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Urinary steroids have been studied during early and late pregnancy in domestic horses or sporadic samples at various stages of pregnancy in wild equidae. In our studies, urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) were monitored throughout pregnancy in six pony mares by enzyme immunoassay (EIA). Both hormones were corrected by creatinine (Cr) index to compensate for the variation in specific gravity. The mean concentration for E1S, (μg/mg Cr), was .38 ± .03 at d 0, decreased to .17 ± .04 at d 1, and maintained at less than .5 μg/mg Cr until d 30. Although, there was an apparent increase to .80 ± .34 at d 34 (NS, P=.122), the first significant increase was .69 ± .15 at d 46 (P=.0275). Mean concentrations remained relatively stable at this approximate level until d 60. This level was followed by a sustained significant increase observed from d 60 onwards. Mean concentrations of E1S increased to 1.11 ± .25, 2.01 ± .45, and 5.48 ± 1.47 at d 64, d 76, and d 86, respectively. Levels of E1S further increased reaching a peak of 143.3 ± 9.51 at d 142 (P=.0006), with maximum for individual mares ranging from d 114 to 170, and also ranging from 115.4 to 286.1 μg/mg. In all cases, maximum concentrations were followed by a

gradual decline toward parturition with a more rapid decrease 1 to 3 days before parturition. The first significant decrease following the maximum concentration was 91.40 ± 13.11 (P=.0024) at d 184. Estrone sulfate was 12.1 ± 3.8 one day prepartum and decreased to .4 ± .1 and .1 ± .01 at d 1 and 4 postpartum, respectively. The mean concentrations of PdG (ng/mg Cr) increased from 14.7 ± 4.3 at d 0 to 50.87 ± .17 (NS, P>.05), 36.8 ± 8.1 (P=.016), and 27.6 ± 7.3 (P=.049) at d 6, 8 and 10, respectively. This increase was followed by a decline and generally the levels fluctuated ranging from 20 to 30 ng/mg Cr until d 80. At d 86, the PdG levels increased to 54.7 ± 11.7 (P=.033). This was followed by a further increase to 141.8 ± 21.4 (P=.0139, compared to d 93) at d 135, then continued to increase to 213.0 ± 25.2 at d 198, and remained at this approximate level until d 303. During the last month of gestation, the mean concentrations of PdG increased from 171.8 ± 9.8 at d 29 prepartum to reach a peak of 388.4 ± 108.6 at d 7 prepartum. Maximum concentrations were followed by a slight decrease to 354.5 ± 84.0 at d 1 prepartum and then decreased to 150.6 ± 23.4 and 39.6 ± 9.3 ng/mg Cr at d 1 and 4 postpartum.

In comparing the two hormones, E1S remained baseline followed by a slight increase at d 35, whereas PdG was relatively stable until both hormones increased after d 70 of gestation. This might be related to secretion of both hormones by the fetus and their rapid metabolism by placenta. Estrone sulfate reached a peak at approximately d 142 followed by a decline toward parturition while PdG showed a rapid increase from d 70 to 150, followed by a slow

sustained increase to d 300 then increased dramatically again before parturition, while E1S continued to decline. The profile of these urinary hormones throughout pregnancy appeared to parallel previously published concentrations in blood. Since the patterns of urinary E1S and PdG are different, their sites and mechanism of metabolism are likely different. The results indicate that the presence of the feto-placental unit is important for the secretion of both estrogens and progestins throughout pregnancy and thus could be utilized as a reliable method for pregnancy determination after three months of pregnancy.

Urinary Estrogens and Progestins in Pregnant Pony Mares

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIX FIGURES

DEDICATION

In Loving Memory of:

My Mother, Mrs. Badria Amer,

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Dr. George Stabenfeldt.

Urinary Estrogens and Progestins in Pregnant Pony Mares

INTRODUCTION

Reproductive physiology of the mare has several unique aspects. These aspects involve the anatomical structure of the reproductive tract and their endocrine roles (for review, see Stabenfeldt et al., 1975; Hyland, 1990; Ginther, 1992). The pattern of hormones during pregnancy are also unique to the mare. Equine chorionic gonadotropin hormone (eCG) begins secretion at d 35 which is produced by the endometrial cups, peaking at d 55 to 65, and decreasing gradually to baseline at d 120 to 150 of gestation in association with sloughing of endometrial cups (Murphy and Martinuk, 1991). Plasma and urinary estrogens, including the ring B unsaturated estrogens, increase slowly during early pregnancy, peak between d 170 (Monfort et al., 1990) and d 210 (Nett et al., 1975; Raeside and Liptrap, 1975), and gradually decline toward parturition. The main estrogens excreted in urine during pregnancy in the mare are the common phenolic estrogens and the ring B unsaturated estrogens. Equilin and equilenin are the main ring B unsaturated estrogens and are unique to the pregnant mare (Bhavnani, 1988). Progesterone increases gradually, peaks at d 64, decreases gradually to baseline at d 180, remains at this level until d 300 and increases again the last 30 days of pregnancy (Holtan et al.,

1975b). Plasma levels of 5α-pregnanes begin between d 30 and 60 and increase gradually to d 300, increasing rapidly during the last 30 days of pregnancy (Holtan et al., 1991). There are limited studies on urinary progestins at specific stages of pregnancy (Kirkpatrick et al., 1990b).

These hormonal profiles are useful for understanding basic physiology and possibly pregnancy diagnosis in the mare. For instance, hormonal methods involve measuring: (a) eCG in plasma (Murphy and Martinuk, 1991) and in urine (Roser and Lofstedt, 1989) between d 40 and 100 of gestation ; (b) estrone sulfate (E1S) in plasma or serum as early as d 37 (Terqui and Palmer, 1979; Sist et al., 1987a,), in urine as early as d 42 (Evans et al., 1984; Daels et al., 1991a; Monfort et al., 1991), in milk after d 60 (Sist et al., 1988), and in feces after d 100 of gestation (Choi et al., 1985; Schwarzenberger et al., 1991); (c) relaxin in plasma after d 80 (Stewart et al., 1992); (d) plasma progesterone after d 28 (Holtan et al., 1975b), and in milk (Laitinen et al., 1981). However, plasma progesterone is unreliable, yet 5α-pregnanes in plasma (Holtan et al., 1991) or urine could be more useful since it is secreted throughout gestation.

There are no studies on urinary progestins throughout pregnancy, only two limited to particular stages of gestation in uncaptured feral horses. There are only three studies of urinary estrogens throughout pregnancy by using

modified Kober reaction (and)or radioimmunoassay (RIA). Therefore, the objective of the current study was to determine the profiles of both urinary estrogens and progestins from the same animals throughout pregnancy using direct enzyme-immunoassay (EIA).

REVIEW OF LITERATURE

ESTROUS CYCLE OF THE MARE

The mare is seasonally polyestrous with normal cyclic activity during spring and summer and reduced or no activity during winter and is defined as the period from one ovulation to the subsequent ovulation and is accompanied by signs of estrus and(or) progesterone levels below 1 ng/ml of plasma (Hughes et al., 1980). The length of the cycle averages 21 to 22 d during the breeding season. The estrus length averages 5 to 7 days, and diestrus is 15 days during the breeding season. Ovulation usually occurs 24 to 48 h before the end of estrus. The day of ovulation is considered to be d 0 of the cycle. Seasonal variations in estrous cycle patterns occur between and within mares. Anestrus varies greatly in length (40 to 120 d and, on occasion, as long as 6 to 8 mo) and usually occurs in the winter and early spring (Ginther, 1974, 1979; Hughes et al.,1972, 1980; Stabenfeldt et al., 1972).

Hormonal Control of the Estrous Cycle

Levels of follicle-stimulating hormone (FSH) have a distinct 10-d rhythm of surges during the breeding season. The first surge occurs during late estrus/early diestrus and the second during late diestrus. The second FSH surge may stimulate growth of the follicle that is destined to ovulate (Hughes et al., 1980).

Concentrations of luteinizing hormone (LH) are persistently low from d 5 to 16 of the cycle (ovulation = d 0). Concentrations then increase slowly, but progressively, and peak two days after ovulation, declining rapidly over the following four to five days to low diestrus values. The increase in the LH level, after the late diestrus surge of FSH has disappeared, may stimulate growth of the ovulatory follicle until ovulation occurs (Evans and Irvine, 1975; Hughes et al., 1980).

The concentration of estrogens is strongly correlated with ovarian activity, behavioral response to the stallion, and grossly observable changes in the reproductive tract (Hughes et al., 1980). Plasma concentrations increase rapidly just before the onset of estrus and peaks two days before ovulation, declining to baseline diestrous levels within two days after ovulation (Makawiti et al., 1983). Estrogens secreted by the follicle cause sexual receptivity and are also responsible for changes in the reproductive tract that allow passage and transport of the sperm, in addition to their partial role in the ovulatory process (Hughes et al., 1980).

Progesterone concentrations are below 1 ng/ml plasma during the follicular phase, increase abruptly after ovulation and peak within five to six days. Concentrations remain high during the luteal phase (6 to 10 ng/ml plasma) and decline rapidly with the regression of the corpus luteum (CL) (Plotka et al., 1972; Stabenfeldt et al., 1972). The life span of the CL varies from 5 to 18 days and averages 12 to 13 days (Hughes et al., 1972, 1980). Progesterone, secreted by the CL, suppresses, but does not completely inhibit, follicular growth. It also suppresses manifestations of estrus and is responsible for sealing off the uterus from the exterior, preparing its tissues for the support of pregnancy (Stabenfeldt et al., 1972; Hughes et al., 1980).

Prostaglandin $F_2\alpha$ ($PGF_2\alpha$) increases between d 14 and 17 after ovulation in the normal estrous cycle, and the duration of the release ranges from 1 to 4 days, declining rapidly following luteolysis (Neely et al., 1979). That is, the secretion of $PGF_2\alpha$ is temporally associated with the regression of the CL (Sharp et al., 1989).

Concentrations of oxytocin are low on d 0 and peak on d 15 after ovulation. Oxytocin is secreted in a pulsatile manner throughout the estrous cycle. Oxytocin may be involved in the regulation of the estrous cycle by promoting luteolysis via the synthesis and release of uterine $PGF_2\alpha$ (Tetzke et al., 1987).

Pregnancy of the Mare

Maternal Recognition of Pregnancy

Successful maternal recognition of pregnancy is defined as maintenance of the embryonic vesicle, as determined by ultrasound, to the time of endometrial cup formation (d 38 to 40 of pregnancy, determined by detection of eCG in peripheral plasma), and maintenance of progesterone concentrations >1 ng/ml (Sharp et al., 1989). The critical period for the maternal recognition of pregnancy in the mare appears to be confined to the period between d 14 and 16 after ovulation (Hershman and Douglas, 1979; Sharp et al., 1980, 1984). Sharp et al. (1989) suggested that maternal recognition of pregnancy in the mare involves suppression of endometrial $PGF_2\alpha$ secretion by conceptus factors, and that conceptus migration in the first 16 days of pregnancy is an important mechanism by which the $PGF_2\alpha$ inhibitory factors are delivered throughout the entire uterus. Maternal recognition of pregnancy also involves prolongation of luteal function some time before the onset of definitive implantation (Heap et al., 1982). Ginther (1983a,b) reported that the conceptus moves back and forth within and between uterine horns many times each day until approximately d 15, and cessation of mobility (vesicle fixation) occurs on d 16 to 17. Ginther (1983b) hypothesized that vesicle fixation is caused by increasing uterine tone and thickening of the uterine wall coupled with continued growth of the vesicle.

Role of the Ovary

When fertilization occurs, the CL formed after ovulation is considered the primary CL. Secondary corpora lutea develop between d 17 to 50 of pregnancy and are derived from ovulation or from luteinization of ovarian follicles. Both the primary and secondary corpora lutea produce progesterone and persist to d 160 to 180 of gestation, then regress (Squires and Ginther, 1975). These luteal tissues are also the main source of estrogens secretion in the first 35 to 45 days of gestation (Terqui and Palmer, 1979; Daels et al., 1990, 1991b). The ovaries and(or) feto-placental unit produce estrogen between d 45 to 70 of gestation (Terqui and Palmer, 1979).

Elevations of progesterone concentrations in circulation (Squires et al., 1979; Stewart and Allen, 1981; Bergfelt et al., 1989) and the increase in estrogen secretion (Daels et al., 1991b) are associated with the presence of eCG, suggesting a luteotrophic role for eCG in the maintenance of the primary and secondary corpora lutea, as will be discussed later. After regression of the primary and secondary corpora lutea, the ovaries remain small, hard, and completely inactive until parturition (Ginther, 1992); the progesterone necessary to maintain pregnancy is apparently secreted by the feto-placental unit (Holtan et al., 1979).

Role of Feto-Placental Unit

The placenta of the mare is a nondeciduate diffuse epitheliochorial type, and its attachment occurs over d 40 to 150 of gestation (Ginther, 1992). The feto-placental unit is the main source of estrogen synthesis and secretion in the maternal peripheral circulation (Raeside et al., 1973; Pashen and Allen, 1979; Stabenfeldt et al., 1991). The development, enlargement, and regression of fetal gonads parallel the increase and decline of estrogen concentrations in the maternal plasma and urinary excretion throughout gestation (Pashen and Allen, 1979; Pashen, 1984). These data suggest the fetal gonads may act as the main source of precursors for the synthesis of estrogen by the feto-placental unit (Raeside et al., 1973, 1979; Pashen and Allen, 1979). Fetal gonadectomy in mid-pregnancy results in a rapid decline to basal levels of estrogen concentrations (Raeside et al., 1973; Pashen and Allen, 1979).

Progesterone is present in high concentrations in both the placenta (Short, 1957) and the umbilical cord (Short, 1959). Ainsworth and Ryan (1969) demonstrated that horse placenta plays an active role in the production of progesterone from pregnenolone. These investigators also reported that mare placental tissue converts progesterone primarily to saturated 5α-pregnane derivatives, suggesting that 5α-dihydroprogesterone in the mare is a metabolite of progesterone and that it derives from the feto-placental unit (Ainsworth and Ryan, 1969). Ganjam et al. (1975) found that progestin levels decline rapidly after parturition and this may be caused by placental separation from the

endometrium. There are also higher levels of progestins in the newly born foal than in the dam, and total progestins are also higher in the umbilical artery than in the umbilical vein. Ganjam et al. (1975) also suggested that progesterone is produced by the fetal gonad . Moss et al. (1979) reported that tritiated progesterone is converted to 5α-pregnane-3,20-dione (5α-DHP), and in summary, they suggest that 5α-DHP is produced by the placenta and a maternal source; 20α-hydroxy-5α-pregnan-3-one (20α-5P) is primarily of maternal origin, and 3ß-hydroxy-5α-pregnan-20-one (3ß-5P) is secreted primarily by the fetus. Holtan et al. (1991) reported that 5α-DHP is the predominant steroid in the fetal vein, and that concentrations are much higher there than in the uterine vein or maternal artery, and that it may be produced in the placenta and metabolized by the fetus. They also found 3ß-5P and 5α-pregnane-3ß,20ß-diol are higher in fetal vessels, and 5α-pregnane-3ß,20α-diol is higher in maternal vessels, suggesting that 3ß- and 20ß-hydroxylation predominate in the feto-placental unit. The 20α-hydroxylation predominate in the maternal side, and 20α-5P is the highest steroid concentration in the uterine vein and is two times as high as concentrations in the maternal artery, suggesting that 20α-5P may be produced by the endometrium. Fetal pregnenolone may be converted rapidly to progesterone and(or) 5α-DHP by the placenta (Holtan et al., 1991).

Hormonal Control of Pregnancy

Equine Chorionic Gonadotropin

Equine chorionic gonadotropin, formerly called pregnant mare serum (PMS) or pregnant mare serum gonadotropin (PMSG), (reviewed by Murphy and Martinuk, 1991), is first detectable on approximately d 35 to 42, increases rapidly to a peak at d 55 to 65, and then decreases slowly to low or nondetectable concentrations by d 120 to 150. The concentration of eCG is temporal to the development and degeneration of the endometrial cups. Equine chorionic gonadotropin may be produced by the trophoblast cells of the endometrial cups, as evidenced by their ability to secret eCG in-vitro and the observation that these cells invade the maternal epithelium to produce the endometrial cups. In addition, eCG is present in cup tissue, and the degeneration of the cups is associated with declines in eCG concentration (Murphy and Martinuk, 1991).

The role of eCG is not completely understood. It is believed to act as an LH-like hormone to induce supplementary ovulation and(or) luteinization of the secondary follicles and to serve as a luteotropin to the primary and secondary corpora lutea (Murphy and Martinuk, 1991). Equine chorionic gonadotropin also may serve as a redundant system to maintain pregnancy until the placenta assumes its role as the principal steroidogenic organ of gestation (Murphy and Martinuk, 1991).

Luteinizing Hormone

After the ovulatory surge of LH, concentrations of LH remain at baseline levels throughout pregnancy (Evans and Irvine, 1975). Direct assay of LH during eCG production has not been done because of assay cross-reactivity (Ginther, 1992).

Follicle Stimulating Hormone

Surges of FSH occur at 10- to 11-d intervals during the estrous cycle and early pregnancy. Considerable follicular development before the production of eCG may be related to FSH activity (Evans and Irvine, 1975).

Estrogens

Two main groups of estrogens occur in the pregnant mare. The first, the common phenolic estrogens, includes mainly estrone (E1) and estradiol-17ß (E2). The second group includes the ring B unsaturated estrogens, mainly equilin and equilenin, which are unique to the mare (Pashen, 1984). Estrogens present in plasma are either conjugated (such as E1S) or unconjugated (such as free E1). Plasma conjugated estrogens are present in 100-fold higher concentrations than unconjugated estrogens (Palmer and Terqui, 1977).

During the first 80 days of pregnancy, plasma concentrations of unconjugated estrogens (E1, equilin, and equilenin) remain low (<20 pg/ml), then increase to a maximum (828 $\pm$ 151 pg/ml) at d 210, decreasing gradually

toward foaling. During the first 90 days of gestation, the concentration of E2 remains low (< 15 pg/ml) and then increases and peaks (71 ± 18 pg/ml) at d 240. This is followed by a gradual decline toward parturition. The concentration of unconjugated estrogens is at the baseline one day postpartum (Nett et al., 1973; 1975). Terqui and Palmer (1979) measured plasma total estrogens (conjugated and unconjugated) from d 0 to 100 of gestation. Concentrations are low, similar to diestrus levels during the first 35 days of pregnancy and increase between d 35 to 40. This is followed by a plateau of 3 ng/ml between d 40 to 60 that is slightly higher than the preovulatory level. This increase occurs in conjunction with the onset of eCG secretion by the endometrial cups. There is a second sharp increase after d 60 to > 5 ng/ml by d 85. The first increase may be ovarian in origin, either from the corpus luteum or from follicles, or may be the result of the indirect stimulation of eCG causing follicular growth. The second increase may be of feto-placental origin. This is supported by the findings that the normal increase in estrogen levels, which occur after d 55, is maintained after ovariectomy (Terqui and Palmer, 1979).

Progestins

Progesterone Plasma progesterone concentration increases from 1 ng/ml on d 0 to 7 ng/ml on d 8 of pregnancy, followed by a transient (nonsignificant) decrease to 5 ng/ml on d 28, and then increasing from d 28 to reach a peak on d 64. The level of progesterone gradually declines after d 64

and remains between 1 to 2 ng/ml from d 180 to 300 (Allen and Hadley, 1974; Holtan et al., 1975b). Progesterone concentration becomes elevated during the last 30 days of pregnancy to 5 ng/ml at d 5 prepartum, followed by a rapid decline to less than .5 ng/ml d 1 to 3 postpartum (Holtan et al., 1975b,c). This rapid decline could be related to placental separation from the endometrium and removal of the fetus (Ganjam et al., 1975). However, a recent study by Holtan et al. (1991) reported that progesterone is not detectable during mid-to late gestation and that other progestins apparently account for increases when using immunoassays.

Other Progestins Levels of 5α-pregnanes are first observed between d 30 and 60 and elevate gradually to d 300, increasing rapidly during the last 30 days prepartum (Holtan et al., 1975a, 1991). The concentration of 17α-hydroxyprogesterone is less than .2 ng/ml throughout pregnancy, except from d 40 to 120 (2 to 4 ng/ml) and the last 30 days (.5 ng/ml) of pregnancy (Holtan et al., 1975b,c; Seren et al., 1981). Using gas chromatography/mass spectrometry (GC/MS), Holtan et al. (1991) identified and measured different progestins during pregnancy in horses. The predominant steroids near term are 20α-hydroxy-5α-pregnan-3-one, 5α-pregnane-3ß,20α-diol, and four other mono- and dihydroxy-5α-pregnanes. These pregnanes begin to be detected between d 30 and 60 of pregnancy, elevate gradually, and increase rapidly in the last 30 days prepartum (Holtan et al., 1991).

Relaxin

There are breed differences in equine relaxin concentration (Stewart et al., 1992). Relaxin is not detected during the estrous cycle and the first 80 days of pregnancy in Thoroughbred mares. The hormone begins to elevate at d 80, increasing sharply to peak levels (5.9 ng/ml) between d 176 to 200, then subsequently declining until d 240 (4.2 ng/ml). This is followed by an increase from d 240 to the day of parturition and a rapid decline after foaling (Stewart and Stabenfeldt, 1981). During gestation in Standardbred mares, the relaxin concentration is first observed at d 80, increasing to a peak (100 ng/ml) on d 150. This is followed by a gradual decline until parturition and then sharply decreases after foaling (Stewart et al., 1992). This pattern shows a closer association to plasma E1 production that is reported by Nett et al. (1973) in both the increase to midpregnancy and the continual decline until parturition than do Thoroughbred mares (Stewart et al., 1992). Relaxin is first observed in pony mares at d 85, increasing rapidly to d 115, followed by a slow increase until parturition (Stewart et al., 1992).

The actions of relaxin in the horse are not known. Relaxin may aid progesterone in the maintenance of pregnancy, stimulating uterine growth and promoting uterine quiescence in a variety of domestic animals; these actions

may all be possible in the mare (Stewart et al., 1992). The horse placenta is the main source of relaxin activity (Stewart and Stabebfeldt, 1981; Stewart et al., 1982, 1992), but factors regulating placental relaxin synthesis in the mare are not well understood (Stewart et al., 1992).

Endocrinology of Parturition

Cortisol levels in the maternal and fetal blood and in fetal fluids begin to increase toward foaling. A steady increase in progestin and a decrease in estrogen concentrations occurs in the maternal peripheral plasma until the onset of foaling. There is a slight increase in the concentration of maternal E2 at foaling and a concurrent decrease in concentrations of E1 and equilin. This increase may stimulate the synthesis and release of $PGF_2\alpha$ during parturition (Pashen, 1984). The concentration of plasma $PGF_2\alpha$ begins to increase during the first stage of foaling and may enhance myometrial activity, sensitizing the uterus to any further $PGF_2\alpha$ increase. This is followed by an explosive increase in $PGF_2\alpha$ secretion in synchrony with the onset of powerful uterine contractions during the second stage of parturition. The concentrations then decline sharply by 90 min after foaling (Pashen and Allen, 1979). The concentrations of oxytocin remain at the baseline throughout pregnancy and increase only at the beginning of the expulsive stage of labour. Maximal concentrations of $PGF2_\alpha$ and oxytocin were observed between rupture of the chorioallantois and the completion of foaling (Haluska and Currie, 1988).

Sources and Roles of Estrogens

The horse embyro is capable of synthesizing estrogens as early as d 7 of gestation in-vitro (Flood et al., 1979; Heap et al., 1982; Zavy et al., 1984), but not in levels high enough to be secreted in the maternal blood (Daels et al., 1991b). The main source of estrogen secretion during the first 35 to 45 days of pregnancy is the ovary (active CL) rather than the feto-placental unit (Terqui and Palmer, 1979; Daels et al., 1990, 1991b). A temporal relationship exists at d 40 of pregnancy between the increase of eCG and plasma estrogen concentrations in the presence of an active CL, suggesting that eCG stimulates luteal estrogen synthesis (Daels et al., 1991b).

The primary source of estrogens between d 45 to 70 of pregnancy is the ovaries and(or) feto-placental unit. The feto-placental unit may be the principal site of estrogen secretion (Kasman et al., 1987, 1988; Jeffcott et al., 1987; Hyland and Langsford, 1990). This may be the result of the correlation between the presence of a viable fetus and high estrogen concentrations in plasma and urine and the rapid decline of the concentrations following the termination of pregnancy at d 45, either by the surgical removal of the fetus (Kasman et al., 1987, 1988) or induction of fetal death by the injection of hypertonic saline solution into the fetal sac (Jeffcott et al., 1987; Hyland and Langsford, 1990). On the other hand, Daels et al. (1991b) and Stabenfeldt et al. (1991) demonstrated that plasma estrogen concentrations, between d 35

and 70 of pregnancy, are not directly correlated with fetal viability. This appears to be reflected by the presence of an active CL and is shown by the rapid decline of estrogen concentrations in the absence of active CL following the administration of $PGF_2\alpha$ on d 16 (Daels et al., 1991b) or injection of endotoxin that releases $PGF_2\alpha$ between d 35 and 70 of gestation (Stabenfeldt et al., 1991). Furthermore, endotoxaemia without fetal death between d 35 and 70 of pregnancy results in marked transient decreases in plasma estrogen concentrations. Fetal death without CL regression after d 35 does not produce an immediate decline in plasma estrogen concentrations, and existing levels are maintained for 10 to 14 days (Stabenfeldt et al., 1991).

The determination of the source of estrogens during early pregnancy is controversial. This controversy may be related to the different techniques used to induce fetal death,including the use of $PGF_2\alpha$ (Kasman et al., 1987, 1988; Daels et al., 1991b), injections of hypertonic saline into the fetal sac (Jeffcott et al., 1987; Hyland and Langsford, 1990), surgical removal of the embryo or conceptus (Kasman et al., 1987, 1988), and ovariectomizing (OVX) the mare (Terqui and Palmer, 1979; Kasman et al., 1987, 1988; Daels et al., 1990). The number of days of gestation before the application of these various techniques may also contribute to this controversy.

After d 70 until parturition, the principal site of estrogen production is the feto-placental unit (Nett et al., 1973; Raeside et al., 1973; Pashen and Allen, 1979; Kasman et al., 1987, 1988). The enlargement and regression of

the fetal gonads parallel the increase and decrease of estrogen concentrations during gestation. The removal of fetal gonads in mid-pregnancy causes an immediate decline to basal levels of estrogen in maternal plasma (Raeside et al., 1973; Pashen and Allen, 1979). Estrogen levels remain low after gonadal removal throughout gestation; however, progestin levels appear to be unaffected (Pashen and Allen, 1979). Estrogens may play a part in normal fetal development because fetal gonadectomy caused retarded growth and development in three of four gonadectomized foals (Pashen and Allen, 1979). Surgical removal of the fetus on d 44, 62, and 88 of pregnancy results in an immediate and continuous decline of serum and urinary estrogens to baseline values (Kasman et al., 1987, 1988). These findings suggest that the primary source of estrogens from the second month of gestation is a viable fetus.

Estrogen Biosynthesis during Pregnancy

The biosynthesis of estrogen in the pregnant mare was reviewed by Bhavanani (1981, 1988). During equine pregnancy, there are two pathways of steroidogenesis through the feto-placental unit: the first is a classical pathway via cholesterol that leads to the formation of the classical estrogens (ring B saturated estrogens), E1 and E2; the second is an alternate pathway that bypasses cholesterol and leads to biosynthesis of the ring B unsaturated estrogens, equilin and equilenin (Bhavnani, 1988). This indicates that ring B unsaturated estrogens are not metabolites of E1 and E2. The structures of

main classical estrogens and ring B unsaturated estrogens are illustrated in Appendix Figures 1. The exact site of ring B unsaturated estrogen production in the pregnant mare is unknown. It is postulated that the feto-placental unit may be involved in the formation of these estrogens (Bhavnani, 1988).

Sources and Role of Progestins

The primary source of progesterone production during early pregnancy is the ovaries (mainly the corpora lutea of pregnancy). Ginther (1992) classifies luteal progesterone secretion during pregnancy as three responses: the first is the period of maternal recognition of pregnancy. The CL gradually decreases in size and function, and progesterone secretion gradually declines until d 35 of gestation. The second response begins at d 35, and progesterone secretion increases as a result of the appearance and stimulation by eCG. This response is also characterized by resurgence (growth and productivity) of the primary CL. The third luteal response involves formation of the secondary corpora lutea between d 40 to 70 and increasing numbers of luteinized follicles (accessory corpora lutea) throughout d 40 to 150. High concentrations of eCG account for the second and third luteal responses, which in turn account for the increasing production of progesterone (Ginther, 1992).

Holtan et al. (1979) demonstrated that ovaries are the primary source of progesterone up to d 45 of pregnancy. The period of d 50 to 70 is transitional because ovariectomy during that period results in abortion in some, but not all mares. The feto-placental unit gradually produces progestins as the luteal progesterone output is gradually declining. The progestins secreted into the maternal circulation are mainly 5α-pregnanes (Holtan et al., 1979).

Ovariectomy at d 140 and 210 (Holtan et al., 1979), d 150 (Amoroso, 1955), and d 200 (Hart and Cole, 1934) does not result in abortion, and ovariectomized mares have carried normal fetuses until foaling. These data indicate that the presence of ovaries is not essential for the production of progesterone and maintenance of pregnancy during this time. Short (1957) reported that equine placenta contain high levels of progesterone, although insufficient for release into the peripheral maternal circulation. Squires and Ginther (1975) found that concentrations of progesterone in the uterine vein are significantly higher than those in the peripheral plasma (jugular vein) at d 80 and 100. The placenta or uterus contributes to progesterone secretion in the maternal circulation by d 80 and continues to produce progesterone until d 220, which is the last day studied (Squires and Ginther, 1975).

Progesterone is metabolized to saturated 5α-pregnane derivatives, and 5α-pregnane-sulfate is identified in urine of mares in late pregnancy. This suggested that horse placenta and fetus contribute to the urinary output of progestins (Hirschman and Williams, 1964; Ainsworth and Ryan, 1969). The

concentration of 5α-DHP was also identified in the blood of pregnant mares (Atkins et al., 1974, 1976; Holtan et al., 1975a). In addition to this pregnane, Holtan et al. (1975a, 1991) detected 3ß-5P and 20α-5P in the peripheral circulation of pregnant mares and suggested that these pregnanes are produced or metabolized by the feto-placental unit (Holtan et al., 1975a, 1991). On the other hand, Hamon et al. (1991) found that the production of 5α-DHP during late pregnancy is derived primarily from the endometrial metabolism of pregnenolone and(or) progesterone.

Urinary Estrogens

General History of Identification and Measurement

Early biological and chemical tests to identify and measure urinary estrogens (previously called estrin) of pregnant and nonpregnant mares began in the 1930's and were modified during the 1940's and 1950's. Most of these studies were reviewed by Cox (1971). From the 1970's to the present, various types of chromatography to identify estrogens, RIA, and EIA to quantify estrogens have been used to study estrogens excretion in the urine of pregnant mares (reviewed by Lasley et al., 1990).

Early biological assays used acid hydrolysis of urine, extracted with 95% ethyl alcohol, and extracts were injected subcutaneously into 4 mo-old spayed virgin female rats, with vaginal smears taken at the time of injection and after

48, 72, and 96 h. Estrin concentration is measured by the number of rat units per liter of urine. The rat unit is determined by halving a dose until the smallest amount is found that will give a smear of nucleated epithelial cells and cornified cells, or the latter alone, within 48 h of the injection (Catchpole and Cole, 1934; Hart and Cole, 1934). Estrin activity is first observed between d 50 and 100 of pregnancy (Cole et al., 1933). Catchpole and Cole (1934) reported that the number of rat units per liter of urine, during the last 2 months prepartum, declined from 16,000 to <200 rat units per liter of urine on the day of foaling and d 2 pospartum. In the 1960's and 1970's, biological assays were rarely used because they are generally more costly and time-consuming than the chemical tests that have replaced them (Cox, 1971).

The use of chemical tests began after the discovery of the Kober reaction in 1931. In the Kober reaction, urine of pregnant mares is heated with a phenolsulforic acid reagent, and when high estrogen concentrations are present a cherry red color develops (Kober, 1931). This colorimetric method has been modified in further studies using different acids and solvents (Cohen and Marrian, 1934; Beall and Edson, 1936; Schachter and Marrian, 1938; Mayer, 1944). In general, these chemical methods include acid hydrolysis of conjugated estrogens in urine, extraction of free and conjugated estrogens from the hydrolysed urine, and development of a fluorescent color reaction. Other chemical methods include the Ittrich method which developed in 1958 (cited in Cox, 1971). In the Ittrich method, the Kober reaction mixture is extracted

with p-nitrophenol and the chromogen is quantified in a spectrophotometer. The Kober-Ittrich method has been used in several studies (Savard, 1961; Raeside and Liptrap, 1975; Hillman and Loy, 1975). Hillman and Loy (1975) determined estrogen excretion in urine during estrous cycles. They analyzed samples for the presence of E1 and E2 after acid hydrolysis, ether extraction, separation of the estrogens by chromatography on alumina columns, and colorimetric measurement using the modified Kober method as described by Brown (1955). Estrone excretion increases from 18 μg/L urine (i.e. 18 ng/mL urine) at d 8 before ovulation to a peak of 60 μg/L (60 ng/mL) on the day before ovulation, followed by a rapid decline to 20 μg/L (20 ng/mL) on the day after ovulation. Estradiol excretion pattern tends to parallel that of E1 at levels that rarely exceed half those of E1 (Hillman and Loy, 1975). Using enzyme hydrolysis and RIA with antiserum against E1-17-hydrazone:BSA, Palmer and Jousset (1975) determined urinary estrogen levels during estrous cycles. Estrogens begin to increase from approximately 100 ng/mL at d 8 before ovulation, peak at 450 ng/mL d 2 before ovulation, and then decrease slightly before ovulation. This concentration is followed by a decline to approximately 200 ng/mL d 1 after ovulation and a further decrease to approximately 100 ng/mL d 5 after ovulation (Palmer and Jousset, 1975). The general pattern of urinary estrogens in these two studies is similar; however, the concentrations are different. The reason for this difference may be related to the methods

used, individual variations between and within mares, and the lack of indexing of creatinine. The importance of creatinine as an index to the concentration of steroids in urine will be discussed later.

Estrone and equilin are the main constituents of urinary estrogens, and equilinin is a minor component beginning the fourth or fifth month of pregnancy until parturition, as determined by the use of celite chromatography and modified Kober reaction (Savard, 1961; Raeside and Liptrap, 1975). In addition, Raeside and Liptrap (1975) found estradiol-17α (E2α) in urinary estrogens as a minor constituent. The levels of equilin increase from the fourth or fifth month of gestation to equal, and in some instances exceed, those of estrone in the late months of gestation (Savard, 1961; Raeside and Liptrap, 1975). Total urinary estrogen concentrations elevate in the third to fourth month (about 100 μg/24 hr) and peaks approximately the sixth to seventh month of gestation (780 μg/24 hr). This peak is followed by a rapid decline, then a more gradual decline toward parturition, and a decrease to the baseline at d 1 postpartum (Raeside and Liptrap, 1975).

In the 1980's and 1990's, high pressure liquid chromatography (HPLC), more advanced RIA, and EIA were used to identify and measure urinary estrogens (free and conjugated) during the estrous cycle, early pregnancy, mid- and late pregnancy. Evans et al. (1984) measured estrogen conjugate (EC) from preconception diestrus to d 78 of pregnancy. They used direct RIA, with antiserum that cross-reacted equally with estrone glucuronide (E1G) and E1S,

and HPLC to assess the contribution of sulfate and glucuronide conjugates of E1, assaying the fractions for immunoreactivity. Estrone sulfate is the predominant peak and accounts for 94 to 97% of the total immunoreactivity after chromatography. The concentration of urinary E1S during diestrus is .15 ± .07 μg/mg Cr and increases to .47 ± .14 μg/mg Cr during estrus. There is also a significant increase (1.21 ± .12 μg/mg Cr) from d 35 to 45 of pregnancy as compared with values (.27 ± .01 μg/mg Cr) obtained from d 25 to 34 of pregnancy (Evans et al., 1984). Daels et al. (1991a) studied urinary and plasma EC, E1, and E2 concentrations during the estrous cycle and early pregnancy. Estrogen conjugate concentrations in plasma and urine were determined by a direct RIA, with antiserum directed against estrone-3-glucuronide:BSA. The contribution of free and conjugated estrogens to the EC immunoreactive profile was assessed using HPLC, and each eluate was assessed for estrogen immunoreactivity. The concentrations of conjugated plus free E1 and E2 were determined in urine samples, with added radiolabelled E1 and E2, by enzyme hydrolysis (ß-glucuronidase), extraction with ether, and separation of free E1 and E2 by celite chromatography. The free E1 and E2 and total free estrogens were measured by RIA. Estrone sulfate represents 95% of the total immunoreactive estrogen in the untreated urine samples, as determined by using HPLC, and 1% co-eluted with free E1 and E2 in all stages of the reproductive cycle. Following enzyme hydrolysis and celite chromatography separation, E1 and E2 are identified as the hydrolytic products

in the urine of non-pregnant and pregnant mares; however, an unidentified estrogen is the major hydrolytic product in both nonpregnant and pregnant mares before d 38 of pregnancy. During the estrous cycle, the mean of EC concentration on d 5 before ovulation is 126 ± 22 ng/mg Cr and peaks to 351 ± 31 ng/mg Cr on d 1 before ovulation. Following ovulation, EC declines rapidly to 140 ± 37 ng/mg Cr on d 1 after ovulation and increases again to 265 ± 72 ng/mg Cr on d 7 after ovulation, decreasing during luteal regression to 89 ± 18 ng/mg Cr on d 15 after ovulation (Daels et al., 1991a). This pattern of urinary EC during the estrous cycle agreed with other findings (Hillman and Loy, 1975; Palmer and Jousset, 1975; Monfort et al., 1991).

During early pregnancy, the concentration of EC in urine remains constant between d 1 and 30 of pregnancy, ranging from 72 to 298 ng/mg Cr. However, the mean concentration of EC begins to increase on d 34 and peaks (1066 ± 219 ng/mg Cr) on d 39, followed by a decline (637 ± 94 ng/mg Cr) on d 44. The decline is followed by a sustained increase through d 60 of pregnancy. Estrogen conjugate concentration in urine, during the estrous cycle and early pregnancy, followed the same pattern as that observed in plasma (Daels et al., 1991a).

Monfort et al. (1991) determined urinary estrogens during different reproductive states (estrus, luteal phase, early, mid- and late gestation) in Przewalski's horses. Urinary estrogens were measured using direct RIA, with antibody directed against estrone-3-glucuronide:BSA, identified using HPLC and

the eluates were assayed for immunoreactivity by direct RIA. The major excreted immunoreactive estrogens in non-pregnant and pregnant mares are E1S and E1G, and free E1 and E2 are minor contributers. The general pattern of urinary EC during the estrous cycle and early pregnancy is similar to that observed previously by Daels et al. (1991a). During mid- and late pregnancy, the concentration of EC is markedly elevated (about 30 μg/mg Cr) from d 90 and peaks (about 100 μg/mg Cr) on d 175 to 180 of pregnancy, declining gradually toward parturition. The concentration of EC decreases to about 7 μg/mg Cr one day prepartum and dropped to baseline (near zero μg/mg Cr) one day postpartum (Monfort et al., 1991). This pattern is similar, in general, to that described in a previous study by Raeside and Liptrap (1975) using celite chrmoatography and modified Kober reaction.

Kirkpatrick et al. (1988, 1990b, 1991) measured E1S and EC in uncaptured feral horses during various stages of pregnancy. Samples were collected from urine-soaked soil at >35 days after the day of natural service to determine pregnancy (Kirkpatrick et al., 1988). Estrone sulfate concentration was determined by direct RIA, with antiserum directed against estrone-3-glucuronide:BSA. The mean concentration of E1S for mares that produced foals is 2.64 ± 1.02 μg/mg Cr and is .44 ± .26 μg/mg Cr for mares that did not foal (Kirkpatrick et al., 1988). Kirkpatrick et al. (1990b) also determined EC in samples from urine-soaked snow and feces at d 180 to 200 postconception from mares on the Pryor Mountain National Wild Horse Range,

and in samples of urine and feces collected directly from the ground at d 120 to 180 postconception from mares on Assateague Island National Seashore. Samples were analyzed for EC by direct EIA, with antiserum directed against estrone-3-glucuronide:BSA and HPLC to identify types of estrogen. The mean concentration of EC for Pryor Mountain mares that produced foals is 7.3 ± 1.39 μg/mg Cr and is .096 ± .084 μg/mg Cr for mares that did not foal. The mean concentration of EC for Assateague mares that produced foals is 3.47 ± .735 μg/mg Cr and is .11 ± .034 μg/mg Cr for mares that did not deliver foals (Kirkpatrick et al., 1990b). In summary, EC and(or) E1S concentrations in uncaptured feral horses are significantly increased in pregnant mares compared with nonpregnant mares.

Urinary Creatinine

Creatine is synthesized in the liver and pancreas from three amino acids, arginine, glycine, and methionine. Creatine becomes phosphorylated primarily in the muscles and forms creatine phosphate. Creatine phosphate acts as a reservoir of high energy and converts to ATP in the muscles. Both creatine and creatine phosphate are metabolized into creatinine (Cr). Creatinine is excreted by the kidneys in the urine without being reabsorbed by the renal tubules (Faulkner and King, 1978). This results in a relatively constant excretion rate of Cr in urine (Vestergaard et al., 1958; Van Niekerk et al., 1963; Hodgen et al., 1967). The determination of Cr is based on the Jaffe orange color reaction

which occurs between Cr and sodium picrate in an alkaline medium (Jaffe, 1886; cited in Owen et al., 1954). The measurement of urinary excretion rate of metabolites, such as steroids, should be based on total volume of urine or expressed as a ratio based on a relatively constant index substance, such as Cr, because of rapid changes in the volume of urine and the concentration of the excreted metabolites (Jones and Erb, 1968; Erb et al., 1970). The collection of urine in 24 hours is time consuming and relatively inaccurate when short-interval changes in the excretion rate of metabolites are determined over periods of days or months in the same animal (Jones and Erb, 1968). Jones and Erb (1968) also reported when the average Cr excretion is determined, the daily excretion rates can be estimated without continuously determining total urine output in 24 hours. Anker and Colo, (1954) noted that the correction for Cr does not result in such a marked change of the curve of urinary pregnanediol levels throughout pregnancy in human, but merely tends to bring extremely high or low values into line. This results in meaningful data that can be derived from many urine specimens which are grossly incomplete and may have to be discarded if Cr corrections are not available (Anker and Colo, 1954). Erb et al. (1970) demonstrated that the ratio, μg steroid per mg Cr, is an accurate method for expressing the rate of urinary steroid excretion.

Fecal Estrogens

Determination of the presence of estrogens in feces has recently been reported as a noninvasive method for pregnancy diagnosis in mares (Bamberg et al., 1984, 1991; Sist et al., 1987b; Kirkpatrick et al., 1990b, 1991; Lucas et al., 1991; Schwarzenberger et al., 1991). Using enzymatic methods and RIA, Bamberg et al. (1984) determined fecal estrogens in non-pregnant and pregnant mares. The enzymatic method was based on an amplifying system, in which the enzyme estradiol-17ß-dehydrogenase, with its transhydrogenase function, used E1 or E2 as substrate and NAD^+/NADH + H^+ or $NADP^+$/NADPH + H^+ as coenzymes. This double specificity toward the coenzyme enabled a continuous and rapid hydrogen transfer from NADPH + H^+ to NAD^+. This transfer was based on the cyclic interconversion of E1 to E2 and vice versa. The mean concentration of estrogens in non-pregnant animals is 3.7 ± 2.2 ng/g feces (enzymatic method) and 4.1 ± 3.4 ng/g feces (RIA), and on d 120 of pregnancy the fecal estrogen concentration is 10.3 ng/g feces (enzymatic method) and 14.3 ng/g feces (RIA). Both methods give similar values; therefore, it is possible to confirm pregnancy in mares after d 120 of pregnancy with either the enzymatic method or RIA (Bamberg et al., 1984). Using commercial kits for EIA of E1S in milk, Choi et al. (1985) measured estrogen concentrations in feces of non-pregnant mares and mares between d 20 to 150 of pregnancy. The mean concentration of estrogen in non-pregnant mares is

7.7 ± 5 ng/g feces, and the estrogen concentrations during estrus are not different from that of the other stages of the estrous cycle. The concentrations of estrogen are approximately 100 ng/g feces on d 110 and are about 300 ng/g feces on d 150 of pregnancy (Choi et al., 1985).

Serum and fecal E1S were determined by RIA during pregnancy in mares (Sist et al., 1987b). The concentrations of serum E1S increase sharply (> 2000 pg/ml serum) after d 60 of pregnancy while the concentrations of fecal E1S remain at the nonpregnant level (< 200 pg/g feces). Mean concentration of fecal E1S in nonpregnant mares is 50.2 ± 3.25 pg/g of feces. The mean concentration of E1S is about 200 pg/g feces on d 100, elevates to 436.2 ± 73 pg/g of feces on d 120, and increases to 872.8 ± 67.84 pg/g feces after d 120 of pregnancy. The mean fecal E1S concentration (592.9 pg/g feces) in mares during heat is higher than in nonestrous mares (50.2 pg/g feces). This higher concentration during heat may give a false-positive for pregnancy; however, there are no false-positive results after d 120 of pregnancy (Sist et al., 1987b). Kirkpatrick et al. (1990b) reported that the mean concentrations of fecal estrogen for Assateague mares that produced foals are 3.18 ± .7 ng/g feces and are .552 ± .08 ng/g feces for mares that did not foal. The correlation coefficient (.928) between urinary EC and fecal EC is high, suggesting that fecal steroids could be an important new parameter for the determination of pregnancy (Kirkpatrick et al., 1990b).

Bamberg et al. (1991) determined fecal EC during late pregnancy in Przewalski's mares. Samples were collected once weekly starting on wk 14 prepartum to wk one postpartum. Fecal EC concentration is 470 ng/g feces on wk 14 prepartum and declines gradually toward parturition. The concentration is 90 ng/g feces at wk one prepartum and decreases to 3 ng/g feces on wk one postpartum. The concentration in the nonpregnant mare is 4.8 ng/g feces (Bamberg et al., 1991). Using RIA, Lucas et al. (1991) studied fecal estrogens in 454 uncaptured feral mares. The antiserum used in the RIA is raised in sheep against estrone-3-hemisuccinate:BSA. The mean concentration of fecal estrogens is not detectable before d 120 postconception and considered as a limiting factor in determining pregnancy loss before d 120. The mean concentration of fecal estrogens is >5 ng/g feces at d 120 (Lucas et al. 1991). Schwarzenberger et al. (1991) measured fecal estrogens and progestagens in pregnant Lipizzan, Trotter, and Thoroughbred mares by using EIA for conjugated estrogens and HPLC followed by EIA for unconjugated estrogens. Fecal estrogens of Lipizzan mares are significantly elevated above the levels of non-pregnant mares after d 90 of pregnancy. There is a marked increase (>20 ng/g feces) in wk 15 of gestation that is similar to previous reports (Bamberg et al., 1984; Choi et al., 1985). The level of fecal estrogens begins declining gradually during the last 14 weeks before foaling and decreases to baseline values three days postpartum (Schwarzenberger et al., 1991).

Urinary and Fecal Progestins

Early studies on the isolation of pregnanediol from pregnant mare urine began in the late 1930's. Brooks et al. (1952) first reported the isolation of a pair of 20-epimeric hydroxysteroids from pregnant mare urine. They isolated both 5α-pregnane-3ß:20ß-diol and 5α-pregnane-3ß:20α-diol, in addition to uranediol. Hirschmann and Williams (1963) demonstrated that 5α-pregnane-3ß:20ß-diol-sulfate yields, on acid hydrolysis, uranediol [17α-methyl-D-homo-5α-androstane-3ß:17 (α,ß-diol)]. Hirschmann and Williams (1963) also suggested that the water-soluble moiety in the precursor of 5α-pregnane-3ß,20ß-diol must either be at C-3, or if it is at C-20, must be a group such as glucuronic acid, which may be removed by acid hydrolysis without rupture of the steroid-oxygen bond.

The first detailed study of urinary progesterone metabolites during the estrous cycle in horses was reported by Kirkpatrick et al. (1990a). Using direct EIA, Kirkpatrick et al. (1990a) determined the concentrations of urinary pregnanediol-3-glucuronide (PdG) and non-specific urinary progesterone metabolites, which was called immunoreactive PdG-like conjugate (iPdG), indexed to Cr, and compared these concentrations to plasma progesterone during the estrous cycle in mares. Three different antibodies were used in this study. The first antibody was a non-specific monoclonal antibody against PdG and was utilized to measure urinary iPdG. The second was a polyclonal

antibody against PdG and was used to measure urinary PdG. The third was a polyclonal antibody against progesterone and was used to measure plasma progesterone. In addition, the urinary iPdG assay was validated by using urine from mid-luteal phase, subjecting the urine samples to HPLC, and analyzing the eluates for iPdG and PdG. Concentrations of urinary PdG are minimal (average 7.04 ng/mg Cr ± .686), remain constant throughout the estrous cycle, and do not reflect luteal regression. The concentration of urinary iPdG is 3.6 ng/mg Cr at the time of ovulation and increases to 390.3 ng/mg Cr at the mid-luteal phase, paralleling plasma progesterone concentrations. The correlation coefficient for plasma progesterone and urinary iPdG is .91, suggesting that urinary iPdG concentrations reflect changing concentrations of plasma progesterone. The results of HPLC show an absence of significant concentrations of PdG and the presence of at least three immunoreactive compounds, all of which are more polar than PdG, confirming the EIA results (Kirkpatrick et al., 1990a).

Few studies of urinary and fecal progestins at different stages of pregnancy in mares have been performed. Using EIA, Kirkpatrick et al. (1990b) measured iPdG, indexed to Cr, in two experiments with uncaptured feral horses. In the first experiment, urine samples were collected from the snow at d 180 to 200 of gestation. The mean concentration of iPdG for mares producing foals is 167 ± 80.33 ng/mg Cr and is 7.04 ± 1.69 ng/mg Cr for mares that did not produce foals. In the second experiment, urine samples

were collected from the ground at d 120 to 180 of gestation. The mean concentration of iPdG for mares producing foals is 215.8 ± 83.4 ng/mg Cr and is 3.6 ± .499 ng/mg Cr for mares that did not produce foals (Kirkpatrick et al. 1990b). Kirkpatrick et al. (1991) determined the concentrations of iPdG in the feces of uncaptured feral horses during pregnancy. Fecal samples were collected at d 120 to 180 postconception and assayed for iPdG by EIA. Samples were also subjected to HPLC and the fractions were assayed for iPdG by EIA, as described by (Kirkpatrick et al. 1990a). Fecal concentration of iPdG for mares producing foals was 1,411 ± 569 ng/g feces and was 32.8 ± 4.5 ng/g feces for mares that did not produce foals. The results of HPLC reveal the presence of several major peaks, which demonstrate immunoreactivity with the iPdG antibody (Kirkpatrick et al. 1991). Schwarzenberger et al. (1991) reported concentrations of fecal progestins throughout pregnancy in Lipizzan, Trotter, and Thoroughbred mares. Fecal samples were assigned into two groups: samples collected 7 to 16 weeks after mating and samples collected from 14 weeks before until parturition. Samples were assayed for total progestagens by EIA, with antibody against 20α-hydroxy and 20ß-hydroxy-progestagen. Samples were also subjected to HPLC and the fractions were measured by EIA. During wk 7 to 16 postconception, the concentrations of 20α-OH-progestagens (20α-G) and 20ß-OH-progestagens (20ß-G) increase slightly, but not as abruptly as do those of estrogens. The mean concentration of progestagens is significantly higher than the nonpregnant values ($P < .01$) from wk 11

onwards; and in wk 16, the mean concentrations of 20α-G and 20ß-G were > 1000 ng/g feces and 500 ng/g feces, respectively. There is a significant difference between Lipizzan, Trotter, and Thoroughbred mares throughout gestation except, the last 4 weeks prepartum. In contrast to estrogens, maximum progestagen concentrations are observed during the last week before foaling. This is followed by a rapid decline after parturition. Fecal estrogens or progestins could be an important noninvasive method of determining pregnancy after d 100 postconception (Schwarzenberger et al., 1991).

Urinary and Fecal Steroids in Other Domestic Species

Sow

Metabolites of estrogen and progesterone in urine of pregnant sows were first studied by Kust (1931, cited in Choi et al., 1987). Estrone sulfate concentration may be used to detect early pregnancy (d 25 to 30) in sow plasma (Velle, 1960; Cupps et al., 1966; Robertson et al., 1978; Guthrie and Deaver, 1979; Hattersley et al., 1980; Saba and Hattersley, 1981; Cunningham et al., 1983) and in urine (Lunaas, 1962; Edgerton and Erb, 1971; Seren et al., 1983). The pattern of estrogen in plasma is similar to that observed in urine (Cunningham et al., 1983). Measurement of E1S levels in sow plasma (Tillson and Erb, 1970; Horne et al., 1983; Stone et al., 1986) and in sow urine (Edgerton et al., 1971; Frank et al., 1987) in early pregnancy may also allow

the prediction of litter size. The direct determination of urinary EC or E1S for early pregnancy diagnosis and litter size in sows may be more practical than determining blood values because urine sampling is relatively simple compared with blood sampling, in addition to being time consuming (Seren et al., 1983).

There is a significant positive correlation between the number of live fetuses and the concentration of urinary estrogens on d 26 of pregnancy (Edgerton et al., 1971). There is also a significant positive correlation between the levels of plasma E1S on d 20, 24, 26, and 32 of pregnancy and the number of live fetuses (Horne et al., 1983). The first study to measure E1S concentration simultaneously in maternal blood and urine by direct RIA, without extraction of samples or hydrolysis, was performed by Frank et al. (1987). Estrone sulfate concentrations in plasma and urine on d 28 of pregnancy correlate with litter size at birth (Frank et al., 1987). These studies support the practical use of E1S to detect early pregnancy in either serum or urine (Guthrie and Deaver, 1979; Hattersley et al., 1980; Robertson et al., 1978 and 1985; Saba et al., 1981; Cunningham et al., 1983; Seren et al., 1983) and to predict litter size, distinguishing sows that will have very small litters from those that may have large litters (Edgerton et al., 1971; Horne et al., 1983; Stone et al., 1986, Choi et al., 1987; and Frank et al., 1987).

Choi et al. (1987) studied estrogens in blood and feces during the estrous cycle and early pregnancy in sows. The concentrations of E1S in blood are low in non-pregnant sows and until d 20 of pregnancy and increase to peak

values between d 25 and 30 of pregnancy. Concentrations of unconjugated estrogen in feces follow the same pattern as in blood, indicating that the levels of estrogen in feces reflect the concentrations in blood (Choi et al., 1987).

Progesterone excretion in urine was studied extensively in Erb's laboratory in the 1960's and early 1970's. The rate of excretion of metabolites of progesterone in urine reflects similar patterns of change during the estrous cycle in the ovarian venous plasma (Schomberg et al., 1966; Jones and Erb, 1968). Four progesterone metabolites have been identified in sow urine during the estrous cycle and early pregnancy. Three metabolites were identified and determined by Schomberg et al. (1966) in the urine of the nonpregnant sow; these are: 5ß-pregnane-3α,20α-diol (pregnanediol); and two pregnanolones, 3α-hydroxy-5ß-pregnan-20-one and 3ß-hydroxy-5α-pregnan-20-one. The identification was performed by applying urine samples to thin-layer chromatography (TLC) after enzyme hydrolysis and ether extraction. Quantification was done by applying the eluates, following TLC, to spectroscopy and gas-liquid chromatography (GLC). Concentrations of ovarian venous plasma progesterone and urinary pregnanediol and pregnanolones are very low near estrus and highest during the luteal phase (10 to 15 days after estrus). Urinary pregnanediol concentration correlates significantly with 3ß-5α- and with 3α-5ß-pregnanolones (Schomberg et al., 1966). The fourth metabolite was 3α,6α-dihydroxy-5ß-pregnan-20-one. It was identified and determined, in addition to the three previous progesterone metabolites, by

Tillson et al. (1970) in urine collected during the estrous cycle and the first 28 days of pregnancy. The methods used were modified from Schomberg et al. (1966). The rate of excretion of 3α-hydroxy-5ß-pregnan-20-one in urine reflects luteal function during the estrous cycle and the first 28 days of pregnancy and provides a reasonable estimate of total metabolites of progesterone excreted in urine. The excretion of 3α,6α-dihydroxy-5ß-pregnan-20-one may be useful in predicting conception and subsequent loss of embryos during early pregnancy because its excretion increases between d 2 and 6 in pregnant sows and does not change significantly during the estrous cylce in unbred sows (Tillson et al., 1970). Pregnanes excretion decrease from d 19 to 54 of pregnancy. On d 75 of pregnancy, there is a significant increase in 3α-hydroxy-5ß-pregnan-20-one,3α,6α-dihydroxy-5ß-pregnan-20-one,andtotal pregnanes. Furthermore, the excretion of 5ß-pregnane-3α,20α-diol is significantly higher during the last half than during the first half of pregnancy. However, the excretion of 3ß-hydroxy-5α-pregnan-20-one does not vary significantly from d 19 to 110 of pregnancy. The excretion of 3α-hydroxy-5ß-pregnan-20-one, but not the other pregnanes, increases from d 75 to d 103 of pregnancy (Tillson et al., 1970).

The concentrations of total estrogens in urine correlate significantly to the concentrations of total pregnanes in urine at d 19, 26, 68, 75, 82, and 110 of pregnancy (Edgerton and Erb, 1971; Edgerton et al., 1971).

Cows

Aschheim and Zondek in 1927 were the first to recognize urinary estrogens in cows (cited in Cole, 1950). The estrogenic activity of cow urine is low compared with mare urine or woman urine (Cole, 1950). The methods used in the following studies to determine and identify various estrogens during the estrous cycle and pregnancy are the same. The methods include enzyme hydrolysis, TLC, extraction with ethanol, and applying GLC. Estrone is the primary urinary estrogen during the estrous cycle and makes up 39% of total estrogens, compared with 34% for E2, and 27% for E2α. Concentrations of total urinary estrogen are highest during a 7-day period, including estrus, and for 3 days preceeding and 3 days following estrus (Garverick et al., 1971). On the day of esturs, concentrations of urinary E2 and total estrogens are higher during the LH surge than when the LH surge is missed (Garverick et al., 1971). Randel et al. (1971) determined urinary estrogens from d 0 to 42 after breeding. On d 7 and 14 after breeding, concentrations of E2α, E1, and total estrogens in cows that returned to estrus are higher compared with values for cows that became pregnant (Randel et al., 1971). Randel and Erb (1971) measured urinary estrogens in cows from d 0 to 260 of pregnancy. The first significant increase in urinary estrogens is in E2α on d 42. Concentrations of urinary E2α and E1 increase and are 20-fold and 6-fold higher, respectively, by d 230. The concentration of urinary E2 is low throughout pregnancy, except on d 155, d 215, and d 245. There is a steady increase in concentrations of

total urinary estrogens toward parturition. Concentrations are approximately 80 ng/mg Cr on d 0 and peak at approximate 800 ng/mg Cr on d 245 (Randel and Erb, 1971). Hunter et al. (1970) determined urinary estrogens in the last 30 days of pregnancy in cows. The concentrations of total estrogen excretion in urine increase steadily in the last 30 days and are followed by a decrease after parturition. Urinary E2α is the major metabolite on d 30 prepartum and represents 50% of the total urinary estrogen while the concentration of E1 is 44% and of E2 6%. The proportion of E2α elevates to 76% wk 2 prepartum and to 93% at .5 day after calving, compared with 21% and 5%, respectively, of E1. The proportions of the three estrogens are nearly the same on d 8 postpartum and are similar to d 30 prepartum, except E2 represents 11% instead of 6% of the total estrogens. The above findings suggest that E2α may be more important than E1 or E2 in the mechanism controlling the time of parturition (Hunter et al., 1970).

Levin (1945) studied the fecal excretion of estrogens in the last few weeks of pregnancy, by using ether extraction and injecting the estrogenic material into rats. Cows excrete 5000 to 10,000 rat units of estrogenic substance per kilogram of dry feces. The major proportion of the estrogenic activity is E2α (Levin, 1945). Recent studies to determine the presence of estrogens in feces of cattle are reported by Möstl et al. (1984). The method is performed by extracting feces with chloroform and NaOH, applying the supernatant to Sephadex column chromatography, and determining the

fractions by RIA, in addition to determining the concentrations of the three estrogens by RIA without chromatography. Estrogen levels are higer in pregnant cows than in nonpregnant cows after d 100 of pregnancy. Although E2α is a weak, phenolic, and non-ketonic estrogen, it is the major urinary and fecal metabolite in cows during pregnancy and is 10 times higher than that of either E1 or E2 (Möstl et al., 1984). The concentration of E2α, between wk 10 and 13 of pregnancy, is not different from that of non-pregnant animals. However, the concentration increases significantly from wk 14 of gestation and is higher than in non-pregnant cows (Möstl et al., 1984).

Pregnanediol (5ß- pregnane-3α,20α-diol) has been detected in the urine of cows at late pregnancy (Klyne and Wright, 1959; Heitzman and Thomas, 1965). The methods used for identification of pregnanediol is different. For instance, Klyne and Wright (1959) determined pregnanediol by using acid hydrolysis, extraction, paper chromatogrphy and spectrometry. Heitzman and Thomas (1965) identified pregnanediol by using acid hydrloysis, extraction, and GLC. The mean concetration of urinary pregnanediol in late pregnant cows is 200 μg/L urine (Heitzman and Thomas, 1965). The first study of excretion of pregnanediol in urine of cows during the estrous cycle was reported by Haraszti et al. (1967). The method used acid hydrolysis, extraction, paper chromatography, and was quantified by spectrophotometry. The mean concentrations of urinary pregnanediol are as follows: 2.0 mg/24 h during estrus, 6.8 mg/24 h on d 7 to 8, and 4.6 mg/24 h on d 14 to 15 of the cycle

These findings are in agreement with progesterone concentrations in ovarian vein and peripheral plasma during the cycle (Haraszti et al., 1967).

Llamas

Using HPLC and EIA, Bravo et al. (1991) determined urinary E1S and PdG in prepartum, postpartum, and through early pregnancy in llamas. The concentration of E1S increases during the last 20 days of pregnancy through 12 h before parturition and declines at the time of delivery. The concentration of PdG follows a pattern similar to that of E1S except that the level of PdG begins to decline d 5 prepartum. The concentration of E1S and PdG declines to the baseline 24 h postpartum. The patterns of urinary E1S and PdG during prepartum, postpartum, and through early pregnancy are similar to those of the blood levels (Bravo et al., 1991).

Urinary and Fecal Steroids in Wildlife Species

The studies of urinary and fecal steroids during the ovarian cycle and pregnancy in wildlife species are reviewed by Lasley and Kirkpatrick (1990). The patterns of urinary EC and(or) PdG, determined by direct RIA or EIA, have been shown to reflect the reproductive activity accurately in various wildlife species, such as okapi, giraffe (Loskutoff et al., 1982, 1986), eld's deer (Monfort et al., 1990a,b), Indian rhinoceros (Kasman et al., 1986), black

rhinoceros (Ramsay et al., 1987), Goeldi's monkeys (Carroll et al., 1990; Ziegler et al., 1990), and gorilla (Lasley et al., 1982; Czekala et al., 1988; and Hodges and Green, 1989). The main advantages in collecting urine and fecal samples is simplicity, efficiency, and is noninvasive without great risk to the animal or the investigator. Time-mating programs, detecting early pregnancy, and monitoring fetal viability are other stated uses (Monfort et al., 1987; Lasley and Kirkpatrick, 1990).

Loskutoff et al. (1982) studied the concentrations of iPdG in urine of the okapi during the estrus cycle and pregnancy. The length of the estrous cycle is approximately 14.5 days. The follicular phase is eight days and the preovulatory concentrations of iPdG is .8 ng/mg Cr. The luteal phase is 6.5 days and the postovulatory concentrations of iPdG is 24 ng/mg Cr. The concentrations of iPdG increase to 163 ng/mg Cr on d 22 of pregnancy and persist until approximately the fourth month of pregnancy when the concentrations decline to 60 ng/mg Cr. The levels of iPdG increase steadily on the sixth month of pregnancy and remain above 100 ng/mg Cr until the 14th month of pregnancy when the levels decline again at approximately three week preparetum. Following parturition, the levels are followed by more decline to 4 ng/mg Cr, and remain low for at least six months during lactation (Loskutoff et al., 1982).

Urinary Steroids in Women

The concentrations of urinary EC and(or) PdG, determined by direct RIA or EIA, have been usefull to study the menstrual cycle, and to detect ovulation and early pregnancy (Samarajeewa and Kellie, 1975; Samarajeewa et al., 1979; Stanczyk et al., 1980; Chatterton et al., 1982; Shah et al., 1984; Gray et al., 1987; Munro et al., 1991). The profiles of urinary EC and PdG have been also used to study causes of infertility in women (Cheesman et al., 1982; Miller et al., 1990), and to detect early pregnancy loss (Baird et al., 1991). Munro et al. (1991) reported the relationship of serum estradiol and progesterone concentrations to the levels of their urinary metabolites as determined by both RIA and EIA. The mean concentrations of urinary EC is parallel to the serum estradiol values. This finding suggests that estrogen production by the ovaries can be monitored accurately through urine or blood samples. In addition, the increase of urinary estrogens during the late follicular phase reflects the preovulatory estrogen secretion and may be useful to predict ovulation. The concentrations of serum progesterone is also parallel to the urinary PdG. This provides comparable information for confirming the occurrence of ovulation and the presence of a functional CL. The RIA and EIA values for urinary EC and PdG are not different (Munro et al., 1991). The use of EIA has more advantages than RIA, including simplicity, flexibility, economy, and lack of health hazards (Shah et al., 1984; Munro et al., 1991).

Objectives of the Current Study

In summary of the above literature, there was no previous studies to determine both profiles of E1S and PdG throughout pregnancy with frequent sampling in same animal in the domestic horses. The present experiments were conducted to: 1) measure the concentrations of both E1S and PdG in unextracted urine throughout pregnancy in pony mares using direct EIA, 2) test the main effects of hormone and its interaction by time, 3) study the significance increase and decrease of each hormone throughout pregnancy.

MATERIALS AND METHODS

Animals

Between September 1990 and July 1991, six pony mares (unknown breeds) were maintained in paddocks and fed grass hay and grain to meet normal husbandary requirements. Each mare weighed approximately 250 kg at the beginning of the study. Animals were teased for estrus and bred either naturally or artficially inseminated from the same stallion. Ovulation and pregnancy were determined by rectal palpation and ultrasonography (Aloka 210-DX with a 5-MHz probe, Corometrics Medical System, Inc. Wallingford, CT) and d 0 of pregnancy was considered as the day of ovulation. Urine samples were collected by inserting a disinfected metallic catheter into the urinary bladder through the urethra. Samples were collected once per day during estrus and through d 2 postovulation, every other day until d 52 of pregnancy, then twice per week until d 99 of pregnancy, once per week until parturition, then daily after parturition for four days. Sampling of two mares began at d 90 of pregnancy and sampling stopped in another two mares at d 303. Samples were collected into 20 mL plastic disposable scintillation vials (Kimble, Division of Owens-Illinois, Toledo, OH) and were stored without preservatives at -20°C until analyzed.

Chemical Analyses

The chemical reagents used for both the estrone sulfate (E1S) and pregnanediol glucuronide (PdG) assays were as follows: methyl alcohol (spectro grade), sodium bicarbonate (NaHCO3), sodium carbonate (Na_2CO_3), dibasic sodium phosphate ($NaH_2PO_4.H_20$), monobasic sodium phosphate (Na_2HPO_4), and sodium chloride (NaCl) (Mallinckrodt, Paris, KY); bovine serum albumin (BSA), polyoxyethylene-sorbitan monolaurate (Tween 20), citric acid, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (H_2O_2) (8 M, 30%), 3α,20α-dihydroxy-5β-pregnane-3-glucronide (pregnanediol glucuronide), 3-hydroxy,1,3,5[10]-estratrien-17-one-3-sulfate (estrone sulfate), and 3-hydroxy,1,3,5[10]-estratrien-17-one-3-glucuronide (estrone glucuronide, E1G) standards (Sigma, St Louis, MO). Ninetysix-well flat- bottom, high-binding ELISA plates (Nunc-Immuno Plate Maxi Sorp F96, InterMed, Naperville, IL) were used throughout the study.

The antibodies against E1G (E1G-Ab) and pregnanediol glucuronide (PdG-Ab) and corresponding hormone-enzyme conjugates (horseradish peroxidase) were obtained from Dr. Stabenfeldt and C. Munro, School of Veterinary Medicine, Davis, CA, and stored at -20°C.

The bicarbonate coating buffer to dilute the antibody (.05 M) consisted of 1.59 g Na_2CO_3 and 2.93 g NaHCO3 in 1000 mL deionized double distilled water (ddH_2O) (pH adjusted to 9.6 with NaOH). The EIA phosphate buffer

stock consisted of two solutions: solution A (.2 M) contained 27.8 g of $NaH_2PO_4.H_2O$ in 1000 mL ddH_2O and solution B (.2 M) contained 28.4 g Na_2HPO_4 in 1000 mL ddH_2O. The working EIA phosphate buffered saline (PBS-BSA, .1 M) consisted of 1.0 g BSA, 8.7 g NaCl, 195 mL solution A, 305 mL solution B, and 500 mL ddH_2O (pH adjusted to 7.0 with NaOH). The EIA wash stock solution was 10 times more concentrated than the working wash solution. It consisted of 87.66 g NaCl (1.5 M), 5.0 mL Tween 20 (.5%) in 1000 mL ddH_2O. The citrate buffer for substrate contained 9.61 g citric acid in 1000 mL ddH_2O (pH adjusted to 4.0 with NaOH). The dilute H_2O_2 (.5 M, 2%) contained 500 μL H_2O_2 (8 M, 30%) in 7.5 mL ddH_2O. The ABTS (40 mM) contained .55 g ABTS in 25 mL ddH_2O (pH adjusted to 6.0). All the above solutions were stored at 4°C. The substrate working solution was made just prior to adding the substrate and consisted of 12.5 mL citrate buffer (.05 M, pH 4.0), 40 μL H_2O_2 (.5 M, 2.0%), and 125 μL diluted ABTS (40 mM, pH 6.0) for each plate.

Estrone Sulfate Assay

The protocols for the E1S and PdG assays were modified from Munro and Stabenfeldt (1984) by varying the concentrations of antibody, conjugates and urine dilution. The antibody (R583) against estrone-3-glucuronide:BSA was raised in rabbits (Munro and Stabenfeldt, 1984). Cross-reactivity of E1S-Ab was reported by Daels et al. (1990, 1991a,b) using E1S as standard. The

antibody cross-reacted with estrone (200%), estradiol-17β (100%), equilin (50%), estrone-3-glucuronide (38%), estradiol-3-sulfate (21%), estradiol-3-glucuronide (6.8%); the cross reaction was less than .5% with all nonestrogenic steroids tested.

The antibody stock was aliquotted 100 μL/vial of 1:100 dilution in coating buffer solution and stored at -20°C. The working dilution was 1:1500 in coating buffer and prepared at the time of use. The hormone-enzyme conjugate was horseradish peroxidase (HRP) and was coupled to the estrone-3-glucuronide via the carboxylic acid group of the 3-glucuronide carbohydrate moiety using the mixed anhydride procedure (Munro and Stabenfeldt, 1984). The E1S-HRP stock was 100 μL/vial of 1:100 dilution in PBS-BSA and stored at -20°C. The working dilution was 1:2000 in PBS-BSA and diluted just prior to use.

Estrone sulfate stock standard was 10 μg/mL in methanol. The highest standard, 10 ng/mL, was made from E1S stock standard, and was serially diluted (halving dilution) to .625 ng/ml, in addition to 0 ng/mL; standards were diluted in PBS-BSA. Samples were prepared as quality control from the E1S stock standard and were used to test quantity and repeatibility. The highest quality control was 5 ng/mL and the lowest was 1.25 ng/mL, in addition to 0 ng/mL. The quality controls were prepared using ovariectomized mare's urine diluted 1:20 in PBS-BSA. Urine samples were diluted in PBS-BSA according to the stage of pregnancy. Urine was diluted 1:200 or 1:400 in the first 45 days

of pregnancy, 1:1000 or 1:2000 from d 45 to 70 of pregnancy; 1:4000 or 1:8000 from d 70 to 85; 1:64,000 from d 85 to 100; 1:80,000 or 1:100,000 from d 100 until parturition; and 1:400 after parturition. Few samples were diluted 1:120,000 and some samples were rediluted due to high concentration of E1S.

The procedure of the assay was performed as follows: 50 μL of the antibody was added to each well of the plate, except two wells to test the non-specific binding, at a working dilution 1:1500 in coating buffer using a multichannel pipette (Flow Laboratories, Covina, CA). The edges of the plate were tapped gently to distribute the antibody evenly across the surface area of the plate. The plate was then gently agitated on an autoplate shaker (Titer Plate Shaker, Lab-Line Instruments, Inc., Melrose Park, IL) for 15 min, covered with an adhesive sealer (Dynatech Lab, Chantilly, VA) and incubated overnight at 4°C. On the next day the plate was washed five times with wash solution and all the remaining drops were shaken to remove the excess unbound antibody. Immediately, 50 μL of PBS-BSA was added to each well using the multichannel pipette, the plate was sealed, and incubated at room temperature for 2 h to allow the solution to equilibrate. The outside row and columns were not used due to plate variability. Forty μL of urine sample, standard solution, and quality control solution were added to duplicate wells, followed by 50 μL of E1S-HRP to each well. The E1S-HRP was not added to two different wells to test non-specific enzyme conjugate binding. The plate was gently agitated

on an autoplate shaker for 60 min and incubated at room temperature for 5 h or overnight at 4°C. Following incubation, the plate was washed five times with EIA working wash solution and all remaining drops were shaken off. The substrate solution was made immediately before use to avoid autoxidation and deterioration of the substrate. Substrate solution (100 μl) was added to each well, except another two wells to test color background, using a multichannel pipette; the plate was sealed tightly and shaken gently on an autoplate shaker until blue color developed in about 30 to 50 min. The plate was read using a Titertech Multiskan Plus II automatic plate reader (Flow Laboratories) at 405 nm. Absorbance was used to calculate concentrations using a logistic-log transform program in a computer connected to the plate reader (Titersoft E.I.A. software version 2.0A, Flow Laboratories). The absorbance of zero wells (E0) was used to determine the maximum binding of the enzyme conjugate to the antibody.

Pregnanediol Glucuronide Assay

The antibody (P70) against pregnanediol-3-glucuronide:BSA was raised in rabbits (Munro and Stabenfeldt, 1984). Cross-reactivity of PdG-Ab was reported by Munro et al. (1991). The antiserum cross-reacted with pregnanediol-3-glucuronide (100%), 20α-hydroxyprogesterone (60.7%), 20β-hydroxyprogesterone (2.5%), 17α-hydroxyprogesterone (<.1%), 11α-hydroxyprogesterone (<.1%), pregnanediol (7.3%); progesterone, pregnenolone, androstenedione, cortisol, estradiol-17β, estrone, and testosterone were all <.1%.

The anti-PdG:BSA stock was 100 μL/vial at 1:50 dilution in coating buffer and was stored at -20°C. The working dilution was 1:15,000 in coating buffer and was prepared only at the time of use. The hormone-enzyme conjugate was horseradish peroxidase (HRP) and was coupled to the PdG via the carboxylic acid group of the 3-glucuronide carbohydrate moiety by the mixed anhydride procedure (Munro and Stabenfeldt, 1984). The PdG-HRP stock was 100 μL/vial at 1:100 dilution and was stored at -20°C. The working dilution was 1:40,000 and was prepared at the time of use. The PdG stock standard solution was 10 μg/ml in methanol. The standards were diluted in urine from an estrous mare, 0 ng/mL, diluted 1:20 in PBS-BSA. The highest standard, 80 ng/ml, was made up from stock standard in urine diluted 1:20 in PBS-BSA and was serially diluted (halving dilution) to 5 ng/mL, in addition to 0 ng/mL. Samples were prepared for quality control from the PdG stock

standard and were used to test quantity and repeatibility. Quality controls were made in urine of 0 ng/mL diluted 1:20 in PBS-BSA. The highest control was 20 ng/mL and the lowest was 5 ng/ml in addition to 0 ng/mL. Urine samples were diluted in PBS-BSA according to the stage of pregnancy. They were diluted 1:5 and 1:10 for the first 90 d of pregnancy and 1:20 from d 90 until parturition, however, a few samples were diluted 1:40 at late pregnancy.

Coating and handling plates, adding the substrate, and calculating the concentrations followed the E1S protocol explained above.

Creatinine Assay

Measuring creatinine was performed to compensate for the variation in specific gravity of the urine. Creatinine standard and picric acid were purchased from Sigma, and NaOH from Mallinckrodt and ninetysix-well flat-bottom, polystyrene plates were purchased from Dynatech.

Urinary creatinine concentrations were determined by modifying the colorimetric method of Tietz (1976), to include measuring smaller quantities and reading results on a microplate reader.

Creatinine stock standard, 1 mg/mL, was prepared by dissolving 100 mg of creatinine in 100 mL .1 M HCl and stored at 4°C. Creatinine working standard, 40 μg/mL, was made from the stock standard in ddH_2O and was serially diluted (halving dilution) to 5 μg/mL in addition to 0 μg/mL in ddH_2O and

was stored at 4°C. Urine samples were diluted 1:100 in ddH_2O and a few samples were diluted 1:50 or 1:20 due to low concentration of creatinine.

Two hundred μL of standards and samples were added to duplicate wells of a microplate, then 50 μL picric acid (.04 M) and 50 μL NaOH (.75 M) to all wells using a multichannel pipette. The plate was shaken on an autoplate shaker for 15 min and was incubated for another 15 min at room temperature. The plate was read using a Titertek Multiskan Plus II automatic plate reader spectrophotometer at 492 nm (Flow Laboratories). The concentrations were calculated using a linear regression transform program (Titersoft E.I.A. software version 2.0A, Flow Laboratories).

Statistical Analyses

Data were divided into 3 stages: (1) d 0 to 86; (2) d 90 to 303; and (3) d 36 prepartum to 4 postpartum. Each stage was analyzed separately. Because weekly samples were taken after d 100 of pregnancy, a few sampling dates were adjusted by ± 3 days for calculation of weekly means. The data for the two hormones represented repeated measurement within mares and were analyzed using the REPEATED statement of the GLM procedure for analysis of variance (PROC GLM, SAS, 1988) to test the main effects of hormone and its interaction by time. The correlation coefficient of the two hormones, at each of the stages, was measured using PROC CORR, SAS (1988). Stepwise paired t-test was performed using SAS to study the significance increase and decrease of each hormone throughout pregnancy.

RESULTS

Hormonal Assays

Estrone Sulfate

Parallelism between the standard curves for E1S, E1G, and serial dilutions of the urine samples at various stages of reproductive activity are presented in Figure 1. Estrone sulfate standard curve was parallel to the curves of serial dilution of urine samples while E1G standard curve was not parallel, suggesting that E1S was the main conrtibuter to urinary estrogens (Figure 1). Sensitivity was calculated from the B_o values minus 2 SD for eight consecutive assays. The sensitivity of E1S assay was .096 μg per well, varying between .05 and .15 μg per well (per mL). The working range of the standard curve was 0 to 10 μg/ml. The overall assay sensitivity was approximately .007 μg/mg Cr, which accounted for urine dilution and creatinine correction. Recovery of known amounts of E1S (1.25, 2.5, and 5.0 μg/mg Cr) was linear, with mean values (μg/mg Cr) $\pm$ standard error (SE) at 1.44 $\pm$ .22, 2.48 $\pm$ .46, 5.23 $\pm$ 1.15, respectively (Figure 2). Utilizing low, medium, and high quality controls, the average interassay coefficient of variation (C.V.) was 17.97% (n = 37) and the average intraassay C.V. was 3.54% (n = 10).

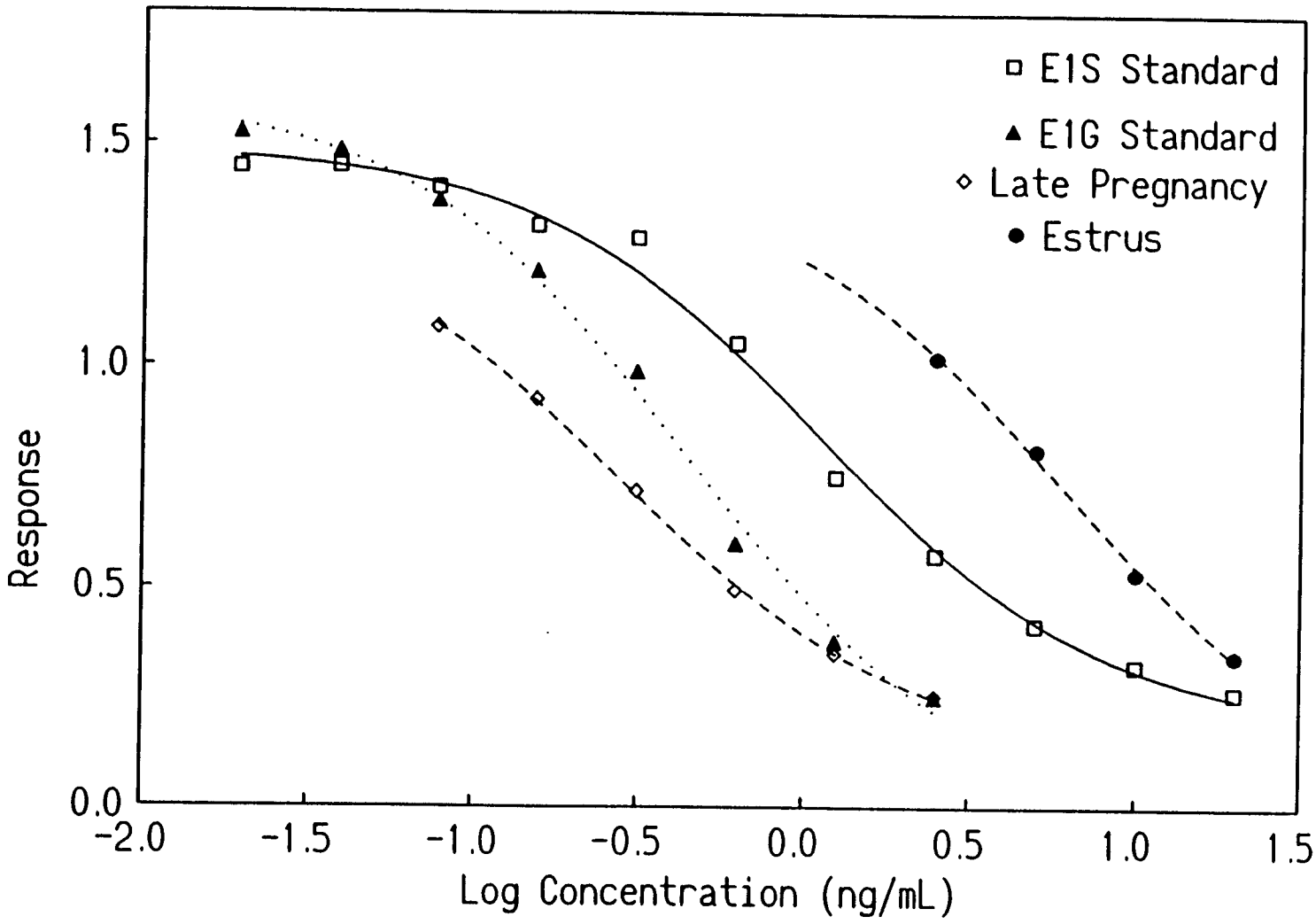

Figure 1. Displacement curves by enzyme immunoassay of estrone sulfate (E1S), estrone glucuronide (E1G), and serial dilutions of urine from pregnant mares at various stages of pregnancy.

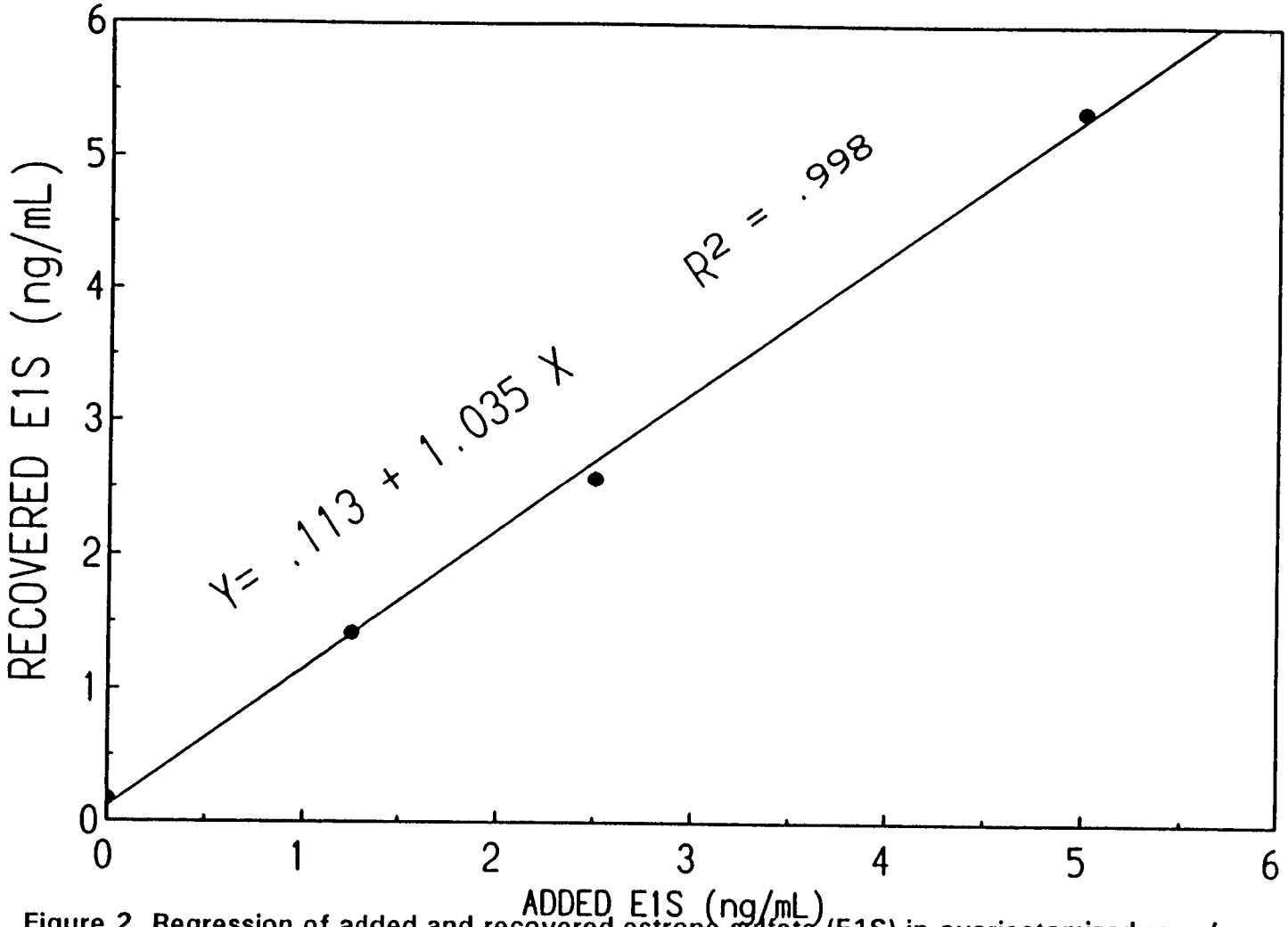

Figure 2. Regression of added and recovered estrone sulfate (E1S) in ovariectomized mare's urine quality controls of 1.25, 2.5, and 5.0 ng/mg Cr using standards diluted in phosphate buffered saline (PBS-BSA).

Pregnanediol Glucuronide

Parallelism between the standard curves for PdG and serial dilutions of the urine samples at various stages of reproductive activity are shown in Figure 3. It appeared that there was similar parallelism between the curves of serial dilution of urine and PdG standard curve (Figure 3). The sensitivity of PdG assay was .54 ng per well, varying between .10 and 1.50 ng per well. The working range of the standard curve was 0 to 80 ng/ml. The overall assay sensitivity was approximately 1.87 ng/mg Cr, which accounted for urine dilution and creatinine correction. Recovery of known amounts of PdG (5, 10, and 20 ng/mg Cr) was linear, with mean values (ng/mg Cr) at 4.9 ± 1.1, 10.6 ± 2.2, 22.3 ± 3.5, respectively (Figure 4). The average (for the three quality controls) interassay C.V. was 19.5% (n = 31) and the average intraassay C.V. was 5.2% (n = 10).

Creatinine Assay

The working range for the standard curve was 0 to 40 μg/mL Cr (equivilant to 4 mg/mL taking in account 1:100 dilution required). Mean creatinine value was 1.43 ± .64 mg/mL with lowest value .23 and highest value 3.66 mg/mL. The average (for the three quality controls) interassay C.V. was 9.59% (n = 13).

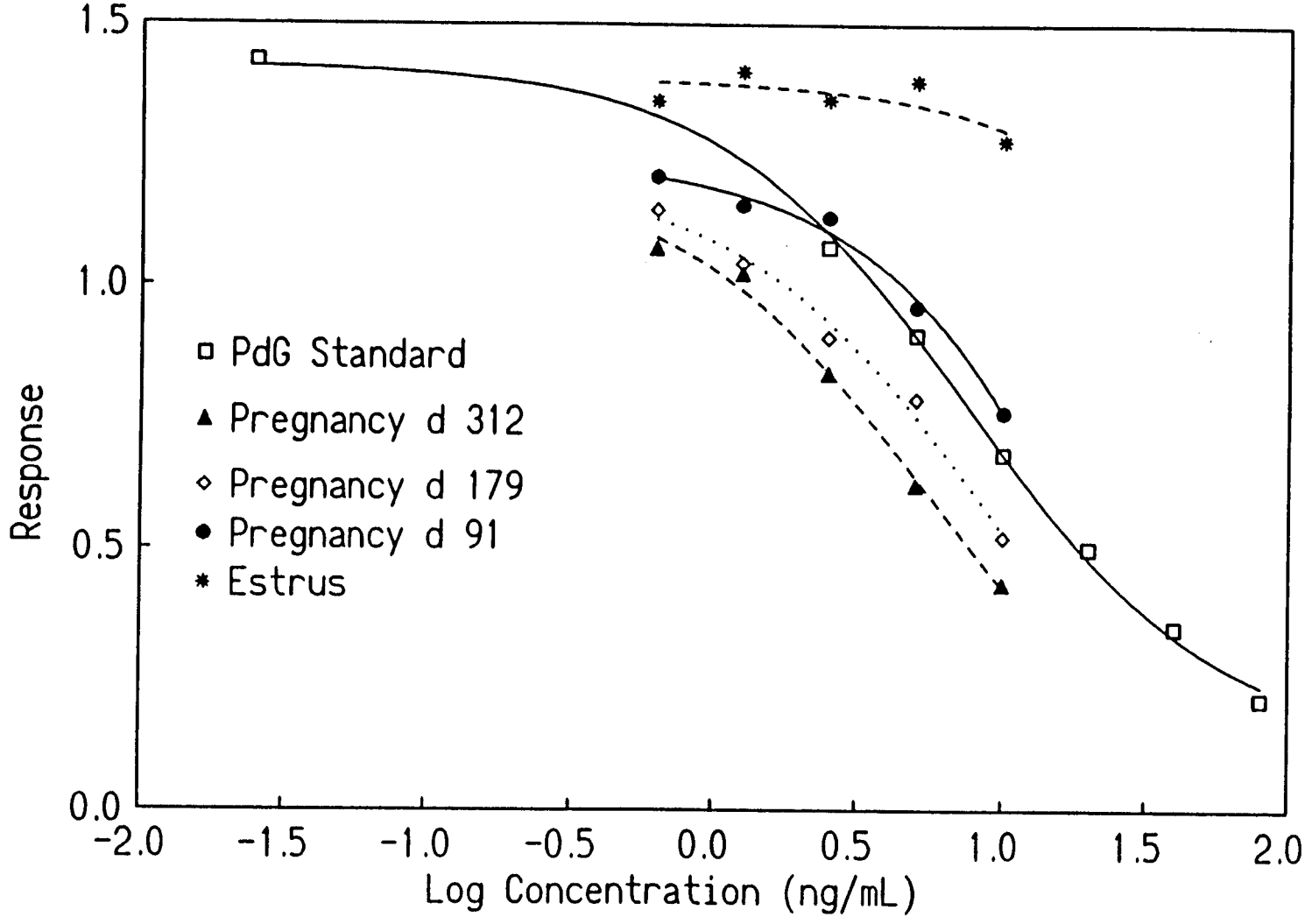

Figure 3. Displacement curve by enzyme immunoassay of pregnanediol glucuronide (PdG) and serial dilutions of urine from pregnant mares at various stages of pregnancy.

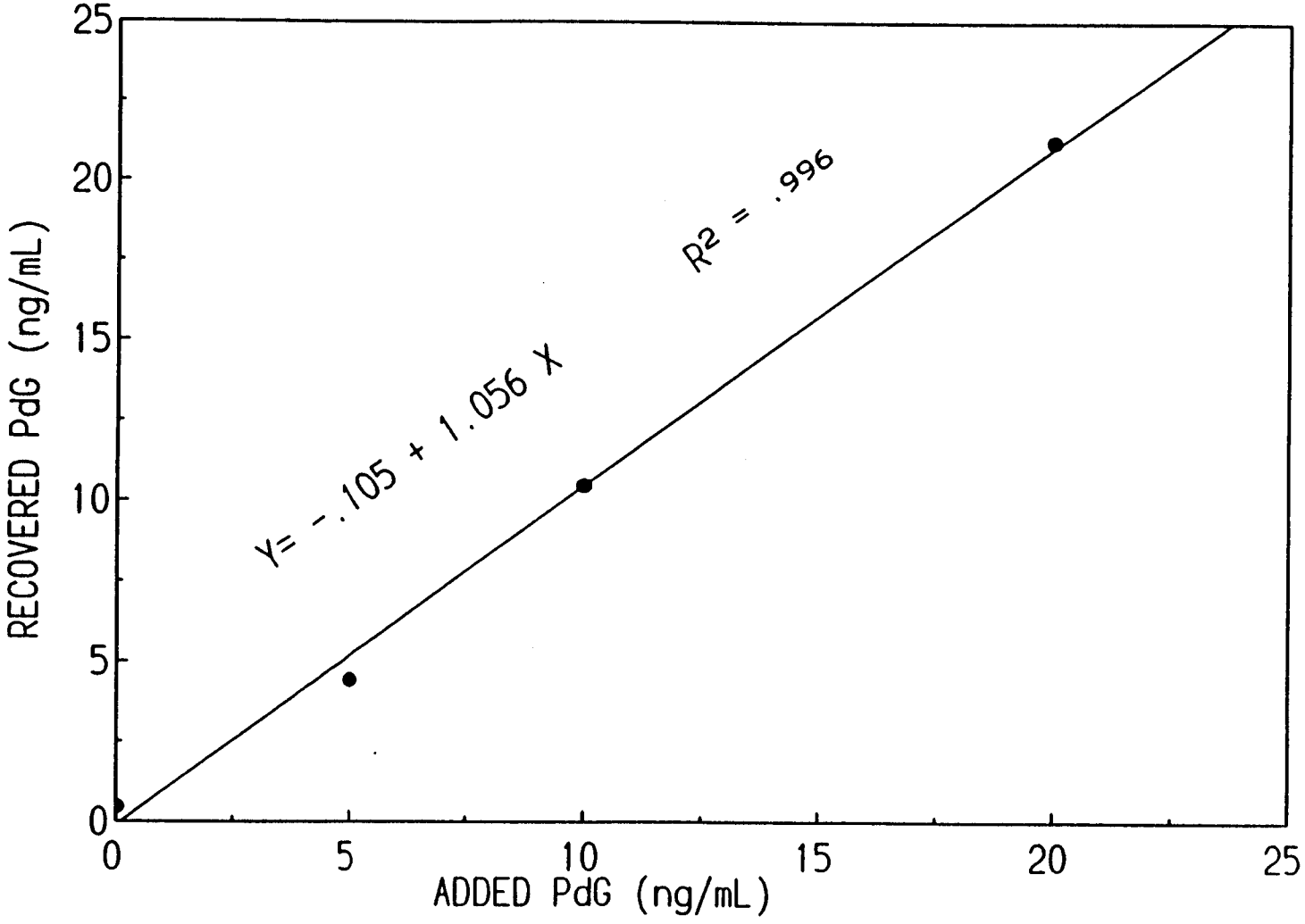

Figure 4. Regression of added and recovered pregnanediol glucuronide (PdG) in estrous mare's urine quality controls of 5, 10, and 20 ng/mg Cr using standards diluted in estrous mare's urine.

Hormonal Levels

Statistical Analyses

Table 1 shows the results for the multivariant ANOVA, using SAS to test the effects for hormones, days, and hormones-by-days interactions throughout pregnancy. Throughout the three stages of pregnancy, the difference between the concentrations of E1S and PdG are significant ($P<.05$), since E1S concentrations were in μg/mg Cr and PdG concentrations were in ng/mg Cr. There was also significant effects of hormone concentration over days throughout pregnancy ($P=.0001$). Significant effects were found throughout the three stages of pregnancy for hormones-by-days interactions ($P=.0001$), indicating that the patterns and profiles of E1S and PdG are significantly different throughout pregnancy. The results of stepwise paired t-test for individual hormones throughout pregnancy are discussed in the section concerned with each hormonal level. The correlation coefficient during early pregnancy (from d 0 to 86) was .28 ($P=.0007$), and during the second stage of pregnancy (from d 92 to 303) was -.14 ($P=.0486$). However, there was a trend of marked increase for both hormones from d 86 to 142.

Table 1. Repeated Measures Analysis of Variance Univariate Tests of Hypotheses for Within Subject Effects

Source	DF	SS	Mean Square	F Value	P > F
First Stage of Pregnancy: From d 0 to d 86					
HORMONE	1	49293683	49293683	16.88	0.0261
Error(HORMONE)	3	8759549	2919850		
DAYS	35	75457593	2155931	8.74	0.0001
ERROR(DAYS)	105	25905526	246719		
HORMONE*DAYS	35	74137946	2118227	8.74	0.0001
ERROR	105	25454903	242428		
Second Stage of Pregnancy: From d 93 to d 303					
HORMONE	1	503292586325	503292586325	90.59	0.0002
ERROR(HORMONE)	5	27778486268	5555697254		
DAYS	30	146647564020	4888252134	12.62	0.0001
ERROR(DAYS)	150	58102651502	387351010		
HORMONE*DAYS	30	146789231197	4892974373	12.65	0.0001
ERROR	150	58019486690	386796578		
Third Stage of Pregnancy: From d -36 to d +4					
HORMONE	1	5147038711	5147038711	12.10	0.0401
ERROR(HORMONE)	3	1276454920	425484973		
DAYS	11	2995885706	272353246	6.85	0.0001
ERROR(DAYS)	33	1311786349	39751101		
HORMONE*DAYS	11	2912615857	264783260	6.71	0.0001
ERROR	33	1301375252	39435614		

Estrone Sulfate

The mean concentration for E1S, (μg/mg Cr), was .38 ± .03 at d 0, decreased to .17 ± .04 at d 1, and maintained at less than .5 μg/mg Cr until d 30 (Figure 5). Although, there was an apparent increase to .80 ± .34 at d 34 (P = .122), the first significant increase was .69 ± .15 at d 46 (P = .0275). Mean concentrations remained relatively stable at this approximate level until d 60. This level was followed by sustained significant increase observed from d 60 onwards. Mean concentrations of E1S increased to 1.11 ± .25, 2.01 ± .45, and 5.48 ± 1.47 at d 64, d 76, and d 86, respectively, (Figure 5). Levels of E1S further increased to 107.11 ± 13.72 at d 121 (P = .017, compared to d 93) and reached a peak of 143.3 ± 9.51 at d 142 (P = .0006) (Figure 6), with maximum for each mare ranging from d 114 to 170, and also ranging from 115.4 to 286.1 μg/mg Cr as shown in Table 2. Although there was extreme variablity between mares at the day of peak and a wide range of maximum concentrations, the pattern for all mares was similar since the maximum concentrations were followed by a gradual decline toward parturition. Logarithmic means of E1S and PdG (Figure 7) shows the magnitude of changes of both hormones and their major significant increase, which began after d 70 of pregnancy. The first significant decrease following the maximum concentration was 91.40 ± 13.11 (P = .0024) at d 184 (Figure 6). During the last month of pregnancy, mean concentrations decreased slightly from 25.65 ± 7.5 at d 29 to 23.32 ± 9.97 at d 7 prepartum (Figure 8). Levels then

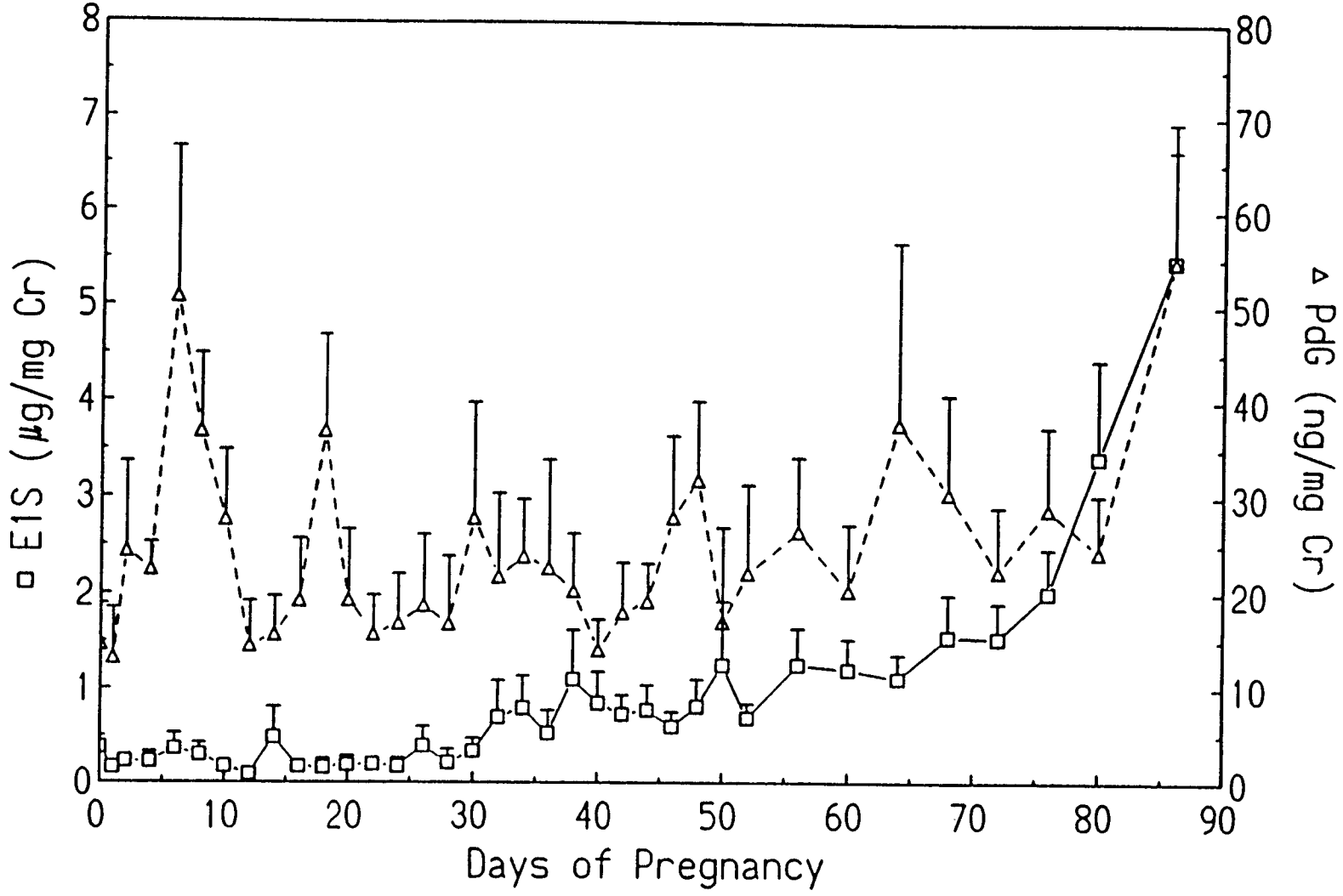

Figure 5. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) during early pregnancy in pony mares (mean ± S.E., n = 4).

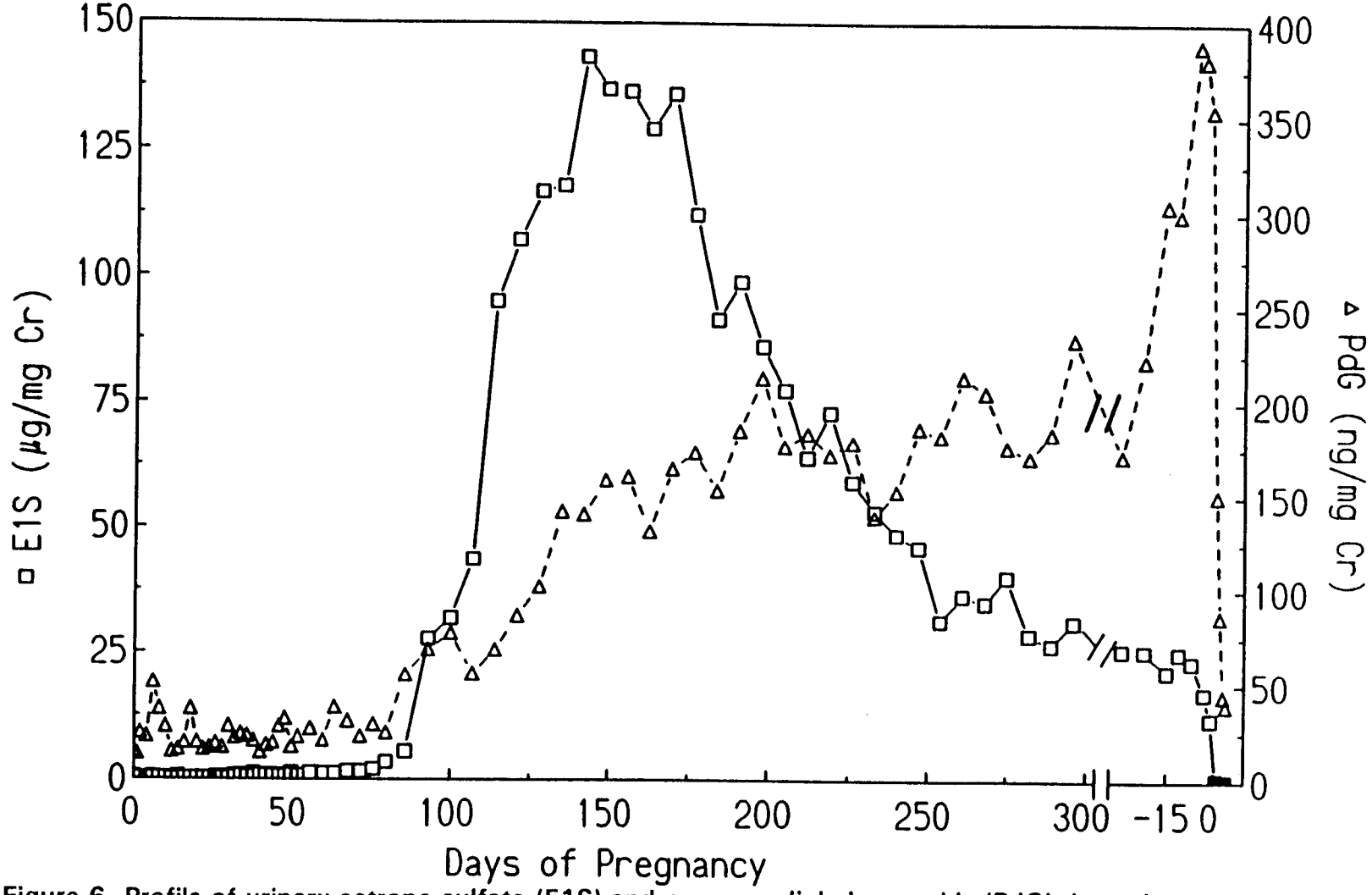

Figure 6. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy in pony mares (mean, n = 4 to 6).

Table 2. Maximum Concentration of E1S of Various Pony Mares during Pregnancy

Mare # Length*	Day of Pregnancy	Maximum (μg/mg Cr)	Gestational (Days)
75	114	206.40	---
35	142	115.38	330
81	142	158.50	---
43	149	138.20	336
60	156	185.70	326
74	170	286.10	318

* Mean Gestational Length was 327.5 days

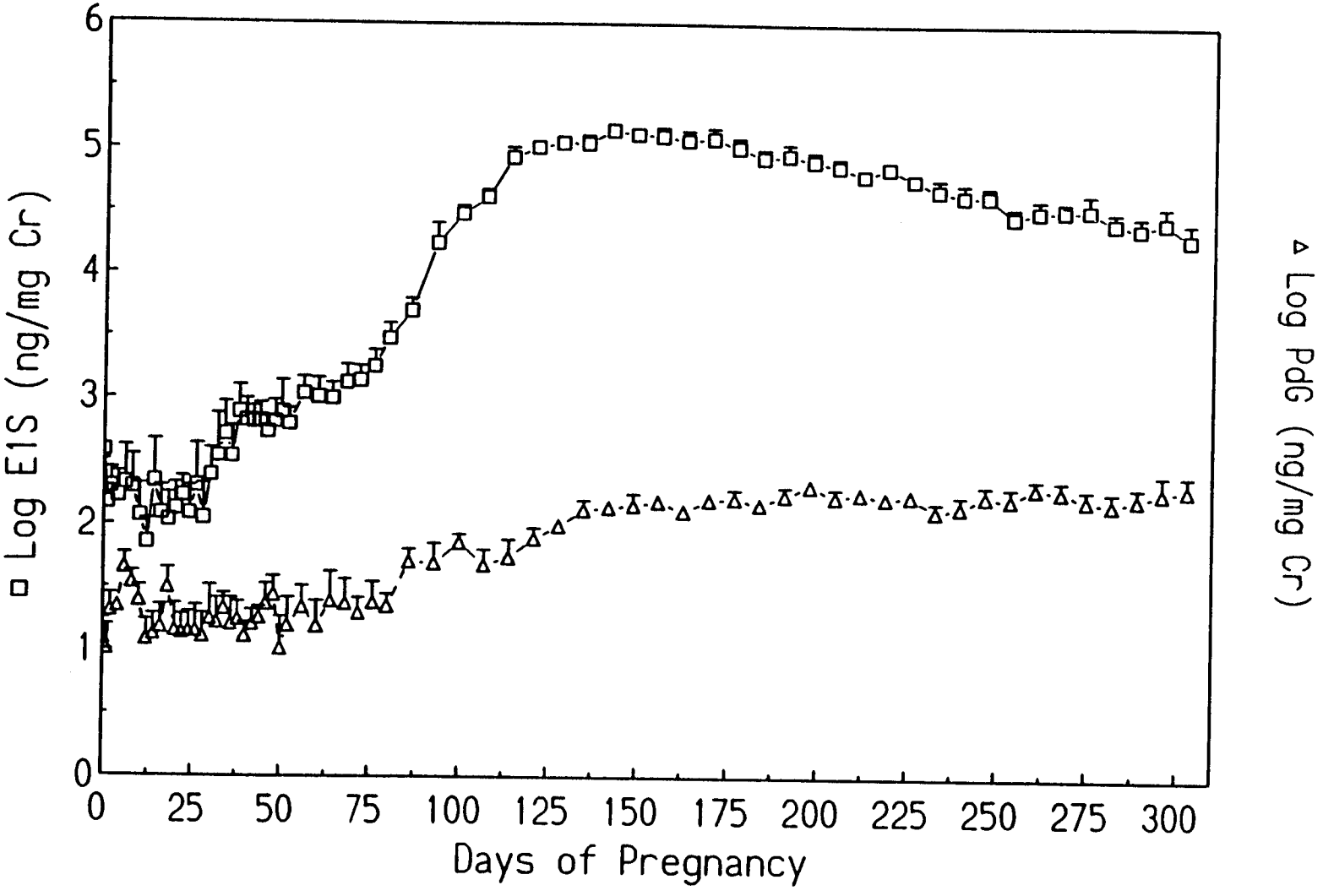

Figure 7. Logisitic means of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) during pregnancy in pony mares (n = 4 to 6).

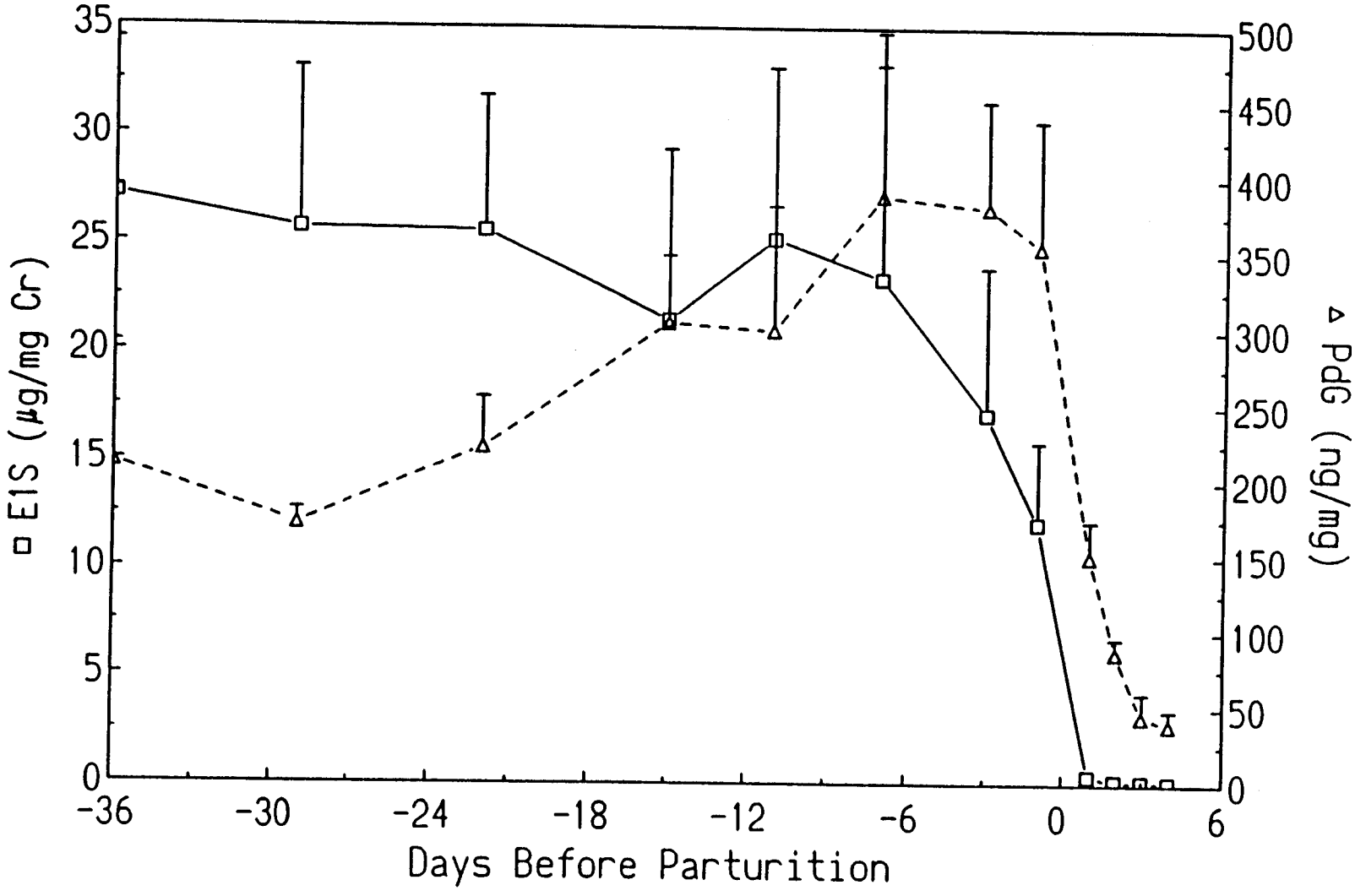

Figure 8. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) during periparturient period in pony mares (mean ± S.E., n = 4).

declined markedly to 12.08 ± 3.76 at d 1 prepartum. This pattern of decline was observed in all mares. Mean concentrations decreased significantly to baseline ($P<.05$) at d 1 postpartum onward (Figure 8).

Pregnanediol Glucuronide

The mean concentrations of PdG (ng/mg Cr) increased from 14.7 ± 4.3 at d 0 to 50.87 ± .17 (NS, $P>.05$), 36.8 ± 8.1 ($P=.016$), and 27.6 ± 7.3 ($P=.049$) at d 6, 8 and 10, respectively (Figure 5). This increase was followed by a decline and generally the levels fluctuated ranging from 20 to 30 ng/mg Cr until d 80. At d 86, the PdG levels increased to 54.7 ± 11.7 ($P=.033$) (Figure 5). This was followed by a further increase to 141.8 ± 21.4 ($P=.0139$, compared to d 93) at d 135, then continued to increase to 213.0 ± 25.2 at d 198, and remained at this approximate level until d 303 (Figure 6). During the last month of gestation, the mean concentrations of PdG increased from 171.8 ± 9.8 at d 29 prepartum to reach a peak of 388.4 ± 108.6 at d 7 prepartum (Figure 8). Maximum concentrations were followed by a slight decrease to 354.5 ± 84.0 at d 1 prepartum. Comparing to d 3 prepartum (380.52 ± 6.7), all days postpartum were lower ($P<.05$); 150.6 ± 23.4, 86.5 ± .02, 39.6 ± .01 at days 1, 2, and 4 postpartum, respectively, (Figure 8).

Figure 9 and 10 are examples of the variations of E1S and PdG patterns between mares throughout pregnancy. The pattern of E1S and PdG are similar in both mares, however, the maximum concentrations and the age of gestation were different.

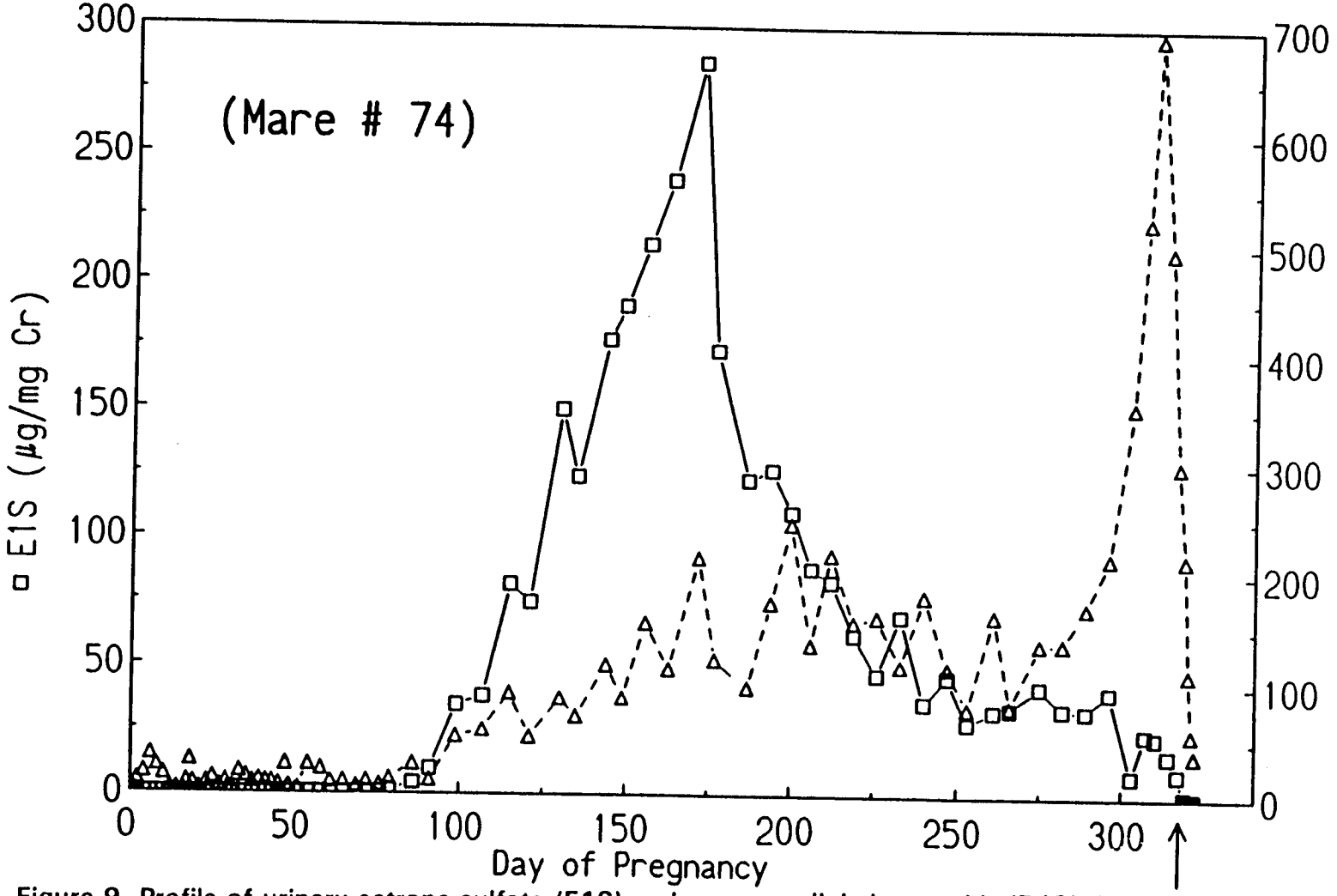

Figure 9. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy of a pony mare (from d 0 of pregnancy to d 4 postpartum, Mare # 74).

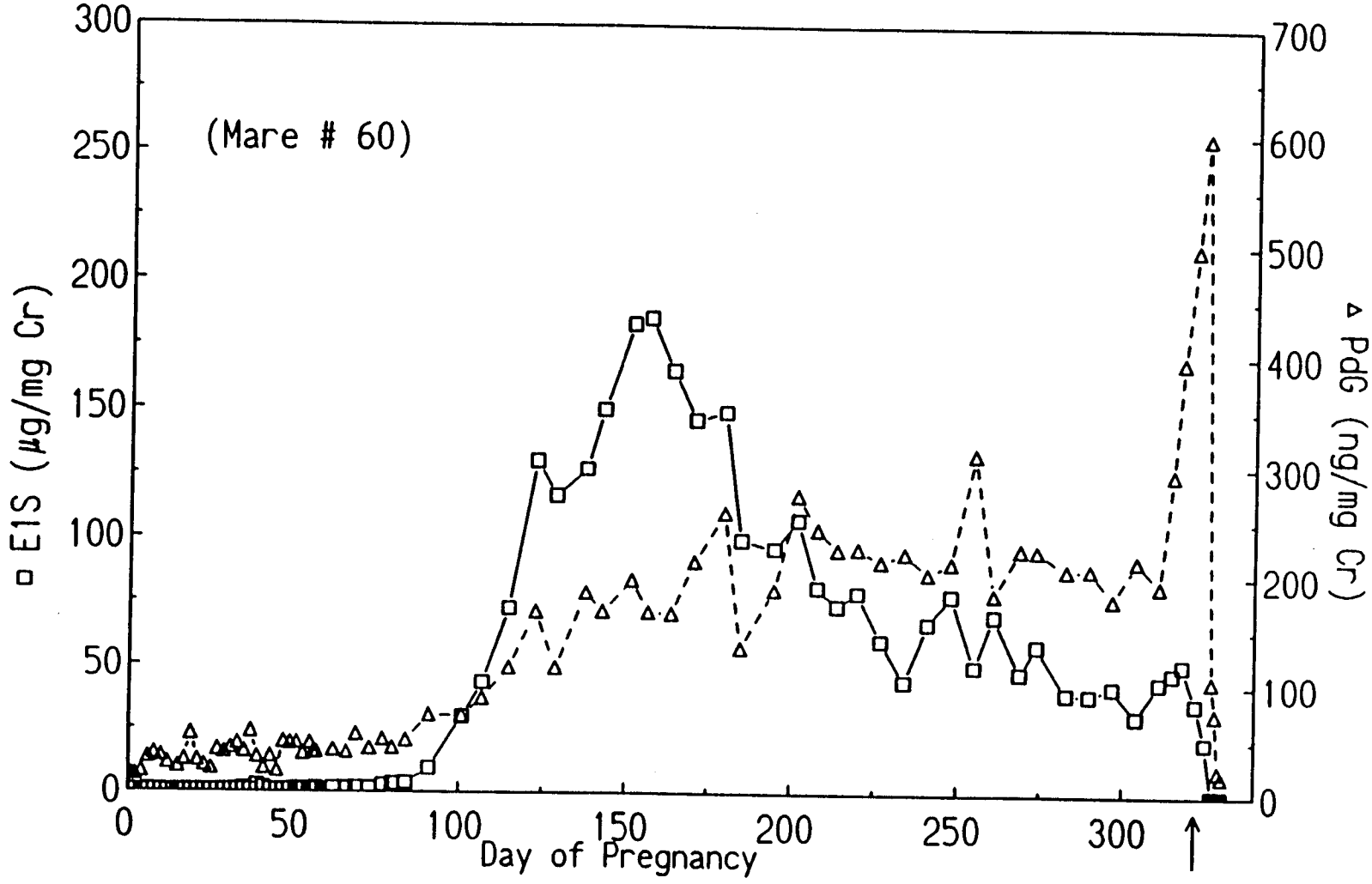

Figure 10. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy of a pony mare (from d 0 of pregnancy to d 4 postpartum, Mare # 60).

DISCUSSION

This study is the first detailed description of both E1S and PdG profiles in urine from the same mares throughout pregnancy in domestic horses using EIA. The general pattern of urinary E1S throughout pregnancy in the current study is similar to urinary EC reported by Raeside and Liptrap (1975), Czekala et al. (1990), and Monfort et al. (1991); it also agrees with plasma E1 observed by Nett et al. (1973). The general pattern of urinary PdG throughout pregnancy is also similar to those observed in plasma 5α-pregnanes (Holtan et al., 1975a, 1991)

Our data showed that the first marked increase in E1S was 2-fold and was observed at d 38 of pregnancy. These results agree with previous studies in urine (Evans et al., 1984; Czekala et al., 1990; Daels et al., 1990, 1991a,b; Hyland and Langsford, 1990; Lasley et al., 1990; Monfort et al., 1991) and in plasma (Terqui and Palmer, 1979; Kindahl et al., 1982; Hyland et al., 1984; Daels et al., 1991a; Stabenfeldt et al., 1991). However, Nett et al. (1973, 1975) were unable to report any significant increase in estrogen secretion around d 37 when determining plasma unconjugated estrogen concentrations by RIA. This could be related to the fact that plasma conjugated estrogens are present in 100-fold higher concentrations than unconjugated estrogens (Palmer and Terqui, 1977). The horse embyro is capable of synthesizing estrogens as

early as d 7 of gestation in-vitro (Flood et al., 1979; Heap et al., 1982; Zavy et al., 1984), but not in levels high enough to be secreted in the maternal blood (Daels et al., 1991b).

Figure 5 shows that, after d 34, the concentrations of E1S were followed by a plateau of approximately 1.0 μg/mg Cr between d 38 to 60. The second marked increase was observed at d 68 of pregnancy and E1S levels increased to 1.6 ± .4 μg/mg Cr (5-fold). These results are in agreement with levels measured in urine (Daels et al., 1990; Monfort et al., 1991) and in plasma (Terqui and Palmer, 1979; Stabenfeldt et al., 1991). The main source of estrogen secretion during the first 35 to 45 days of pregnancy is the ovary (active CL) rather than the feto-placental unit (Terqui and Palmer, 1979; Daels et al., 1990, 1991b). A temporal relationship exists at d 40 of pregnancy between the increase of eCG and estrogen concentrations in the presence of an active CL, suggesting that eCG stimulates luteal estrogen synthesis (Daels et al., 1991b).

Figure 5 also illustrates that, after d 68, the mean concentrations of E1S increased gradually to 3.4 ± 1.0 μg/mg Cr at d 80. This finding agreed with previous reports (Terqui and Palmer, 1979; Daels et al., 1990; Stabenfeldt et al., 1991). The primary source of estrogens between d 45 to 70 of pregnancy is the ovaries and(or) feto-placental unit (Kasman et al., 1987, 1988; Jeffcott et al., 1987; Hyland and Langsford, 1990). This may be the result of the correlation between the presence of a viable fetus and high estrogen

concentrations in plasma and urine and the rapid decline of the concentrations following the termination of pregnancy at d 45, either by the surgical removal of the fetus (Kasman et al., 1987, 1988) or induction of fetal death by the injection of hypertonic saline solution into the fetal sac (Jeffcott et al., 1987; Hyland and Langsford, 1990). On the other hand, Daels et al. (1991b) and Stabenfeldt et al. (1991) demonstrated that estrogen concentrations, between d 35 and 70 of pregnancy, are not directly correlated with fetal viability. This appears to be reflected by the presence of an active CL and is shown by the rapid decline of estrogen concentrations in the absence of active CL following the administration of $PGF_2\alpha$ on d 16 (Daels et al., 1991b) or injection of endotoxin that releases $PGF_2\alpha$ between d 35 and 70 of gestation (Stabenfeldt et al., 1991). Furthermore, endotoxaemia without fetal death between d 35 and 70 of pregnancy results in marked transient decreases in plasma estrogen concentrations. Fetal death without CL regression after d 35 does not produce an immediate decline in plasma estrogen concentrations, and existing levels are maintained for 10 to 14 days (Stabenfeldt et al., 1991).

Figures 6 and 7 show that, after d 80, the mean concentration of E1S increased abruptly and peaked (143.3 ± 9.5 μg/mg Cr) at d 142 (range d 114 to 170), and then followed a gradual decline toward parturition. This pattern, in general, agrees with previous studies in plasma (Nett et al., 1973, 1975) and urine (Raeside and Liptrap, 1975; Monfort et al., 1991). However, the peak urinary E1S of our results was higher than those measured by Czekala et al.

(1990) and Monfort et al. (1991). Monfort et al. (1991) measured urinary E1S using direct RIA with antibody (R583) directed against estrone-3-glucuronide:BSA and this antibody was the same as that used in the current study. The maximun concnetration was less than our study and this might be related to the breed of horses and type of immunoassay (RIA vs EIA). Maximum urinary E1S in our findings was also about 30 to 45 days earlier in gestation than those determined in urine (d 180, Raeside and Liptrap, 1975) and in plasma (d 210, Nett et al., 1973, 1975). This difference might be related to different techniques or infrequent sampling in previous studies.

The mean concentrations of E1S was at the baseline one day postpartum (figure 8). This finding agreed with previous studies (Nett et al., 1973, 1975; Raeside and Liptrap, 1975; and Monfort et al., 1991). After d 70 until parturition, the principal site of estrogen production is the feto-placental unit (Nett et al., 1973; Raeside et al., 1973; Pashen and Allen, 1979; Kasman et al., 1987, 1988). The enlargement and regression of the fetal gonads parellel the increase and decrease of estrogen concentrations during gestation. The removal of fetal gonads in mid-pregnancy causes an immediate decline to basal levels of estrogen in maternal plasma (Raeside et al., 1973; Pashen and Allen, 1979). Estrogen levels remain low after gonadal removal throughout gestation; however progestin levels appeared to be unaffected (Pashen and Allen, 1979).

Estrogens may play a part in normal fetal development because fetal gonadectomy caused retarded growth and development in three of four gonadectomized fetuses (Pashen and Allen, 1979).

To the best of our knowledge, there were no previous studies determining the profile of urinary PdG throughout pregnancy in equine, except few studies limited to particular stages of gestation in uncaptured feral horses. Therefore, the current results can be compared to plasma progestins throughout pregnancy, and urinary and fecal progestins at specific stages of gestation in the horse. Our data showed that the mean concentrations of PdG increased 3-fold on d 6 and followed by a decline and remained low until d 80 of pregnancy (Figure 5). This first increase could be related to the presence of CL. After d 80, the concentrations increased rapidly until d 198 of pregnancy, maintained at this level until d 300 (Figure 6, 7). This pattern did not follow the reported pattern of plasma progesterone. Plasma progesterone concentration increased from 1 ng/ml on d 0 to 7 ng/ml on d 8 of pregnancy, followed by a transient (nonsignificant) decrease to 5 ng/ml on d 28, and then increasing from d 28 to reach a peak on d 64. The level of progesterone gradually declined after d 64 and remained between 1 to 2 ng/ml from d 180 to 300 (Allen and Hadley, 1974; Holtan et al., 1975b,c). However, our data were somewhat similar to 5α-pregnanes in plasma. Levels of 5α-pregnanes in plasma are first observed between d 30 and 60, elevate gradually to d 300, and increase rapidly in the last 30 days prepartum (Holtan et al., 1975a, 1991).

Our findings also showed that the concentrations of PdG increased abruptly in the last 4 weeks prepartum, followed by a decrease one day prepartum, and then a rapid decline 1 to 3 days postpartum (Figure 8). This pattern agreed with the concentration of plasma progesterone (Ganjam et al., 1975; Holtan et al., 1975b), plasma 5α-pregnanes (Holtan et al., 1975a, 1991), and fecal progestagens (Schwarzenberger et al., 1991).

Progesterone is metabolized to saturated 5α-pregnane derivatives and 5α-pregnane sulfate (and)or glucuronide was identified in urine of mares in late pregnancy. This suggested that horse placenta and fetus contribute to the urinary output of progestins (Hirschman and Williams, 1964; Ainsworth and Ryan, 1969). The source of 5α-DHP and 3ß-5P during early pregnancy may be maternally derived. During mid-and late pregnancy, the source of other progestins, including 5α-DHP and 3ß-5P, may be the feto-placental unit (Holtan et al., 1991). On the other hand, Hamon et al., (1991) found that the production of 5α-DHP during late pregnancy is derived primarily from endometrial metabolism of pregnenolone and(or) progesterone. Our findings suggested that PdG might be produced and(or) metabolized by the feto-placental unit and this could be related to the high level of PdG in mid- and late gestation and the low level of PdG 1 to 3 days postpartum.

Using EIA, Kirkpatrick et al. (1990b) studied iPdG, indexed to Cr, in uncaptured feral horses. In the first experiment, urine samples were collected from the snow at d 180 to 200 of gestation with iPdG for mares producing

foals 167 ± 80.33 ng/mg Cr and 7.04 ± 1.69 ng/mg Cr for mares that did not produce foals. In the second experiment, urine samples were collected from the ground at d 120 to 180 of gestation with iPdG for mares producing foals 215.8 ± 83.4 ng/mg Cr and 3.6 ± .499 ng/mg Cr for mares that did not produce foals (Kirkpatrick et al. 1990b). These results showed that the concentrations of iPdG in pregnant mares was significantly higher than the concentrations of iPdG in non-pregnant mares. Our data showed essentially the same concentration in domestic horses (Figure 6).

Although the current study did not assay urinary E1S and PdG during the estrous cycle, it appeared from previous studies that urinary E1S correlates with plasma E1S (Daels et al., 1991a) and urinary PdG correlates with plasma progesterone (and)or progestins (Kirkpatrick et al., 1990a). This suggests that the assay of current study could be used for measuring urinary E1S and PdG throughout the estrous cycle.

In summary, in comparing the two hormones, E1S remained baseline followed by a slight increase at d 35, whereas PdG was relatively stable until both hormones increased after d 70 of gestation. This might be related to secretion of both hormones by the fetus and their rapid metabolism by placenta. Estrone sulfate reached a peak at approximately d 148 followed by a decline toward parturition while PdG showed a rapid increase from d 70 to 150, followed by a slow sustained increase to d 300 then increased dramatically again before parturition, while E1S continued to decline. Since the

patterns of urinary E1S and PdG are different, their sites and mechanism of metabolism are likely different. The results indicate that the presence of the feto-placental unit is important for the secretion of both estrogens and progestins throughout pregnancy and thus could be utilized as a reliable method for pregnancy determination after three months of gestation.

A recent report indicated that the pattern of plasma relaxin throughout gestation correlates with plasma E1 in Standardbred mares (Stewart et al., 1992) and thus with urinary E1S in the current study. However, the pattern of plasma relaxin in Thoroughbred mares (Stewart and Stabenfeldt, 1981) was not well correlated with E1S and seems to correlate better with urinary PdG in the current study.

IMPLICATIONS

The presented study is the first to determine the levels of both urinary E1S and PdG throughout pregnancy in pony mares. The results from this study may be used to determine pregnancy and viablity of a live fetus in domestic and wild horses. The safety, economy, and simplicity of use of direct EIA in unextracted urine samples made it more convenient than using RIA. These methods could also be useful in wildlife species, without capture, to determine pregnancy and abortion, and to study infertility. The identification and measurement of urinary estrogen and progestin metabolites throughout

pregnancy by using GC/MS should be used to identify specific hormones that are measured by immunoassays. Further studies on both E1S and PdG should be performed to determine if urinary values reflect plasma values throughout pregnancy; fecal hormonal analyses may also be included in this comparison.

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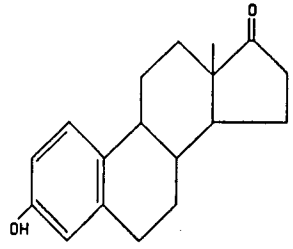
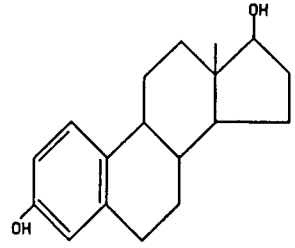
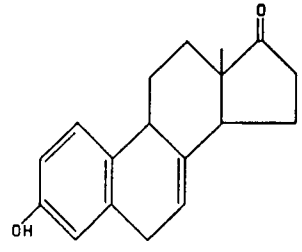
APPENDIX



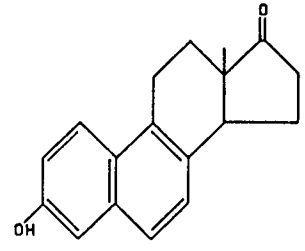
a) ESTRONE (E1)



b) 17β-ESTRADIOL (E2)

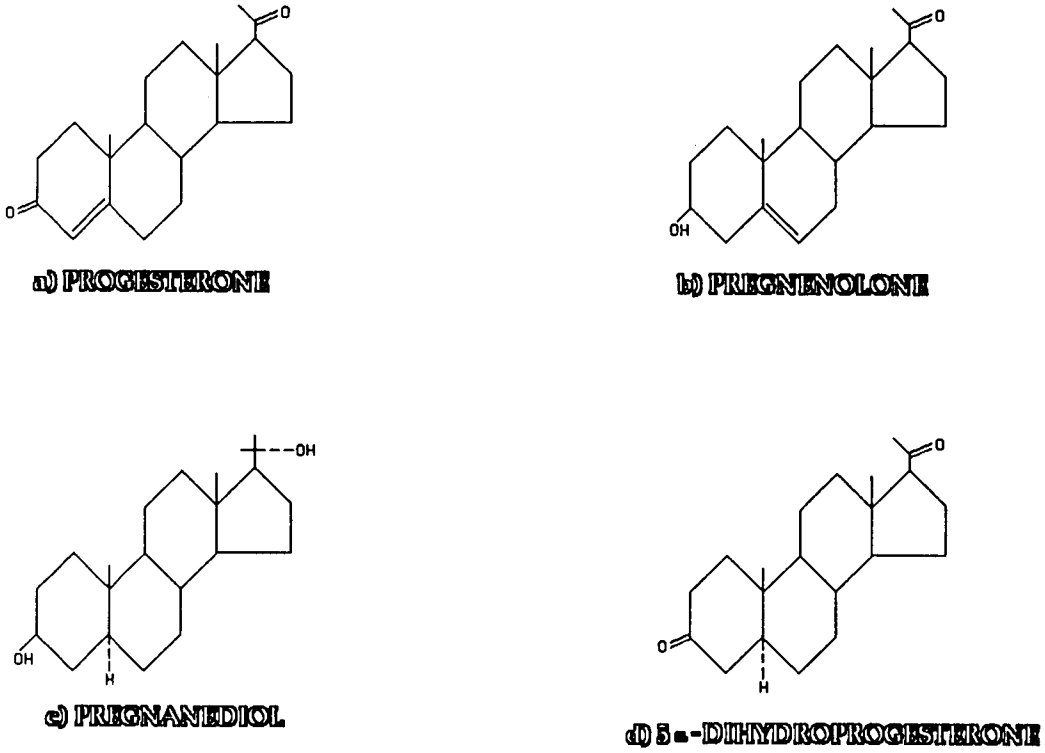


c) EQUILIN

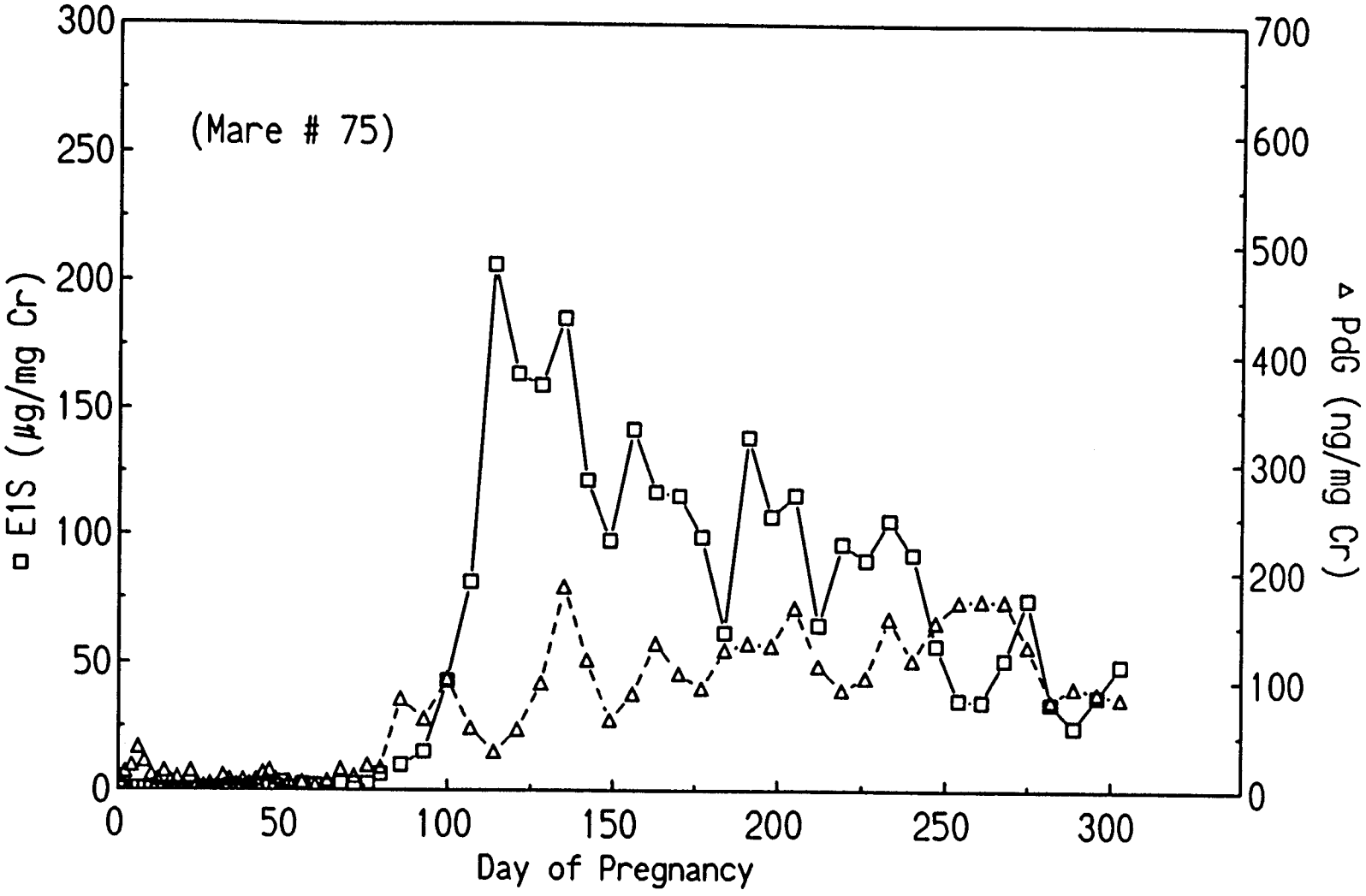


d) EQUILENIN

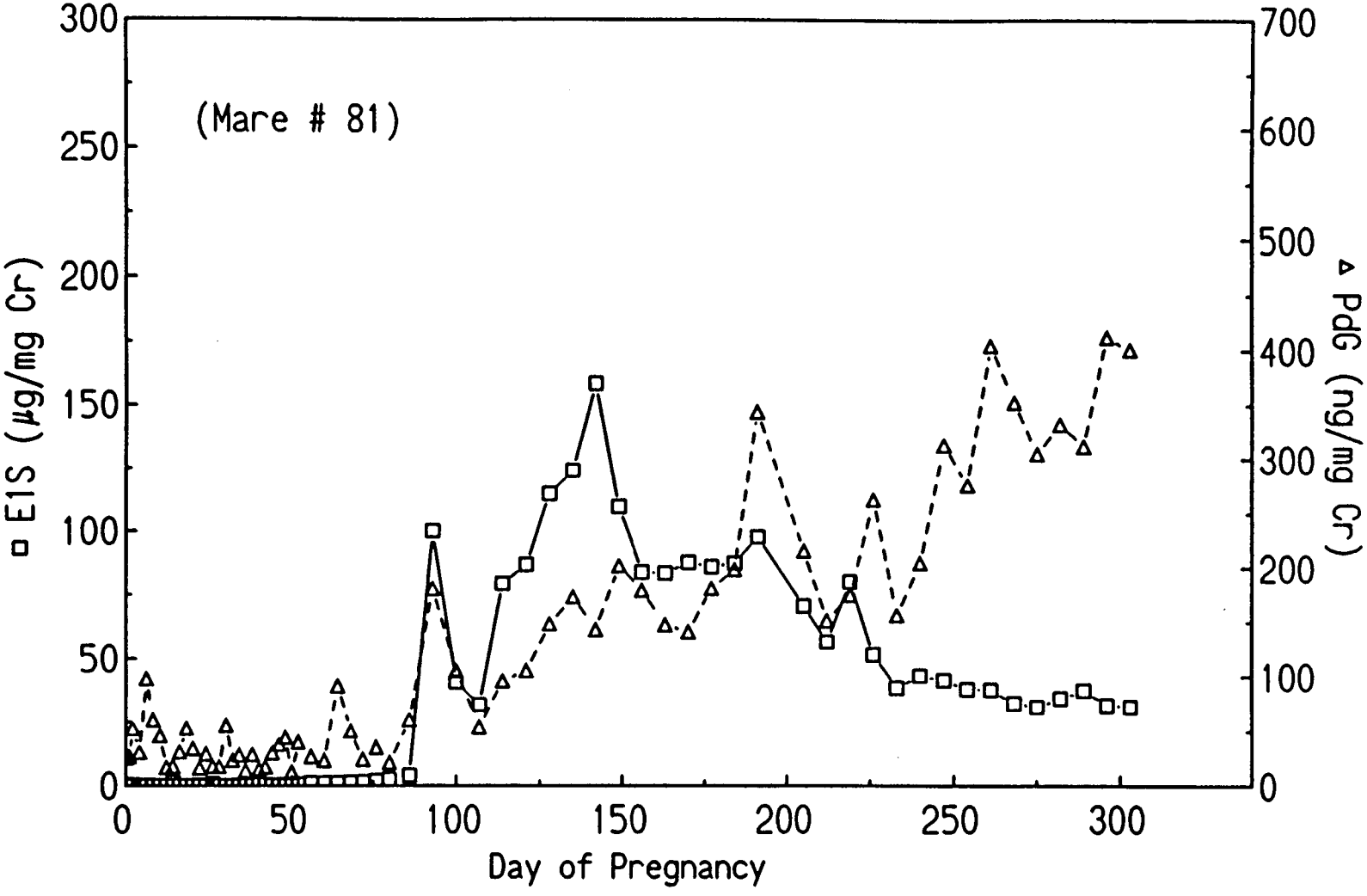
Appendix Figure 1. The structures of some estrogens in pregnant mare.



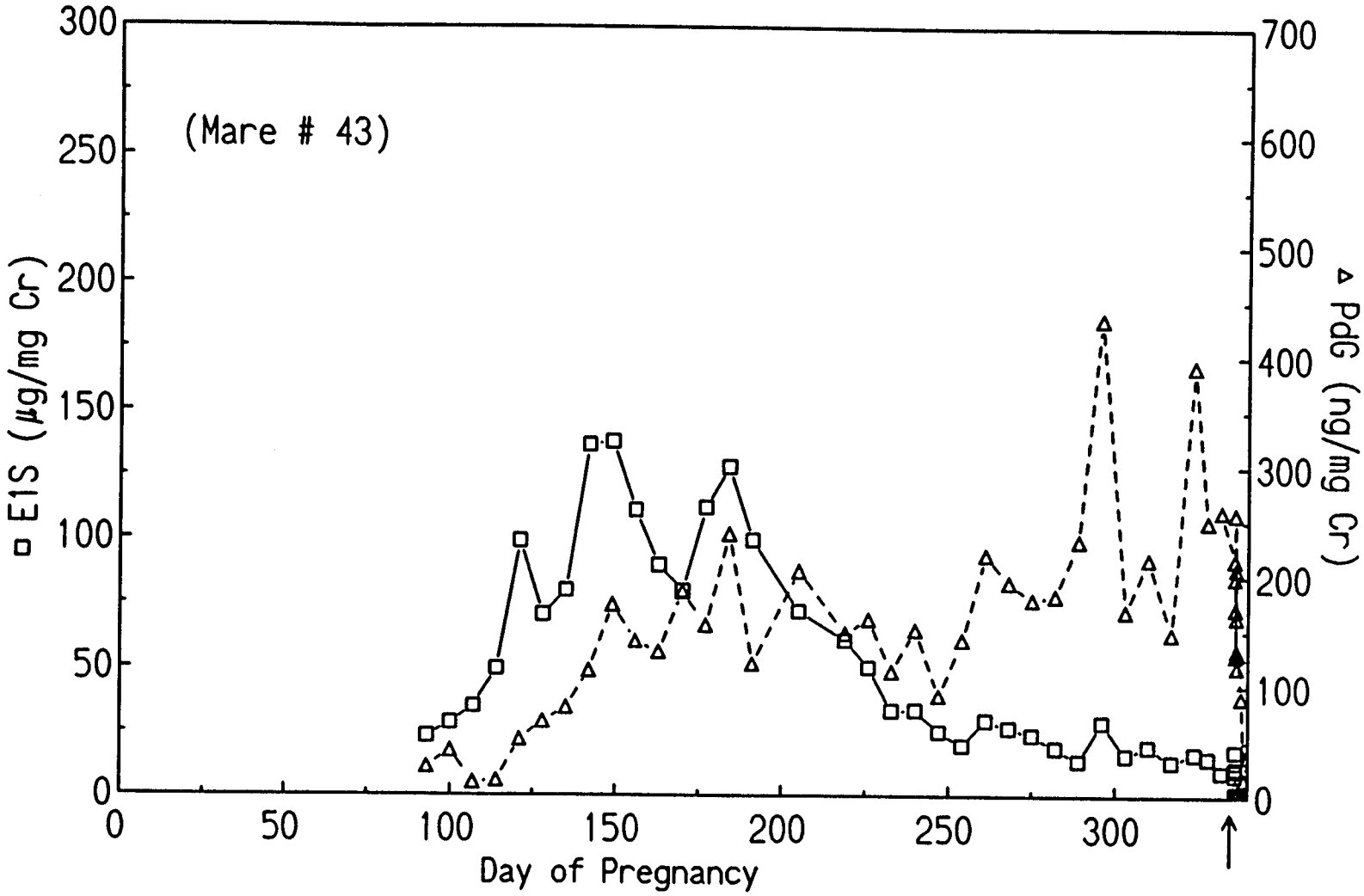
Appendix Figure 2. The structures of some progestins in pregnant mare.



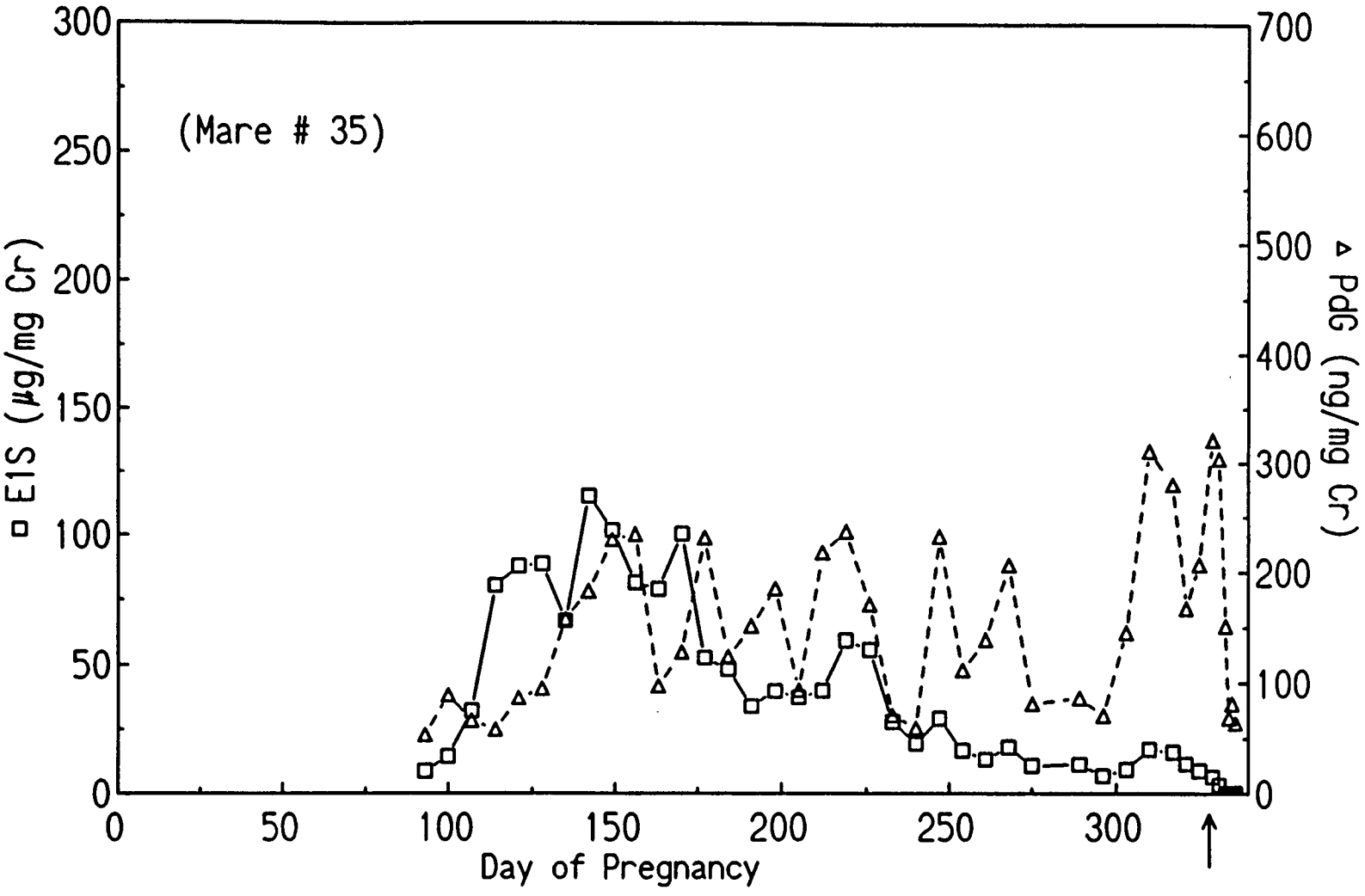
Appendix Figure 3. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy of a pony mare (from d 0 to d 303 of pregnancy, Mare # 75).



Appendix Figure 4. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy of a pony mare (from d 0 to d 303 of pregnancy, Mare # 81).



Appendix Figure 5. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy of a pony mare (from d 93 of pregnancy to d 4 postpartum, Mare # 43).



Appendix Figure 6. Profile of urinary estrone sulfate (E1S) and pregnanediol glucuronide (PdG) throughout pregnancy of a pony mare (from d 93 of pregnancy to d 4 postpartum, Mare # 35).