EVALUATION OF SALIVARY PROGESTERONE PROFILES AS AN INDICATOR OF REPRODUCTIVE STATUS IN EQUINES

BY

SWANAND RAMESH SATHE

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS - Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Master's Committee:

Clinical Associate Professor John Arthur Herrmann, Chair
Professor Emeritus Janice Bahr
Clinical Assistant Professor Debra S Sauberli
Associate Professor Sherrie Clark (VA-MD Regional College of Veterinary Medicine)

ABSTRACT

Diagnostic assays of reproductive hormones are usually performed by veterinarians on mares after inconclusive findings from trans-rectal palpation or ultrasound genital examination, or on intractable mares. Of the reproductive hormones, progesterone (P4) is one of the most commonly measured hormones in the field of equine reproduction. Serum concentration of P4 from mares is usually evaluated using an extracted radioimmunoassay (RIA). The use of RIA for serum P4 measurement usually involves an extraction process to dissociate the steroids in blood from carrier proteins, which bind more close to 90 to 95% of the steroid hormone present. Estimation of salivary steroid hormones presents an attractive alternative, since the steroids are not protein bound and represent the free fraction which appears to represent the biologically active form. The objective of this study was to develop and validate a sensitive and specific RIA for estimation of salivary P4 concentration and evaluate its usefulness in identifying the stage of the estrous cycle and early pregnancy in mares. Serum and saliva samples were collected on selected days of the estrous cycle and early pregnancy (up to day 65) from mares and were validated using a liquid phase RIA. Saliva samples did not require solvent extraction and were run without this processing step. The inter-assay coefficient of variation (CV) for low and high controls was 6.91% ± 0.81 (Mean ± S.E.) and 5.06 % ± 0.57 (Mean ± S.E.), while the intra-assay CV averaged 13.19% for saliva and 11.71% for serum. Serum P4 showed statistical significance with Friedman's One Way ANOVA ($p < 0.0001$) but, no significance was noted for the saliva samples ($p > 0.05$). Saliva: Serum ratio was elevated during the follicular phase of the cycle (35%), remained between 8 to 12% for the remaining duration of the cycle and showed a statistical significance ($p > 0.05$). As anticipated, there was a significant difference in the serum

P4 levels by day of observation in the pregnant mares ($p<0.0001$). Statistical significance for salivary P4 concentration for pregnant mares was seen ($p > 0.05$), only for days 1 and 3. Sa: Se ratio for pregnant mares showed significance ($p > 0.05$) but with only day 1 contributing to the significance. Pearson's correlation analysis showed that the correlation between salivary and serum progesterone levels in cycling mares was not significant except on day 5 for the cycling mare group ($p >0.05$), whereas for pregnant mares only days 5 and 8 ($p < 0.05$) were seen to correlate significantly. The study shows that salivary P4 concentration in mares was not statistically correlated with serum values, but nevertheless can be utilized to monitor the luteal phase of the estrous cycle as well as for estimating P4 concentrations during early pregnancy.

To my wife Malavika for always being there.

ACKNOWLEDGEMENTS

I am extremely thankful to my advisor, Dr. John Herrmann for not only guiding me during my research, but also for all the support he showed during my residency training. He truly got me interested in the bovine side of Theriogenology. Many thanks to my advisory committee members, Dr. Sauberli, for teaching me the finer nuances of equine reproduction and Dr. Clark for her patience and unstinted support during my research and training program.

And special thanks especially to Dr. Janice Bahr, who students so rightly admire and call the RIA Queen. This research would not have been completed without her guidance and her generosity.

A big thank you to Dr. Yvette Johnson and Dr. San Myint for taking time and helping me understand the statistical analysis of my data.

I am extremely grateful to Dr. Cliff Shipley for teaching me the importance of being a better person first and then a clinician. You are the coolest theriogenologist around!

A big thanks to the U of I horse barn staff, Dr. Kevin Kline, Ivy Hilliker, Jamie and Lacey who so patiently helped me to catch mares in the paddocks during my sample collection. It was a wonderful four years working the breeding season with you all.

Special thanks to Dr. Romana Nowak and her lab, Annie, Fae, Victoriya, Andrea and Jia Jia for all their help.

Most importantly my wife, mother and sister who have always been the source of my inspiration, for their love and support.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION…………………………………………………………....1

CHAPTER 2: LITERATURE REVIEW……………………………………………………..…5

CHAPTER 3: MATERIALS AND METHODS……………………………………..……….13

CHAPTER 4: RESULTS………………………………………………………………...….24

CHAPTER 5: DISCUSSION……………………………………………………………….29

CHAPTER 6: CONCLUSIONS……………………………………………...…………….33

CHAPTER 7: TABLES AND FIGURES……………………………………..…………….34

REFERENCES ...……………………………………………..……………………………59

CHAPTER 1

INTRODUCTION

Equine reproduction is a highly demanding and performance based field for veterinarians due to the limited time available in trying to get the mare bred and in foal as early as possible in the breeding season. To meet these demands, veterinarians are largely dependent on various diagnostic modalities to accurately assess a mare's reproductive status, among which trans-rectal palpation and ultrasound of the mare's reproductive tract and immunoassays for various reproductive hormones are the most commonly utilized.

Diagnostic assays of reproductive hormones are usually performed on mares after inconclusive findings from genital examination, on irregularly cycling mares or on intractable mares to evaluate their reproductive status. Of the reproductive hormones, progesterone (P4) a C21 steroid hormone produced primarily by the corpus luteum, is one of the most commonly measured hormones in the field of equine reproduction. It is estimated that as many as 120 assays for serum P4 are run per day at the BET labs in Kentucky during the peak breeding season using radioimmunoassay (RIA) technique (Douglas, 2004). In equines, the plasma P4 concentrations increase significantly within the first 12 hours (Plotka et al., 1975) and are usually greater than 2 ng/ml by 48 hours, post ovulation (Townson et al., 1989; Koskinen et al., 1990). P4 concentration continues to rise until it reaches a plateau by around day 6-8 post ovulation (Nagy et al., 2004). In non-pregnant mares there is a sharp decrease in plasma P4 beginning by around day 14 due to a lack of a maternal recognition of pregnancy factor. However in pregnant mares the P4 concentration remains fairly high in serum until the second half of gestation when

the feto-placental unit produces a range of 5α-reduced metabolites of progesterone, most notably dihydroprogesterone (5α-DHP) and 20α-dihydroxy-5-pregnan-3-one (20α-5P), which predominate in the maternal circulation, and P4 itself is barely detectable (Short, 1959; Holtan et al., 1991).

In routine breeding management of the mare there are a number of clinical applications for serial P4 assays which include determination of the adequacy of the corpus luteum in a pregnant mare, determination of the day of ovulation, presence or absence of luteal tissue, and assessment of the end of seasonal transition (Douglas, 2004). Serial P4 assays are also run on mares that are in danger of early or late pregnancy loss or on mares that have a history of experiencing early embryonic death and are suspected of suffering from luteal insufficiency. It is due to an often unsubstantiated concern, that thousands of mares are administered exogenous progestagens to assuage owner and/or veterinarian fears that a failure of production of sufficient endogenous progesterone might cause embryonic or fetal death and abortion (Allen, 2001).

Serum concentration of P4 from these mares is usually evaluated using an extracted radioimmunoassay (RIA) or an enzyme linked immunosorbent assay (ELISA), of which the RIA is generally considered as the gold standard in reference laboratories. The use of RIA for serum P4 measurement usually involves an extraction process which is both labor intensive and biohazardous as it involves use of petroleum ether, methanol, and dry ice that must be used in a fume hood. This extraction procedure is required because it has been well documented that in species like humans (Tallon et al., 1984) and cows (Kanchev, 1976) more than 80% of circulating P4 is bound to plasma proteins and only the free fraction which appears to be biologically active (Riad-Fahmy et al., 1982; McGarrigle et al., 1984) is found in saliva. In equines, only about 1-3% of P4 in the blood of mares is free (ie. unbound) and the remainder is

bound to cortisol binding globulin (CBG) or albumin, which are carrier proteins that interfere with the antigen-antibody reaction thus necessitating solvent extraction (Carriere and Lee, 1994). Serum proteins like albumin or corticosteroid binding globulin are too large to pass through the membranes of the salivary cells. Since 90-99% of steroids in the blood are bound by these specific or non-specific proteins, only the free, non-bound steroid molecules are able to pass into saliva. Thus salivary concentration of steroid hormones such P4 reflects the free or unbound fraction.

It has been shown that there is a good correlation between serum and salivary P4 levels, and salivary steroid concentrations appear to be closely related to unbound steroid levels in serum, which may reflect the biological activity of the hormone (Choe et al., 1983). Measurement of salivary P4 concentration appears to be an attractive alternative to serum analysis since sample collection is easy and noninvasive and hormone levels in saliva are thought to be a better measure of steroid exposure to target organs (Chiappin et al., 2007). The non-invasive nature and ease of sample collection is especially useful when multiple samples have to be collected over a period of time to monitor hormone levels in cases of high risk pregnancies or as a part of research studies.

Measurements of steroid hormones in saliva have been popular in human medicine for the past 30 years (Lima et al., 2010). Since that time its popularity has increased due to the attractiveness of non-invasive, repeated and simple stress-free sampling and it has proven to be a popular sampling fluid for psychobiology, sports medicine, pharmacology and pediatric studies as well as in the area of complementary medicine. In the diagnostic laboratory, salivary P4 and estradiol have been used for assessing ovarian function and 17α-OH P4 for the diagnosis of congenital adrenal hyperplasia (CAH). In human medicine, salivary cortisol is used for

investigating adrenal function and recently there has been considerable interest in the use of bedtime salivary cortisol levels as a screening test for Cushing's disease (Lewis 2006). In veterinary medicine, the diagnostic value of saliva as a body fluid has been realized. Studies in a wide variety of species ranging from bovine (Kanchev and Stankov, 1988) to the Indian rhinoceros (Gómez et al. 2004) have been performed to evaluate the usefulness of salivary steroids in the assessment of ovarian function, with encouraging results. In equines, studies validating saliva-sampling as noninvasive techniques for cortisol level assessment have been performed (Peeters et al. 2011). However, no validated studies comparing salivary P4 profiles to serum levels have yet been published (Douglas, 2004). A quantitative and accurate P4 assay that involves the least invasive method of sample collection would be a valuable addition to the various commercial assays currently available to the equine industry. Such an assay would preclude the need for personnel trained in venipuncture for repeated sample collection and would be more receptive to the horse owner or the barn manager. Thus, the objective of the current study was to develop a quantitative, sensitive and specific P4 RIA assay that could be used on non-extracted saliva.

The aims of the study were as follows:

1. To validate a radioimmunoassay for non-extracted equine salivary P4
2. To compare and correlate the serum and salivary P4 profiles in cycling mares and mares in early gestation (up to day 65).
3. To evaluate the usefulness of salivary P4 in the identification of the proper stage of estrous cycle and pregnancy.
4. To evaluate if salivary progesterone could replace serum progesterone measurements to assess reproductive status in mares.

CHAPTER 2

LITERATURE REVIEW

2.1 Endocrinology of the equine estrous cycle and early pregnancy

Mares are classified as polyestrous long day breeders having an estrous cycle that is approximately 21 days in length on an average. The normal length of estrus and the inter-estrous interval range from 5 to 7 days and from 14 to 16 days, respectively (Evans et al., 2007). Individual variations in estrous cycles exist and can be dependent on seasonal influences, body condition, number of follicular waves, lactation, and the possibility of multiple diestrus ovulations (Pycock, 2003). During estrus the predominant reproductive steroid in the blood is estradiol of the follicular origin, which is primarily responsible for the display of signs of estrus by the mare when teased with a stallion. P4 levels are low in the blood at this time (<1 ng/ml). However P4 levels have been shown to increase significantly within the first 12 hours of ovulation (Plotka et al., 1975) to reach levels more than 2 ng/ml by 48 hours post-ovulation (Townson et al., 1989; Koskinen et al., 1990). In fact, Belin et al. (2000) have shown that the intra-follicular concentrations of P4 increase as early as about 2 days before ovulation. Mares are known to increase levels of P4 secretion in plasma earlier than in any other species and continue to rise until day 6 of the cycle when they reach a plateau (Nagy et al., 2004). In cycling non-pregnant mares, P4 levels rapidly decline after day 14 of the cycle due to lack of maternal recognition of pregnancy signal and due to luteolysis associated with peak peripheral blood levels of Prostaglandin F_2 alpha (PGF_2 α) resulting in luteolysis and return to estrus 1 to 3 days later.

In pregnant mares, the primary CL which develops from the ovulation that led to the pregnancy, persists beyond its normal cyclical lifespan of 15-16 days due to the actions of a undefined maternal recognition of pregnancy factor secreted by the still spherical (Van Niekerk, 1965) and still mobile (Ginther, 1983) conceptus. The mare during this period is able to suppress the normal cyclical upregulation of oxytocin receptors in the endometrium (Stout et al., 2000) and prevent the release of PGF2 α in response to oxytocin released from the endometrium (Watson et al., 1997; Stout et al., 2000). Having escaped luteolysis the primary CL persists, but also declines in its P4 output over the next 20 or so days, such that the P4 levels may decrease from a peak of 12 to 20 ng/ml to as low as 3 to 5 ng/ml (Allen, 2001). The CL then merely exists without true LH support till day 38-40 when it is rescued the second time by equine chorionic gonadotropin (eCG) produced by the endometrial cups. Due to the LH like action of eCG, secondary corpora lutea form on the ovaries resulting in a sharp rise in the P4 values in blood, and continue to form until about day 100 -120 of gestation, when the level will typically be as high as 25 to 30ng/ml. Between days 40 and 120 of gestation the trophoblast cells, besides producing eCG, also acquire a comprehensive repertoire of hormone synthetic capabilities. These also include production of P4 and the 5a-reduced metabolites, 5aDHP and 20a-hydroxy-5a-pregnan-3-one, from around day 70 but utilizing exclusively maternal sources of cholesterol (Holtan et al., 1991; Pashen and Allen, 1979). Between days 70 to 150 of gestation in the mare, the peripheral blood represents a mixture of P4 of ovarian origin and related progestagens secreted by the enlarging allantochorion. Studies by Holtan et al., (1979) have shown that these progestagens are sufficient to maintain pregnancy to term even in ovariectomized mares.

2.2 Endocrine diagnostics in mares for P4 estimation

Mares, more than pregnant females of any other species are administered P4 or progestins during part or all of their pregnancy due to fear of luteal insufficiency during pregnancy. Pregnancy loss in mares occurs in 10 to 15% during gestation and the majority of these losses occur during the first 40 days of pregnancy when the primary CL is the sole source of P4 (Allen, 2001). It is because of this fear, that it has become common practice for mares bred in the United States and several other countries to receive P4 or synthetic progestagens early in the postovulatory period, especially in mares with a history of early embryonic loss or after embryo transfer. Mean serum P4 concentrations of 4 ng/mL or greater are considered necessary to maintain pregnancy consistently in mares (Shideler et al., 1981). P4 is by far the most common reproductive hormone assayed in broodmare practice. Because of the physiological patterns of change in P4 and progestagen concentrations during gestation, single-point measurements of either hormone are not necessarily helpful and serial sampling for determination of trends or mean concentrations during a given interval is recommended (Troedsson, 2003). The most common endocrine assays used for the purpose are RIA and ELISA which are highly specific for P4, whereas others (competitive protein binding) reflect total progestagen concentration. The most common assay used for P4 estimation is the RIA as it can measure extremely small quantities with greater accuracy. Allen et al., (1974) and Sato K. et al., (1977) have provided reference ranges for P4 in cycling and non-cycling mares as well as relationship of P4 with estradiol during the estrous cycle. In 1980, Gunther et al. experimented to measure and compare P4 in the milk and plasma of mares using RIA in order to try and establish an alternative test for pregnancy diagnosis in mares and concluded that milk could indeed be used by day 18 conclusively to differentiate pregnant and non-pregnant mares. Since then various quantitative

and qualitative ELISA (EIA) tests have been evaluated to measure P4 in serum and plasma of mares with the aim of providing a rapid and reliable P4 estimation. In the mare, values obtained with quantitative ELISA are usually greater than values with RIA, but the two assays show similar profiles for plasma progesterone concentration (Giguere, 1994). Correlation between the two types of assay is high (coefficient of correlation = 0.94), especially for the detection of the low concentrations of progesterone found during estrus and the subsequent rise during the luteal phase of the cycle (Eckersall et al., 1987). A recent study by Relave et al., (2007) also has shown that a semi-quantitative EIA that they developed was a comparable and reliable test to RIA and chemiluminescent assays for measuring P4 in the blood of mares. In spite of these various studies, no quantitative EIA commercial kits capable of measuring extremely minute quantities of P4 are available in the market and hence reference laboratories still continue to use solid phase RIA kits developed for humans as a reliable assay for monitoring P4 in mares.

2.3 Saliva, an alternative to serum/plasma for P4 estimation.

Salivary analysis has become an important resource for the evaluation of salivary conditions with physiologic and pathologic implications and is a useful tool for disease diagnosis, mainly due to its origin, composition, functions, and interactions with other organ systems (Chiappin et al., 2007). Additionally, it is a simple and non-invasive collection method, easy to store, and is inexpensive when compared to blood collection. With the addition of modern techniques and chemical instrumentation equipment, there has recently been an increase in its use for laboratory investigations, applications for basic and clinical purposes in dentistry and other medical areas. Oral fluid sampling is safe for the operator and the patient, and has easy

and low-cost storage. These characteristics make it possible to monitor several biomarkers in infants, children, elderly and uncooperative subjects, and in many circumstances in which blood and urine sampling is not available. Saliva analyses have been used mainly in dentistry and for studies in oral diseases to help assess the risk of caries, by measuring saliva buffer capacity and bacterial contents (Van Nieuw Amerongen et al., 2004). Oral fluid is mainly utilized for research and diagnostic purposes concerning systemic diseases that involve the salivary glands and oral cavity, such as Sjögren syndrome (Pedersen et al., 2005) Beçhet syndrome, benignant and malignant oral tumors (Li et al., 2004). Studies that use saliva for the diagnosis of HIV using specific antibodies as biological markers (Holm et al., 1993; Burgess-Casseler et al., 1996) have been demonstrated to be successful and reproducible. This method made the first quick test for the detection of HIV-1 infection possible, a test that the US Centers for Disease Control and Prevention (CDC) has been using a saliva-based test for the detection of HIV that provides results in only 20 minutes (Burgess-Casseler et al., 1996). .

Saliva is used for the detection of different biomarkers such as electrolytes, hormones, drugs and antibodies in human and veterinary medicine. The measurement of steroid hormones in saliva is a widely accepted alternative to the determination in plasma or serum (Riad-Fahmy et al., 1982). Salivary steroids correlate very well with the non-protein-bound fraction in plasma samples (Vining et al., 1987). Thus saliva can be analyzed as a part of evaluation of endocrine function. The majority of hormones enter saliva by passive diffusion across the acinar cells. Most of these hormones are lipid-soluble (i.e., steroids). Small polar molecules do not readily diffuse across cells and instead enter saliva through the tight junctions between cells via ultrafiltration (Quissell, 1993; Read, 1993). The molecular-weight cut-off for ultrafiltration is 100-200, which prevents many hormones from entering saliva from serum by means of ultrafiltration. In

addition, active transport does not appear to facilitate hormone transfer into saliva (Vining and McGinley, 1986). For neutral steroids which diffuse readily into saliva, salivary hormone levels represent the non-protein-bound (free) serum hormone levels. In contrast due to their size, protein hormones do not enter saliva through passive diffusion, but primarily through contamination from serum as a result of outflow of gingival crevicular fluid (GCF) or from oral wounds. In blood 95-99% of the steroids are bound up by binding proteins such as sex hormone binding globulin (SHBG), cortisol binding globulin (CBG), and albumin. The remaining unbound fraction (1-5%) is referred to as "free" hormone, and is generally regarded as bioavailable, since steroid hormone molecules must interact with sites on DNA unencumbered by binding proteins. Saliva hormone levels are inherently more reflective of bioavailability no matter which hormone delivery mode is used, because the salivary hormones represent hormone that has been delivered to tissue (Lima et al., 2010).

One of the most common assays used in humans is 17a-hydroxyprogesterone (17P) in serial saliva samples for the diagnosis and treatment monitoring of congenital adrenal hyperplasia. Likewise, serial salivary progesterone analysis to monitor ovarian function in subfertile women has also been a popular application since P4 values do not fluctuate with the circadian rhythms, enabling sampling at different times of the day possible. Salivary progesterone measurement can be used for longitudinal monitoring of luteal function (Lipson et al., 1994), as it displays both free and total serum progesterone levels during normal menstrual cycle and in pregnancy. Vimpeli et al., (2001) evaluated salivary P4 levels during two luteal support treatment regimens for in-vitro fertilization (IVF) in humans. Serum progesterone levels were significantly lower in patients who received vaginal progesterone than in those who received Human Chorionic Gonadotropin (hCG). But this group also had high salivary P4

concentrations suggesting that vaginally administered progesterone might have extraordinary pharmacokinetics compared with other administration routes. Efforts have been made in human medicine to validate and establish cutoff levels for P4 in saliva during the follicular and luteal phases of the menstrual cycle. Ishikawa et al., (2002) have suggested such cutoff values for P4 during the mid-luteal stage of the cycle and have recommended daily salivary progesterone profiles during the luteal phase and a simple estimation of mid-luteal salivary progesterone useful for the diagnosis of luteal phase defects. In addition, several studies reported that a good correlation exists between the plasma and salivary concentrations of progesterone, since salivary progesterone is thought to reflect the levels of free serum progesterone (Walker R.F. 1985; Choe et al., 1983; Tallon et al., 1984; Ellison et al., 1993). Thus, salivary progesterone measurements can provide a practical way of allowing longitudinal studies of the corpus luteum function.

2.4 Applications of salivary P4 estimation in veterinary medicine

Studies on use of salivary assays for hormonal estimation in veterinary medicine have been very limited and have generally focused on its use in early pregnancy diagnosis for farm animals. Kanchev et al., (1988) attempted to set up a simple, sensitive and reproducible direct RIA method for the determination P4 in bovine saliva for the assessment of the ovarian function and early pregnancy diagnosis in cows and heifers. They found that salivary levels measured by this method reflected changes in plasma progesterone concentrations. The saliva/plasma progesterone ratio X 100 during the follicular phase was 15% while during the luteal phase it was 12%. The assay was sensitive enough to differentiate non-pregnant animals from pregnant ones. In the same year a comparative study between P4 concentrations in plasma, milk and saliva

of cows in different reproductive stages was conducted (Gao et al., 1988) showing that salivary progesterone could be used to assess ovarian function in cows and that the salivary progesterone concentrations in the luteal phase and during pregnancy reflected the plasma concentrations more closely than the values obtained in milk. Applications of salivary P4 assays have also been employed in the swine industry. Moriyoshi et al., (1996) tried validating a P4 salivary assay using bovine milk P4 EIA kits and managed to prove that qualitative measure of salivary P4 could be a practical method of diagnosing early pregnancy in sows. Likewise Needham et al. (2007) have demonstrated that similar results can be obtained in sheep as well. There are also unpublished references to measurement of salivary P4 in horses (Smith, 2005) using milk P4 EIA kits.

Due to the ease and non-invasive nature of sample collection salivary P4 measurement has been found to be useful in captive wild animal species such as the Black Rhinoceros (Czekala et al., 1996). Using RIA and High Performance Liquid Chromatography (HPLC) it was possible to measure salivary estradiol and 20 alphahydroxypreg-4-en-3-ones in diagnosing pregnancy and even predicting impending parturition. Based on the same study, ovarian cycles were monitored in the Indian Rhinoceros at the National Zoo using salivary steroids (Gomez et al., 2004). Analyses of salivary androgens, progestins and estrogens were found to reliably monitor follicular activity. Salivary P4 estimation in marine mammal species such as captive false killer whales (Atkinson et al., 1999) and Hawaiian Monk Seal (Pietraszek et. al., 1994) have shown that salivary concentrations of estrogen and progesterone may provide an accurate, less-invasive method of monitoring reproductive hormones. In equines, no published data providing detailed information of P4 measurements in saliva is available to date, though salivary cortisol measurements are fast gaining popularity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Objectives

The study was conducted to evaluate whether salivary levels of P4 in mares could be used to identify the stage of their reproductive status. RIA'-s were set up to compare the serum and salivary P4 profiles collected at selected days of the estrous cycle of non-pregnant cycling mares and during early gestation of pregnant mares. Hypothesis one was that the levels of P4 in saliva would rise and fall concurrently along with levels in the serum during the selected days of the estrous cycle and during early pregnancy of these mares. Hypothesis two was that the salivary P4 levels would be consistently in the range of 1 to 5 % of the levels found in serum and that these findings would prove that saliva could be a useful alternative to serum in monitoring P4 profiles in mares.

3.2 Selection of Animals for the Study

Fifteen Standardbred mares included in the study were obtained from the University of Illinois Horse Farm and were divided in two groups; non-pregnant cycling (8 mares) and pregnant (7 mares). The study was conducted during the breeding season of 2011 and the serum and saliva samples were obtained between the months of June and September. Mares were selected randomly and their ages ranged between 4 to 16 years. Prior to sample collection, the reproductive tract of each non-pregnant mare was palpated trans-rectally using manual and

ultrasound techniques and the mare was selected for the study only if she displayed signs of cyclicity. This included evidence of growth of multiple follicles with at least one follicle greater than 35 mm in diameter, signs of uterine edema, receptivity upon teasing with a stallion, and also presence of corpora hemorrhagica (CH) or corpora lutea (CL) on the ovary indicating a previous ovulation. Mares selected for the pregnant group were bred with semen from stallions housed at the same farm by artificial insemination technique. Pregnant mares were identified by examining them with a trans-rectal ultrasound on day 14 post-ovulation and identifying an embryonic vesicle in the reproductive tract.

3.3 Sample Collection and Initial Processing

Serum and saliva samples were collected from each mare included in the study on different days of the estrous cycle and early pregnancy at the same time of the day (approximately 10:00 a.m.) to avoid any effects of the circadian rhythm on the hormone profiles. Blood (10 ml) was drawn from the left jugular vein of each mare in a red top BD Vacutainer® blood collection tube with a conventional stopper, using a BD Vacutainer® 21 G x 0.75 inch needle and holder (Becton Dickinson Blood Collection Products, NJ USA 07417). The tubes were centrifuged at 2000 x G rpm for 10 minutes within 2 hours of collection to separate the serum. The serum was transferred to separate 5ml Snap Cap Falcon tubes (BD Biosciences, MA 01730 USA) and then stored at -20°C until further processing.

Saliva samples were collected from individual mares immediately after collection of the blood sample. A 7.5 ml capacity plastic transfer pipette (Cole-Palmer Disposable Transfer Pipettes, IL 60061 USA) was used for saliva collection. The procedure involved proper restraint of the head

of the mare and collection of the pooled saliva from the buccal commissures by aspiration with the pipette. In cases where sufficient saliva was not obtained, secretion was stimulated by offering feed such as alfalfa hay, and then collecting the samples after the mare had finished chewing and the mouth was relatively free of feed material. All samples were transferred from the collecting pipette to a standard red top vacutainer sterile blood collection tube (Becton Dickinson Blood Collection Products, NJ USA 07417). Every effort was made to collect as much saliva with minimal feed or blood contamination. Saliva was then frozen at -20^{0}C and were subjected to four freeze-thaw cycles which helped in degradation of mucins present in saliva and allowed a cleaner separation of the aqueous phase. The sample was centrifuged to precipitate any particulate matter, and the supernatant was then transferred to a red top blood collection tube and stored at -20^{0}C.

Saliva and serum samples were collected from the cycling and pregnant mares on various days of the estrous cycle and early gestation (up to day 65). The collection time-points are as shown in Table 1 and Table 2.

3.4 Preparation of Reagents for the Radioimmunoassay

1) Phosphate Buffered Saline 0.01 M (PBS)

The following reagents were measured and added to Distilled Water:

- 1.3 g sodium phosphate, monobasic ($NaH2PO4-H2O$)*
- 10.85 g sodium Phosphate, dibasic ($Na2HPO4-7H20$)*
- 42.5 g sodium Chloride (NaCl)*
- 5 liters of distilled water

*(Fisher Chemicals, Fisher Scientific NJ 07410)

2) <u>0.1% PBS Gelatin</u>

The following reagents were added to 1 liter of PBS (0.01M) to prepare PBS Gelatin (PBS-Gel)

- 1 g gelatin (Bio-Rad laboratories, Richmond, CA 94804)
- Heat to boiling to dissolve the gelatin
- Add 0.1 g of Na azide (ACROS, NJ, USA) to the solution and keep in the cold room (4^0C) overnight at all times.

3) <u>Charcoal Dextran</u>

To 500 ml of 0.01 M PBS the following were measured and added:

- 3.5 g of charcoal (Sigma Chemical Co. St. Louis, MO 63103)
- 0.35 g dextran (Sigma Chemical Co. St. Louis, MO 63103)
- Stir for 1 hour before use and store at 4^0C up to one month.

4) <u>Preparation of Tracer</u>

For preparation of the tracer, Tritiated 3H-Progesterone (3HP4) (250 mCu/ 250 µl, Perkin-Elmer, Boston, MA 02118) was used. This isotope was diluted with absolute alcohol to make the stock solution. Original isotope was removed from the freezer and brought to room temperature and 100µl of the isotope were added to 0.9 ml of absolute alcohol (stock solution). Next, 40 µl of this stock solution were added to 20 ml of PBS-Gel to make the tracer for RIA assays. This solution was mixed well and counted in the liquid scintillation counter after addition of 3 ml of scintillation fluid. The final counts desired were 10,000 count per minute (cpm)/100 µl. This solution was then stored in the cold room for overnight incubation and future use. A RIA assay was set up to evaluate the binding efficiency which should be close to

40% percent binding (Table 3). The calculations for binding efficiency were calculated as follows:

$$\text{Percent Binding} = \frac{\text{Bound (Bo) cpm} - \text{Non-specific Binding}}{\text{Total cpm} - \text{Non-specific Binding}} \times 100$$

5) Preparation of the Spike

Spike (3HP4) was prepared by adding 9 ml of PBS-Gel to 1ml of the prepared tracer (10,000 cpm/100 µl) to give a radioactivity of 1000 cpm/100 µl. The spike so prepared was mixed gently and kept in the cold room for overnight incubation and future use. The spike was used as a known source of radioactivity which was added to the serum samples before the solvent extraction process and to the hot recovery vials to account for procedural losses during extraction.

6) Preparation of the P4 Antibody

A P4 antibody validated and prepared at the Animal Sciences Department, University of Illinois at Urbana-Champaign (UIUC) was graciously provided by Dr. Janice Bahr's laboratory. This P4 antibody (GS #253), generated against preogesterone-11-hemisuccinate: bovine serum progesterone cross-reacts 22% with 11 α-hydroxy-progesterone, 4% with 5 α-pregnane-20-dione, 2% with 17 α-hydroxy-progesterone and <0.1% with Testosterone and Estradiol.

7) Preparation of Standards

P4 standards of various concentrations were prepared, and were used for determination of the standard curve as well as for assay validation. Based on the results obtained after determination

of the standard curve the sensitivity of the assay was calculated and standards falling on the linear part of the standard curve were used for the estimating P4 in serum and saliva samples. Table 4 shows the different concentrations and dilutions used to prepare the standards.

3.5 Validation of the Radioimmunoassay

1) Determination of a Standard Curve

A P4 standard curve was validated by using increasing known amounts of unlabeled antigen (P4 standards as prepared above) and constant amounts of antiserum (P4 antibody) and labeled antigen (3H-Progesterone). Thus various concentrations of unlabeled antigen were reacted with constant amounts of antiserum and the labeled antigen to obtain a sigmoid curve after running the values in universal computerized assay calculator software AssayZap. The linear portion of the curve was identified the only valid part of the curve. The values falling on the flat part at the beginning and end of the curve were rejected as these values are usually subject to large errors and are not very reproducible with greater than 10% variability. Thus the range of the standard curve was determined by identifying the 80% and 20 % bound values and accepting values falling between these two points. As a general rule the standard curve should have at least five points which fall on the linear part of the curve (Refer to Fig. 1). The RIA for the standard curve was set up in the following manner.

Day 1

Twenty two 12 x 75 mm culture tubes (Fisher Brand Disposable Culture Glass Tubes, NJ, USA) were numbered and labeled as outlined in Table 5. The amount of standard, PBS-Gel, P4-antibody (P4-Ab) and the labeled antigen 3H-P4 were added to the tubes using a Repeating

Pipetter (Fisher Brand NJ, USA) to minimize error during pipetting. The tubes were then vortexed to ensure thorough mixing and were incubated at 4^0C in the cold room overnight for equilibration.

Day 2

Before carrying out the separation of bound and free steroid, the charcoal dextran solution was subjected to at least 30 minutes of stirring on a magnetic stirrer to ensure thorough mixing. 200 µl of charcoal dextran solution was added to the tubes (except tube no. 3 and 4 which represented the Total amount of labeled radioactivity). The tubes were vortexed gently to ensure thorough mixing and were incubated in the cold room for 15 minutes at 4^0C to ensure proper separation of the unbound fraction of steroids by the charcoal dextran and the tubes were then centrifuged in a refrigerated centrifuge (Beckman Model J-6B, Beckman-Coulter Inc. CA, USA) at 4^0C and 3000 X G RPM for 10 minutes. Using a Digiflex CX Automatic Pipette (Micromedic Systems Inc., Seattle WA) 300 µl of the clear supernatant was aspirated and transferred to plastic scintillation vials (Fisher Brand, Fisher Scientific, NJ) and 3ml of Scintiverse ™ BD Cocktail (SX18-4) (Fisher Scientific, NJ) were added. The vials were capped, thoroughly shaken to mix the contents and placed in a Liquid Scintillation Analyzer Tri-Carb 2200 CA (Packard Instrument Co. CT 06450, USA) to determine the radioactivity in cpm. The readings (in cpm) were then analyzed using a universal computerized assay calculator software AssayZap v.3 1 (Copyright® Biosoft & PL Taylor, Cambridge, UK).

2) Validation for parallelism and recovery of unlabeled ligand (cold recovery) for serum samples

There are two critical validation steps. First, it is necessary to determine that the volume of the sample does not affect the amount of hormone measured. In other words if, 50, 100 or 200 µl (or more) are assayed the final concentration of hormone (in pg/ml or ng/ml) is the same in each sample when expressed per ml. This step is also done in order to determine that non-specific materials in the sample did not interfere with the accurate determination of the sample. The presence of interfering substances such as fat or cholesterol in the sample can result in measurement of lesser hormone as the sample volume is increased. A pooled serum sample was used for the assay, prepared by mixing 100 µl of serum of the seven pregnant mares from day 35 of gestation, as by this day the P4 levels in the mare's blood would theoretically be high enough to be detectable easily by the assay. Four different volumes of the sample tested for parallelism were 50 µl, 100 µl, 200 µl and 300 µl.

Validation of cold recovery was done by measuring known amounts of unlabeled ligand (cold hormone) added to the samples before extraction. The percent of the unlabeled ligand recovered would be indicative of the percent endogenous hormone measured. Thus three different amounts of unlabeled ligand (in pg) were added to 100 µl of sample in separate tubes viz. 50 pg, 100 pg, and 200 pg.

A set of internal controls using 125 and 250 pg standards were included in the assay to determine the inter-assay coefficient of variability. All standards and samples and internal control tubes were run in duplicates.

Step 1: Solvent Extraction

The serum samples were subjected to solvent extraction procedure to dissociate the P4 in the sample from the binding proteins and dissolve it in the organic solvent. A starting volume of 500 µl of each sample to be tested for parallelism was pipetted into in 16 x 100 mm disposable culture glass tubes (Fisher Brand Disposable Culture Glass Tubes, NJ, USA). To each sample tube, 100 µl of the spike (1000 cpm/100 µl) were added to account for procedural losses. In addition, 100 µl of the spike were added to two scintillation minivials which were used as Spike controls in the RIA. The samples remained at room temperature for 1 hour before starting the extraction process. 6ml (approximately 10 times the serum sample volume) of petroleum ether (E 120-4) Optima ® (Fisher Scientific, NJ) were added to the each sample. The samples were then agitated vigorously in a multi-vortexer for 10 minutes inside a fume hood. The aqueous phase in each sample was frozen with methanol and dry ice and the organic phase containing the steroids was decanted into new 12 x 75 mm disposable culture glass tubes. These were then placed in a hot water bath (40^{0}C) inside the fume hood and the organic solvent was evaporated by gentle flow of air. After the organic solvent was evaporated, the sides of the tubes were washed several times with petroleum ether and again evaporated. After complete evaporation an equal amount of PBS-Gel (500 µl) was added to each tube and was vortexed for 1 minute. The samples were incubated overnight at 4 ^{0}C in a cold room.

Step 2: Setting up the RIA

Before setting up the RIA on Day 2, 1/5th (100 µl) of the original sample was transferred to a minivial to account for hot recovery, to which 3ml of scintillation fluid were added. The cpm in the sample when compared to cpm of the hot recovery minivials will indicate the percent of sample recovered. The RIA was set up in a similar fashion as the set up for standard curve. The

assay plot for the RIA is displayed as Table 6. After adding the reagents the tubes were then vortexed to ensure thorough mixing and were incubated at 4^0C in the cold room overnight for equilibration.

Step 3 Charcoal Dextran Separation.

The separation of bound and free steroid with charcoal dextran solution and the estimation of radioactivity on the Liquid Scintillation counter were performed as described previously.

3) Validation for parallelism and recovery of unlabeled ligand (cold recovery) for saliva samples

The procedure for validation of the saliva samples was carried out in a similar manner to that of the serum. The pooled saliva sample assayed was from mares at 65 days of gestation because of limited volume of saliva samples available at day 35. An experiment was conducted to evaluate the difference in recoveries with and without subjecting the saliva to the solvent extraction process. Based on the results it was decided that the saliva samples need not be extracted as these gave better estimation of the P4. Also there was no need for estimating the hot recoveries. This confirmed the hypothesis that the P4 in saliva represents the free (unbound) fraction found in the body and hence does not require extraction.

3.6 Estimation of progesterone in serum and saliva samples

Upon validation of the respective assays, the actual serum and saliva samples were run in a similar fashion. The samples were run in triplicates and as before internal controls were maintained for evaluation of intra- and inter-assay coefficients of variation. The serum P4 values obtained on AssayZap were corrected for hot recovery in the following example:

Spike cpm = 978 cpm. 1/5th of 978 = 978/5 = 195.6

For Sample X: Hot Recovery (cpm) = 164

Hence, $\frac{164 \times 100}{195.6} = 83.84\ \%$

Mean Result of Sample X (pg) = 35.65

Corrected result for Sample X = $\frac{35.65 \times 100}{83.84} =$

This is the corrected value after accounting for the hot recovery.

3.7 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics Desktop software version 20.0.0. Friedman's non-parametric one-way analysis of variance was used to identify differences in serum P4 concentration over time, salivary P4 level over time, and the ratio of salivary to serum P4 concentration over time in the two groups of mares (pregnant and open). Post-hoc pairwise comparisons of each sampling period were conducted using the Wilcoxon-Signed Ranks test. To assess the correlation between serum P4 levels and salivary P4 concentration Pearson's Correlation Analysis was conducted using the Proc Corr statement of SAS 9.2 software (SAS Institute Incorporated).

CHAPTER 4

RESULTS

Validation of the Standard Curve.

The standard curve generated had a sigmoid shape with five standards ranging from 32 pg to 500 pg (Figure 1) fitting on the linear part of the curve. The lowest concentration that the assay could detect was 16 pg which still fell on the upper flat part of the slope. The curve wasn't sensitive enough to detect standards 4 pg and 8 pg consistently as these were shown out of range. Hence it was decided to omit these standards in the subsequent assays and only keep the standards that range between 16 to 1000 pg, which was satisfactory because the expected P4 range in serum and saliva was expected to range between approximately 50 pg/ml and 10 ng/ml. The curve had an overall good quality of fit (R^2= 0.999905). The percent binding was close to 40% as desired (42.64%) and the non-specific binding was quite low (1.86%). The calculated means for the standards were close to the estimated values indicating a very low pipetting error and high specificity of the assy. The values are shown in Table 7.

Validation for Parallelism and Recovery of the Unlabeled Ligand (cold recovery) for serum.

Parallelism for the pooled serum sample was performed to determine that non-specific materials in the sample did not interfere with the accurate determination of the sample. Four different volumes (50 µl, 100 µl, 200 µl and 300 µl) had been assayed and the cpm obtained was entered into the AssayZap program to determine if the P4 concentration in each sample showed a proportionate increase. The recovery from the 100 µl was 99.5 pg. Likewise the values obtained from 50, 200 and 300 µl were 44 pg, 212 pg and 286 pg The same assay had also been set up for

the recovery of the cold or unlabeled ligand and three different amounts of unlabeled ligand (in pg) were added to 100 µl of sample in separate tubes viz. 50 pg, 100 pg, and 200 pg. The recovery for these different amounts was 132 pg (88.29%), 211 pg (105.76%) and 306 pg (102.17%).

Validation for Parallelism and Recovery of the Unlabeled Ligand (Cold Recovery) for Saliva Samples.

Saliva samples were assayed without extraction with petroleum ether. This change in procedure was based on the fact that after running an extracted vs. non-extracted sample, it was observed that the hormone estimation without extraction of saliva was almost 25 % more than that of extracted sample. The increasing volumes (50 µl, 100 µl, 200 µl and 300 µl) used for parallelism gave proportionate recoveries at 35.5 pg, 72.0 pg, 138.2 pg, and 208.4 pg. Cold recoveries for saliva samples assayed also showed a proportionate recovery at 118.4 pg (96.33%), 181.09 pg (104.73%), and 289.41pg (106.04%) for the 50, 100 and 200 pg added to 100 µl of saliva sample.

Validation for Hot Recovery for Serum

Hot recoveries were estimated to account for the procedural losses that could occur during the extraction process. The spike containing a known amount of radioactivity (3H-P4; 1000 cpm) was added to the serum samples before the solvent extraction process and 1/5 of this original amount (500 µl) was transferred to a scintillation vial after the extraction process was over. The average percent hot recovery obtained after extraction was 86.22 ± 0.65 (Mean ± S.E.).

Inter-assay and Intra-assay Coefficients of Variation

To express the precision, or repeatability, of the radioimmunoassay test results two measures of the Coefficient of Variability (CV: the Inter-Assay CV and the Intra-Assay CV) were measured. The inter-assay CV for the low and high internal controls was 6.91% ± 0.81 (Mean ± S.E.) and 5.06 % ± 0.57 (Mean ± S.E.). Likewise the intra-assay CV was calculated for all assays and averaged to 13.19 % and 11.71%, respectively, for saliva and serum.

Progesterone Profiles in Serum of Cycling Mares

Serum P4 concentrations in cycling mares averaged 1.52 ng/ml on day 1 post ovulation and rose steadily to peak at day 8 of the cycle at 9.06 ng/ml. The serum P4 concentrations then steadily decreased by day 14 and fell sharply by day 17 post ovulation and reached baseline levels again by day 20 (1.40 ng/ml). Friedman's non-parametric one-way analysis of variance revealed significant differences in serum P4 levels by day of observation (Friedman's Chi-Square=47.37 $p<0.0001$). A Wilcoxon Signed Rank Test revealed significance between all days except days 17 and 20. The test statistics and the rank tests are as shown in Table 8. i) to vi).

Progesterone Profiles in Saliva of Cycling Mares

The P4 levels in the saliva of cycling mares rose proportionately to the rise in the serum levels as the estrous cycle progressed. Saliva P4 concentrations also peaked by day 8 of the cycle (Avg. 0.76 ng/ml) and then fell steadily as the serum levels also decreased post day 14 of the cycle. However salivary P4 as a percentage of serum P4 levels did not follow this general pattern. Salivary P4 concentration was at its peak at the beginning of the cycle (35%) and then fell sharply by day 3. The proportional percentage then remained between 8 to 12 % between

days 3 and 14 post ovulation and then rose sharply again by day 17 (29.98%) and peaked again by day 20. Table 9 shows comparative serum and salivary P4 levels and the ratio of saliva: serum in cycling mares. Figure 2 is a graphical representation of P4 profiles in serum and saliva of cycling mares.

Friedman's one way ANOVA showed no significant difference in salivary P4 levels by day of observation (Friedman's Chi-Square = 8.46, p = 0.206).

A significant difference was however seen with the saliva to serum ratio in cycling mares (Friedman's Chi-Square = 28.82 $p<0.0001$) as seen in Figure 4. Table 10 i) to iv) shows the pairwise comparisons.

<u>Progesterone Profiles in the Serum of Pregnant Mares</u>

Progesterone levels in pregnant mares rose sharply after ovulation, peaked around day 8 (7.85 ng/ml), decreased slightly by day 25, but again started rising post day 35 of gestation and remained constant till day 65. The percent P4 levels in saliva as compared to serum were high (24.13%) immediately after ovulation but fell sharply by day 3 post-ovulation and were maintained in a steady range between 8 to 11% till day 65 of gestation. As anticipated, there was a significant difference in the serum P4 levels by day of observation in the pregnant mares (Friedman's Chi-Square = 47.60, $p<0.0001$). Besides, the post-hoc pairwise comparison of each sampling period also showed significance for almost all sampling days. Table 11 i) to viii) shows the Chi-Square values and the Wilcoxon Signed Rank Values for serum P4 concentrations in pregnant mares. Figure 3 is a graphical representation of P4 profiles in serum and saliva of cycling mares.

Progesterone Profiles in Saliva of Pregnant Mares

Progesterone levels in the pregnant mare saliva rose concurrently along with serum levels and peaked at day 8 similar to serum values. Levels were maintained consistently above 0.5 ng/ml past day 14 up to day 65. Percent P4 in saliva compared to serum was approximately 25% (24.13%) on day 1 post ovulation and then values dropped sharply and were maintained between 8 to 11% till the last day of sample collection. Figure 3 is a graphical representation of P4 profiles in serum and saliva of pregnant mares along with table showing the days of significance.

Statistical significance was seen after Friedman's one way ANOVA, however only days 1 and 3 seemed to contribute to this significance. Table 12. i) to iii) shows values for the Friedman's one way analysis and Wilcoxon Signed Ranks for day 1 and 3.

Ratios of saliva: serum P4 concentrations in pregnant mare's shows statistical significance however the Wilcoxon Signed Ranks Test showed that only day 1, serum: saliva P4 concentration ratio was of significance (Figure 5). Table 13 shows the values for the serum: saliva ratio for pregnant mares.

Pearson's Correlation Analysis

Pearson's correlation analysis showed that the correlation between salivary and serum progesterone levels in cycling mares was not significant except for day 5 of the cycle ($r = 0.784$, p-value = 0.021). Likewise, there was no significant correlation found in the pregnant mare group as well except for days 5 ($r = 0.959$, p-value = 0.0006) and 8 ($r = 0.771$, 0.042) which showed a strong positive correlation. Table 14 shows detailed results of the analysis.

CHAPTER 5

DISCUSSION

The present study evaluated whether salivary levels of Progesterone (P4) in mares could be used to identify the stage of their estrous cycle and to differentiate non-pregnant and pregnant animals. As per the authors knowledge this is the first attempt to validate and compare salivary P4 to serum concentration in cycling and pregnant mares using RIA. Based on the results and statistical analysis of the data the following findings were considered important:

1) Overall, salivary P4 values were not found to be of statistical significance nor did they correlate significantly with serum P4 values.
2) During the estrous cycle salivary P4 values as a percentage of serum levels were seen to be much higher immediately post-ovulation in cycling mares, after which they fell and were maintained within a consistent range. Salivary P4 concentrations increased again towards the end of the cycle as the mares entered their follicular phase of the cycle.
3) On day 1 post ovulation salivary P4 values as a percentage of serum levels, in pregnant mares. These values then declined sharply and were again maintained within a consistent range till day 65 which was the last day of sample collection.
4) The study demonstrated that salivary P4 can be estimated by Radioimmunoassay (RIA) using a non-extracted (neat) saliva sample.
5) The study is first of its kind and the findings could be used as a benchmark for further studies in the area.

Serum P4 values in cycling and pregnant mares have been studied since 1970's. Serum P4 concentrations in both cycling and pregnant mares in this study rose rapidly after ovulation with levels well above 2ng/ml by day 3, and peaked by day 8 for both cycling and pregnant mares (8.1 ng/ml and 7.8 ng/ml). In cycling mares, these levels plateaued till day 14 after which a sharp decline was seen in serum P4 levels. These measurements are in accordance with the findings published in previous studies (Allen, 1984). The sharp decline after day 14 is due to a lack of maternal recognition of pregnancy factor, which if present would prolong the lifespan of the corpus luteum. By day 17 serum P4 levels had decreased below 2 ng/ml as the mares entered the follicular phase of the estrous cycle. The last day of sampling for the non-pregnant cycling mare group was day 20±1. By this time most mares had ovulated again and resumed their next cycle, and the P4 levels had started rising again. In the pregnant mare group, the P4 levels were maintained and reached a plateau past day 14 of gestation. P4 levels in this group in fact declined slightly after day 14 but were maintained above 4 ng/ml. The P4 levels then rose again to plateau past day 35 of gestation. These findings reflect the decline in secretory activity of the corpus luteum from as early as day 14-16 (also seen in pregnant mares) and rescue of the pregnancy by formation of secondary corpora lutea between 35 to 45 days of gestation (Allen, 1984).

When salivary P4 levels were compared to the serum P4 levels, no significant correlation was found between them, except on day 5 in pregnant and cycling groups and day 8 in the pregnant mare group only. These findings are contrary to studies in humans (Walker et al. 1985) and bovine species (Kanchev et al. 1988) in which a strong positive correlation was found between these two values for the length of the study. In cycling mare's saliva P4 levels rose concurrently with serum P4 levels after ovulation, peaked by day 8 and subsequently declined after day 14. In pregnant mares the decline wasn't significant; however at no point during the

entire study did salivary P4 values drop below 0.5 ng/ml after day 14. This level could be useful as a cutoff level to monitor the luteal phase or presence of an active corpus luteum in mares. Thus studies validating this cutoff value, with even greater number of mares in each group and a greater frequency of sampling need to be done.

When percent Saliva: Serum ratio (Sa:Se ratio) was calculated, that percent salivary P4 values were much higher during follicular phase, up to the day of ovulation. The values for cycling mares were 35% while those seen in the pregnant group of mares were around 25% of those seen in serum on day 1 post-ovulation. The percentages then dropped sharply by day 3 to less than half and then were maintained steadily between 8 to 11% till day 17 in cycling mares and throughout the study period (up to day 65) in pregnant mares. Similar findings have been noted by Kanchev et al. (1988) in their bovine salivary P4 study where they have seen values up to 15% in salivary levels of P4 during the follicular phase. These values are much higher than the values usually found in human saliva, where 2-10 % levels are considered as a thumb rule (Riad-Fahmy et al. 1981). It may be possible to use this information for timing of ovulation studies in mares by monitoring the saliva: serum ratio. There however is a lot of debate as to why percent follicular levels are higher than luteal values during the estrous cycle. It may be possible that there may be a threshold level that needs to be maintained for salivary P4 and or that rapidly decreasing serum P4 levels during follicular phase somehow causes the dissociation of P4 from the carrier proteins it is bound to. Another explanation could be that the amount of P4 transferred across the salivary gland membrane is not determined only by the unbound concentration in plasma or serum. Similarly the ability of steroids to move across capillary membranes appears to depend on both the steroid and the organ containing the capillary bed (Verheugen et al. 1984).

Hence the study of passage of progesterone across salivary capillary beds may provide an idea as to why this phenomenon occurs.

In humans, salivary P4 levels can be useful for the prediction of ovulation, demonstrating a correlation of 0.75 with serum P4 levels, and salivary estradiol and P4 levels can be used for the evaluation of ovarian function (Lu et al., 1997, 1999) or as a predictor of pre-term delivery (Darne et al., 1987). In mares the serum P4 levels usually decrease in the last 24-48 hours before parturition (Fowden et al. 2008). Thus assays studying the P4: Estradiol ratio might give valuable information regarding the impending parturition.

The RIA set up for the present study showed a high specificity and a sensitivity of 16 pg. /tube. Volumes as less as 500 µL was sufficient to provide enough sample to run the assay. The saliva collection was done using disposable plastic pipettes instead of the conventional salivette system used commercially. This was done specifically to reduce any spurious increase in salivary P4 due to presence of cotton based absorptive material as demonstrated in previous studies (Shirtcliffe E.A. et al. 2001). The assay also demonstrated that salivary P4 could be assayed reliably without subjecting the sample to the lengthy extraction process. The present study has made an attempt to compare salivary and serum P4 in cycling and pregnant mares, because serum is usually used as a reference fluid for most assays. However, it should be remembered that the P4 in both these different body fluids is in a free (saliva) and bound (serum) state. Our findings that saliva P4 levels do not reflect serum levels should not be interpreted as information of little use. Based on the results of the present study, more studies could in fact help establish the normal range of salivary P4 values for mares in different physiological stages of reproduction. The present study could be regarded as a pilot reference study, results of which could benefit similar studies in the future.

CHAPTER 6

CONCLUSIONS

The present study allowed us to conclude:

- Salivary P4 can be reliably estimated in mare saliva during different stages of the estrous cycle and early gestation using a simple liquid phase RIA.
- Saliva need not be subjected to lengthy solvent extraction for P4 estimation. Instead a neat sample can be assayed, thus reducing the time and labor.
- Salivary P4 was not found to correlate statistically with serum P4 values.
- Salivary P4 levels however do rise and fall in conjunction with serum levels during the estrous cycle in cycling mares and also during early gestation in pregnant mares.
- Sa: Se ratio was seen to rise sharply during the follicular phase and drop post-ovulation. This ratio was found to be much higher in mares, when compared to similar data in humans and cattle.
- Luteal phase saliva P4 levels were observed to be consistently above 0.5 ng/ml in both the both groups.
- This preliminary data could be used for further studies in monitoring luteal function in mares.

CHAPTER 7

TABLES AND FIGURES

Table1. Time-points for saliva and serum sample collection in the Cycling Mare Group and their corresponding reproductive status.

Day of the Sample Collection during the Estrous Cycle	**Reproductive Status**
1	Day of Ovulation
3	CL still immature and forming
5	Mature CL formed
8	Peak P4 levels
14	Maternal Recognition of Pregnancy
17	Follicular phase
20-22	Day of Ovulation

Table2. Time-points for saliva and serum sample collection in the Pregnant Mare Group and their corresponding reproductive status.

Day of the Sample Collection during Estrous Cycle and Pregnancy	Significance
1	Day of Ovulation
3	Immature CL
5	Mature CL formed
8	Peak P4 levels
14	Maternal Recognition of Pregnancy and Pregnancy Diagnosis
17	Fixing of the Embryo
25	Heart Beat Check
35	Endometrial Cups Formed
45	Assess Fetal Viability
65	Fetal Sexing

Table3. RIA assay plot for setting up a Binding Efficiency Assay

Tube No.	ID	STD or SAMPLE	PBS-Gel	P_4-Ab	3H-P_4	Charcoal Dextran
1-2	BKGD		400ul		100ul	200ul
3-4	Total		600ul		100ul	
5-6	Bound-Bo		300ul	100ul	100ul	200ul

Table4. Preparation of Standards for the RIA

Solution ID and Conc. (pg/100 µl)	PBS Vol. (ml)	Solution to Add (ml)	Final Volume (ml)
A (100,000)			0.5
B (1000)	0.45	0.05 ml of A	0.5
C (500)	7.125	0.375 ml of B	7.5
D (50)	6.75	0.75 ml of C	7.5
E (250)	2.5	2.5 ml of C	5
F (125)	3.75	1.25 ml of C	5
G (64)	4.37	0.63 ml of C	5
H(32)	1.80	3.2 ml of D	5
I (16)	3.40	1.6 ml of D	5
J (8)	4.20	0.8 ml of D	5
K (4)	4.60	0.4 ml of D	5

Table5. Assay Plot for Setting up a Standard Curve for the RIA

Tube No.	ID	STD	PBS-Gel	P_4-Ab	3H-P_4	Charcoal Dextran
1-2	BKGD		400ul		100ul	200ul
3-4	Total		600ul		100ul	
5-6	Bound-Bo		300ul	100ul	100ul	200ul
7-8	Std 4 pg	100ul	200ul	100ul	100ul	200ul
8-10	Std 8 pg	100ul	200ul	100ul	100ul	200ul
11-12	Std 16 pg	100ul	200ul	100ul	100ul	200ul
13-14	Std 32 pg	100ul	200ul	100ul	100ul	200ul
15-16	Std 64 pg	100ul	200ul	100ul	100ul	200ul
17-18	Std 125 pg	100ul	200ul	100ul	100ul	200ul
19-20	Std 250 pg	100ul	200ul	100ul	100ul	200ul
21-22	Std 500 pg	50ul	250ul	100ul	100ul	200ul
23-24	Std. 1000 pg	100ul	200ul	100ul	100ul	200ul
25-26	Bound-Bo		300ul	100ul	100ul	200ul

Table6. Assay Plot for Serum Sample Parallelism and Hot Recovery. Tube no. 21-30 represent the samples validated for parallelism. As the sample volume is increased, it is compensated by a decrease in the amount of PBS-Gel used. Tubes 36- 43 represent the samples used for validation of the unlabeled ligand (cold recovery). Here the sample volume is constant across the tubes. Tubes 50-55 represent the hot recovery vials containing $1/5^{th}$ of the original mixture of sample and spike whereas tubes 56-57 are the spike controls.

NAME	DAY 1	DAY 2				DAY 3
Tube No	ID	STD or SAMPLE	PBS-Gel	P_4-Ab	3H-P_4	Charcoal Dextran
1-2	BKGD		400ul		100ul	200ul
3-4	Total		600ul		100ul	
5-6	Bound-Bo		300ul	100ul	100ul	200ul
7-8	Std 16pg	100ul	200ul	100ul	100ul	200ul
9-10	Std 32pg	100ul	200ul	100ul	100ul	200ul
11-12	Std 64pg	100ul	200ul	100ul	100ul	200ul
13-14	Std 125pg	100ul	200ul	100ul	100ul	200ul
15-16	Std 250pg	100ul	200ul	100ul	100ul	200ul
17-18	Std 500pg	100ul	200ul	100ul	100ul	200ul
19-20	Std 1000pg	100ul	200ul	100ul	100ul	200ul
21-22	**Serum**	**100ul**	**200ul**	100ul	100ul	200ul
23-24	**Serum + 50 ul**	**150ul**	**150ul**	100ul	100ul	200ul
25-26	**Serum + 100 ul**	**100ul**	**200ul**	100ul	100ul	200ul
27-28	**Serum + 200 ul**	**200ul**	**100ul**	100ul	100ul	200ul
29-30	**Serum + 300 ul**	**300ul**	**-**	100ul	100ul	200ul
31-32	Internal Control 64pg	50ul	250ul	100ul	100ul	200ul
33-34	Internal Control 125pg	100ul	200ul	100ul	100ul	200ul
35-36	Bound-Bo		300ul	100ul	100ul	200ul
36-37	**Serum**	100ul	200ul	100ul	100ul	200ul
38-39	**Serum + 50 pg**	100ul	200ul	100ul	100ul	200ul
40-41	**Serum + 100 pg**	100ul	200ul	100ul	100ul	200ul
42-43	**Serum + 200 pg**	100ul	200ul	100ul	100ul	200ul
44-45	Internal Control 64pg	50ul	250ul	100ul	100ul	200ul
46-47	Internal Control 125pg	100ul	200ul	100ul	100ul	200ul
48-49	Bound-Bo		300ul	100ul	100ul	200ul
50-55	**Hot Recovery Minivials**	100 ul				
56-57	**SPIKE Serum (minivial)**	**100 ul**				

Table7. Calculated means of standards as determined from the semi-log plot generated by assaying actual amounts of P4

Standards Assayed (pg.)	Amount Detected by the RIA (pg.)
16	17.65
32	29.04
64	66.95
125	124.19
250	239.60
500	558.52
1000	937.55

Table8. Friedman's Non-parametric One-way Analysis of Variance (ANOVA) for serum progesterone concentration's in cycling mare group and Wilcoxon Signed Rank Test comparing each day to other days in the group. Statistical significance was set at $p<0.05$.

i) Friedman's Non-Parametric One-way ANOVA

Test Statistics[a]

N	8
Chi-Square	43.736
df	6
Asymp. Sig.	**.000**

a. Friedman's Test

ii) Wilcoxon Signed Rank Test for Day "1" with all other days'

	D3 - D1	D5 - D1	D8 - D1	D14 - D1	D17 - D1	D20 - D1
Z	-2.530[b]	-2.546[b]	-2.539[b]	-2.271[b]	-1.000[c]	-.378[c]
Asymp. Sig. (2-tailed)	**.011**	**.011**	**.011**	**.023**	.317	.705

iii) Wilcoxon Signed Rank Test for group day "3" with other days'

	D5 - D3	D8- D3	D14 - D3	D17 - D3	D20 - D3
Z	-2.546[b]	-2.539[b]	-.085[c]	-2.527[c]	-2.530[c]
Asymp. Sig. (2-tailed)	**.011**	**.011**	.932	**.012**	**.011**

iv) Wilcoxon Signed Rank Test for day "5" with other days

	D8 - D5	D14 - D5	D17 - D5	D20 - D5
Z	-1.890[b]	-2.375[c]	-2.539[c]	-2.565[c]
Asymp. Sig. (2-tailed)	.059	**.018**	**.011**	**.010**

v) Wilcoxon Signed Rank Test for day "8" with other days

	D14 - D8	D17 - D8	D20 - D8
Z Asymp. Sig. (2-tailed)	-2.524[b] **.012**	-2.527[b] **.012**	-2.536[b] **.011**

vi) Wilcoxon Signed Rank Test for day "14" with other days

	D17- D14	D20 - D14
Z Asymp. Sig. (2-tailed)	-2.536[b] **.011**	-2.379[b] **.017**

a. Wilcoxon Signed Rank Test

b. Based on negative ranks.

c. Based on positive ranks.

D. Day

Numbers highlighted in red are statistically significant ($p<0.05$).

Table9. Mean progesterone concentrations in serum and saliva of cycling mares and pregnant mares and percent progesterone in saliva as compared to serum.

i) Cycling Mares

Day of Cycle	Mean P4 in serum (ng/ml) ± S.E.	Mean P4 in saliva (ng/ml) ± S.E.	Saliva/Serum*100
1	1.523 ± 0.378	0.533 ± 0.102	35.00%
3	4.869 ± 0.841	0.622 ± 0.097	12.77%
5	8.102 ± 0.744	0.731 ± 0.105	9.02%
8	9.064 ± 0.675	0.762 ± 0.082	8.41%
14	4.324 ± 0.566	0.539 ± 0.083	12.47%
17	1.114 ± 0.190	0.334 ± 0.056	29.98%
20	1.404 ± 0.177	0.507 ± 0.082	36.11%

ii) Pregnant Mares

Day of Cycle	Mean P4 in serum (ng/ml) ± S.E.	Mean P4 in saliva (ng/ml) ± S.E.	Saliva/Serum*100
1	1.322 ± 0.100	0.319 ± 0.025	24.13%
3	3.755 ± 0.418	0.332 ± 0.037	8.84%
5	5.928 ± 0.740	0.675 ± 0.106	11.39%
8	7.858 ± 0.751	0.888 ± 0.129	11.30%
14	5.658 ± 0.325	0.523 ± 0.040	9.24%
17	5.041 ± 0.418	0.563 ± 0.049	11.17%
25	4.769 ± 0.253	0.547 ± 0.059	11.47%
35	5.877 ± 0.350	0.618 ± 0.043	10.52%
45	7.304 ± 0.345	0.686 ± 0.060	9.39%
65	7.066 ± 0.493	0.758 ± 0.140	10.73%

Table10. Friedman's Non-parametric One-way Analysis of Variance (ANOVA) for saliva to serum progesterone concentrations ratios in cycling mare group and Wilcoxon Signed Rank Test Statistical significance was set at $p<0.05$.

i) Friedman's Non-Parametric One-way ANOVA

Test Statistics[a]

N	8
Chi-Square	28.821
df	6
Asymp. Sig.	**.000**

a. Friedman Test

ii) Wilcoxon Signed Rank Test for Saliva: Serum (Sa: Se) ratio of Day "1" and other day groups of cycling

	D3 – D1	**D5 – D1**	**D8 - D0**	**D14 – D1**	**D17- D1**	**D20 – D1**
Z	-2.380[b]	-2.521[b]	-2.521[b]	-2.240[b]	-.840[b]	-.420[b]
Asymp. Sig. (2-tailed)	**.017**	**.012**	**.012**	**.025**	.401	.674

iii) Wilcoxon Signed Rank Test for Sa: Se ratio of Day "3" and other day groups of cycling

	D5 - D3	**D8 - D3**	**D141 - D31**	**D17 - D3**	**D20 - D3**
Z	-1.540[b]	-2.100[b]	-.140[b]	-1.820[c]	-2.100[c]
Asymp. Sig. (2-tailed)	.123	.036	**.889**	**.069**	**.036**

iv) Wilcoxon Signed Rank Test for Sa: Se ratio of Day "8,14,17 and 20" of cycling groups

	D14 - D8	D17 - D8	D20- D8	D17 - D14	D20 - D14	D20 - D17
Z	-1.612[b]	-2.521[b]	-2.380[b]	-2.521[b]	-2.240[b]	-.560[b]
Asymp. Sig. (2-tailed)	**.107**	**.012**	**.017**	**.012**	**.025**	**.575**

a. Wilcoxon Signed Rank Test

b. Based on negative ranks.

c. Based on positive ranks.

D. Day

Number's highlighted in red are statistically significant ($p<0.05$)

Table11. Progesterone profiles in pregnant mare serum were statistically significant with a Friedman's Chi-Square as well as a post-hoc comparison of each sampling period. The only day not contributing to significance levels was day 5. Statistical significance was set at $p<0.05$.

i) Friedman's Non-Parametric One-way ANOVA

Test Statistics[a]

N	7
Chi-Square	47.605
df	9
Asymp. Sig.	**.000**

ii) Wilcoxon Signed Rank Test

	D3 – D1	D5 – D1	D8 – D1	D14 – D1	D17 – D1
Z	-2.388[b]	-2.384[b]	-2.371[b]	-2.388[b]	-2.375[b]
Asymp. Sig. (2-tailed)	**.017**	**.017**	**.018**	**.017**	**.018**

iii) Wilcoxon Signed Rank Test for Day "3" and all other days

	D5 - D3	D8 - D3	D14 - D3	D17 - D3
Z	-2.401[b]	-2.207[b]	-2.333[b]	-2.041[b]
Asymp. Sig. (2-tailed)	**.016**	**.027**	**.020**	**.041**

iv) Wilcoxon Signed Rank Test for Day "3" and other days

	D25 - D3	D35 - D3	D45 - D3	D65 - D3
Z	-2.271[b]	-2.132[b]	-2.410[b]	-2.232[b]
Asymp. Sig. (2-tailed)	**.023**	**.033**	**.016**	**.026**

v) Wilcoxon Signed Rank Test for Day "3" and other days

	D14 - D80	D17 - D8	D25 - D8	D35 - D8	D45 - D8	D65 - D8
Z	-2.023[b]	-2.214[b]	-2.214[b]	-2.414[b]	-.862[b]	-1.095[b]
Asymp. Sig. (2-tailed)	**.043**	**.027**	**.027**	**.016**	**.389**	**.273**

vi) Wilcoxon Signed Rank Test for Day "14" and other days

	D17 - D14	D25 - D14	D35 - D14	D45 - D14	D65 - D14
Z	-1.633[b]	-2.121[b]	-.447[c]	-2.232[c]	-2.264[c]
Asymp. Sig. (2-tailed)	**.102**	**.034**	**.655**	**.026**	**.024**

vii) Wilcoxon Signed Rank Test for Day "17" and other days

	D25 - D17	D35 - D17	D45 – D17	D65 - D17
Z	-.816[b]	-1.289[c]	-2.232[c]	-2.226[c]
Asymp. Sig. (2-tailed)	**.414**	**.197**	**.026**	**.026**

viii) Wilcoxon Signed Rank Test for ay "25" and other days

	D30 - D25	D45 - D25	D65 - D25	D45 - D35	D65 - D35	D65 - D45
Z	-1.823[b]	-2.388[b]	-2.214[b]	-2.041[b]	-2.060[b]	-.816[c]
Asymp. Sig. (2-tailed)	**.068**	**.017**	**.027**	**.041**	**.039**	**.414**

a. Wilcoxon Signed Rank Test

b. Based on negative ranks.

c. Based on positive ranks.

D. Day

Number's highlighted in red are statistically significant ($p<0.05$)

Table12. Progesterone profiles in pregnant mare saliva were statistically significant with a Friedman's Chi-Square and a post-hoc comparison of each sampling period showed that values from Days 1 and 3 seemed to be contributing to that significance. Statistical significance was set at $p<0.05$.

i) Friedman's Non-Parametric One Way ANOVA

Test Statistics[a]

N	7
Chi-Square	27.778
df	9
Asymp. Sig.	**.001**

a. Friedman Test

ii) Wilcoxon Signed Rank Test for day "1" and other days

	D3 – D1	D5 – D1	D8- D1	D14 – D1	D17 – D1
Z	.000[b]	-2.000[c]	-2.646[c]	-2.000[c]	-2.000[c]
Asymp. Sig. (2-tailed)	**1.000**	**.046**	**.008**	**.046**	**.046**

	D25 – D1	D35 – D1	D45 – D1	D65 – D1
Z	-2.000[b]	-2.449[b]	-2.449[b]	-2.236[b]
Asymp. Sig. (2-tailed)	**.046**	**.014**	**.014**	**.025**

iii) Wilcoxon Signed Rank Test for day "3" and other days

	D5 - D3	D8 - D3	D14 - D3	D17 - D3	D25 - D3
Z	-2.000[b]	-2.646[b]	-2.000[b]	-2.000[b]	-2.000[b]
Asymp. Sig. (2-tailed)	**.046**	**.008**	**.046**	**.046**	**.046**

	D35 - D3	D45 - D3	D65 - D3
Z	-2.449[b]	-2.449[b]	-2.236[b]
Asymp. Sig. (2-tailed)	**.014**	**.014**	**.025**

a. Wilcoxon Signed Rank Test

b. Based on negative ranks.

c. Based on positive ranks.

D. Day

Number's highlighted in red are statistically significant ($p<0.05$)

Table13. Friedman's Non-parametric One-way Analysis of Variance (ANOVA) for levels saliva to serum progesterone ratio in pregnant group of mares and Wilcoxon Signed Rank Test. Statistical significance was set at $p<0.05$.

i) Friedman's Non-Parametric One Way ANOVA

Test Statistics[a]

N	7
Chi-Square	25.514
df	9
Asymp. Sig.	**.002**

ii) Wilcoxon Signed Rank Test for day "1" and other days

	D3 – D1	**D5 – D1**	**D8 – D1**	**D14 – D1**	**D17 – D1**
Z	-2.371[b]	-2.366[b]	-2.366[b]	-2.366[b]	-2.366[b]
Asymp. Sig. (2-tailed)	**.018**	**.018**	**.018**	**.018**	**.018**

	D25 – D1	**D35 – D1**	**D45 – D1**	**D65 – D1**
Z	-2.366[b]	-2.366[b]	-2.366[b]	-2.366[b]
Asymp. Sig. (2-tailed)	**.018**	**.018**	**.018**	**.018**

a. Wilcoxon Signed Rank Test

b. Based on negative ranks.

c. Based on positive ranks.

D. Day

Number's highlighted in red are statistically significant ($p<0.05$)

Table 14. Pearson's Correlation Analysis to demonstrate overall correlation between salivary and serum progesterone concentrations in Cycling and Pregnant mares.

i) Correlation between salivary P4 and serum P4 in cycling mares.

Reproductive Status: Cycling	Day 1	Day 3	Day5	Day 8	Day 14	Day17	Day20
Correlation Coefficient	0.698	-0.518	**0.784**	0.187	0.446	0.451	0.530
p-value	0.054	0.189	**0.021**	0.165	0.268	0.262	0.175

ii) Correlation between salivary and serum P4 in pregnant mares.

Reproductive Status: Pregnant	Day 1	Day 3	Day5	Day 8	Day 14
Correlation Coefficient	0.524	0.417	**0.959**	**0.771**	-0.020
p-value	0.226	0.351	**0.0006**	**0.042**	0.965

Reproductive Status: Pregnant	Day 17	Day 25	Day 35	Day 45	Day 65
Correlation Coefficient	0.266	0.387	0.596	0.155	0.574
p-value	0.563	0.391	0.157	0.739	0.177

Figure1. Standard curve generated shows a typical sigmoid shape with at least five standards fitting on the linear part of the curve, from 32pg. to 500 pg. Standards 4 and 8 pg. were below the sensitivity of the assay and were out of range. Hence these standards were not used. This standard curve gives a good quality of fit ($R^2 = 0.999905$) with an overall 42.64 % binding and low 1.86 % non-specific binding (NSB) as calculated by AssayZap.

The X-axis represents the content of the standards (pg) whereas the Y-axis represents the percent bound in absence or presence of standards. With increasing amounts of unlabeled P4, lesser amount of 3H-P4 is bound to the P4 antibody.

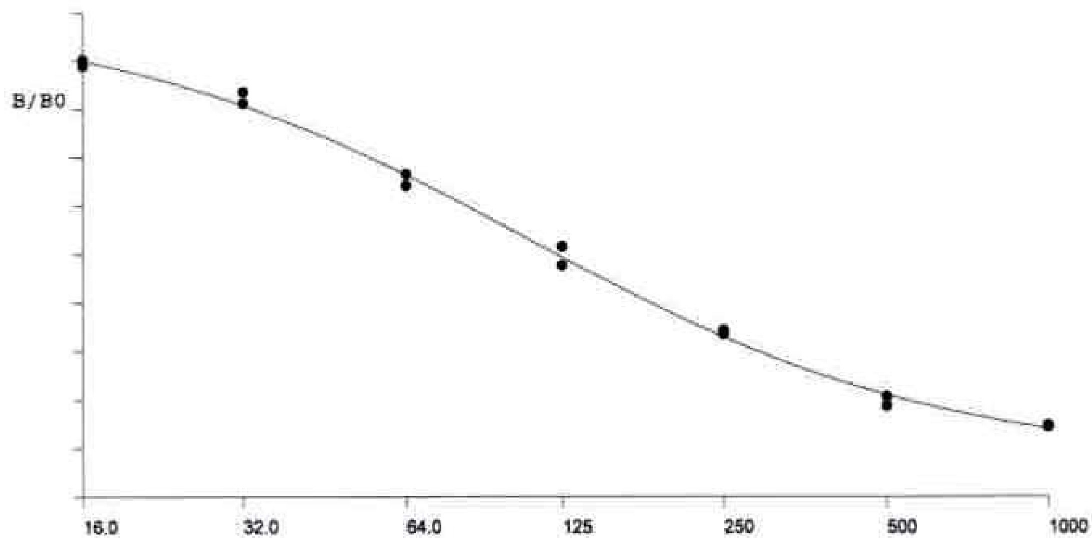
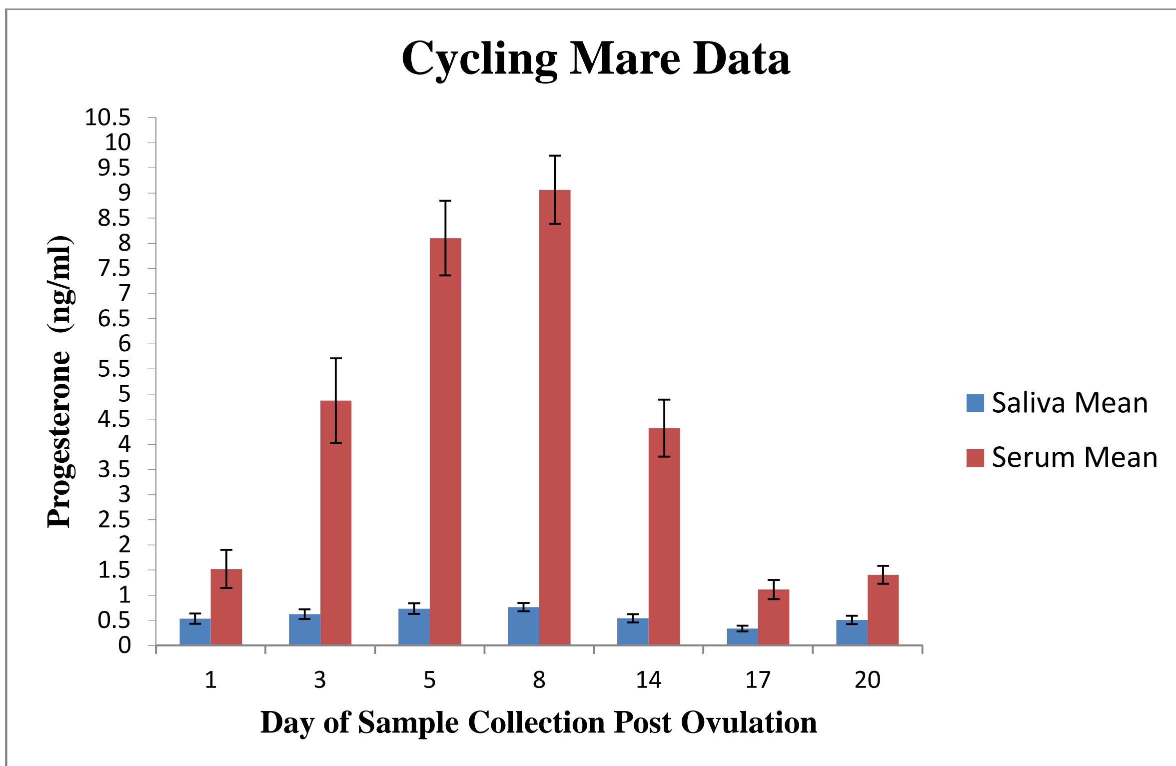


Figure2. Graphical representation of progesterone profiles in serum and saliva of cycling mares (n =8). Friedman's non-parametric one-way analysis of variance revealed significant differences in serum progesterone concentration by day of observation (Friedman's Chi-Square=47.37 $p<0.0001$). A Wilcoxon Signed Rank Test revealed significance between all days except day 17 and 20.

Friedman's one way ANOVA showed no significant difference in salivary progesterone concentration by day of observation (Friedman's Chi-Square = 8.46, $p = 0.206$).

Cycling Mare Data

Mean ± SE shown here for serum and saliva progesterone levels for each time-point



Serum progesterone concentration comparison between time-points in cycling mares (p = 0.05)

	se3	**se5**	**se8**	**se14**	**se17**	**se20**
se1	0.011	0.011	0.011	0.023	0.317	0.705
se3		0.011	0.011	0.932	0.012	0.011
se5			0.059	0.018	0.011	0.01
se8				0.012	0.012	0.011
se14					0.011	0.017
se17						0.083

Saliva progesterone concentration comparison between time-points in cycling mares (p = 0.05)

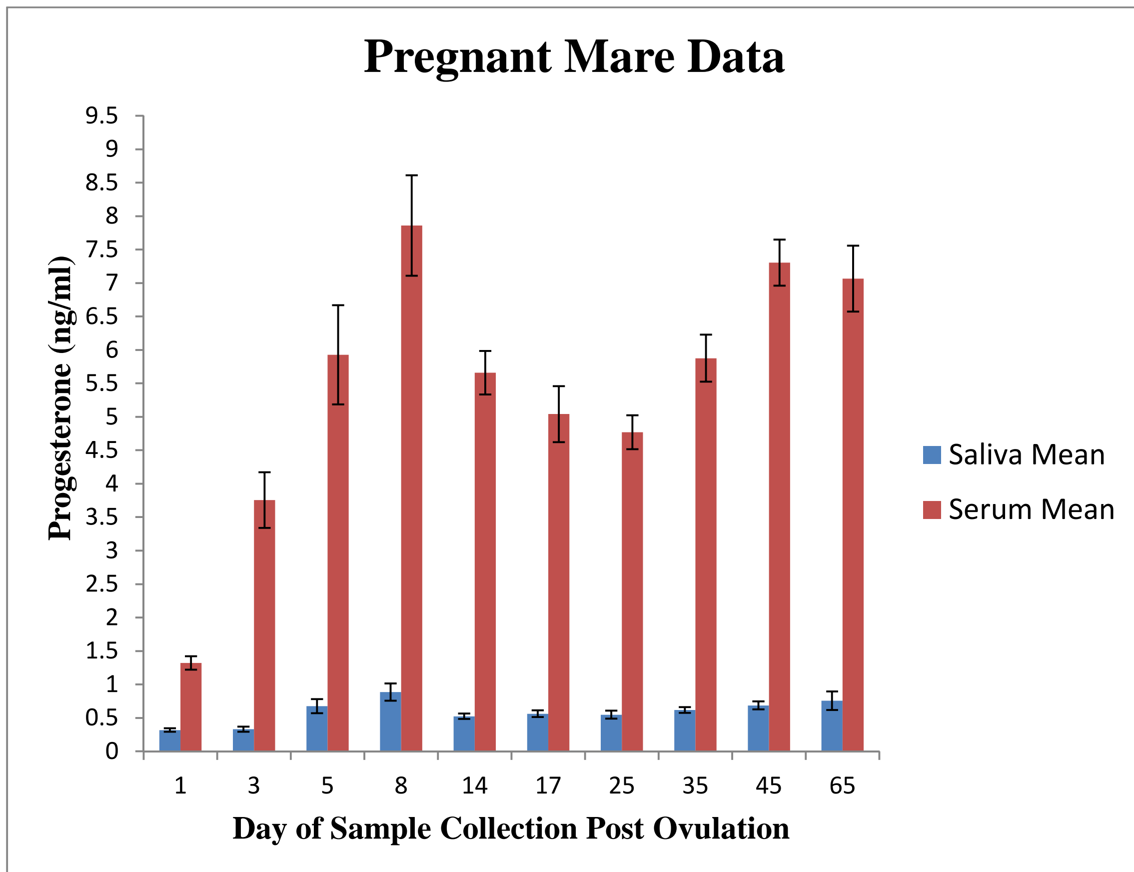
	sa3	**sa5**	**sa8**	**sa14**	**sa17**	**sa20**
sa1	1	0.083	0.317	0.317	0.157	1
sa3		0.083	0.317	0.564	0.414	1
sa5			0.564	0.157	0.025	0.18
sa8				0.564	0.102	0.317
sa14					0.083	0.655
sa17						0.317

Figure3. Graphical representation of progesterone profiles in serum and saliva of pregnant mares (n=7). There was a significant difference in the serum P4 concentration by day of observation in the pregnant mares (Friedman's Chi-Square = 47.60, $p<0.0001$). Besides, the post-hoc pairwise comparison of each sampling period also showed significance for almost all sampling days.

Statistical significance in salivary P4 was seen after Friedman's one way ANOVA, however only days 1 and 3 seemed to contribute to this significance.

Pregnant Mare Data

Mean ± SE for serum and saliva progesterone concentration for each time-point



Serum progesterone concentration comparison between time-points in pregnant mares (p = 0.05)

	se3	se5	se8	se14	se17	se25	se35	se45	se65
se1	0.017	0.017	0.018	0.017	0.018	0.016	0.017	0.016	0.016
se3		0.016	0.027	0.02	0.041	0.023	0.033	0.016	0.026
se5			0.058	0.891	0.336	0.059	0.891	0.206	0.347
se8				0.043	0.027	0.027	0.016	0.389	0.273
se14					0.102	0.034	0.655	0.026	0.024
se17						0.414	0.197	0.026	0.026
se25							0.068	0.017	0.027
se35								0.041	0.039
se45									0.414

Saliva progesterone concentration comparison between time-points in pregnant mares (p = 0.05)

	sa3	sa5	sa8	sa14	sa17	sa25	sa35	sa45	sa65
sa1	1	0.046	0.008	0.046	0.046	0.046	0.014	0.014	0.025
sa3		0.046	0.008	0.046	0.046	0.046	0.014	0.014	0.025
sa5			0.083	1	1	1	0.317	0.317	0.317
sa8				0.083	0.083	0.083	0.317	0.317	0.157
sa14					1	1	0.317	0.317	0.564
sa17						1	0.317	0.157	0.317
sa25							0.157	0.317	0.564
sa35								1	0.564
sa45									0.564

Figure4. Graph showing the saliva to serum ratio (%) in cycling group mares. Days 1, 17 and 20 of sample collection were seen to be statistically similar and differed from rest of the days. Days ranging from 3 to 14 were statistically similar to each other. Friedman's one way ANOVA showed a Chi-square value of 28.82 $p<0.0001$.

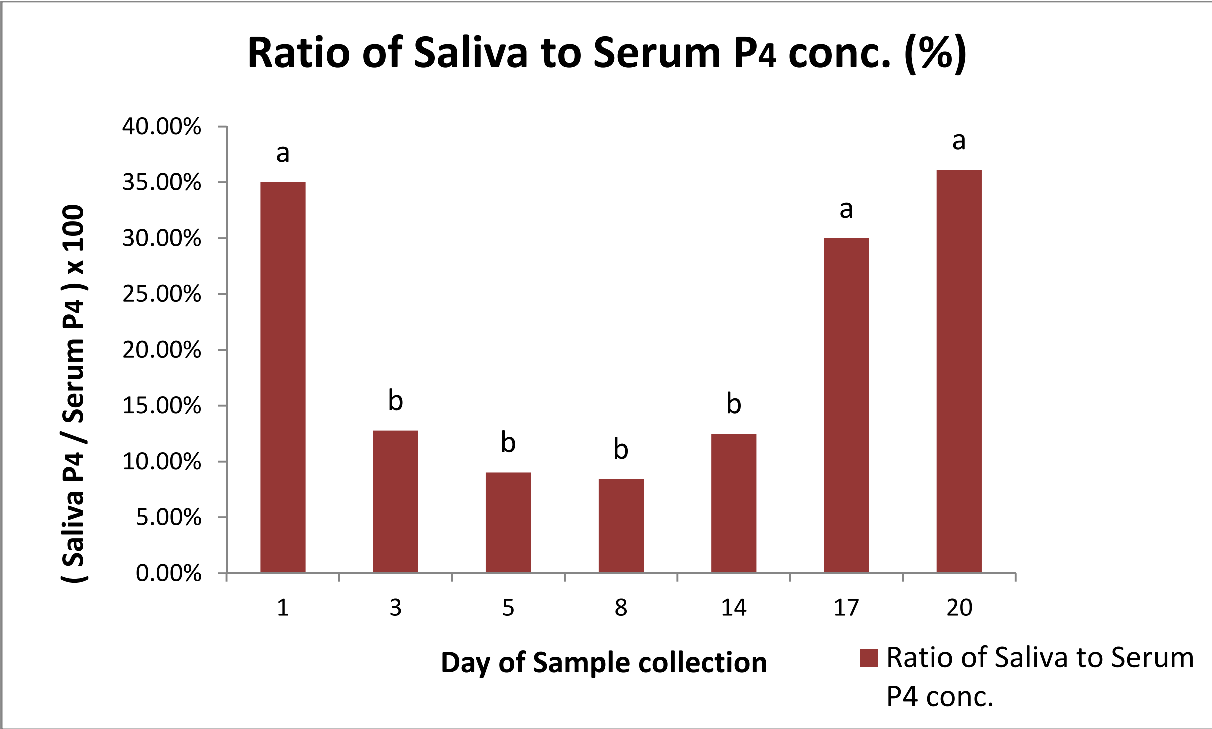
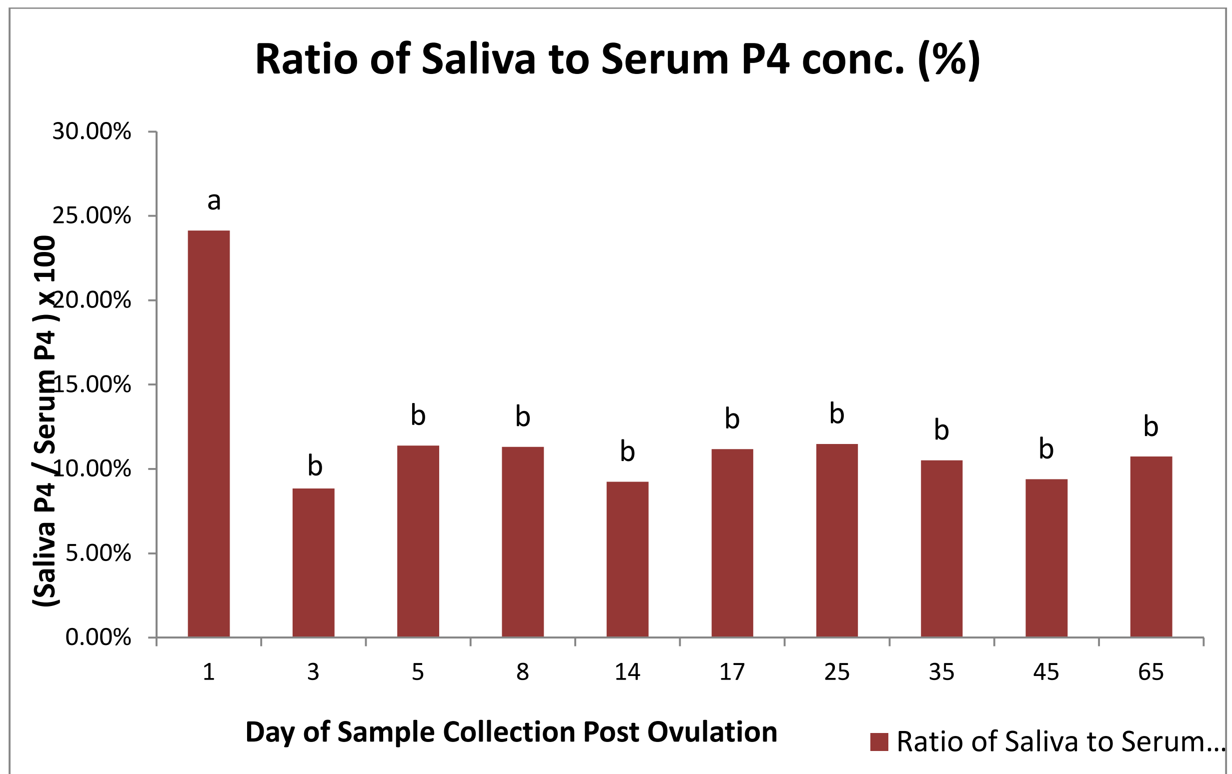


Figure5. Graph showing the saliva to serum ratio (%) in pregnant group mares. Day 1differed statistically from rest of the sampling days. All other sampling days were statistically similar to each other. Friedman's one way ANOVA showed a Chi-square value of 25.514 p<0.002.



REFERENCES

Allen, W.R. (2001). “Luteal Deficiency and Embryo Mortality in the Mare”. Reproduction in Domestic Animals **36**(3-4), 121-131.

Allen, WR (1984). “Hormonal Control of Early Pregnancy in the Mare”. Animal Reproduction Science, **7**(1-3): 283—304.

Allen. W. E. and J.C. Hadley (1974). “Blood progesterone concentrations in pregnant and non-pregnant mares”. Equine Veterinary Journal **6**(2), 87-93.

Atkinson S., M. Breese et al., (1999). “Monitoring of Progesterone in Captive Female False Killer Whales, *Pseudorca crassidens*”. General and Comparative Endocrinology **115**(3):323-332.

Belin F., N. Gérard et al., (2000). “Intrafollicular concentrations of steroids and steroidogenic enzymes in relation to follicular development in the mare”. Biology of Reproduction **62**(5):1335–1343.

Burgess-Casseler A., Schramm W. et al., (1996). “A field-test for the detection of antibodies to HIV-1/2 in serum or plasma”. Clinical and Diagnostic Laboratory Immunology **3**(4):480–482.

Carriere, P.D., and B. Lee (1994). “Direct Radioimmunoassay of Progesterone in Bovine Plasma Using Danazol (17-Alpha-2, 4-Pregnadien-20-Yno (2, 3-D) Isoxazol-17-Ol) as a Displacing Agent”. Canadian Journal of Veterinary Research **58**(3): 230-233

Chiappin S., and E.F. De Palo, (2007). "Saliva specimen: a new laboratory tool for diagnostic and basic investigation". Clinica Chimica Acta **383**(1–2):30–40.

Chiappin S., E.F. De Palo et al., (2007). "Saliva specimen: a new laboratory tool for diagnostic and basic investigation". Clinica Chimica Acta **383**(1-2):30-40.

Choe, J.K., M.Y. Dawood et al., (1983). "Progesterone and estradiol in the saliva and plasma during the menstrual cycle". American Journal of Obstetrics and Gynecology **147**(5):557–562.

Czekala N.M. and L. Callison, (1996). "Pregnancy Diagnosis in the Black Rhinoceros (*Diceros bicornis*) by Salivary Hormone Analysis". Zoo Biology **15**(1):37-44.

Darne J., and G.C.L. Lachelin, (1987). "Increased saliva oestriol to progesterone ratio before idiopathic preterm delivery: a possible predictor for preterm labour"? British Medical Journal **294**(6567):270-273.

Douglas, R.H. (2004). "Endocrine Diagnostics in the Broodmare: What You Need to Know". Proceedings of the Society of Theriogenology 106-115.

Eckersall P.D. and J.A. Harvey, (1987). "The use of a bovine plasma progesterone ELISA kit to measure progesterone in equine, ovine and canine plasmas". Veterinary Record **120**: 5-8.

Ellison P.T., (1993). "Measurements of salivary progesterone". Annals of New York Academy of Science **694**:161–171.

Evans J.J. (1986). "Progesterone in Saliva Does Not Parallel Unbound Progesterone in Plasma". Clinical Chemistry **32**(3):542-544.

Evans T.J., V.K. Ganjam et al., (2007). Clinical Reproductive Anatomy and Physiology of the Mare. Current Therapy in Large Animal Theriogenology. R. S. Youngquist and W. R. Threlfall. St. Louis, MO, Saunders Elsevier**:** 47-67.

Fowden A.L., J.C. Ousey et al. (2008). “Endocrinology of Equine Parturition”. Experimental and Clinical Endocrinology and Diabetes **116**(7):393-403

Gao Y., T.P. Fletcher et al., (1988). “Progesterone concentrations in plasma, saliva and milk of cows in different reproductive states”. British Veterinary Journal **44**(3):262-268.

Giguere S., and D.Vaillancourt, (1994). “Evaluation of two qualitative enzyme immunoassays for the rapid assessment of progesterone in equine plasma”. Canadian Veterinary Journal **35**:643-645.

Ginther O.J., (1983). “Mobility of the early equine conceptus”. Theriogenology **19**(4):603-611.

Gomez A., J.L. Brown et al., (2004). Use of Salivary Steroid Analyses to Assess Ovarian Cycles in an Indian Rhinoceros at the National Zoological Park. Zoo Biology **23**(6):501–512.

Gómez, A., J. Brown, (2004). “Use of salivary steroid analyses to assess ovarian cycles in an Indian rhinoceros at the National Zoological Park”. Zoo Biology **23**:510-12.

Gunther J.D., E. D. Plotka et al., (1980). “Concentrations in Cycling and Pregnant Mares Comparison of Milk and Blood Plasma Progesterone”. Journal of Animal Science **51**(5):1131-1138.

Holm H.C. and N.T. Constantine, (1993). “Saliva and HIV testing”. Lancet **341**(8841):382–383.

Holtan, D.W., E.Houghton et al. (1991). “Plasma progestagens in the mare fetus and newborn foal”. Journal of Reproduction and Fertility (Supplement) **44**:517-528.

Ishikawa M., P.F. Fottrell et al., (2002). “The Clinical Usefulness of Salivary Progesterone Measurement for the Evaluation of the Corpus luteum Function”. Gynecologic and Obstetric Investigation **53**(1):32–37

K. Sato K. and M. Miyake, (1977). “Studies on serum estrogen and progesterone levels during the estrous cycle and early pregnancy in mares”. Equine veterinary Journal **9**(2):57-60

Kanchev L., B. Stankov (1988). “Bovine salivary progesterone: Application to the assessment of ovarian function and early pregnancy diagnosis”. Animal Reproduction Science **17**(1-2):1-8

Kanchev L.N., B.M. Stankov et al., (1988). “Bovine salivary progesterone: Application to the assessment of ovarian function and early pregnancy diagnosis”. Animal Reproduction Science **17** (1-2):1-8.

Kanchev, L.N., (1976). “Determination of the binding percentage of 17p-oestradiol, progesterone and testosterone to plasma proteins in cattle”. Proceedings of the 8th Congress on Animal Reproduction and A.I. Krakow, **3**: 141-143.

Koskinen, E, H. Lindeberg et al. (1990). “Milk and serum progesterone levels in mares after ovulation”. Acta Veterinaria Scandinavica **31**(4):441–444.

Lewis J.G., (2006). “Steroid Analysis in Saliva: An overview”. Clinical Biochemistry Reviews **27**(3):139–146.

Li Y., D.T. Wong, et al., (2004). “Salivary transcriptome diagnostics for oral cancer detection”. Clinical Cancer Research **10**(24):8442–8450.

Lima D.P., A.C. Okamoto et al., (2010). “Saliva: reflection of the body”. International Journal of Infectious Diseases **14**(3): 184-188.

Lipson S.F. and P.T. Ellison, (1994). “Reference values for luteal progesterone measured by salivary radioimmunoassay”. Fertility and Sterility **61**:448–454.

Lu Y., R.T. Chatterton et al., (1999). “Salivary estradiol and progesterone levels in conception and nonconception cycles in women: evaluation of a new assay for salivary estradiol”. Fertility and Sterility **71**(5):863-868.

Lu Y.C., L.K. May et al., (1997). “Direct radioimmunoassay of progesterone in saliva”. Journal of Immunoassay **18**(2):149-163.

McGarrigle H.H.G. and G.C.L. Lachelin, (1984). “Increasing saliva (free) oestradiol to progesterone ratio in late pregnancy: a role for oestriol in initiating spontaneous labour in man”? British Medical Journal (Clinical Research Edition) **289**(6443):457-459.

Moriyoshi M., K. Kawata et al., (1996). “Early pregnancy diagnosis in the sow by salivary progesterone measurement using a bovine milk progesterone qualitative test EIA kit”. Journal of Veterinary Medical Science **58**(8):737-741.

Nagy P, D. Guillaume et al., (2004). “Factors affecting plasma progesterone concentration and the retrospective determination of time of ovulation in cyclic mares”. Theriogenology **61**(2-3):203–214.

Needham C., and A. Flint (2007). “An Investigation into the Use of Salivary Progesterone to Diagnose Pregnancy in Sheep”. University of Nottingham Press (Unpublished Data) 1-6.

Pedersen A.M., B. Nauntofte et al., (2005). “Salivary changes and dental caries as potential oral markers of autoimmune salivary gland dysfunction in primary Sjögren's syndrome”. BMC Clinical Pathology **5**(1):4.

Peeters M., M.Vandenheede et al., (2011). “Comparison between blood serum and salivary cortisol concentrations in horses using an adrenocorticotropic hormone challenge”. Equine Veterinary Journal **43**(4):487-493

Pereira Lima D.P., A.C. Okamoto et al., (2010). “Saliva: reflection of the body”. International Journal of Infectious Diseases **14**(3):184–188.

Pietraszek J. and S. Atkinson (1994). “Concentrations of Estrone Sulfate and progesterone in plasma and saliva, vaginal cytology, and bioelectric impedance during the estrous cycle of the hawaiian monk seal (*Monachus schaunslandi*)”. Marine Mammal Science **10**(4):430-441.

Plotka, E.D., C.W. Foley, et al., (1975). “Periovulatory changes in peripheral plasma progesterone and estrogen concentrations in the mare”. American Journal of Veterinary Research **36**(9):1359-1362.

Pycock J.F., (2003). “Use of Ultrasound to Stage Estrus and Predict Ovulation”. Current Therapy in Equine Medicine. 5th ed. N.E. Robinson edited, Philadelphia, PA, Saunders: 242-245.

Quissell D.O., (1993). “Steroid hormone analysis in human saliva”. Annals of the New York Academy of Science **694**:143–145.

Read G.F., (1993). “Status report on measurement of salivary estrogens and androgens”. Annals of New York Academy of Science **694**:146–160.

Relave F., C. Price et al., (2007). “Accuracy of a rapid enzyme-linked immunosorbent assay to measure progesterone in mares”. Canadian Veterinary Journal **48**(8): 823–826.

Riad-Fahmy D., R.F. Walker et al., (1981). “Steroid Immunoassays in Endocrinology”. In: A. Voller, A. Bartlett and D. Bidwell {Editors), Immunoassays of the 80s. MTP Press Ltd., Fallon House, Lancaster, U.K., pp. 205-256.

Riad-Fahmy, D., K. Griffiths et al., (1982). “Steroids in saliva for assessing endocrine function”. Endocrinology Reviews **3**(4): 367-395.

Shideler R.K., J.L. Voss, et al., (1981). “Exogenous progestin therapy for maintenance of pregnancy in ovariectomized mares”. Proceedings of the 27th Annual Convention of the American Association of Equine Practitioners. New Orleans (LA) 211–9.

Shirtcliffe E.A., M.J Curran et al., (2001) “Use of salivary biomarkers in bio-behavioural research: cotton based sample collection methods can interfere with salivary immunoassay results”. Psychoneuroendocrinology **26**(2)165-173.

Short, R.V., (1959). “Progesterone in blood. IV. Progesterone in the blood of mares”. Journal of Endocrinology 19: 207-210.

Stout T.A.E., W.R. Allen, (2000). “The uterus as a source of oxytocin in cyclic mares”. Journal of Reproduction and Fertility. Supplement **56**:281-287.

Tallon D.F., E.M. Odwyer et al., (1984). "Direct solid phase enzyme immunoassay of progesterone in saliva". Clinical Chemistry **30**(9):1507–1511.

Tallon, D.F., P.F. Fottrel et al. (1984). "Direct solid-phase enzyme immunoassay of progesterone in saliva". Clinical Chemistry **30**(9): 1507-1511.

Townson, D.H., R.A. Pierson et al. (1989). "Characterization of plasma progesterone concentrations for two distinct luteal morphologies in mares". Theriogenology **32**(2):197-204.

Troedsson M.H.T., (2003). "Placentitis". In Robinson N.E.R., ed: Current Therapy in Equine Medicine V. St Louis, WB Saunders 297-300.

van Niekerk C. H., (1965). "The early diagnosis of pregnancy in mares". Journal of South African Veterinary Association **36**:53-58.

Van Niekerk C.H., (1965). "The early diagnosis of pregnancy, the development of foetal membranes and nidation in the mare". Journal of South African Veterinary Medical Association 36, 483-488.

Van Nieuw Amerongen A.V., E.C.I. Veerman et al. (2004). "Salivary proteins: protective and diagnostic value in cariology"? Caries Research **38**(3):247–53.

Verheugen C., G. Chaudhuri et al. (1984). "Differential permeability of uterine and liver vascular beds to estrogens and estrogen conjugates". Journal of Clinical Endocrinology and Metabolism **59**(6):1128-32

Vimpeli T., E. Kujansuu et al., (2001). "Salivary and serum progesterone concentrations during two luteal support regimens used in in vitro fertilization treatment". Fertility and Sterility **76**(4):847-848.

Vining R.F. and R.A. McGinley (1986). "Hormones in saliva". Critical Reviews Clinical Laboratory Sciences **23**(2):95–146.

Vining R.F. and R.A. McGinley (1987). "The measurement of hormones in saliva: possibilities and pitfalls". Journal of Steroid Biochemistry **27**(1-3):81–94.

Walker R.F., D. Riad-Fahmy et al. (1985). "Characterization of profiles of salivary progesterone concentrations during the luteal phase of fertile and subfertile women". Journal of Endocrinology **104**(3):441–448.

Watson E.D., E. Nikolakopoulos et al., (1997). "Immunolocalisation of oxytocin in the uterus of the mare". Journal of Reproduction and Fertility Abstract Series **20**:31.