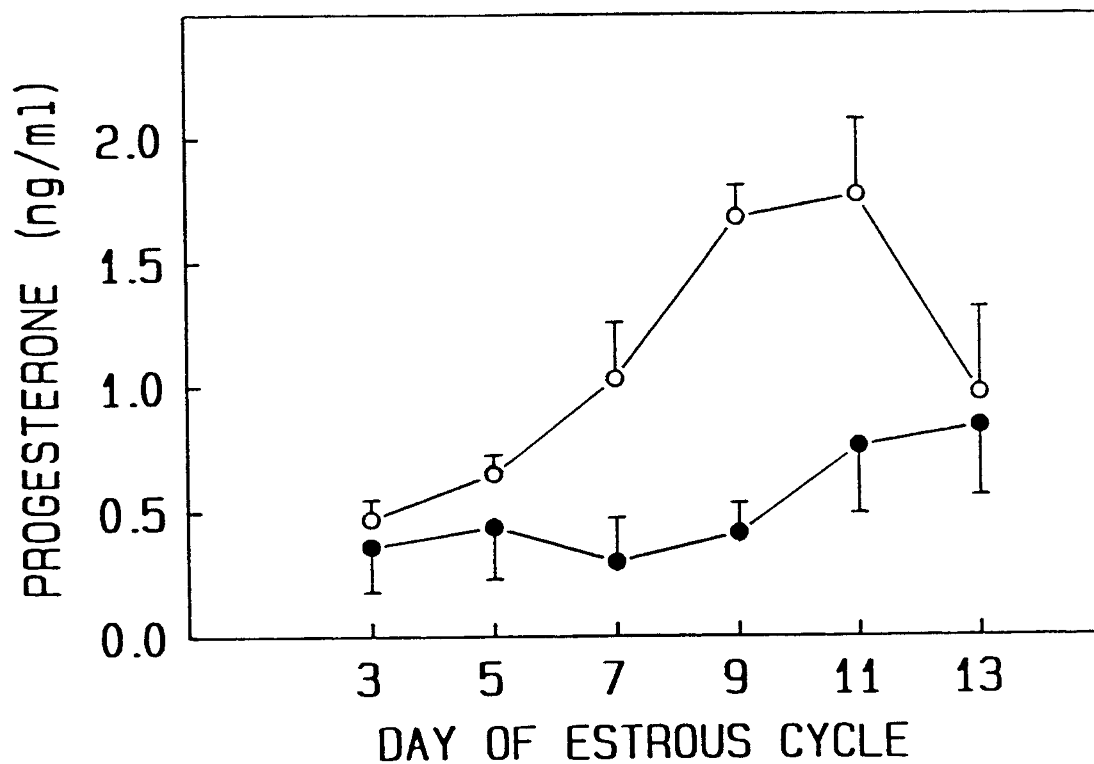


Figure 3-1. Serum concentrations of progesterone (mean ± SE) in ewes injected with 500 ug SA (●——●) or vehicle (○——○) on day 2 of the estrous cycle (treatment x day interaction; $P < 0.01$).

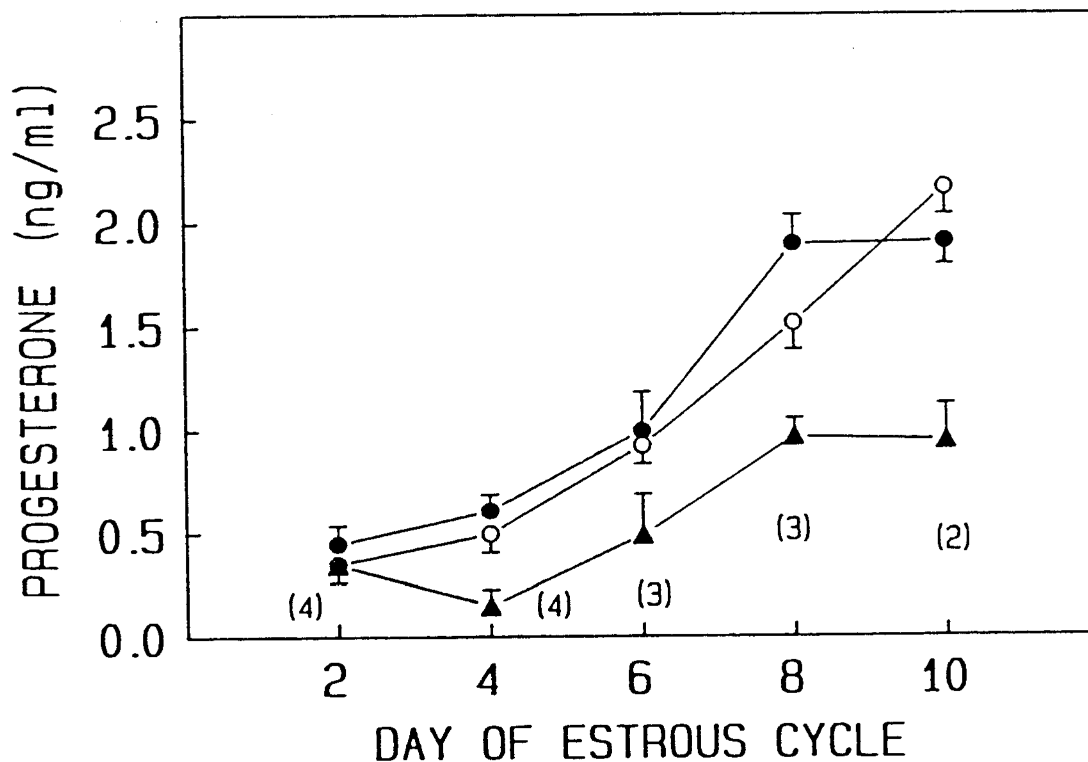
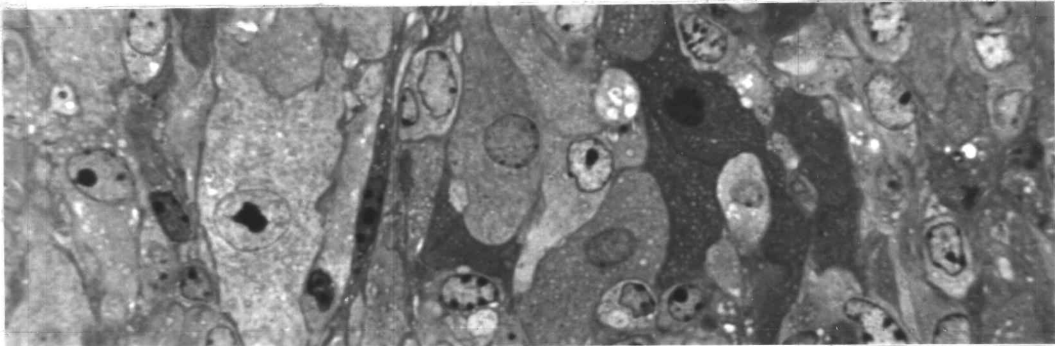
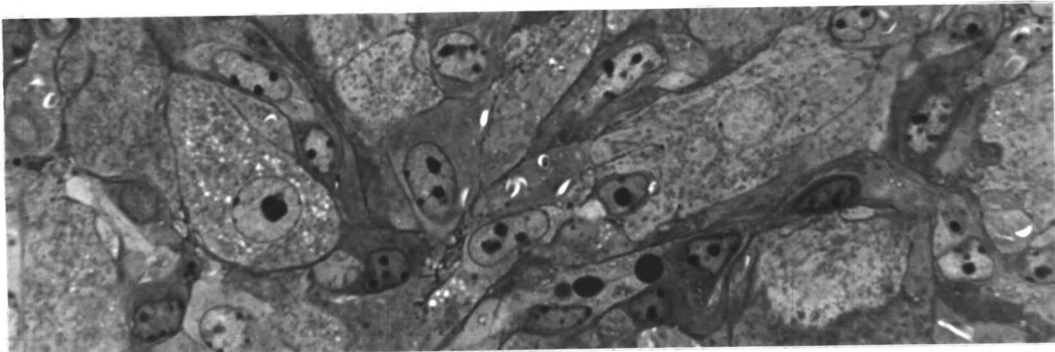


Figure 3-2. Serum concentrations of progesterone (mean ± SE) in ewes injected with either 750 ug SA (▲——▲), 750 ug oleic acid(●——●), or vehicle (○——○) on day 2 of the estrous cycle. Because 3 of 4 ewes injected with SA returned to estrus before day 10 of the cycle compared with those injected with either vehicle or oleic acid, the effect of SA was not analyzed statistically. Values in parentheses represent the number of SA-treated ewes at each stage of the cycle that had not exhibited estrus. Serum concentrations of progesterone in ewes injected with oleic acid or vehicle did not differ.

A



B



C

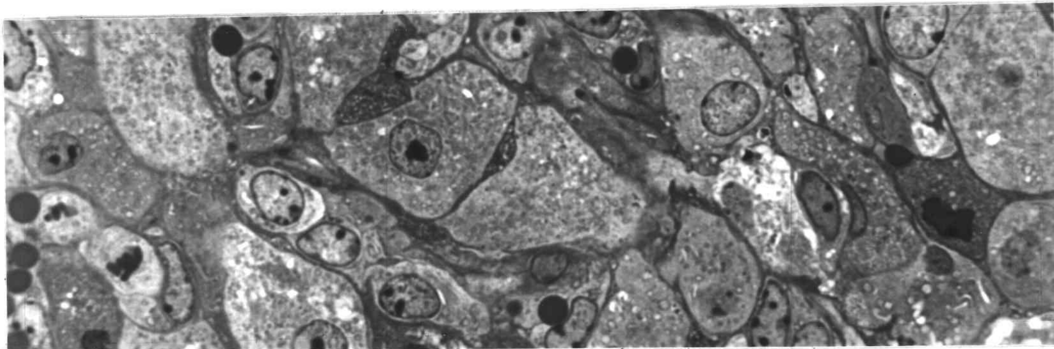


Figure 3-3. Photomicrographs (900 x) of luteal tissue collected from ewes on day 6 of the estrous cycle 4 days after treatment in vivo with either 750 ug SA (A), 750 ug oleic acid (B) or vehicle (C).

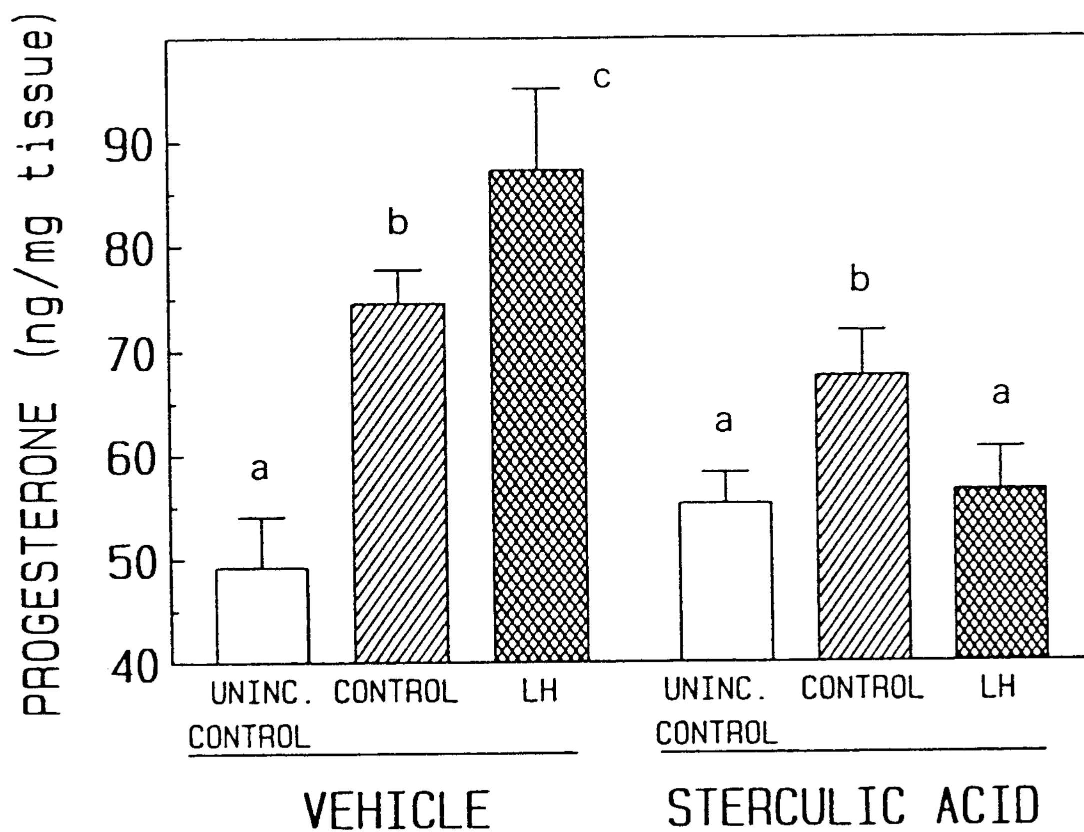


Figure 3-4. Tissue plus medium concentrations of progesterone (mean ± SE) in luteal slices reincubated for 90 min with ovine LH (10 ng/ml) or saline (control) after preincubation with either 100 ng/ml SA or vehicle (SA x LH interaction; $P < 0.01$). Means with different superscripts differ ($P < 0.05$)