11-AND 3-HYDROXYPROGESTERONE AS A PREGNANCY TRACKING HORMONE-METABOLITE IN 13-LINED GROUND SQUIRRELS (*ICTIDOMYS TRIDECEMLINEATUS)*

By Amy G. Miller

Thirteen-lined ground squirrels (13LGS; *Ictidomys tridecemlineatus*) are small, omnivorous, fossorial, hibernating sciurid rodents. As favored models for studies of hibernation and cone-based visual function, they have only recently been bred in captivity on any sort of scale, and only here at UW Oshkosh. 13LGS are reflex ovulators, mating only in spring with a ~28 day gestation period. Increasingly, the UW Oshkosh 13LGS Colony fields client requests for pregnant females, in order to facilitate the first steps of transgenic modification and for embryological studies. These aims require a far better understanding of the captive 13LGS's reproductive endocrinology than we currently have. This project was to ascertain whether enzyme-linked immunosorbent assay (ELISA) of squirrel fecal samples can be used to non-invasively detect pregnancy in a low-stress manner. To this end, competitive ELISAs for 11- and 3-hydroxyprogesterone were conducted on a group of thirteen females, comprising proven dams bred in the Colony or captured from the wild. Feces were collected thrice weekly during the spring of 2016 and frozen for subsequent steroid hormone extraction. Feces collections ceased as soon as a litter was noted. Competitive ELISA tests against the metabolite 11- and 3-hydroxyprogesterone were run using kits (Arbor Assays, Ann Arbor, MI), setting data against seven different time points between hibernation exit and birth of a litter. An ANOVA with repeated measures was run on the ELISA data to identify any significant differences in fecal hormone-metabolite between these time points. Then a pairwise t-test with a Bonferroni correction was run to determine where the differences lay. A Wilcoxon rank sum test was run on the captive-bred *vs.* wild-caught animals to determine significant differences; a Spearman's rank correlation was performed to determine a possible correlation between litter size and progesterone concentration; and a Student's t-test was run on data from pregnant and non-pregnant animals to determine significant differences. Eleven of 13 dams produced litters in the spring of 2016. Fecal ELISA data from the 2 non-pregnant females demonstrated no rise in fecal hormone-metabolite at any time point over four weeks. In contrast, data from the 11 parous females all demonstrated a pronounced rise in fecal hormone-metabolite, with most animals displaying progesterone withdrawal in the final week of gestation. Compared to baseline fecal hormone-metabolite levels on the day of pairing with a male, this rise was statistically significant halfway through gestation, with a >20-fold increase just a week into gestation. No significant differences were noted based on whether a pregnant female was wild-caught or captive-bred, nor with size of the litter. A technical replicate revealed the apparent decomposition of fecal 11- and 3-hydroxyprogesterone over 14 weeks, despite being stored frozen. This study has yielded a step-by-step protocol by which 11- and 3-hydroxyprogesterone may be monitored non-invasively through competitive ELISA of fecal pellets, permitting the detection of pregnancy at least 2 weeks prior to the birth of a litter. It further confirms the 13LGS as consistent with other sciurids in its status as a placental mammal that undergoes progesterone withdrawal late in gestation.

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Background

My thesis research entailed detection of progesterone in fecal samples from female 13-lined ground squirrels (*Ictidomys tridecemlineatus*, 13LGS). To properly introduce this study, I present here background about the hormone progesterone, its sites of synthesis, its receptors, its target tissues, and its action/function in pregnancy, and how it ends up in feces. Major sources of this information are Tarraborrelli (2015) and Graham and Clarke (1997). I also present information on the method known as competitive ELISA (enzyme-linked immunosorbent assay), including how data are generated, validated, and presented.

Progesterone

Progesterone is a hormone that is released by the ovary's corpus luteum, following ovulation, and the placenta, during gestation occurring at specific time intervals of the female mammalian reproductive cycle. Progesterone is classified as a steroid, which means that its base structure is a lipid derived from cholesterol. Because of its lipophilic nature, progesterone cannot dissolve directly in body fluids including blood, and so it is carried by sex hormone binding globulins (SHBGs). In contrast, progesterone readily passes through cell membranes because they are also lipid-based.

Progesterone receptors are located within the target cell's nucleus. Once bound, progesterone typically alters gene transcription and translation, beginning a cascade of physiological events that support reproduction. Receptors are found in many mammalian tissues, including the uterus, the ovary, mammary glands (breasts), brain, and bone. Receptor abundance in target cells is increased by estrogen and down regulated by

progesterone itself. Also, after progesterone binds to intracellular receptors of target cells, it is commonly inactivated by intracellular enzymes. Regulation of progesterone signaling may be accomplished by control of its release or by altering receptors in preparing the female body for reproduction. The hypothalamic-pituitary-gonadal axis (HPGa) hormone cascade is as follows: The hypothalamus releases gonadotropin releasing hormone (GnRH) in pulses into the portal circulation that carries GnRH to the anterior pituitary. The frequency of the pulses determines whether the anterior pituitary releases follicle stimulating hormone (FSH) or luteinizing hormone (LH). These two gonadotropins subsequently control ovarian hormone release, which in turn has large effects on the ovaries (self-regulation), the uterus, and the breasts.

Ovarian Production of Progesterone

Luteal production of progesterone begins with the hypothalamic-pituitary-axis (HPa). The hypothalamus receives neurotransmitter signals based on external and internal stimuli. In seasonal breeders like 13-lined ground squirrels, such signals include information about circannual cycle and day length. In response, the hypothalamus secretes hormones to either the anterior or posterior pituitary gland.

Axonal tracts shuttle intracellular hypothalamic hormones to the posterior pituitary, where they are released into the general circulation. Their functions include the control of uterine contractions, lactation, social bonding, and salt balance in the blood.

In contrast, inhibiting and releasing hormones from the hypothalamus are delivered to the anterior pituitary by the hypophyseal-portal vasculature. These antagonistically control the corpus luteum (plural “corpora lutea”). In addition to

releasing estrogen, the corpora lutea now also release significant amounts of progesterone. The chief function of progesterone at this time is to stabilize the uterine endometrium which, during the follicular phase, was in a state of mitotic proliferation. Progesterone helps maintain the endometrium at the optimal thickness for eventual blastocyst implantation, should fertilization of the ova occur. Note that the progesterone produced by corpora lutea in the days following ovulation is not a sign of actual pregnancy, because this release occurs even if there is no fertilization event.

If there is no fertilization, after a few days the corpora lutea degenerate and estrogen and progesterone levels fall. In a human, the menstrual phase would ensue, with visible drainage of the discarded uterine lining from the vulva. In the 13LGS, the discarded uterine lining is absorbed internally, along with the unused ova. In the wild this is an unusual occurrence since the majority of females are impregnated.

If there is fertilization, the embryos secrete chorionic gonadotropin (CG), which shares much of the same structure as FSH and LH. This CG signal further stabilizes the corpora lutea and their ongoing production of progesterone. In this way, CG also stabilizes the uterine endometrium, ensuring that it lasts long enough for the embryos (at blastocyst stage) to successfully implant. The 13LGS has a Y-shaped uterus with two horns.

Placental Production of Progesterone

After implantation, the embryonic chorion and the maternal endometrium begin a partnership to form a placenta for each embryo. Estrogen and progesterone will then begin the anterior pituitary's release of hormones into the general circulation for

downstream actions. These functions include the control of growth hormone, thyroid hormone, the adrenal stress response, and reproductive targets including the gonads, uterine tract, and breasts. For the latter functions, the HPGa is of great importance.

During the follicular phase, FSH stimulates some ovarian follicles containing ova to enlarge, which enhances their release of estrogen. A positive feedback loop ensues, which culminates in a burst of FSH and estrogen, along with a burst of LH. The LH burst in turn stimulates ovulation, which is the release of ova from their follicles. In my project's species, multiple births of 4-14 pups are the norm, so multiple follicles rupture at ovulation. The released ova enter the uterine tubes, where they join up with sperm if copulation has recently occurred.

In mammals, there are generally two different types of ovulation: spontaneous and induced ovulation. Thanks to HPGa activity, post-pubertal and pre-menopausal humans spontaneously ovulate once during their 28 day cycle, whether there is copulation or not. In contrast, the 13LGS is an induced long-day ovulator. Females mate anywhere from 1-14 days after emerging from hibernation in spring, as daytimes lengthen. The act of copulation is required for ovulation to occur (Foster, 1934). A second, non-reproductive estrus is entered in late summer (Strauss *et al.*, 2009; Millesi *et al.*, 2008). It is non-reproductive largely because, that late in summer, male squirrels have lost testicular function. Therefore, female 13-lined ground squirrels enter hibernation in fall with fairly well-developed follicles.

Induced ovulation marks the beginning of the luteal phase. The now-empty follicles (still stimulated by the LH burst) continue to enlarge, but change their phenotype

into corpora lutea. Once the ova are fertilized, have implanted, and placental tissue forms, the placentas will release progesterone in ever-increasing amounts throughout pregnancy. In humans, the ovarian contribution to estrogen and progesterone falls dramatically during pregnancy, being wholly replaced by placental release. In 13LGSs, however, persistent corpora lutea are thought to supplement placental progesterone (Foster, 1934).

At term, the pregnancy-promoting effects of circulating progesterone must be interrupted in order to permit the placenta's separation from the uterus and exit the mother's body during labor and delivery (Nnamani *et al.*, 2013). In the 13LGS, blood progesterone levels fall in the latter half of pregnancy. This is a ligand-based mechanism to decrease progesterone signaling, and is termed progesterone withdrawal (progesterone is the ligand). This contrasts with humans and guinea pigs, where blood progesterone levels remain high, and instead receptors are down regulated instead of the ligand (progesterone) (Nnamani *et al.*, 2013). This mechanism is called functional progesterone withdrawal.

Some Sciuridae (the family that represents all squirrel species) exhibit a second burst of progesterone during lactation that exceeds that of actual pregnancy, apparently from persistent corpora lutea (Belding's ground squirrel (*Urocitellus beldingi*), California ground squirrel (*Otospermophilus beecheyi*), prairie dog (*Cynomys*), woodchuck (*Marmota monax*); Millesi *et al.*, 2008; Holekamp *et al.*, 1988). This second progesterone burst's proposed purpose is to prevent conception of a second litter in the same summer. A second litter would not have time to wean, grow, and fatten for hibernation entry, and would only exhaust the mother's resources for her own upcoming hibernation season. To

my knowledge, no one has yet determined if the 13-lined ground squirrel also undergoes a lactational progesterone burst.

How Progesterone Enters the Feces

Progesterone released from corpora lutea or placenta into the general circulation is bound by SHBGs, because progesterone itself is so lipophilic. These globulins deliver progesterone to targets tissues but also to the liver, which alters the structure of native progesterone by conjugation. Using enzymes, liver cells typically add sulfate or glucuronide moieties to sex steroids, making them far more water soluble. This in turn means the SHBGs are no longer needed. Conjugated progesterone is thought to be inactive and slated for urinary or fecal excretion. The lag time between progesterone in the blood and its excretion is not yet determined in 13LGS, but Mateo (2006) has demonstrated a 6-12 hour lag time for fecal stress steroids in Belding's ground squirrels. Preliminary data from the D. Merriman laboratory at the University of Wisconsin Oshkosh support this lag time for estrogen (Duncan and Jentz, unpublished).

Some evidence exists that conjugated progesterone can be converted back to "free" form by target cells and gut microbes. This de-conjugated progesterone may return to the blood (Honour, 2015), re-associate there with SHBGs, and have a second chance at activating receptors. For the work presented here, I made the simplifying assumption that the more progesterone reaches the blood, the more is conjugated (by the liver), and the more is excreted.

While most conjugated progesterone is assumed to exit the body via urine, urine collection is impractical in my project species. Therefore I focused on fecal 11-and 3-hydroxyprogesterone detection (its conjugated form when it is in feces).

Enzyme-Linked Immunosorbent Assays

Enzyme-linked immunosorbent assays (ELISAs) are one way researchers make use of antibodies to detect molecules of interest (or antigens) in sample tissues. The "antigen" is so named because it generates an antibody response. Antibodies can be elicited from the immune systems of "production" animals simply by immunizing them with purified antigen. The antibody produced will be specific to the shape of the triggering antigen. Antibody molecules can be purified from the production animal's blood, or else immortal cell cultures can be made from the production animal's immune cells that produce the desired antibody and release it into culture medium for purification.

Immune cells called B-lymphocytes produce antibody molecules that bind to the antigen with great specificity — specifically binding to the epitope of the antibody — to mount an immunological response to the antigen. This specific binding of the antigen to epitope of the antibody is mainly due to the shape of the antibody itself, and its various fragments (Parham, 2009). Antibodies have a "Y" shape; the stem is known as the heavy chain constant region, and the arms are known as the variable region (made up of heavy and light chains). The variable region is where the antigen will bind, or the Fab (**f**ragment **a**ntigen **b**inding). The heavy chain constant region is often called the Fc region (**f**ragment **c**rystallizable region). The Fab regions of the antibody are connected to the Fc region by "hinges" that allow for the Fab region to adopt many orientations with respect to the Fc

region. This flexibility allows for the variable regions of the antibody to bind at various distances on the surface of the antigen (conforming to the specific shape of the antigen), allowing for the tight binding of the antibody and antigen (Parham, 2009).

Since these molecules that are bound to one another are still too small to see, it is necessary to make antibody-antigen binding visible. With any ELISA, the basic premise of the assay is to link antibody-antigen binding to enzyme action that creates a colored product. That colored product can then be quantified and used as a proxy measure of antigen content.

There are different types of ELISA assays: direct, indirect, sandwich, and competitive. The competitive ELISA is the only choice for detecting fecal hormone metabolites. This is because fecal samples are complex, and the competitive ELISAs are more sensitive to smaller concentrations of antigen that are present within these complex samples. ELISA is plate-based (reagents are placed into wells of a polystyrene plate with samples in question) and uses an antibody competitor to produce an absorbance (in wavelengths) that reflects the concentration of the antigen within different samples.

Competitive ELISA Theory

For my research, 11- and 3-hydroxyprogesterone was the antigen and my ELISAs were based on using an anti-11- and 3-hydroxyprogesterone. The first part of any assay is to prepare the samples that contain the 11- and 3-hydroxyprogesterone. This requires extracting the 11- and 3-hydroxyprogesterone in a standard way from some crude preparation obtained from each subject of the study, such as tissue biopsies, urine, or feces. Since 11- and 3-hydroxyprogesterone is lipid-based, an alcohol makes a good

solvent for extracting it from feces whether it is in native form or conjugated form. Hereafter, the term "sample" will refer to fecal extracts.

To develop 11- and 3-hydroxyprogesterone profiles during pregnancy in 13-lined ground squirrels, I used competitive ELISA kits to keep all procedures highly standardized. The ELISA kit that I used in this study was designed as a species-independent assay, and the wells of the plate were pre-coated with goat anti-mouse IgG antibody to facilitate the binding process. The goal was for 11- and 3-hydroxyprogesterone in the samples to bind to this anti-11- and 3-hydroxyprogesterone within each well.

The exposed plastic surfaces of the wells are not 100% covered with anti-11- and 3-hydroxyprogesterone and may artificially bind 11- and 3-hydroxyprogesterone in my samples, which would create a false positive. To avoid this false positive signal, the exposed plastic must be pre-coated with another molecule, such as protein like bovine serum albumin (BSA). This step is known as blocking. Blocking with BSA is permanent, and it coats the potential binding sites with a non-relevant protein (BSA) which decreases the chances of signal-producing antibodies binding non-specifically. Blocking also helps to stabilize the antigen molecules that will be bound to the plate (Block *et al.*, 1990). At this point, the wells are ready to bind any 11- and 3-hydroxyprogesterone molecules that are added to them.

Next, samples (containing unknown amounts of 11- and 3-hydroxyprogesterone) are pre-mixed with known amounts of 11- and 3-hydroxyprogesterone that are

conjugated to an enzyme — or simply the competitor. This enzyme (horseradish peroxidase, or HRP) creates the colored product in the final step of the assay.

Either of these forms of 11- and 3-hydroxyprogesterone are equally capable of binding to the anti-11- and 3-hydroxyprogesterone that coats the bottom of the plate wells. Think of them as different "teams" of 11- and 3-hydroxyprogesterone that are "competing" for a limited number of binding sites in each well.

The mixture of sample and conjugate progesterone are added to the wells and given two hours to compete, after which any unbound molecules are washed away. The enzyme's substrate (tetramethylbenzidine, TMB) is added and, after 30 minutes, the colored product forms. The reaction is stopped using hydrochloric acid with a pH of zero. A plate reader is then used to measure the amount of colored product, based on its known absorbance (optical density).

If the sample happens to contain *only a little* 11- and 3-hydroxyprogesterone, then by chance most of the binding will be done by the 11- and 3-hydroxyprogesterone-enzyme conjugate. With all that enzyme-conjugate binding, a darkly-colored product in the well will result, clearly indicating that the sample 11- and 3-hydroxyprogesterone "lost" because it was in the minority.

If the sample happens to contain *a lot* of 11- and 3-hydroxyprogesterone, then by chance *most* of the binding will be done by the sample 11- and 3-hydroxyprogesterone, *not* by the enzyme conjugate. With so little enzyme-conjugate binding, a barely-colored product in the well will result, clearly indicating that the sample 11- and 3-hydroxyprogesterone "won" because it was in the majority.

An important step in any ELISA is the initial creation of a standard curve using known amounts of 11- and 3-hydroxyprogesterone instead of the unknown amounts found in actual samples. Therefore, the kit includes a supply of pure 11- and 3-hydroxyprogesterone for this purpose. The 11- and 3-hydroxyprogesterone kit (Arbor Assays, Ann Arbor, MI) used in my study had seven standards, prepared by taking the pure 11- and 3-hydroxyprogesterone and adding it to kit assay buffer in an Eppendorf tube. The contents of this tube are then serially diluted six more times to give a range of 1:50-1:3200 pg/ml for the standards. Each assay that is run needs to have its own standard curve to calibrate the plate reader's measurements of color development. Then, when sample readings are obtained, their values can be compared to the standard curve and an accurate idea is gained of the 11- and 3-hydroxyprogesterone content of each sample. If for some reason the sample values don't initially fall on the standard curve, adjustments to sample dilution are done until they do. Figure 1 shows an example of an actual standard curve that was produced during this study.

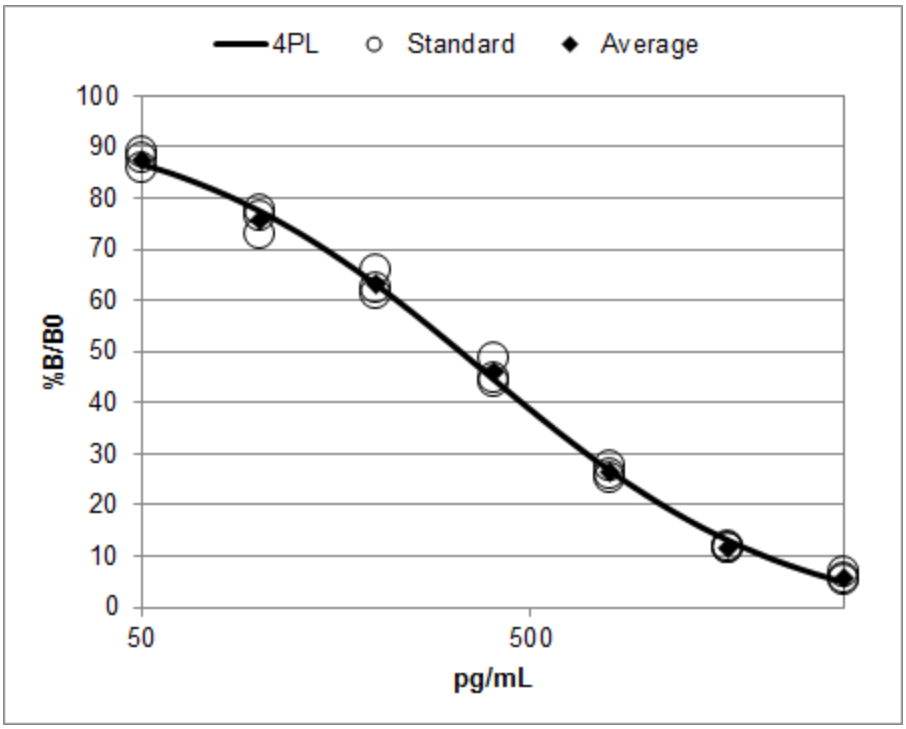

Figure 1. Example of a standard curve run during the study. The x-axis shows the concentration of 11- and 3-hydroxyprogesterone in pg/mL; the y-axis shows the percent binding (or the absorbance) at each concentration. The circles are the values of the standards for this particular assay, the triangles are the averages for this assay, and the thick black line represents the 4-parameter logistic curve that is generated with the standard curve.

Units of measure from the plate reader are picograms of 11- and 3-hydroxyprogesterone per milliliter of sample extract (pg/ml), but are then converted via a simple calculation to nanograms of 11- and 3-hydroxyprogesterone per gram of dry fecal material used in the extraction step.

In my work, the plate reader gave all of the original concentrations (pg/ml) with the dilution factor already calculated. To standardize methods as much as possible, I used 0.1g of dried feces. The only time this did not occur is when the fecal sample was so

small that using less than 0.1g of dried feces was unavoidable. A sample conversion would be as follows:

- Plate reader gives a value of 106,050 pg/ml
- Original amount of dried feces was 0.1 g
- Divide 106,050 pg/ml by 0.1g
- Final concentration is 1,060.5 ng/g

Triplicate wells (including standards and controls) were also performed with each of the plates run. The reason triplicate wells were chosen over duplicate wells is that, if only two wells are run per sample or standard and something does not run appropriately within that well, then there is only one other well with which to compare to determine accuracy and/or precision. If, however, the wells are run in triplicate, the three wells can be compared to each other. If one well is drastically different from the other two, we can eliminate that well and compare the other two wells.

We can then determine the coefficient of variation between the useful wells, and if the coefficient of variation is under a certain percentage (I used $\leq 15\%$), then you can determine that the wells are showing a precise reading.

A technical replicate was also performed in this study. Normally a technical replicate would run all of the samples at least twice to determine any variability in the testing protocols. In my study, because of constraints in time and resources, a technical replicate was only performed on samples from one time point for each female. This particular time point was decided on because of its potential significance of higher progesterone levels compared to the other time points during the gestation period.

Introduction

The 13-lined ground squirrel (13LGS; *Ictidomys tridecemlineatus*; Rodentia; Sciuridae) is an important model species for studies of hibernation physiology (Jastroch *et al.*, 2016; Reilly & Franklin, 2016; Tupone *et al.*, 2016; Ratigan & McKay, 2016; Berg von Linde *et al.*, 2015; Van Breukelen & Martin, 2015), both for its own sake and also for possible translation into medical treatment. It has also been central to our understanding of diurnal, cone-dominant vision (Van Hooser & Nelson, 2006; Krubitzer *et al.*, 2014; Merriman *et al.*, 2016).

As previously described (Vaughan *et al.*, 2006; Merriman *et al.*, 2012), 13LGS prove challenging but not impossible to breed in captivity. They are not sexually mature until near their first birthday, but in captivity may produce offspring for at least six years thereafter (D. Merriman, unpublished). Ground squirrel physiology is strongly influenced by circannual rhythms (Helm *et al.*, 2013) so, from the establishment of the animal colony's beginnings in 2003, the 13LGS have been bred with the philosophy of replicating their circannual cycle by putting all breeding animals through winter hibernation.

Each fall in the UW Oshkosh Squirrel Colony, adult and juvenile 13LGS are placed into a dark 4°C hibernaculum ("autumnal immergence") where they undergo torpor and interbout arousals. Each spring, they are returned *en masse* to regular animal rooms ("vernal emergence") (Vaughan *et al.*, 2006; Merriman *et al.*, 2012). In common with wild animals, captive male emergence is scheduled before female emergence, to give time for the resumption of spermatogenesis (Streubel & Fitzgerald, 1978).

Mechanisms controlling the sex-specific timing of emergence in the wild are somewhat plastic, converging with annual weather patterns, and are incompletely understood (Sheriff *et al.*, 2013).

In the UW Oshkosh Squirrel Colony, emerged males and females are housed in the same room, permitting immediate transfer of visual, auditory, and olfactory cues thought to promote estrus (Millesi *et al.*, 2000). All females are observed for 24-48 hours after emergence to ensure they are healthy, after which physical exposure to a male begins. Under the constraints of captivity, the Colony has experimented with different modes of physical exposure to the male, including co-housed pairs (1M1F); co-housed trios (1M2F); quads co-housed every other day (1M2F2F); and large enclosure mating in which mate choice is possible (5M7F). All methods have yielded litters (D. Merriman, unpublished data).

Upon normal emergence in the wild, 13LGS ovaries already contain antral follicles (Foster, 1934). As described by others (Landau & Holmes, 1988) and based on recorded litter birth dates in the UW Oshkosh Colony, some females copulate within a day or two of vernal emergence while others may not mate until three weeks later. A similar range of birth dates is also observed in the wild at various latitudes (Streubel & Fitzgerald, 1978).

In the wild and in the UW Oshkosh Colony, each year a male will mate multiple females, but each female produces a single litter of 4-14 young. To date, a second litter has only been produced by Colony females when the first litter perished and the females were immediately re-bred. Notably, as summer progresses, 13LGS testes regress and

become nonfunctional (Johnson, Foster, & Coco, 1933). This seasonal male infertility is a major factor in limiting female 13LGSs to one litter per year, though a second litter has been reported in the wild in their southernmost range (McCarley, 1966).

The 13LGS's natural mating strategy is scramble competition polygyny, with first male advantage (Foltz & Schwagmeyer, 1989). Behavioral estrus lasts only a few hours once copulation has occurred. Females are considered induced ovulators, with a lag time of 10-36 hr after copulation (Foster, 1934). A gestation period of 27-30 days is variously claimed, possibly due to this range in ovulatory lag (Barr & Musacchia, 1968; Bridgewater, 1966; Foster, 1934; Johnson, Foster & Coco, 1933; Johnson & Wade, 1931; Landau & Holmes, 1988; Merriman *et al.*, 2012; Vaughan *et al.*, 2006). Copulatory plugs do occur in 13LGS, but not often enough nor do they last long enough (Koprowski, 1992) to be reliable reporters of copulation in a colony of >100 females.

In placental mammals like the 13LGS, serum progesterone rises throughout gestation. To permit parturition, progesterone signaling is thought to fail in one of two basic ways: either progesterone ligand falls in concentration (a.k.a. "progesterone withdrawal"), or progesterone receptors desensitize in some manner (a.k.a. "functional progesterone withdrawal") (Mitchell & Taggart, 2009). Progesterone withdrawal has been demonstrated in several sciurid species (California ground squirrel (*Otospermophilus beecheyi*), Holekamp *et al.*, 1988; alpine marmot (*Marmota marmota*), Exner *et al.*, 2003; woodchuck (*Marmota monax*), Concannon *et al.*, 1984) and this is believed to be generalized to all sciurids (reviewed in Nnamani *et al.*, 2013). In an unusual twist, serum progesterone has been shown to rise again during ground squirrel

lactation, a phenomenon proposed to prevent a second pregnancy, thus giving the dam plenty of time to fatten after weaning her first litter so that she can survive the ensuing winter (Holekamp *et al.*, 1988; Exner *et al.*, 2003). A second, non-reproductive estrus occurs in ground squirrel dams after weaning (13LGS, Foster, 1934; European ground squirrel (*Spermophilus citellus*), Millesi, 2008), when male fertility is long past. This second estrus may give fall females a head start to the following spring's reproductive readiness.

The accurate detection of pregnancy is a concern in the UW Oshkosh Colony, in part because we increasingly receive client requests for pregnant female 13LGS. Survival blood draws to detect the progesterone rise of pregnancy are invasive and stressful and, considering the 13LGS's active season ~150 g body weight, are limited in volume and frequency by best practices. We therefore turned to fecal sample analysis as a non-invasive, less stressful method.

Fecal testing requires a sensitive assay that can detect hormone metabolites lost in feces (Palme, 2005). Fecal enzyme linked immunosorbent assays (ELISAs) have been validated for stress steroid measurements in rats (Cavigelli *et al.*, 2005) and various sciurids (Belding's ground squirrel (*Spermophilus beldingi*), Mateo and Cavigelli, 2005; Mateo, 2006; Richardson's ground squirrel (*Urocitellus richardsonii*), Clary *et al.*, 2014). Fecal ELISAs have also been used for sex steroid measurements in sciurids (Pettit *et al.*, 2006), including hibernating induced ovulators like the 13LGS (Vancouver Island marmot (*Marmota vancouverensis*), Keeley *et al.*, 2012).

During the 2015 breeding season, we performed a pilot study on 11- and 3-hydroxyprogesterone profiles during pregnancy using ELISA testing of female 13LGS fecal samples. Here we provide the results of a full study conducted during the 2016 breeding season.

Methods

Animals and Housing

All animal procedures were preapproved by the University of Wisconsin Oshkosh Institutional Animal Care and Use Committee and conformed to USDA, OLAW, and AAALAC guidelines for rodent care. Per standard operating procedures (Vaughan *et al.*, 2006; Merriman *et al.*, 2012), 13LGS were caged in clear plastic shoebox type cages measuring 17”x24”x8” and provided water, a base diet of dog kibble, and daily rotating enrichment treats (live mealworms, sunflower seeds, dried vegetables, peanuts in the shell, dried corn). All cages were on the same cage rack in the same animal room throughout the study. Photoperiod in the ~68°F room matched that of ambient, *i.e.* day length increased as the study progressed. Animals were uniquely identified by microchips.

Fourteen female 13LGSs were selected for this spring 2016 study. Late in the study, one animal displayed prolonged inappetance and lethargy, was euthanized on the veterinarian’s recommendation, and was eliminated from the data set. Necropsy was unremarkable.

Of the remaining 13 females, 7 had been captured from the wild as pregnant adults in either spring 2013 or 2014, so were at least 3 years old. Four had been born in the Colony in 2013 and were 3 years old. Two had been born in the Colony in 2014 and were 2 years old. All had weaned litters the year before (2015), so were considered proven dams.

All females were removed from the hibernaculum and single-housed on 9 April after having hibernated the previous winter for periods ranging from 28-32 weeks. All 13 were observed to arouse from hibernation normally and resumed eating the same day. On 11 April, an initial fecal sample was collected from each female while she was still single-housed (see below for details). Thereafter, fecal sample collection occurred every Monday, Wednesday and Friday, either until a litter was observed or until 1 June, whichever came first.

After initial fecal collection in the morning of 11 April, exposure to a male began, either in a trio cage (1M2F) or in a "quad" (1M2F2F). In the latter case, on alternate days the male was moved back and forth between 2 pairs of co-housed females. All males selected for this study had hibernated normally; had been brought out of the hibernaculum on 1 April; had aroused normally from hibernation; had normal-appearing breeding season testes; and had sired at least one litter the prior season (2015). As is standard practice in the UW Oshkosh Squirrel Colony, females were bred to the same male as the prior season whenever possible (6 of the 13 study females). While some females rejected the immediate approach of some mates upon introduction, inter-individual aggression has been an extremely rare occurrence in the Colony and was not a problem in this study.

As is standard in the Colony, females were moved to individual housing and male exposure ended 3.5 weeks after it began, ~3 days short of the earliest possible litter birth date. Of the 13 females in this study, 11 bore litters and 2 did not. This gave a rate of 85% fecundity in the study (11 of 13), compared to 42% in the rest of the Colony (39 of

91). Of the 2 study females that did not give birth, the male's fertility was a possible factor in only one case, as all other males used in this study sired 1-3 litters in 2016. Despite thrice-weekly examination, none of the 11 parous females was ever observed with a copulatory plug.

Litter birth dates within the study ranged from 10-28 May. From litter birth dates, we counted back 28 days to estimate the date of presumed copulation. From this, we determined that lag time from first male exposure (11 April) to copulation ranged from 1-19 days in the study group, in line with what we have historically observed in the Colony as a whole.

One of the 7 wild-caught females, and 2 of the 6 captive-bred females, cannibalized their litters after birth. This gave a 2016 litter loss rate of 23% (3 of 13 animals) in the study group, compared to 10% in the rest of the Colony (9 of 91 animals).

Sample Collection and Storage

Fecal sample collection always commenced at 0800 hours, approximately one hour after lights on. Squirrels were handled the same way, by the same person, in the same order each time. For collection, each female was taken from her home cage. A digital image of her lower abdomen and genitalia was quickly captured and then she was placed into a clear plastic shoebox cage measuring 10"x19"x8" containing a plastic hiding tube and water bottle, but no bedding. A highly palatable enrichment treat (shelled peanut) was offered to occupy her and to encourage GI motility.

Females were left in collection cages for 2 hours and then all available fecal samples were collected. Due to ongoing microbial metabolism, fecal steroids can drop by

17% after 2 hours at room temperature (Pettitt *et al.*, 2007; Schutt *et al.*, 2012), so this time lag may have introduced some sample variability. If feces were not produced by 2 hours, that date's sample was deemed not available (N/A). Fecal samples were not screened for urine contamination. Fecal samples were placed into Eppendorf tubes labeled by animal ID and date of collection, and stored in a -80°C freezer where they remained until extraction. Samples frozen in this manner are stable for up to twelve months (Pettit *et al.*, 2007), but all 2016 samples were processed within four months.

Sample Selection, Extraction, and Reconstitution

For the 11 females that gave birth, we assayed fecal samples at particular landmarks with reference to the consensus 28-day span between copulation and litter birth date. These landmarks were:

- DOP (date of pairing but prior to male exposure, 11 April in all cases),
- DOP+2 (DOP plus 2 days, 13 April in all cases);
- eDOF (estimated day of fertilization, *i.e.* 28 days prior to litter birth date),
- DOB-21 (litter birth date minus 21 days),
- DOB-14 (litter birth date minus 14 days),
- DOB-7 (litter birth date minus 7 days), and
- 1-3 Days Before Birth (so-called because dams are not disturbed once a litter is born, limiting me to the MWF fecal sample that had been most recently collected, *i.e.* from 1-3 days prior to birth date).

Extraction of frozen fecal samples occurred between 18 May and 29 July. Samples were removed from their freezer tube, laid onto a labeled square of aluminum

foil, and placed into a drying oven at 75°C for 2-3 hr depending on the number of samples. Dried samples were then immediately ground into a powder with a clean mortar and pestle. Macro-impurities were removed using clean forceps and the powdered samples were weighed to the nearest 0.01 g.

For extraction, a 0.1 g measure of each powdered sample was combined with 1 mL 200 proof ACS-grade ethanol in a labeled Eppendorf tube, which was then capped and mixed on an orbital shaker set on high for 30 min. Remaining powdered sample was stored in the -80°C freezer. After 15 min of centrifugation at 5000 rpm, a single 100 µl aliquot of supernatant and the remainder (containing extracted steroids) were separately transferred to new labeled tubes and placed back into the freezer. ELISA testing of the 100 µl aliquot occurred within one week of sample extraction. Thawing of the remainder for replicate use occurred within four months of sample extraction.

Tubes containing 100 µl aliquots of extracted steroids were uncapped and evaporated to dryness using a SpeedVac (Labconco Centrivap Concentrator & Cold Trap, model #225638, Kansas City, MO with a Precision Scientific Vacuum Pump, model #D 75, Chicago, IL) and then covered and stored in a desiccator in the freezer for up a week before reconstitution the day of use in an ELISA. Reconstitution was done by removal from the freezer, adding back 100 µl of room temperature ethanol, and vortexing 3 times for 10-20 seconds during at least a 15 minute period (if samples sat longer, that was acceptable).

Competitive ELISA Procedures

For competitive ELISA, we used 11- and 3-Hydroxyprogesterone EIA kits designed for use with fecal extracts (11- and 3-Hydroxyprogesterone, Arbor Assays DetectX item K025, Ann Arbor, MI). These kits include an 11- and 3-hydroxyprogesterone standard which was processed for the standard curve per kit instructions. A 50 µl measure of 11- and 3-hydroxyprogesterone standard, diluted in kit assay buffer, was added to each well of the kit's plate, followed by 25 µl of kit anti-11- and 3-hydroxyprogesterone and 25 µl of kit 11- and 3-hydroxyprogesterone conjugate. Ultimately, the dilution series for standards ranged from 1:50 to 1:3200.

Per the manufacturer, after loading, the plates were shaken for 2 hr at room temperature. The excess was then aspirated and discarded, and the wells were washed four times with kit wash buffer. A 100 µl measure of the kit's TMB substrate was placed in each well for 30 min at room temperature. The reaction was stopped in each well using 50 µl of kit HCl (pH of zero), and the plates were read immediately in a BioRad iMark plate reader (Hercules, CA). Raw data were imported into an online analysis package (www.myassay.com for Arbor Assays Progesterone EIA kit). Wells were always run in triplicate. If the coefficient of variation among triplicate wells was >15%, the outlier was excluded from the results or the same assay was re-run on a new plate. A fresh set of triplicate standard curve wells was run each day that ELISA was conducted.

The average percent for extraction efficiency for exogenous hormones from 11- and 3-hydroxyprogesterone was 82%. The average percent recovery for exogenous

hormones from 11- and 3-hydroxyprogesterone was 75.8%. Average intra- and inter-assay coefficients of variation were 9.3% and 14% respectively.

Parallelism of pooled fecal extracts (n=5) was shown by comparing the mean optical densities of the serially-diluted pool to the standard curve that the kit provided. Parallelism was run using pooled ethanolic extracts from feces collected from representative females. The samples chosen had been collected on 11 April and 13 April because this was prior to or just after exposure to a male, when progesterone levels were predicted to be at their lowest. Ethanolic fecal extracts were diluted in a range from 1:2 - 1:2,048 using kit assay buffer, and wells were run in triplicate. Results from a pool of ethanolic samples were averaged by the assay software and are shown in Figure 2. Conversion to ng/g is not done for parallelisms since the purpose is to compare samples to progesterone standards that did not come from feces.

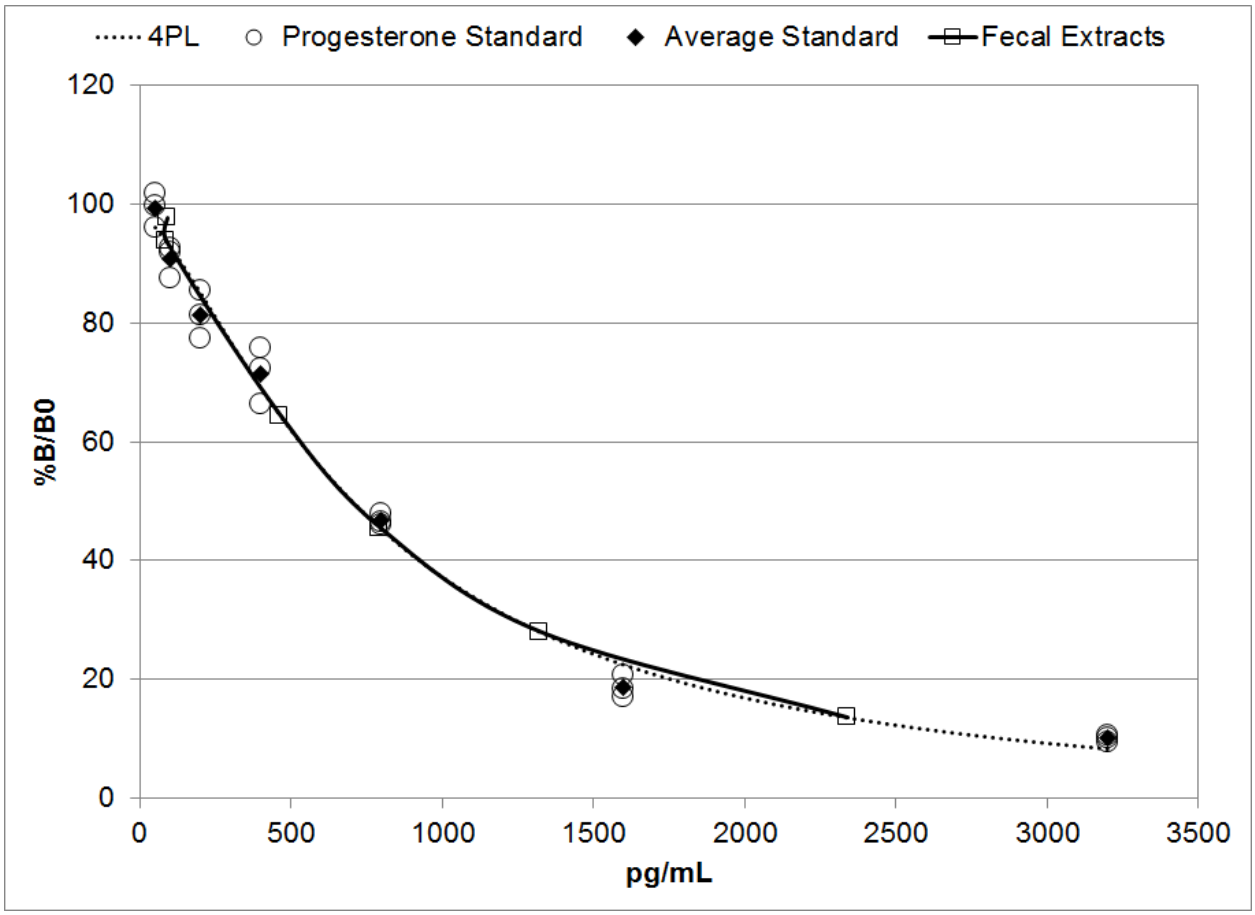

Figure 2. Parallelism between the standard curve and a pool of five ethanolic samples. The x-axis shows the concentration (pg/ml), and the y-axis shows the binding percentage (0-120%). Standards (in triplicate) are shown in circles; the mean of the standards are diamonds on the dashed line (4PL). The squares on the solid black line represent fecal samples.

Parallelism determined that the fecal extract samples from 13LGS were immunologically similar to assay kit progesterone. Parallelism also determined that fecal extracts showed binding percentages in range (20-80%) when run with 1:32 - 1:128 dilution factors. Below a 1:32 dilution, binding percentages were <14%, whereas above 1:128, binding percentages were >94%. The study began with samples being diluted 1:40 (this was because samples in the previous year's pilot study showed in-range binding

percentages with a 1:40 dilution). Some samples needed to be diluted more or less than this range (1:32-1:128) based on the actual binding percentages that were obtained on the first run of the samples. The actual range of dilutions ended up being 1:5 - 1:300 depending on binding percentages from first runs, and dilutions were adjusted accordingly.

If samples needed to be diluted more than 1:80, samples were split up and diluted 1:100, 1:200, and 1:300. For example, if a sample had a very low binding percentage (10%), that sample was run again with both dilutions (1:100 and 1:200) and each of these dilutions were run in duplicate wells. If the sample binding was still too low, the sample was split again and run in the same method but with 1:200 and 1:300. A second parallelism was not conducted, and no samples needed to be run with a dilution greater than 1:300.

From these data, a fecal 11- and 3-hydroxyprogesterone profile for each female was generated (Appendix). Then, aligned to the consensus time points, profiles of pregnant *versus* non-pregnant females were combined and averaged.

Technical Replicate

To test assay variability, a technical replicate for the DOB-21 time point was run using remaining samples from both of the non-pregnant females and for 9 of the 11 pregnant females (insufficient sample remained for the 2 excluded pregnant females). This replicate was run six months after the samples were collected, and all samples were run from powdered fecal samples that had been stored in the -80°C freezer until the test was run. The DOB-21 time point was chosen because, at one week into pregnancy, most of the females had progesterone concentrations well above baseline.

On 9 September, dried powdered fecal samples were removed from the freezer after no more than three months in storage, and extracted and processed as described above. Initial dilution in kit assay buffer was 1:40. Usefulness was based on a 50% binding goal (with a range of 20%-80% as acceptable), and wells were run in triplicate with an acceptable ≤ 15% coefficient of variation between wells. If any sample yielded data outside of these ranges, that sample was re-assayed with adjusted dilutions until data fell within range.

Statistical Analysis

All statistical analyses were performed in R (R Core Team 2016), and Excel version 1609 7369.2120 (Microsoft Corporation, Redmond, WA). An ANOVA with repeated measures was run to determine significant differences between fecal progesterone at landmark time points for each female. Then, a pairwise t-test with a Bonferroni correction ($\alpha=0.01$) was run to determine where the differences lie. This correction was due to the fact that at least one of the t-tests was found to be significant

and rejected the null hypothesis, so an adjustment was made to the alpha based how many comparisons were run.

The Wilcoxon rank sum test was run on the DOB-21 time point data for both wild-caught and captive-bred females, because captive-bred female data turned out to not be normally distributed, whereas wild-caught female data were.

To determine if litter size had any effect on progesterone concentration during gestation, a Spearman's rank correlation test was run on 7 females and 4 litter sizes (only these 7 had both a full fecal sample set *and* a confirmed litter size).

Finally, to determine whether or not the fecal ELISA for 11- and 3-hydroxyprogesterone is an appropriate test to determine levels above baseline in this species, a Student's t-test was used to identify any significant differences between the 11 females that produced litters and the 2 females that did not.

Because progesterone was tracked over a wide range from baseline to peak pregnancy amounts, sample dilutions varied considerably. Samples were run as follows: DOP & DOP+2 (1:10), eDOF (1:25), DOB-21 (1:40), DOB-14 (1:100 & 1:200) DOB-7 (1:100 & 1:200), and DOB-1-3 days (1:80). Within these time point groups, occasional samples needed to be diluted even further (i.e. 1:200 or 1:300), or diluted less (1:5) due to their low or high absorbance within the assay.

Results

Breeding outcomes for spring of 2016 are shown in Table 1. Fecundity in the study population (n=13) was roughly double that of the rest of the Colony (n=91), but litter loss in the study population was nearly triple that of the rest of the Colony. The small sample size of the study population is a factor, as is the fact that members of the study population were purposely selected from younger proven dams, whereas the rest of the Colony included virgin yearlings and elderly animals. The combined figures of fecundity and litter loss for the year are not atypical in the Colony.

	Fecundity	Litter Loss
Study population (n=13)	85% (11/13)	27% (3/11)
Rest of Colony (n=91)	42% (38/91)	10% (4/38)
Combined (n=104)	47% (49/104)	14% (7/49)

Table 1. Production by female 13LGS bred in the Colony in 2016. Fecundity is calculated as those bred who bore a litter or were euthanized and found to be pregnant with a normally-developing litter. Litter loss was attributed to postnatal cannibalism.

Fecal 11- and 3-hydroxyprogesterone of the 2 females who did not give birth during the study did not rise above this low initial concentration throughout collections that lasted until 30 May. Their averaged results are shown in Figure 3. The implication is that neither female had a pregnancy nor a pseudopregnancy during the study.

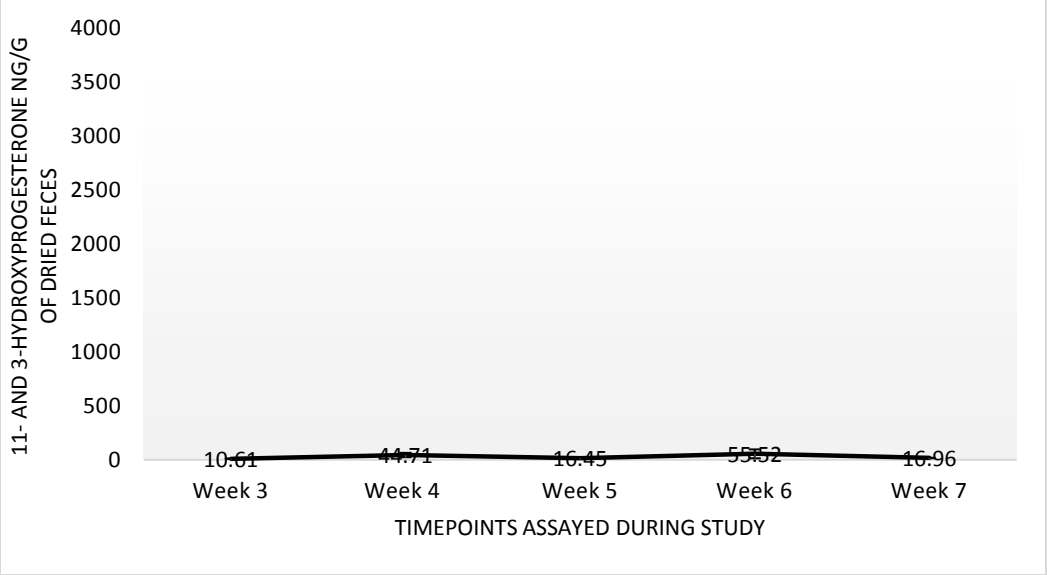

Figure 3. Mean fecal 11- and 3-hydroxyprogesterone measurements of the 2 (of 13) females who did not give birth during the study, despite 3+ weeks of exposure to a male (see x-axis). Standard error of the mean are shown as bars.

In contrast, fecal 11- and 3-hydroxyprogesterone of the 11 females who did give birth during the study rose substantially above the low initial concentration measured on DOP; averaged results are shown in Figure 4.

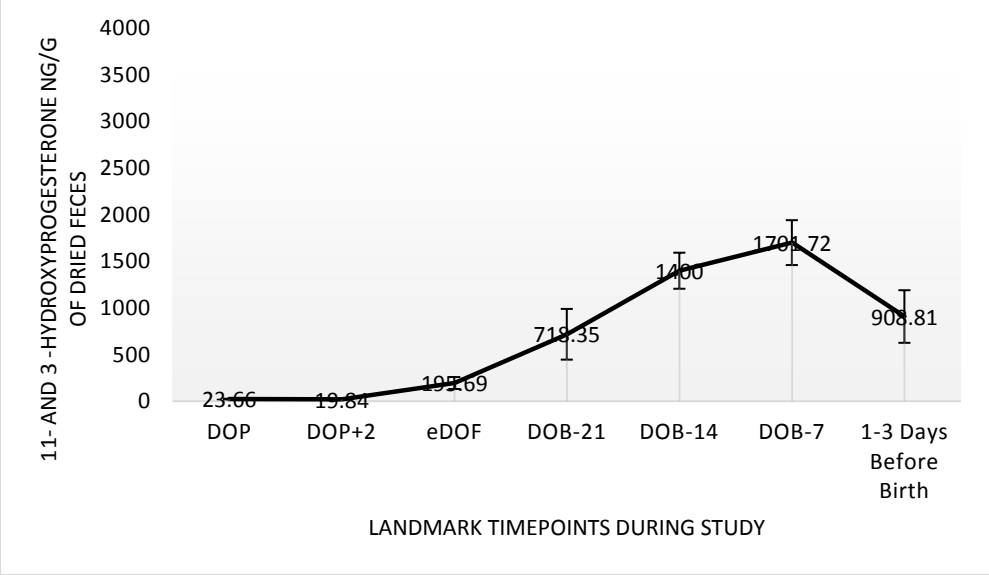

Figure 4. Mean fecal 11- and 3-hydroxyprogesterone measurements of the 11 (of 13) parous females. Note steady rise to peak at 1701.72 ng/g on DOB-7. Fall-off thereafter indicates normal progesterone withdrawal. Standard error of the mean are shown as bars.

When individual results from the 11 parous females (see Appendix) were visually compared *post hoc*, three distinct 11- and 3-hydroxyprogesterone profiles became immediately apparent. I have termed them the "high 11- and 3-hydroxyprogesterone withdrawal" group (n=6; shown averaged in Figure 5), the "low 11- and 3-hydroxyprogesterone withdrawal" group (n=3; shown averaged in Figure 6), and the "no withdrawal" group (n=2; shown averaged in Figure 7).

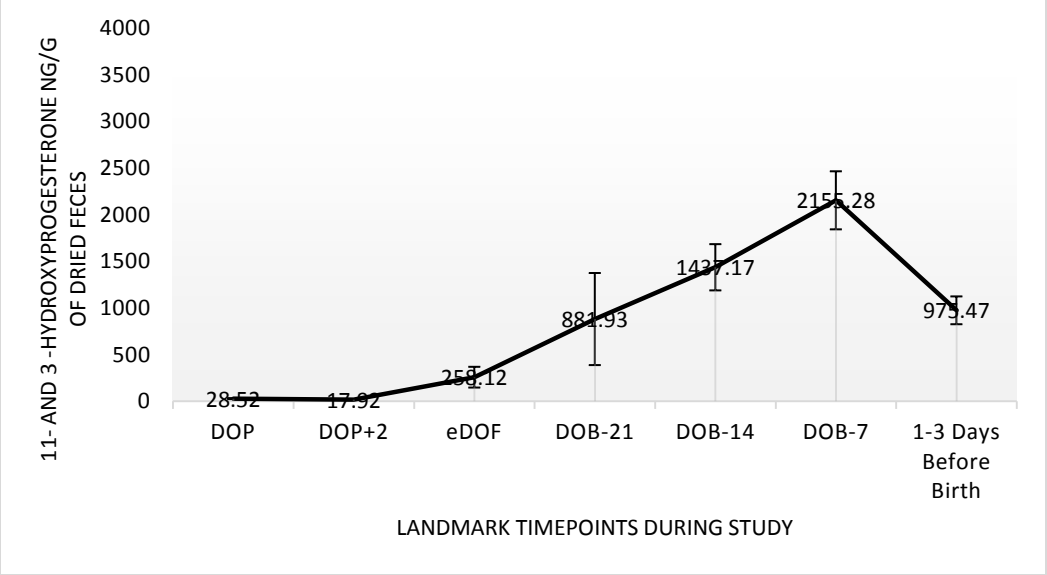

Figure 5. Mean fecal 11- and 3-hydroxyprogesterone measurements of 6 (of 11) parous females with "high 11- and 3-hydroxyprogesterone withdrawal" profiles. Note rise to peak at ~2155 at DOB-7, followed by fall-off indicating normal withdrawal. Standard error of the mean are shown in bars.

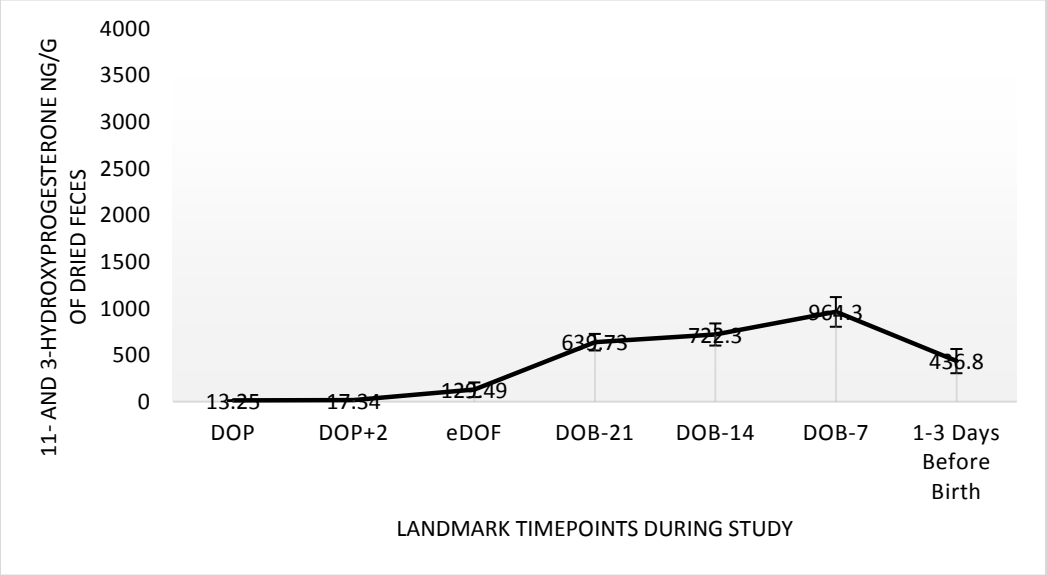

Figure 6. Mean fecal 11- and 3-hydroxyprogesterone measurements of 3 (of 11) parous females with "low 11- and 3-hydroxyprogesterone withdrawal" profiles. Note rise to peak at ~964 at DOB-7, followed by fall-off indicating normal withdrawal. Standard error of the mean are shown in bars.

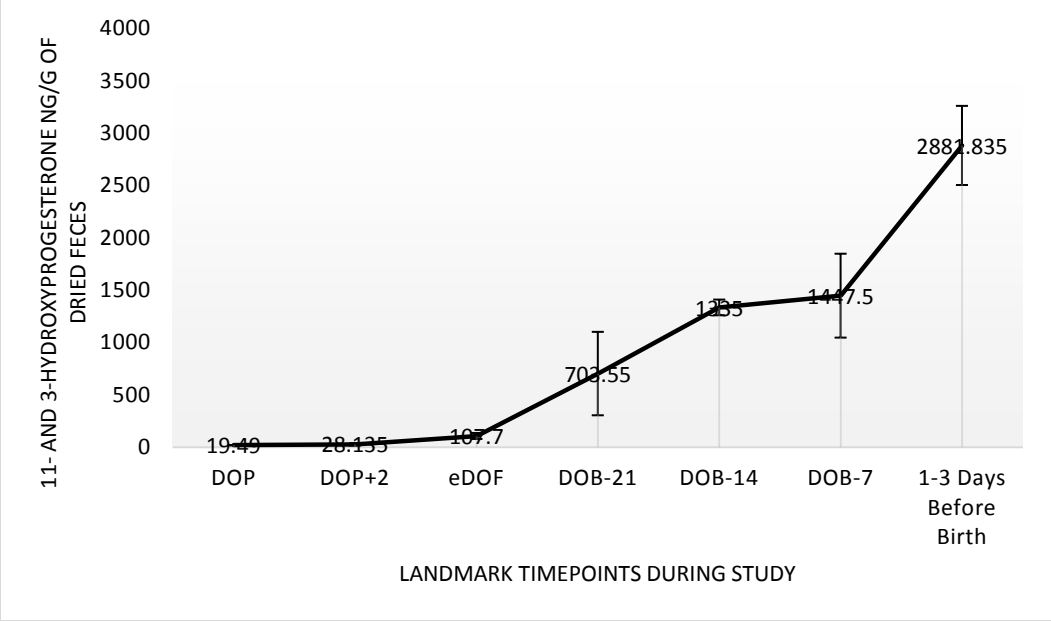

Figure 7. Mean fecal 11- and 3-hydroxyprogesterone measurements of 2 (of 11) females of the "no withdrawal" group. Note rise to peak at ~2800 at 1-3 days before birth, but no sign of fall-off over the last ~10 days of gestation. Standard error of the mean are shown as bars.

Technical Replicate

The results of a technical replicate comparing assay results from dried frozen fecal samples at the DOB-21 landmark date are shown in Figure 8. Replicate values are consistently lower than original values for all but the non-parous females.

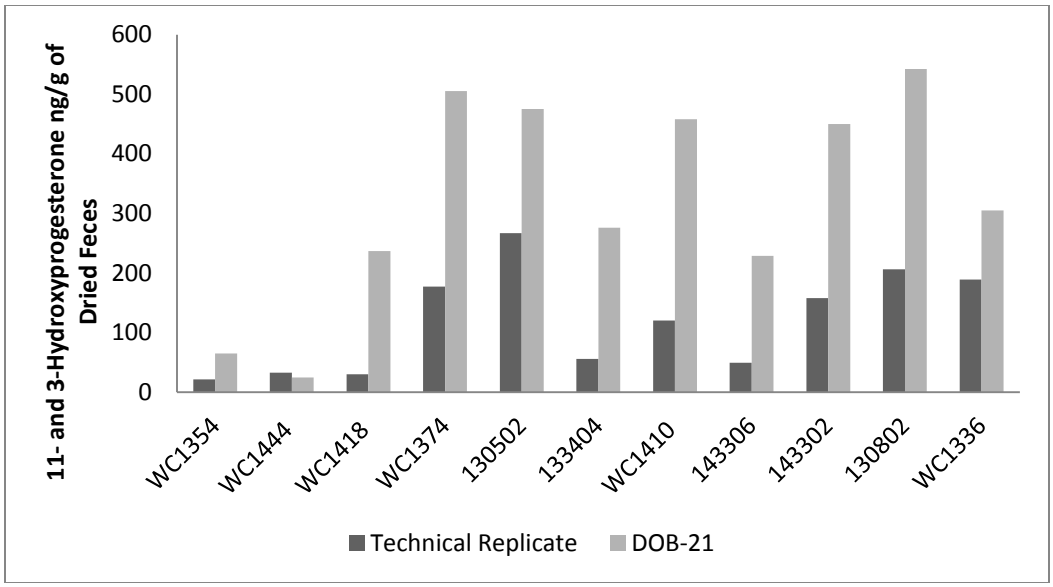

Figure 8. Results of a technical replicate comparing mean assay results from time point DOB-21 for the 11 parous females in the study. First-run results are shown by the lighter gray bars. Replicate results run 3.5 months later are shown by the darker gray bars. The females are in this particular order from left to right: no pregnancy (n=2), high peak (n=6), low peak (n=3), and no withdrawal (n=2) (see text for definitions of these groups).

Effects of Pregnancy on Progesterone Concentration

To determine the overall effect of pregnancy on fecal progesterone, data obtained from the 2 non-pregnant females during the 4 weeks following male exposure were averaged, and data obtained over the 4 week pregnancies of 10 pregnant females were averaged (the eleventh parous female was omitted because we did not obtain fecal samples at every landmark time point from her). A t-test was then run to identify any statistically significant difference between the two groups ($\alpha = 0.05$). Figure 9 shows the median and variances from the non-pregnant females, compared to the pregnant females. The lower median fecal progesterone exhibited by non-pregnant females was statistically lower than the higher median fecal progesterone exhibited by pregnant females ($p = 0.02$, $t_{13} = -3.63$).

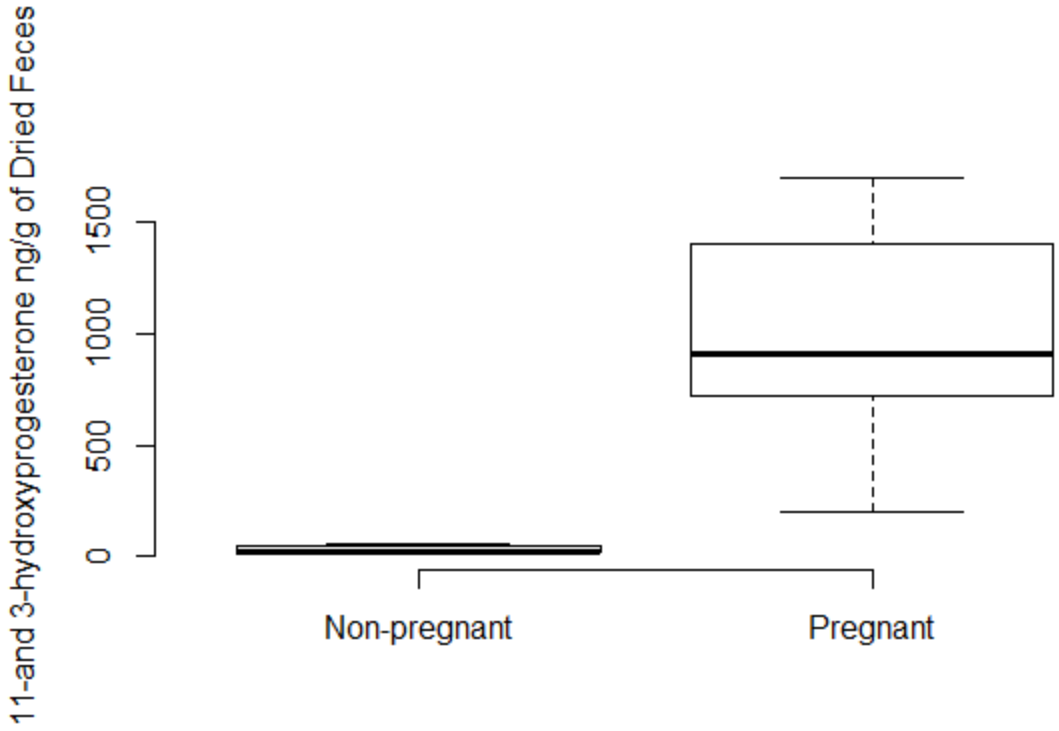

Figure 9. Fecal 11- and 3-hydroxyprogesterone data from two non-pregnant females compared to ten pregnant females; mean over four weeks in both group. Boxes show the first and third quartile; dark lines show the medians (non-pregnant at 16.96 ng/g, pregnant at 908.81 ng/g); and lines above or below the box show data minima and maxima.

Effect of Landmark Time Point on Progesterone Concentration

A major objective of this study was to build a fecal 11- and 3-hydroxyprogesterone profile for female 13LGS, and to test for significant differences between time points to see if an increase could reliably indicate pregnancy. Landmark data from 10 of the 11 parous females were statistically compared (the eleventh parous female was omitted because we did not obtain fecal samples at every landmark time point from her). Figure 10 shows the fecal 11- and 3-hydroxyprogesterone results of the 10 parous females over their respective 28-day gestation periods. After the Bonferroni *post hoc* test was performed on these means, significant differences were found between

multiple timepoints. Significant differences were found between fecal 11- and 3-hydroxyprogesterone at:

- DOP *versus* DOB-14 ($p = 0.00013$), DOB-7 ($p = 2.2e-6$), and 1-3 days before birth ($p = 0.00124$);
- DOP+2 *versus* DOB-14 ($p = 0.00012$), DOB-7 ($p = 2.0e-6$), and 1-3 days before birth ($p = 0.00118$); and
- eDOF *versus* DOB-14 ($p = 0.00133$), DOB-7 ($p = 2.7e-5$), and 1-3 days before birth ($p = 0.01093$)

From among individual results, the lowest 11- and 3-hydroxyprogesterone concentration measured by competitive ELISA was 4.98 ng/g at DOP+2 days and the highest was 3340.4 ng/g at DOB-21.

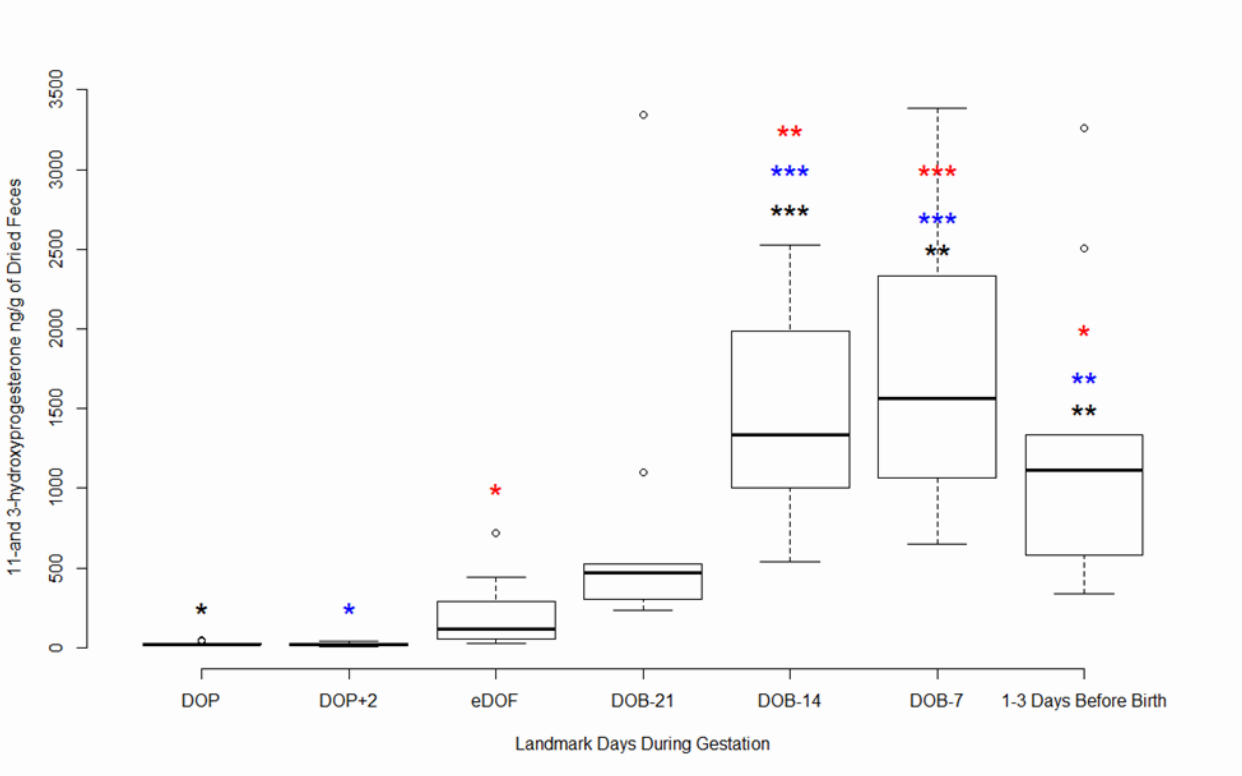

Figure 10. Fecal 11- and 3-hydroxyprogesterone data among 10 parous females averaged over 4 weeks for all groups. Boxes show the first and third quartile of the data; the dark lines show the median of the data (DOP at 19.57 ng/g, DOP+2 at 19.03 ng/g, eDOF at 115.89 ng/g, DOB-21 at 466.45 ng/g, DOB-14 at 1335 ng/g, DOB-7 at 1567 ng/g, 1-3 Days Before Birth at 1110.65 ng/g); and the lines above or below the box show the data minima and maxima. Dots that appear above the lines are outliers. Asterisks show significant differences: * = 0.01, ** = 0.001, *** = 0.0001, with color coding showing corresponding differences.

Effect of Female Provenance On Early-Gestation Progesterone Concentration

This study employed 7 wild-caught and 6 captive-bred breeding females. Figure 11 compares 11- and 3-hydroxyprogesterone concentrations between these 2 groups at DOB-21, a timepoint when 11- and 3-hydroxyprogesterone was consistently well above the pre-mating DOP baseline. A Wilcoxon rank sum test obtained a p-value of 0.6991, indicating no significant difference between captive-bred and wild-caught female squirrels.

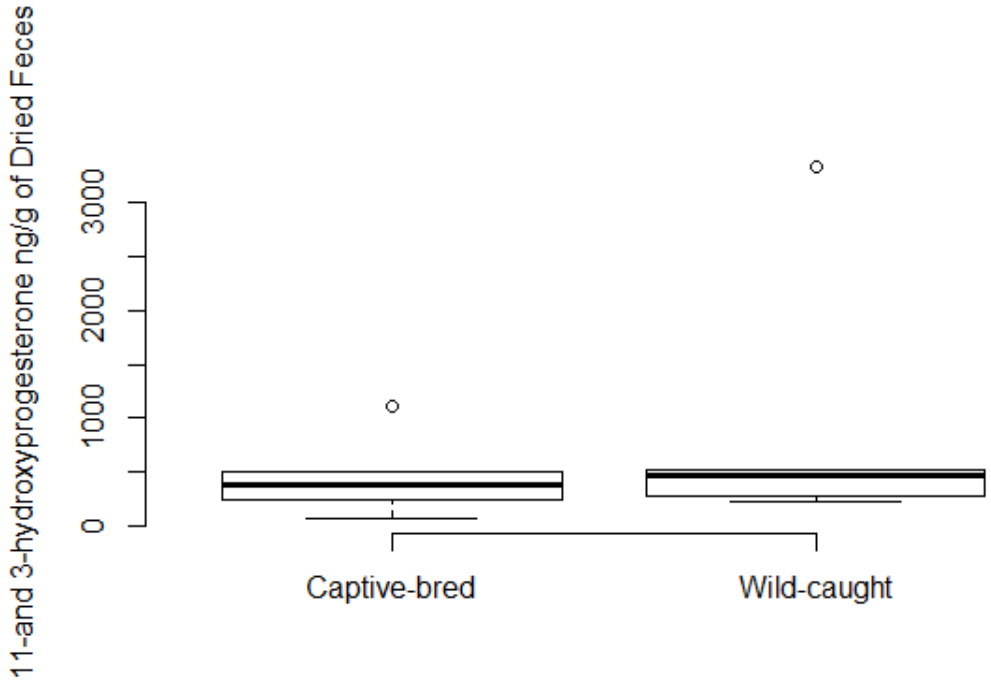

Figure 11. Fecal 11- and 3-hydroxyprogesterone at the DOB-21 landmark, comparing 7 wild-caught to 6 captive-bred parous females. Boxes show the first and third quartiles of the data; the dark lines show the median of the data (captive-bred at 462.55 ng/g, wild-caught at 381.5 ng/g); and the lines above and below the box show the data minima and maxima. Dots that appear above the lines are outliers.

Effect of Litter Size on Progesterone Concentration

To determine if litter size had any effect on 11- and 3-hydroxyprogesterone concentration, results from the 7 parous females who had both a complete set of fecal samples and a verified litter size were compared. A Spearman's rank correlation test was run on these data and revealed that there was a weak negative correlation between litter size and progesterone concentration.

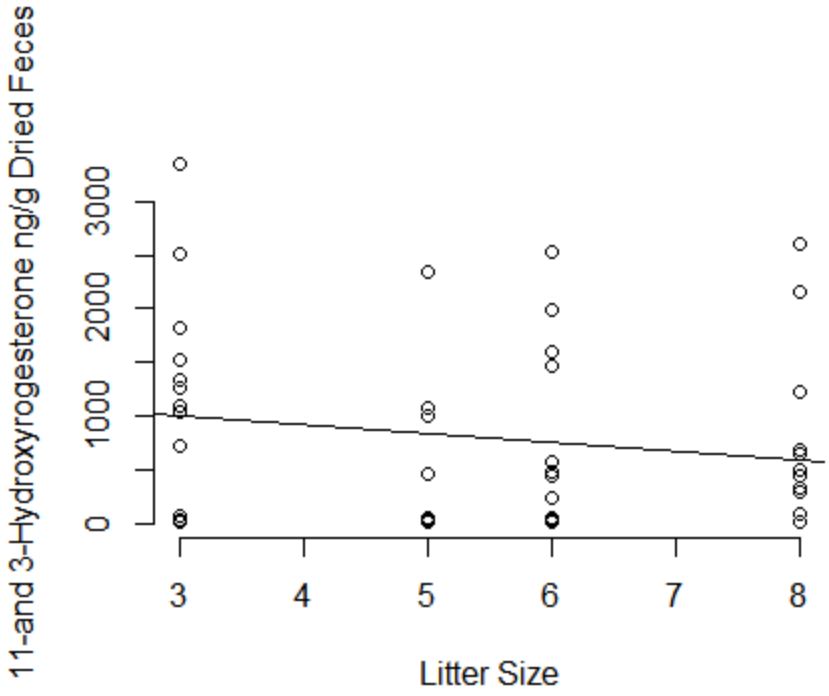

Figure 12. Results of the Spearman's rank correlation test. There were 4 different litter sizes compared, from 7 females altogether. This graph shows a weakly negative correlation between litter size and progesterone, with a *rho* of -0.1642815.

Discussion

The specific aim of this study was to examine fecal 11- and 3-hydroxyprogesterone profiles of pregnant 13LGS, yet its first principal finding was not related to that aim at all. Handling stress during mating and gestation is a concern in any breeding operation. The female 13LGS employed in this study experienced handling for the purposes of fecal collection and vaginal photography three times a week for at least four weeks. In contrast, 91 Colony females not part of this study did not receive that handling. Unexpectedly, the study females had twice the fecundity (85% *vs*. 42%) of the Colony females (Table 1). Indeed, the Colony historically averages around 50% fecundity in any given year (D. Merriman, unpublished results). Counteracting this higher fecundity, the study females had nearly 3 times the litter loss rate (27%/n=3 *vs*. 10%/n=4) as Colony females (Table 1). Handling ceased the day the litter was detected so, while handling during mating and gestation seems to do no harm to fecundity, it may somehow predispose litter loss.

Second, this study did not determine the lag time between any change in serum hormone level and the corresponding change in fecal hormone level. In a study of fecal stress hormones of Belding's ground squirrel, Mateo (2006) reported a 6-12 hour lag time, suggesting that fecal sampling should always occur at the same time of day. That was indeed the method employed in the present study; fecal samples were always collected from 0800-1000 hours.

In this study, we were able to compare data from 2 non-pregnant females to data from 10 pregnant females. Compared to the 4 weeks of gestation by pregnant females, 4

weeks of data from non-pregnant females exhibited consistently (Figure 3) and significantly (Figure 9) lower fecal 11- and 3-hydroxyprogesterone. This is in contrast to fecal estrogen, which vacillates widely in pregnant and non-pregnant sciurids (A. Miller, unpublished; Keeley *et al.*, 2012). This progesterone rise was equally detected in wild-caught and captive-bred female 13LGS (Figure 11). Therefore, we have confidence in the general notion that fecal 11- and 3-hydroxyprogesterone testing has the capacity to detect pregnancy in 13LGS.

Next, the mean11- and 3-hydroxyprogesterone profile from 11 pregnant 13LGS over the mating and gestation period (Figure 4) demonstrated steadily rising fecal hormone starting on the estimated day of fertilization (eDOF), peaking about a week before the litter's birth (DOB-7), and then falling to about half-maximal levels in the 1-3 days before birth. This mean 13LGS profile shows clear evidence of classic progesterone withdrawal and agrees well with an earlier fecal 11- and 3-hydroxyprogesterone study of another hibernating sciurid bred in captivity, the Vancouver Island marmot (Keeley *et al.*, 2012; Figure 13). As in our 13LGS, progesterone level is minimal and flat until the marmot's eDOF, at ~30 days before parturition, when the progesterone rise begins.

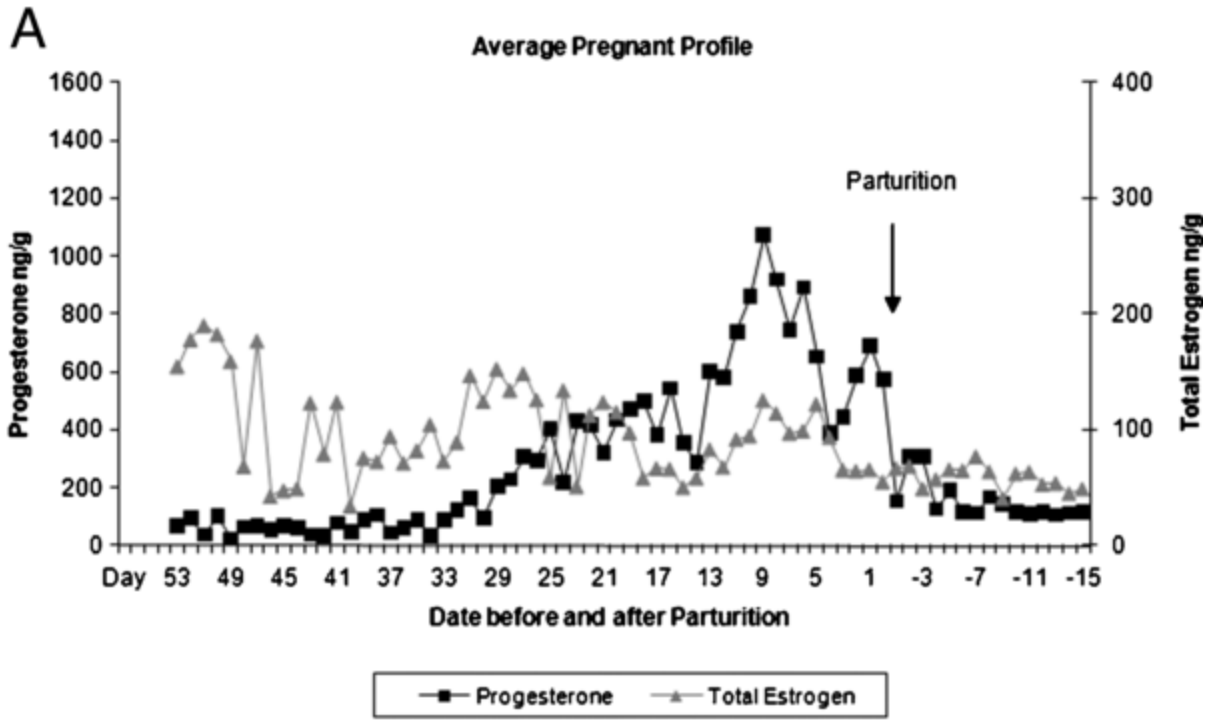

Figure 13. From Keeley, T. *et al.* (2012). *Zoo Biology*,*31*,275-290. Mean fecal hormone values per day from a study of 17 female Vancouver Island marmots. Progesterone data are tracked by the dark line whereas estrogen data are tracked by the light line. Marmot gestation is 30 days so, on this graph, "eDOF" falls between 33 and 29 days before parturition, where progesterone levels begin to rise above baseline. *Cf.* Figure 4 of this thesis.

Another noteworthy finding was the apparent division of pregnant 13LGS into three distinct 11- and 3-hydroxyprogesterone profiles that we have unofficially termed "high withdrawal" (Figure 5), "low withdrawal" (Figure 6), and "no withdrawal" (Figure 7). These groupings could not be explained by litter size (Figure 12), so we sought other explanations.

The late-peaking time points in the "no withdrawal" profiles of 2 of 11 females (Figure 7) may stem from a procedural issue mentioned in Methods: urine-contaminated samples were not excluded from the study. Such contamination is known to artifactually

elevate “fecal” hormone levels (Cavigelli *et al*., 2005; Wasser *et al.,* 1988). Most urinary steroids are conjugated (98%), whereas most fecal steroids are unconjugated (98%) (Adlercreutz *et al.*, 1976). An enzyme called glucuronidase is secreted by gut bacteria into feces, where it de-conjugates steroids, thus artificially increasing the level of unconjugated fecal steroids (Jarvenpaa *et al.*, 1980). Immediate addition of ethanol to fecal samples the moment they are collected from the cage halts this enzymatic action (Wasser *et al.*, 1988), but this step was not taken in our study. Therefore, urine artifacts in our data set could explain the abnormally high hormone levels that suggest “no withdrawal”, as well as in other data.

Another problem identified in this study appeared in the technical replicate (Figure 8). For this particular study, fecal samples were collected with forceps, placed dry into a freezer vial, and placed into a -80° C freezer. Sample extraction occurred 5 weeks to 14 weeks later. Comparing data from samples processed at both extremes gave clear evidence of 11- and 3-hydroxyprogesterone signal loss over a 14 week period, presumably due to degradation by fecal microbes. This contradicts a report that freezing preserves ground squirrel fecal sex steroids (Pettit *et al.*, 2007). We conclude that ethanol-immersed, frozen fecal samples should be processed for competitive ELISA as soon as possible and all at once (rather than over a period of weeks) to avoid variability introduced by signal loss over time.

Despite these difficulties, the data did indicate how soon after mating we can reliably use elevated fecal 11- and 3-hydroxyprogesterone data to distinguish pregnant from non-pregnant females. Because of the normal withdrawal profiles obtained in the

"high" *vs*. "low" groups, we would argue that a comparison against pre-mating baseline (DOP) appears to be a more reliable approach than the selection of some threshold ng/g value of fecal hormone. From all 11 pregnant animals, mean fecal 11- and 3-hydroxyprogesterone was 5-to-10-fold elevated at eDOF over DOP (Figure 10). Even more dramatically, it was >20-fold elevated at DOB-21 over DOP (Figure 10). The individual variation recorded at eDOF makes DOB-21 the more conservative choice.

In practice, then, this study suggests the following as a standard operating procedure for using competitive ELISA for fecal 11- and 3-hydroxyprogesterone as a pregnancy test in the 13LGS:

1. On the date that females are paired with males (DOP), collect a baseline fecal sample. Immerse in 100% ethanol and freeze.
2. The earliest possible mating date is the date of pairing so, one week later, collect another fecal sample into 100% ethanol and freeze.
3. Run competitive ELISA immediately. If the 1-week sample's steroid content is 10-to-20-fold higher than that of the date of pairing, the female is judged to be pregnant. If not:
4. 2 weeks after pairing, collect another sample and freeze; immediately run competitive ELISA comparing newest sample to that of the date of pairing. If the 2-week sample's steroid content is 10-to-20-fold higher than that of the date of pairing, the female is judged to be pregnant. If not:
5. Repeat step 4 up to 3 more times; after that, conception is unlikely.

European ground squirrel females undergo a *post-partum* estrus cycle after delivering their litters (Millesi *et al.*, 2008; Strauss *et al.*, 2009), akin to the "foal heat" of equines. The squirrel's *post-partum* estrus is non-reproductive, however. First, this late in summer the males' testes have already begun to regress, so the availability of live sperm is low. Second, serum (and thus fecal) progesterone rises abruptly after birth, which probably suppresses further ovulation which would only produce a second litter far too late in the season to wean and fatten for hibernation. This phenomenon yields a bimodal oscillation in progesterone over the entirety of a breeding European ground squirrel's (non-hibernating) active season. The method described here can now be used to document whether a similar progesterone phenomenon occurs in the 13LGS.

APPENDIX

APPENDIX: Supplemental Data

The following pages illustrate the individual 11- and 3-hydroxyprogesterone profiles from all 13 females in the study. Results from the non-parous females are shown first (Figures A1-A2), followed by results from the parous females (Figures A3-A13).

As described in the text, abbreviations used in graphing data from parous females are as follows:

DOP, day of pairing with a male.

DOP+2, two days following DOP.

eDOF, estimated day of fertilization, beginning a 28 day/4 week pregnancy.

DOB-21, 21 days/3 weeks prior to birth.

DOB-14, 14 days/2 weeks prior to birth.

DOB-7, 7 days/1 week prior to birth.

1-3 Days Before Birth, self-explanatory.

As discussed in the text, results from the 11 parous females fell into three main groups and their respective results are shown in order as follows:

- "High peak" 11- and 3-hydroxyprogesterone, at roughly 2,000-3,000 ng/g (6 of 11; Figs. A3-A8).
- "Low peak" 11- and 3-hydroxyprogesterone, at roughly 700-1,100 ng/g (3 of 11; Figs. A9-A11).
- "No withdrawal" 11- and 3-hydroxyprogesterone, lacking the expected drop-off just prior to parturition (2 of 11; Figs A12-A13).

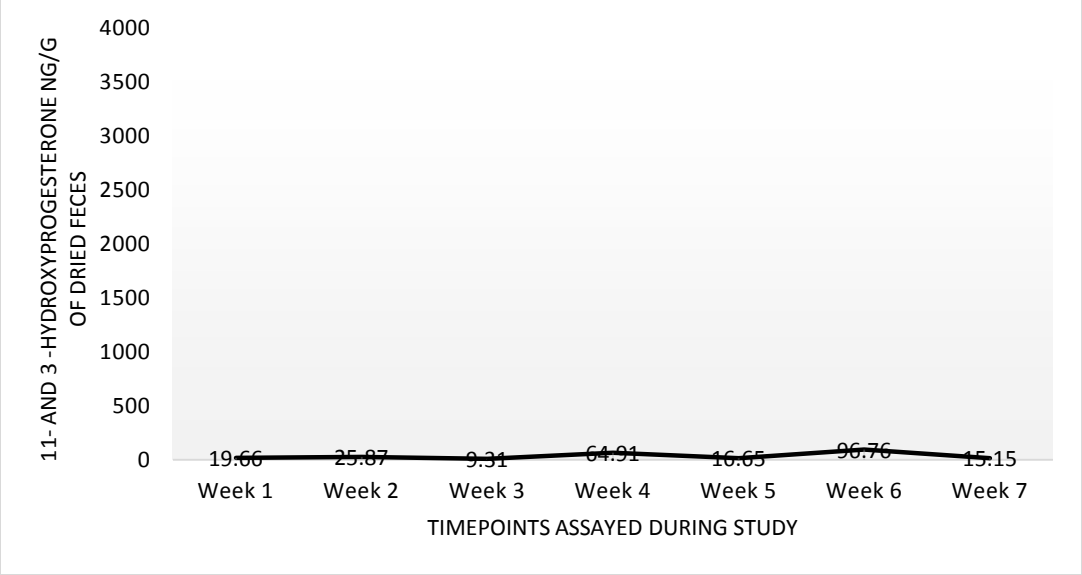

Figure A1. 11- and 3-hydroxyprogesterone profile for WC1354, from whom no litter was ever detected.

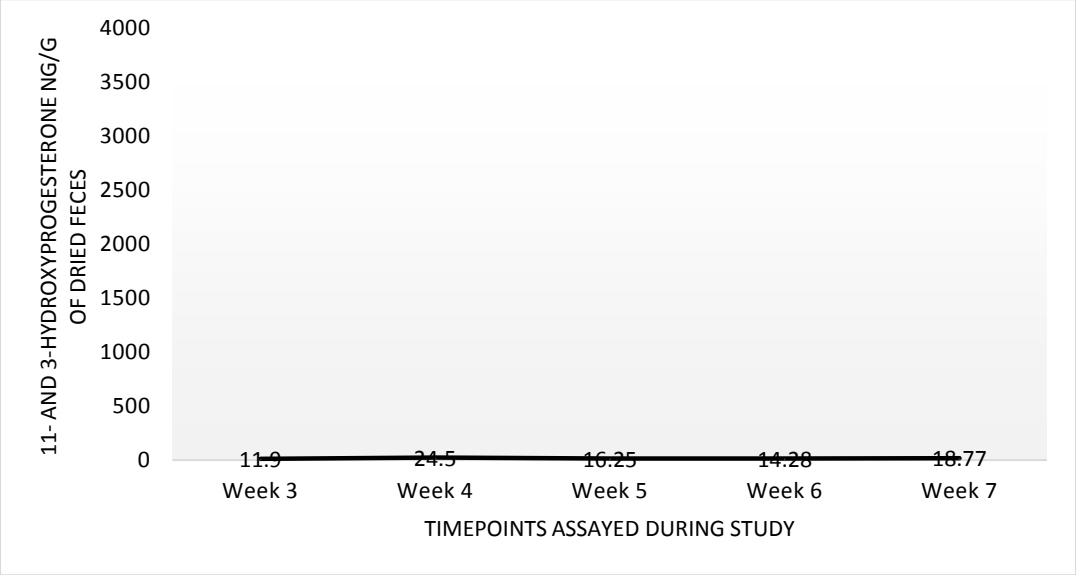

Figure A2. 11- and 3-hydroxyprogesterone profile for WC1444, from whom no litter was ever detected. The first two weeks are missing because this female failed to defecate at those time points.

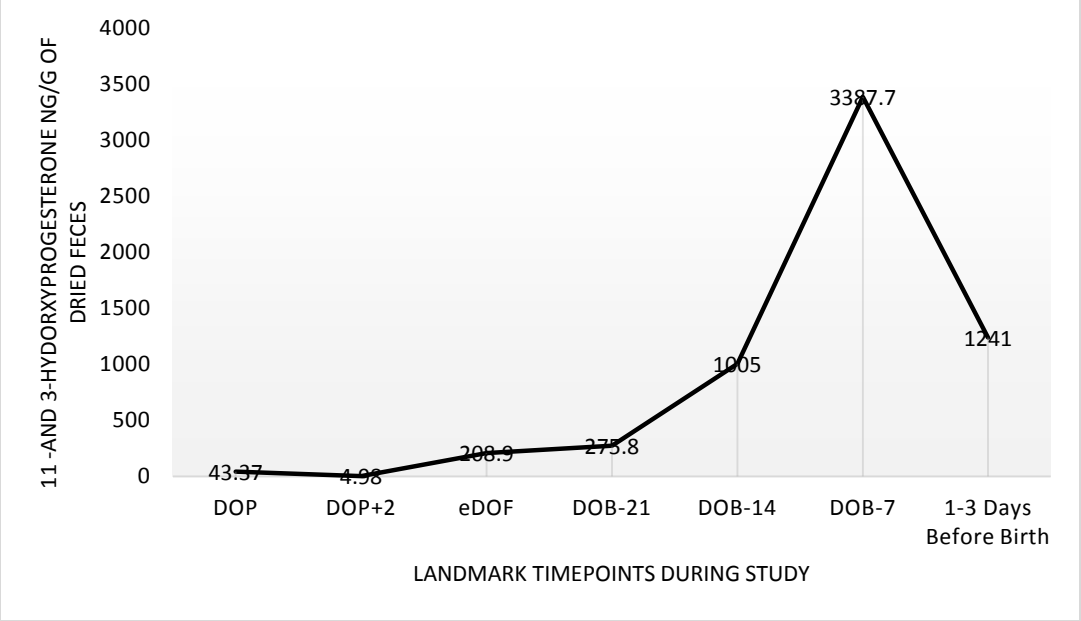

Figure A3. 11- and 3-hydroxyprogesterone profile for 133404. Mating lag time, 11 days. Litter DOB May 19th (n = 6 pups). Example of a "high peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 2,000-3,000 ng/g).

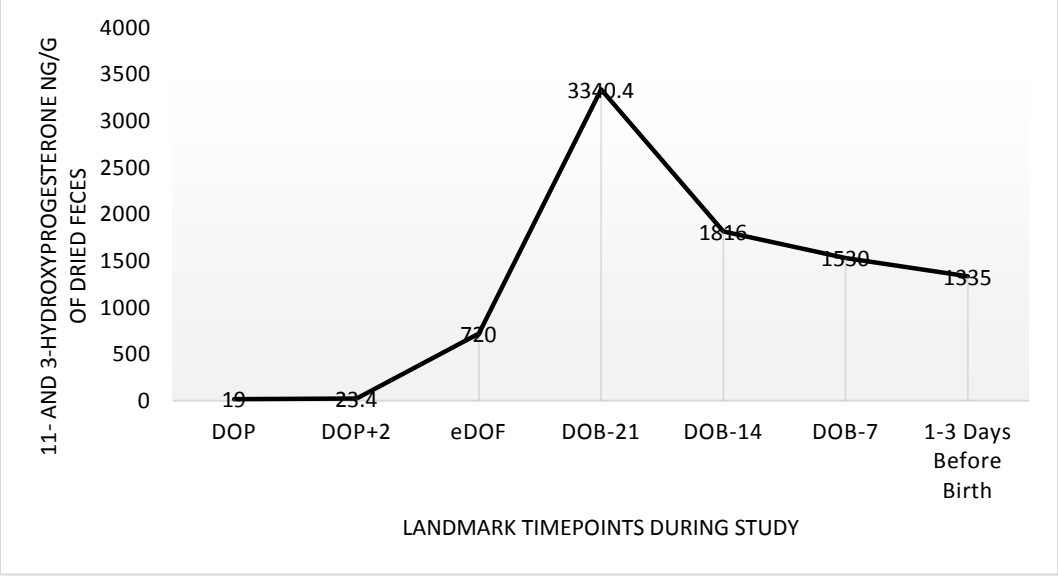

Figure A4. 11- and 3-hydroxyprogesterone profile for 134404. Mating lag time, 10 days. Litter DOB May 19th (n = 3 pups). Example of a "high peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 2,000-3,000 ng/g).

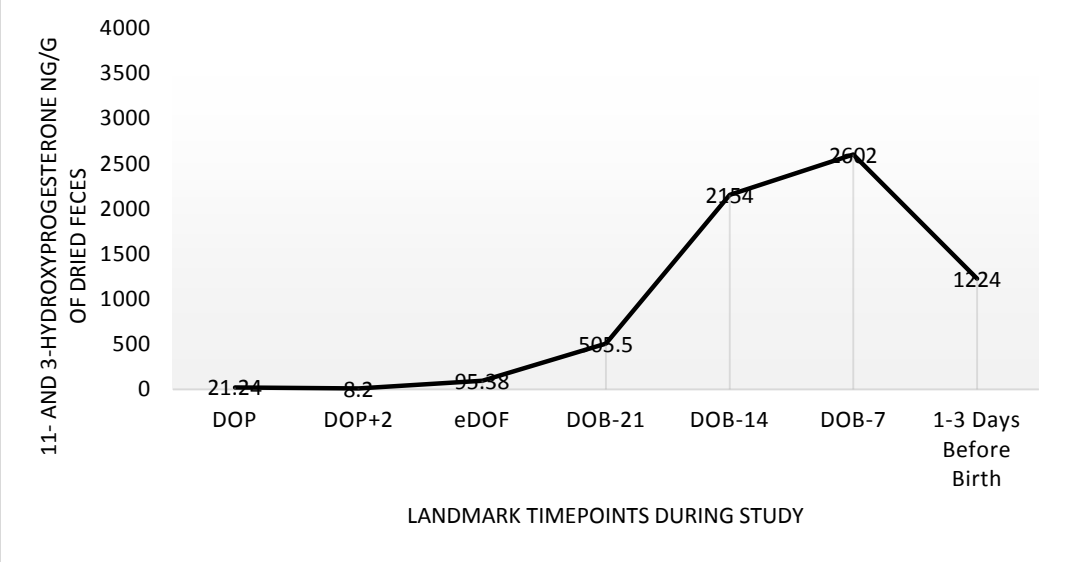

Figure A5. 11- and 3-hydroxyprogesterone profile for WC1374. Mating lag time, 19 days. Litter DOB May 28th (n = 8 pups). Example of a "high peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 2,000-3,000 ng/g).

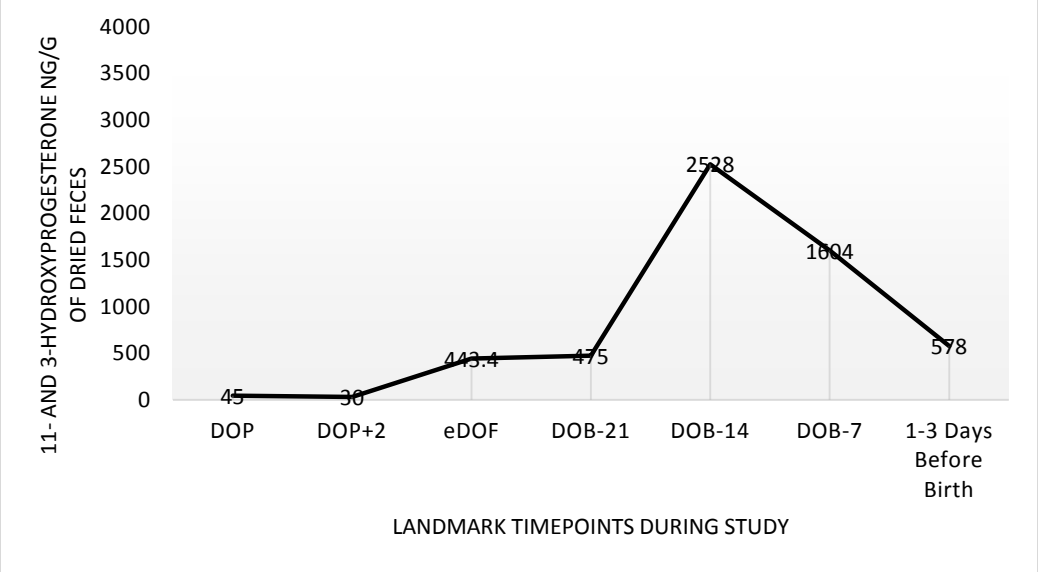

Figure A6. 11- and 3-hydroxyprogesterone profile for 130502. Mating lag time, 12 days. Litter DOB May 17th (n = 6 pups; cannibalized). Example of a "high peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 2,000-3,000 ng/g).

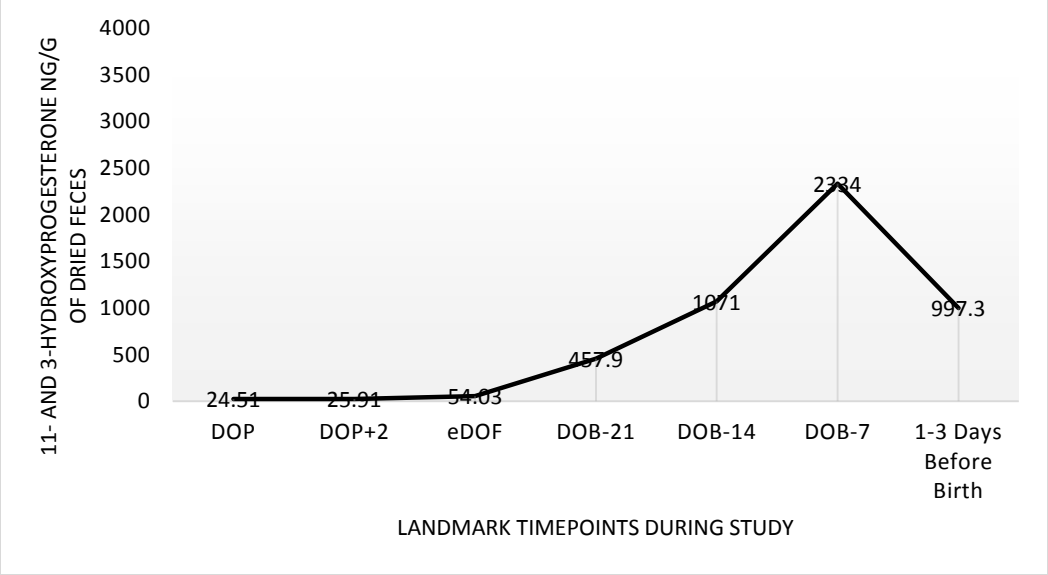

Figure A7. 11- and 3-hydroxyprogesterone profile for WC1410. Mating lag time, 1 day. Litter DOB May 10th (n = 5 pups). Example of a "high peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 2,000-3,000 ng/g).

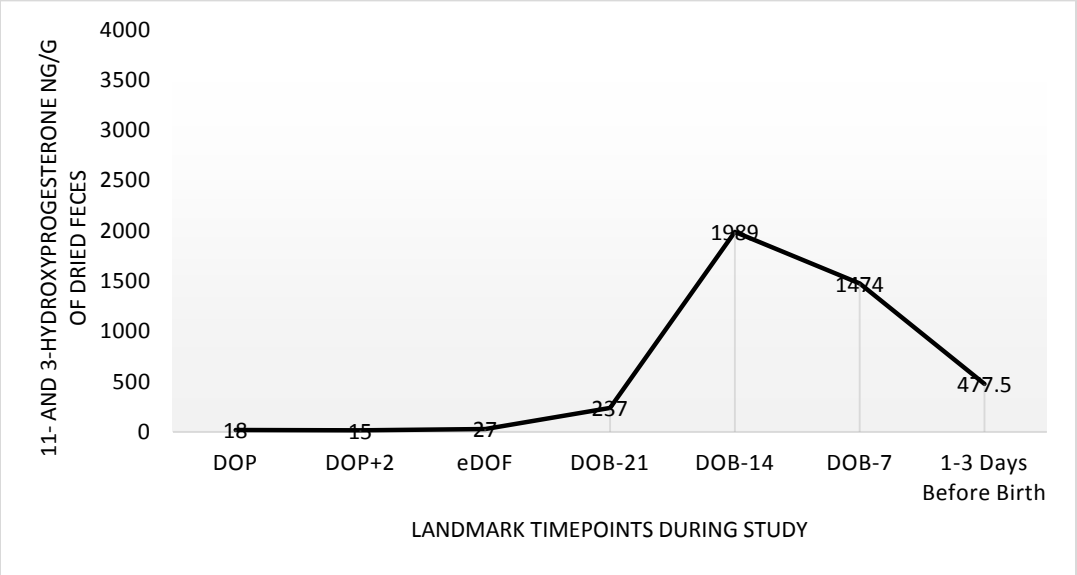

Figure A8. 11- and 3-hydroxyprogesterone profile for WC1418. Mating lag time, 3 days. Litter DOB May 12th (n = 6 pups). Example of a "high peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 2,000-3,000 ng/g).

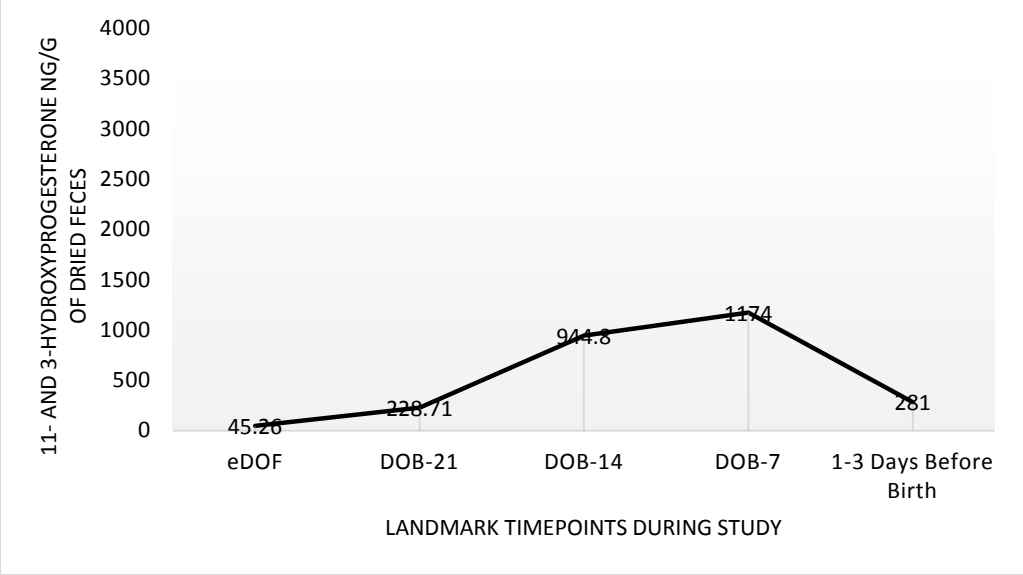

Figure A9. 11- and 3-hydroxyprogesterone profile for 143306. Mating lag time, 6 days. Litter DOB May 15th (n = 8 pups). Example of a "low peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 700-1,100 ng/g).

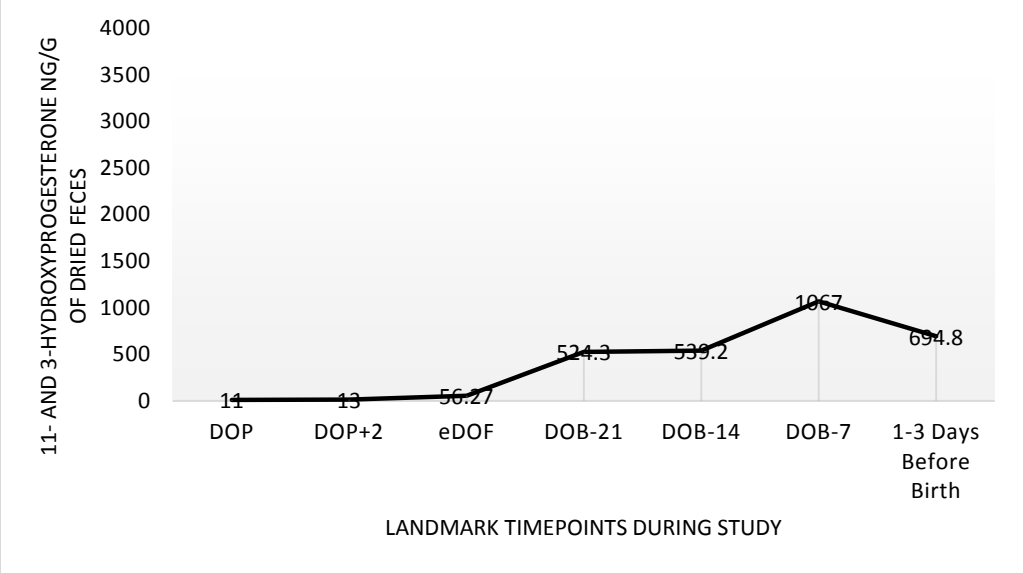

Figure A10. 11- and 3-hydroxyProgesterone profile for 130802. Mating lag time, 12 days. Litter DOB May 21th (pups detected but cannibalized before a head count was obtained). Example of a "low peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 700-1,100 ng/g).

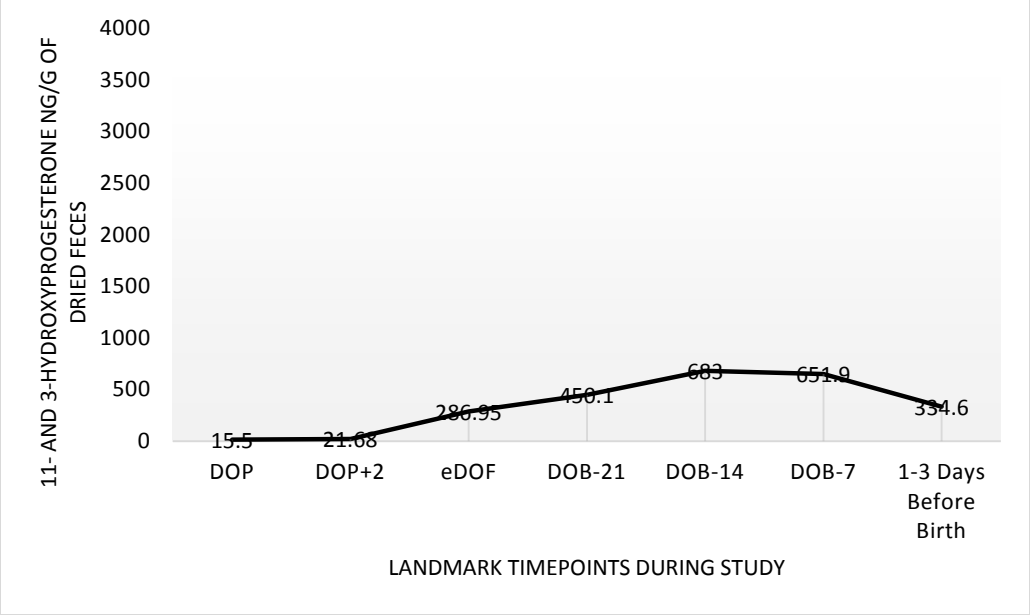

Figure A11. 11- and 3-hydroxyProgesterone profile for 143302. Mating lag time, 12 days. Litter DOB May 20th (n = 8 pups). Example of a "low peak" result (peak 11- and 3-hydroxyprogesterone *ca.* 700-1,100 ng/g).

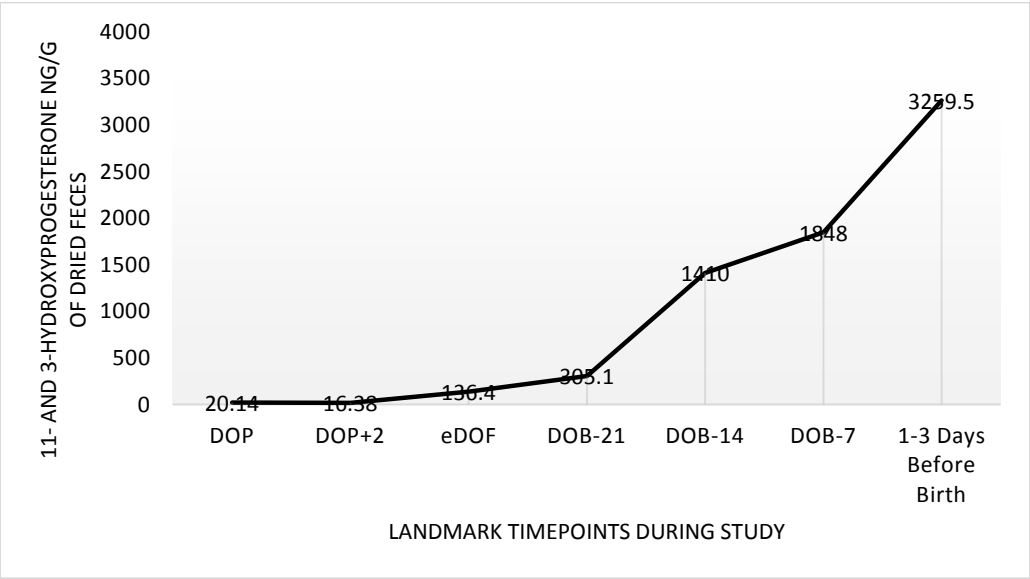

Figure A12. 11- and 3-hydroxyprogesterone profile for WC1336. Mating lag time, 19 days. Litter DOB May 24th (pups detected but cannibalized before a head count was obtained). Example of a "no withdrawal" result.

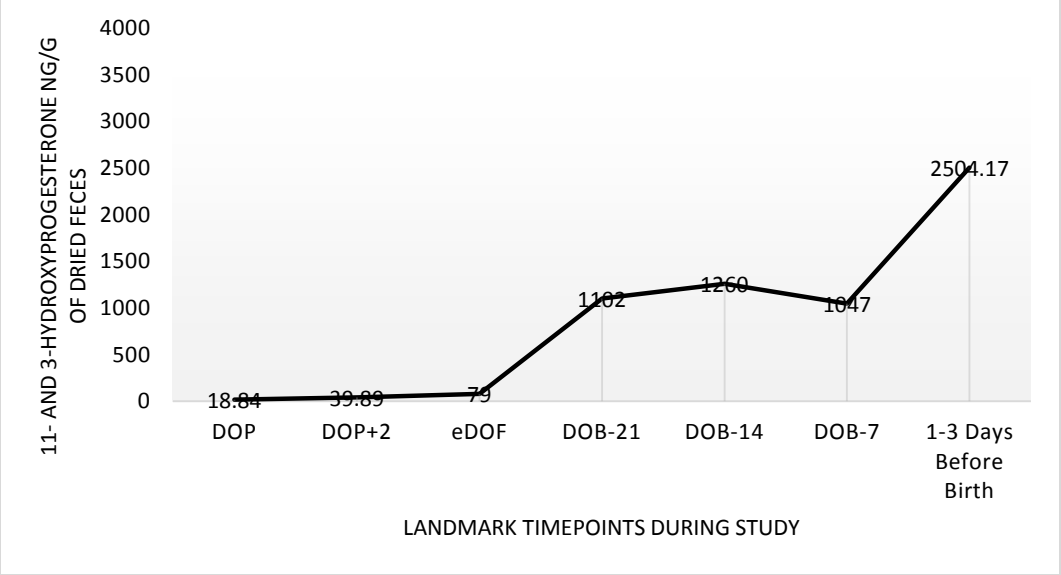

Figure A13. 11- and 3-hydroxyprogesterone profile for WC1344. Mating lag time, 17 days. Litter DOB May 26th (n = 3 pups). Example of a "no withdrawal" result.

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