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Artículo

VARIATION OF VAGINAL CYTOLOGY, PROGESTERONE AND ESTRADIOL METABOLITES IN SEBA'S SHORT-TAILED FRUIT BAT DURING THE ESTROUS CYCLE AND GESTATION

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ABSTRACT. Although analyzing reproductive hormones provides insight into reproduction, reproductive endocrinology remains understudied in most free-ranging organisms due to difficulties obtaining and preserving biological samples. This study examined differences in fecal hormone (progesterone and estradiol) metabolites at different reproductive stages and determined the probability that a female bat (Seba's short-tailed fruit bats, *Carollia perspicillata)* was in metestrus or pregnancy by relating vaginal cytology with hormone concentrations. We examined fifty-three females collected throughout the reproductive season for the presence of a conceptus, and an additional fifteen that were lactating. In addition, groups of cells (superficial, intermediate, and parabasal cells) in exfoliate vaginal cytology were quantified for each individual to further characterize stage of reproduction. Progesterone and estradiol concentrations increased from estrus through early pregnancy and then decreased into late pregnancy. From late pregnancy to early lactation progesterone levels continued to decrease whereas estradiol levels increased. Superficial cell proportions were higher during metestrus than during pregnancy and were an effective predictor for these two phases of the reproductive cycle.

RESUMEN. Variación de la citología vaginal y los metabolitos de progesterona y estradiol en el murciélago frutero común durante el ciclo estral y la gestación. Aunque el análisis de las hormonas reproductivas provee información precisa acerca de la reproducción, la endocrinología reproductiva sigue siendo aún poco estudiada en la mayoría de la fauna silvestre debido a las dificultades de obtener y conservar muestras biológicas. En este estudio se examinaron las diferencias en los metabolitos de hormonas en materia fecal (progesterona y estradiol) en diferentes etapas reproductivas, y en particular se examinó la probabilidad de que una hembra de murciélago (*Carollia perspicillata*) esté en metaestro o preñez al correlacionar la citología vaginal con las concentraciones de hormonas reproducivas. Examinamos cincuenta y cinco hembras colectadas a lo largo de la temporada reproductiva con evidencia de embarazo (presencia de un feto), y otros quince murciélagos lactantes. Además, se cuantificaron los grupos de células (células superficiales, intermedias y parabasales) a partir de citologías vaginales de cada individuo para caracterizar su estado reproductivo. Las concentraciones de progesterona y estradiol aumentaron desde el estro hasta el inicio del embarazo, y luego disminuyeron hasta el final del embarazo. Desde la etapa final del embarazo hasta la lactancia temprana los niveles de progesterona continuaron disminuyendo, mientras que los niveles de estradiol aumentaron. Las proporciones de células

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superficiales fueron más altas durante el metestro que durante el embarazo y permitieron predecir de manera efectiva estas dos fases del ciclo reproductivo.

Key words: Bat. *Carollia*. Estradiol. Progesterone. Vaginal cytology.

Palabras clave: *Carollia*. Estradiol. Murciélago. Progesterona. Citología vaginal.

INTRODUCTION

Variation in gonadal sex hormones, such as estradiol (an estrogen sex hormone) and progesterone, are key indicators of reproductive status, infertility, influences of environment on patterns and timing of pregnancy, unique reproductive strategies (e.g., embryonic diapause, reflex ovulation, etc.), or progression through the estrous cycle (i.e., early pregnancy, multiple cycles, lengths of cycles; Buchanan & Younglai 1986; Wasser et al. 1991; De Rensis & Scaramuzzi 2003). Although hormone analysis is a useful tool for understanding reproductive characteristics, information is lacking for many taxa in particular, free-ranging wildlife species.

For a few mammals such as rodents (Dey et al. 2004), pigs (Robertson & King 1974), cows (Desaulniers et al. 1989), dogs (Jöchle & Andersen 1977), common ferrets (Marshall 1904) and primates (Wasser et al. 1991, 1993), reproductive hormones have been extensively examined. While there are differences in reproductive strategies (e.g. embryonic diapause), reproductive cyclicity tends to be fairly similar among mammals. The estrous cycle (menstrual cycle for primates and humans; Tulsiani & Khan-Dawood 2003) integrates complex hormonal and physiological changes and can be divided into four stages: proestrus, estrus, metestrus, and diestrus. Anestrus falls outside the estrous cycle and is a period of reproductive inactivity (Daniel 1978; Vaughan et al. 2013). Proestrus is characterized by follicle development and estradiol secretions, which then leads into estrus, a sexually receptive period. During estrus, estradiol peaks in conjunction with ovulation (Jöchle & Anderson 1977; Vaughan et al. 2013). Females enter metestrus when the corpus luteum develops from the theca and granulosa cells of the ovarian follicle (Jöchle & Andersen 1977; Tulsiani & Khan-Dawood 2003). The corpus luteum produces some estradiol and large amounts of progesterone (Tulsiani & Khan-Dawood 2003). Progesterone is crucial for maintaining pregnancy (Piccinni et al. 1995). Due to its critical role in uterine maintenance and placentation, there are higher concentrations of progesterone in pregnant than non-pregnant females (Burns & Easley 1977; Hoffman et al. 1978; Buchanan & Younglai 1986). Successful pregnancy initiates when the ovum is fertilized. If it is not fertilized, the female will repeat the estrous cycle (Tulsiani & Khan-Dawood 2003).

Although hormone levels can indicate reproductive status, collecting viable samples for analysis can be difficult. Once a biological sample (e.g. plasma, feces, urine, etc.) is collected, it must be frozen, which may be challenging under field conditions. In addition, pilot studies may be required to assess presence or absence of hormones within particular biological materials. In addition, adequate serum samples, from which plasma must be isolated by centrifugation, are notoriously difficult to obtain from small and free-ranging mammals without sacrificing them (Voigt & Schwarzenberger 2008). As a result, many free-ranging species have been understudied with respect to reproductive phenology and endocrinology (Pukazhenth & Wildt 2004). Nonetheless, fecal samples are purported to have steroid concentrations similar to blood (Reeder & Widmaier 2009). Therefore, fecal samples are one of the best biological material for hormone analysis of wild-caught small mammals (Voigt & Schwarzenberger 2008). Fecal samples are easy to collect and can be done non-invasively in field settings. However, there are drawbacks

when analyzing feces. Depending on the species, there is a lag time from hormone excretion to feces absorption (Ziegler & Wittwer 2005). Dietary components, such as fiber, can have negative effects on steroid measurements in feces (Wasser et al. 1993). Moreover, purifying the sample for analyses is expensive and time-consuming (Ziegler & Wittwer 2005).

Another way to estimate female reproductive status that may be more practical and feasible under field settings is the use of vaginal cytology. Estradiol and progesterone concentrations and reproductive status can be analyzed by examining the epithelial lining of the vagina (De Bonilla & Romero 1988; McEndree 1999; Frederick et al. 2010). The abundance of maturate cells (nucleated and enucleated superficial cells and intermediate cells) expressed by the vaginal wall is an indicator of the systemic estradiol effect (McEndree 1999). Dominance of superficial cells is a strong indicator of estradiol stimulation (McEndree 1999) and reproductive status (De Bonilla & Romero 1988; Frederick et al. 2010; Vela-Vargas et al. 2016). Nucleated superficial cells are dominant during proestrus, whereas enucleate superficial cells are dominant during estrus (Vela-Vargas et al. 2016). As the female transitions from estrus to metestrus, intermediate cells become most abundant due to progesterone stimulation, with superficial and parabasal cells (least mature cell type) present in reduced numbers (Bekyürek et al. 2002). In non-model organisms, if pregnancy occurs, parabasal and intermediate cells tend to be found at median densities and superficial cells decrease in abundance (Bekyürek et al. 2002). If the parabasal cells are highly abundant this is indicative of reduced sexual steroid hormone stimulation and the female is in anestrus (Mills et al. 1979; McEndree 1999; Vela-Vargas et al. 2016).

Although vaginal cytology is a cost-effective tool for determining reproductive status, it may be difficult to determine if a female is truly pregnant (Racey 2009; Byers et al. 2012). For this reason, some researchers may group females that are in metestrus or early pregnant together even though they are physiologically and energetically very different (Gittleman & Thompson 1988; Badwaik & Rasweiler 2000). Metestrus is characterized by the development of the corpus luteum, whereas pregnancy spans from fertilization of the blastocyst all the way to parturition. Therefore, simply entering metestrus does not guarantee pregnancy. Energetic demands during metestrus and mating are minimal when compared to gestation (Gittleman & Thompson 1988). Because energetic demand increases throughout pregnancy into lactation (Loudon & Racey 1987), it is essential for females to reproduce during periods when resources are available to satisfy those demands (Thompson 1992; Bronson 1985). Better determination of when a female is in metestrus or pregnancy may improve understanding of how ecological phenomena such as predator-prey dynamics (Creel et al. 2007), intra- and interspecific competition (Berger 1983; Watts & Holekamp 2008), environmental variation (Foley et al. 2001; Greiner et al. 2011) and resource demand may relate to reproductive success.

Bats, especially those from the New World family Phyllostomidae, are highly diverse regarding reproductive strategies but little is known about their reproductive endocrinology (Voigt & Schwarzenberger 2008). Less precise external reproductive characteristics (e.g. vulva size and appearance, palpation of a fetus, balding nipples, etc.) are often used to indicate reproductive status instead of using uterine histology. Recently, the efficacy of analyzing reproductive hormones and vaginal cytology for determining reproductive status in some bats has been demonstrated (Voigt & Schwarzenberger 2008; Vela-Vargas et al. 2016).

We analyzed reproductive endocrinology and vaginal cytology of Seba's short-tailed fruit bats (*Carollia perspicillata*) in Santander, Colombia. *Carollia perspicillata* is well-studied and one of the most common species within the family Phyllostomidae. *Carollia perspicillata* has a range that extends from Mexico to northern Argentina (Cloutier & Thomas 1992). It has two breeding periods, from May to February and June to August, in Costa Rica and Colombia (Fleming 1988; Cloutier & Thomas 1992; Alviz-Iriarte 2014). At the Macaregua Cave, the diet of *C. perspicillata* is composed mostly of the genera *Myrcia*, *Neosprucea*, and

Piper (Bohlender et al. 2018). Reproduction tends to be asynchronous (Fleming 1988), but a few populations have been found to be highly synchronized in Central America and Trinidad (Rasweiler et al. 2009). Rasweiler & Badwaik (1997) demonstrated that females can delay development when under stress, but most females undergo nondelayed gestation. Gestation lasts typically 115-120 days and the fetus is palpable after 5 to 6 weeks of pregnancy (Kleiman & Davis 1979). After parturition, the lactation period will occur during the fruiting season (Fleming 1988), with most females experiencing postpartum estrus (Cloutier & Thomas 1992; Rasweiler et al. 2009).

We examined differences between non-pregnant and pregnant *C. perspicillata* regarding estradiol concentrations. Such differences have been found in other Neotropical species such as *Saccopteryx bilineata* (Voigt & Schwarzenberger 2008). We also examined differences in progesterone concentrations among bats in early pregnancy, late pregnancy, and lactation. Along with feasibility of using enzyme-linked immunosorbent assays to analyze hormones from fecal material of wild-captured animals, we predict that hormone (progesterone and estradiol) concentrations and vaginal cytology can be used to differentiate metestrus from implantation, early pregnancy and late pregnancy. We predict that females in metestrus and pregnancy (implantation, early and late pregnancy) have a high abundance of intermediate cells, with variable proportions of superficial and parabasal cells. Finding a cellular or hormonal difference between these two stages will facilitate more detailed analyses of bat reproduction in the future. This is especially important for free-ranging individuals, where pregnancy is not detectable until the latter part of gestation.

MATERIAL AND METHODS

Sample collection

Fecal and vaginal lavage samples were collected from June to August 2015 following the American Society of Mammalogists guidelines (ASM; Sikes et al. 2016) and Texas Tech University IACUC protocol No. 15027-004. Our study colony was at the Macaregua Cave in Santander, Colombia (6°39′36.2″ N, 73°06′32.3″ W). There are 9 bat species present at the cave and an estimated 7000 to 10 000 individuals present (Pérez-Torres et al. 2015). It is considered to have the highest species richness of bats of any cave in Colombia, with *C. perspicillata* being the most abundant species (Pérez-Torres et al. 2015). The study was conducted during the wet season, which extends from April to October. Temperature throughout the year ranges between 12-30 °C, and rainfall averages around 1550mm (Pérez-Torres et al. 2015).

Bats were captured in mist nets and placed individually into clean cloth bags. They were fed banana in the morning, and fecal samples and vaginal lavage samples were collected in the afternoon. Fecal samples were placed in Eppendorf tubes and stored in liquid nitrogen. Samples were then lyophilized with a Labconco 2.5 L Freezone Lyophilizer and stored frozen until analysis at the Functional Ecology Laboratory in the Pontificia Universidad Javeriana in Bogota, Colombia. Bats were tattooed and fitted with an individually numbered metal band on their forearm. Sixty-six females were euthanized from June to August 2015 and were deposited at the Collection of Mammals of the Museo Javeriano de Historia Natural of the Pontificia Universidad Javeriana (MPUJ-MAMM). All collected females were necropsied for the presence of a conceptus. Forearm and crown-to-rump length were measured for each conceptus. Sixty-eight individuals (samples from fifty-five potentially pregnant females and fifteen lactating females were collected; two samples were removed from analyses [see below]) produced sufficient quantity/quality (>0.01 g for bats) of fecal material for analyses.

Hormone assay

Estradiol and progesterone metabolites were extracted using a methanol and diethyl ether procedure modified from Greiner et al. (2011) and Voigt & Schwarzenberger (2008). Fecal sample dry weights ranged from 0.01 to 0.74 g. Each sample was placed into 0.5 mL of distilled water plus 0.5 mL of 5% $NaHCO_3$ and pulverized with a Kinematrica polytron homogenizer/sonicator. Four (4) mL of methanol were added to each homogenate and extracted for at least 48 hours. Samples were centrifuged at 800xg and 1 mL of supernatant was transferred to a clean tube. Three (3) mL of diethyl ether were added to each supernatant sample for re-extraction 24 hours later. The ether phase of each extract was transferred to a clean tube and air dried. Dry samples were re-dissolved in 100 microliters of 1X assay buffer prepared from a 10X concentrate (i.e. Tris

buffer [Enzo Catalog #80-2079] and Tris-buffered saline [Enzo Catalog # 80-0010]) for estradiol and progesterone, respectively, by dilution with deionized water. Concentrations were measured with an Enzo Life Sciences 17 β-estradiol kit and a modified progesterone enzyme-linked immunosorbent assay (ELISA) kit. The ELISA for estradiol was performed according to manufacturer's instructions. The ELISA for progesterone was performed with a CL425 antibody to measure all progesterone metabolites. Values for each sample were converted to hormone concentrations (pg/mL) from a standard curve for each assay. Hormone concentrations were corrected for dilution, standardized to fecal dry weight and expressed as ng/g.

To analyze hormonal and cytological differences during stages of the estrous cycle and gestation, groups were organized according to presence/absence of a fetus, fetus size, and progesterone levels. Females placed in the estrus group had low levels of progesterone (1.42 to 8.52 ng/g), females with intermediate levels of progesterone (10.13 to 15.7 ng/g) were grouped as metestrus, and females with high levels (24.46 to 64.74 ng/g) were grouped as implantation. These criteria correspond to hormone expression trends during mammalian reproduction (Robertson & King 1974; Oxberry 1979; Van der Merwe & Van Aarde 1989; Hurn & Macrae 2000; Tulsiani & Khan-Dawood 2003; Dey et al. 2004). Due to progesterone levels being part of the criteria for grouping, progesterone concentrations of estrus, metestrus and implantation were not compared statistically. Females in early pregnancy had an unpalpable fetus that had a crown-to-rump length < 19 mm (reproductive stages 18-21 [out of 24 stages], 60-70 days post coitum [out of 90 days]; Cretekos et al. 2005), whereas late pregnancy consisted of females with palpable fetuses with crown-to-rump length > 19 mm (reproductive stages 22-24, 80-90 days post coitum; Cretekos et al. 2005). Early lactating bats were also included in analyses, and were characterized as bats producing milk and carrying a pup.

Vaginal cytology

Exfoliate vaginal cytology was assessed by vaginal lavage (Vela-Vargas et al. 2016). Saline solution (0.5 μl) was pipetted into the vagina, aspirated, placed on slides, air dried, fixed with 90% ethanol, and then stained for microscopic evaluation with hematoxylin. Cells were then classified according to their morphology (**Fig. 1**) and a minimum of

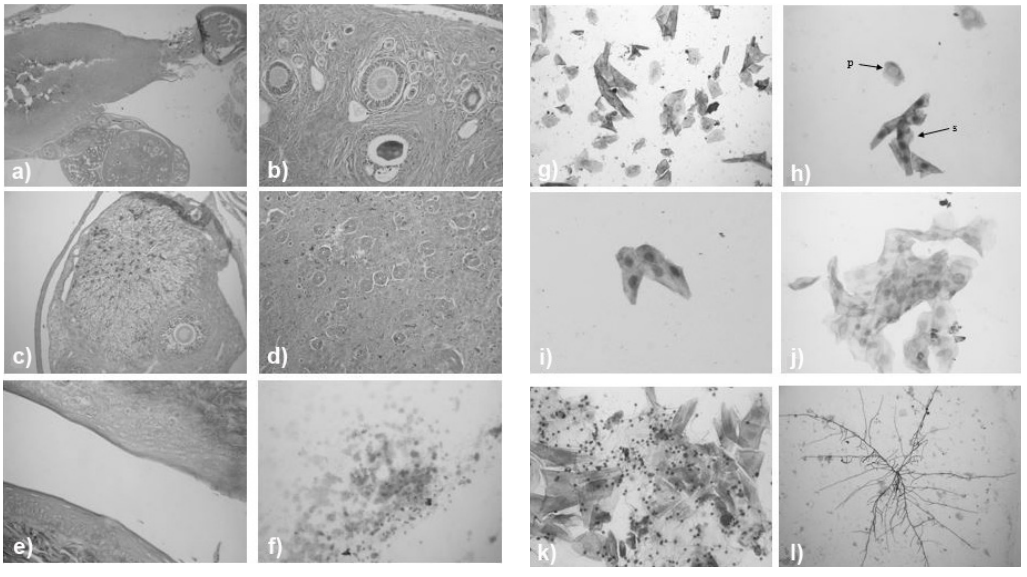

Fig. 1. Reproductive tract of a bat in metestrus and vaginal cytology of bats throughout reproduction. a) Stages of folliculogenesis distinguish the left ovary from the b) contralateral ovary c) with a corpus hemorrhagicum. Formed immediately after ovulation, the corpus hemorrhagicum will become a corpus luteum of pregnancy and produce progesterone. d) Straight tubular glands in the endometrium and e) superficial cells lining the vaginal canal confirm estradiol was the dominant hormone prior to ovulation. Changes in exfoliate cytology are shown as follows: f) a mass of parabasal cells, g) superficial cells and intermediate cells, h) parabasal cell (p) and superficial cell (s), i) intermediate cells, j) a mass of intermediate and superficial cells, k) sperm and l) fungus found within the vaginal cytology lavage of bats captured in Santander, Colombia during the breeding season.

85 cells per individual were counted. Parabasal cells are the least mature cell. They are round with the largest nucleus to cytoplasm ratio (Bekyürek et al. 2002). Intermediate cells are two to three times larger than parabasal cells. They tend to be round in shape and have a moderate nucleus to cytoplasm ratio. Superficial cells are large, flat, and irregular in shape. Depending on their cornification, some cells may not have a nucleus. If a nucleus is present, the nucleus to cytoplasm ratio of superficial cells is the smallest (Bekyürek et al. 2002). Presence of fungus, neutrophils, sperm (which was found in only one sample), and possible plant matter was also noted (**Fig. 1k**, **l**).

Statistical analyses

All analyses were conducted using SPSS 23. Due to violations of the assumptions of homogeneity of variances, among group differences regarding estradiol and progesterone (comparisons only done on early pregnant, late pregnant, and lactating bats) concentrations and log transformed vaginal cytology ratio of parabasal/superficial cells for reproductive stages were examined with Analysis of Variance with a Welch correction (ANOVA; Welch 1947) and Games-Howell post hoc tests (Games & Howell 1976). Normality was violated, however, according to Glass et al. (1972), ANOVA is robust given other data distributions. To determine the predictability of a female being pregnant or in metestrus, we conducted a logistic regression with independent variables being superficial cell proportions, parabasal cell proportions, and progesterone and estradiol metabolites using a forward conditional variable entry method.

RESULTS

Of the bats captured (n = 68) during the breeding season, 24% were not pregnant (n = 16), 54% were pregnant (including implantation, early and late pregnant females; n = 37), 22% were lactating (progesterone: n = 15; estradiol: n = 13). Two pregnant females were removed from analyses due to aberrantly low progesterone and estradiol concentrations. These were an early pregnant female (fetus full body length 9.9 mm) that had progesterone concentrations of 0.93 ng/g and estradiol concentration of 1.37 ng/g, and a late pregnant female (fetus full body length 30 mm) with progesterone concentration of 0.25 ng/g and estradiol concentrations of 0.45 ng/g.

Visual observations suggest that patterns of estradiol and progesterone concentrations had a similar trend across reproductive stages (**Table 1**, **Fig. 2**). Concentrations rose from estrus to pregnancy then decreased in late pregnancy. Progesterone continued to decrease into lactation, while estradiol increased during lactation.

ANOVA with a Welch correction indicated significant differences in estradiol concentrations ($F_{5,\ 23.6} = 14.19$, $P < 0.001$) among reproductive stages (**Fig. 2a**). According to Games-Howell post hoc tests, estradiol concentrations during estrus (14.69 ± 4 ng/g) were lower than metestrus (21.92 ± 4.5 ng/g, $P = 0.048$) and during implantation (76.80 ± 24.4 ng/g,

Table 1

Progesterone (P) and estradiol (E) metabolite concentrations from fecal material obtained from euthanized *C. perspicillata* in different reproductive stages from June to August 2015 (Santander, Colombia).

Reproductive Stage	No. of bats	P range (ng/g)	P $\bar{x} \pm$ SD	E range (ng/g)	E $\bar{x} \pm$ SD
Estrus	9	1.42 - 8.5	6.24 ± 2.4	9 - 20.3	14.7 ± 4
Metestrus	7	10.1 - 15.7	12.8 ± 2.2	17.8 - 29.9	21.9 ± 4.5
Implantation	10	24.5 - 64.7	39.2 ± 14.4	47.5 - 105.4	76.8 ± 24.4
Early Pregnancy	7	11.6 - 42.8	21.4 ± 11.76	17.7 - 96.4	39.7 ± 27.8
Late Pregnancy	20	6 – 61.1	16.9 ± 12.46	9.6- 102.1	27.9 ± 20.8
Early Lactation	15(P) 13(E)	3.1 – 56.9	16.9 ± 15	8 – 98	32.7 ± 30.9

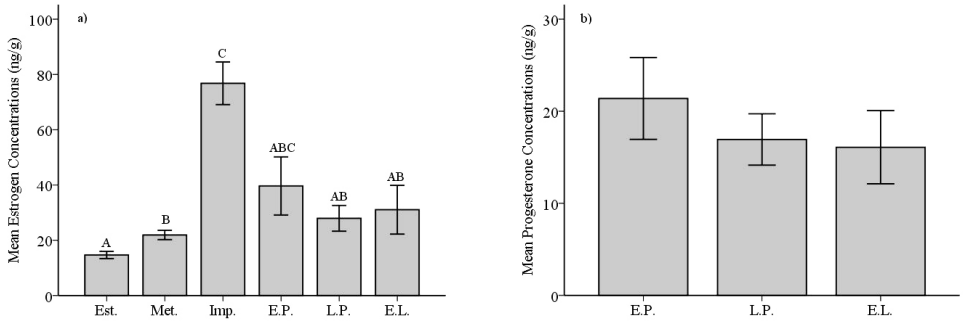

Fig. 2. (a) Mean estradiol concentrations and (b) mean progesterone concentrations during reproductive stages of *C. perspicillata* in Colombia from June to August 2015. Est. = estrus, Met. = metestrus, Imp. = implantation, E.P. = early pregnancy, L.P. = late pregnant, E.L. = early lactation. Different letters indicate significant differences between groups. Standard Error ± 1

$P < 0.001$). Estradiol concentrations during metestrus were similar during early and late pregnancy. Estradiol concentrations peaked at implantation, and were significantly different than all other reproductive stages, except early pregnancy (**Fig. 2a**). There was no significant difference in progesterone concentrations between early pregnant, late pregnant, and early lactation groups ($F_{2,\ 17.2} = 0.68$, $P = 0.648$, **Fig. 2b**).

Although intermediate cells were more abundant than superficial and parabasal cells during all five reproductive stages (**Fig. 3**), most variation was present in parabasal and superficial cell proportions. Four out of the eight females in estrus had greater proportions of superficial cells than intermediate cells. There was a significant difference in parabasal/superficial cell ratios among reproductive stages ($F_{5,\ 19.61} = 5.6$, $P < 0.001$, **Table 2**). Parabasal/superficial cell ratios were significantly lower in females during estrus than early and late pregnancy (mean difference -1.27 to -1.29; $P = 0.004$ to 0.005). The difference between females in stages of implantation were nonsignificant to females in estrus (mean difference -0.98, $P = 0.053$). Females during metestrus, implantation, early and late pregnancy, and early lactation had equivalent parabasal/superficial cell ratios. Observationally, superficial cells were moderately or highly abundant during estrus, and then decreased during metestrus, implantation, early pregnancy, and late pregnancy. Superficial cells increased during early lactation. Parabasal cells

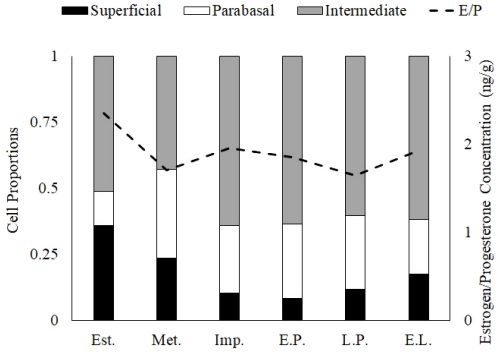

Fig. 3. Mean vaginal cell proportions and mean estradiol/progesterone concentrations during different reproductive stages of *C. perspicillata* during the reproductive period in Colombia. Est. = Estrus, Met. = Metestrus, Imp. = Implantation, E.P. = Early Pregnancy, L.P. = Late Pregnancy, E.L. = Early Lactation.

were the least abundant during estrus and metestrus, and then increased during implantation, early pregnancy, and late pregnancy.

The logistic regression model to determine predictability of females in pregnancy or metestrus successfully classified a significant proportion (87.8%) of individuals into their appropriate groups (females in metestrus and pregnancy). The model was able to significantly predict group membership ($\chi^2 = 4.94$, $d.f. = 1$, $P = 0.026$). A Hosmer and Lemeshow goodness of fit test ($\chi^2 = 5.59$, $d.f. = 8$, $P = 0.694$) indicated that observed values of superficial cells are statistically indistinguishable from expected values. Area under the curve (AUC) was 0.71,

Table 2

Results from Games-Howell post hoc tests of differences in log parabasal/superficial cell ratio among different reproductive stages. P-values are in the top right of the matrix, mean difference is in the bottom left. E.P. = early pregnancy, L.P. = late pregnancy, and E.L. = early lactating. Bold value was significant P-value.

	Estrus	Metestrus	Implantation	E. P.	L.P.	E.L.
Estrus		0.612	0.053	**0.005**	**0.004**	0.109
Metestrus	-0.632		0.935	0.494	0.491	0.994
Implantation	-0.984	-0.352		0.837	0.831	0.995
E.P.	-1.292	-0.659	-0.307		1	0.458
L.P.	-1.274	-0.642	-0.290	0.017		0.414
E.L.	-0.832	-0.200	0.152	0.459	0.442	

indicating that the model was a good fit from the logistic model

$$PBP = \frac{e^{(2.75+(-5.47*Superficial\ cell\ proportions))}}{1+e^{(2.75+(-5.47*Superficial\ cell\ proportions))}}$$

where PBP is Probability of Being Pregnant.

Parabasal cell proportions, estradiol and progesterone concentrations were nonsignificant predictors for determining whether a female was pregnant or in metestrus, and therefore not included in the model. Superficial cell proportions were a significant predictor of whether a female was in metestrus or pregnant ($\beta = -5.47$, Wald = 3.94, *d.f.* = 1, $P = 0.047$; **Fig. 4**). Non-pregnant females were more likely to have higher abundances of superficial cells compared to pregnant females.

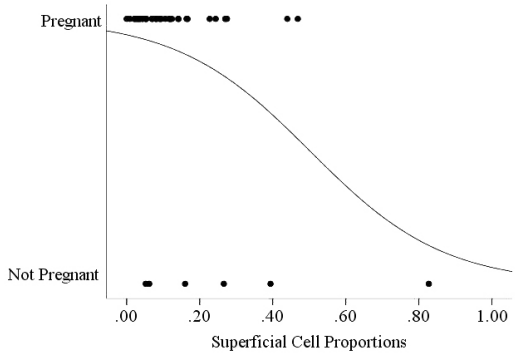

Fig. 4. Predictability of female *C. perspicillata* being in metestrus or pregnancy (implantation, early and late pregnancy) based on superficial cell proportions. The line is a tendency line to indicate probability of pregnancy in response to superficial cell proportions.

DISCUSSION

This is the first study to analyze fecal estradiol and progesterone metabolites in free-ranging female *C. perspicillata* during reproduction. We demonstrated a consistent pattern of variation across reproductive stages. We did exclude two individuals from analyses. In these two cases, individual bats exhibited abnormally low progesterone and estradiol concentrations. Concentrations for pregnant mammals are much higher than concentration assayed from feces of these two bats (Buchanan & Younglai 1986; Van der Merwe & Van Aarde 1989; Bernard et al. 1991; Hosken et al. 1996; Gudermuth et al. 1998; Chelini et al. 2005). Progesterone concentrations for pregnant bats, which is more readily studied than estradiol concentrations, had values ranging from 4.64 to 64 ng/mL (Buchanan & Younglai 1986; Van der Merwe & Van Aarde 1989; Bernard et al. 1991; Hosken et al. 1996). For non-model mammals, estradiol concentrations during pregnancy averaged around 6.4 ± 0.6 ng/g, and concentrations ranged between 3.91 to

319.89 ng/g for superovulated females (Muir et al. 2001; Chelini et al. 2005). The lowest progesterone estradiol concentrations for early pregnant and late pregnant bats in this study were 12x times higher than hormonal concentrations assayed from the excluded bats. The progesterone concentrations assayed from these bats were similar to ranges found in nonpregnant Gould's wattled bats (*Chalinolobus gouldii*; 0.3 to 2.39 ng/mL; Hosken et al. 1996). Possible reasons may be that these females were stressed (De Rensis & Scaramuzzi 2003), they had already entered parturition where hormone levels tend to drop (Robertson & King 1974), or because these two samples may have been compromised in the field.

Estradiol and progesterone concentrations during estrus were not different between stages of early pregnancy and early lactation, and these concentrations during metestrus were not significantly different between early and late pregnancy. Such observations contradict our original hypotheses of estradiol and progesterone having statistically different concentrations throughout the reproductive cycle. This may be due to high variability of hormone expression, which could be caused by a multitude of factors, such as environmental factors (Behie et al. 2010) or diet variation (Wasser et al. 1993). Estradiol concentrations exhibited a rise from estrus into implantation, then a decrease into late pregnancy and a second rise during early lactation. This peak at implantation suggests the possible importance of estradiol for blastocyst attachment, which has also been found in *Macrotus waterhousii* (Burns & Wallace 1975). Estradiol is essential for the blastocyst to implant within the uterus, at least for other mammals such as rodents (Dey et al. 2004). Progesterone alone maintains a state of embryonic diapause until there is a surge of estradiol that allows for implantation (Dey et al. 2004). Once the blastocyst is implanted, estradiol plays an important role in fetal development (Albrecht et al. 2000), explaining why estradiol concentrations may be high during implantation and throughout pregnancy.

However, it must be noted that levels of estradiol that are too high may impede implantation (De Catanzaro et al. 1991). Women and mice with high levels of estradiol compared to progesterone failed to become pregnant (Gidley-Baird et al. 1986). Higher levels of progesterone compared to estradiol increased the likelihood of pregnancy (Gidley- Baird et al. 1986). Therefore, when analyzing stages like implantation, it may be better to analyze the ratio of estrogen to progesterone than the whole values (Gidley-Baird et al. 1986). For *C. perspicillata*, there was an increase in estradiol, but progesterone also increased, keeping the ratio of estradiol to progesterone low (**Fig. 3**).

When a female enters into parturition, there is a shift in the estradiol/progesterone ratio. An increase in this ratio allows for an increase in oxytocin receptors and prostaglandins, both essential for contractions and cervix softening in other mammals (Johnson & Everitt 2000). The increase in estradiol from late pregnancy into early lactation, along with *C. perspicillata* potentially going through postpartum estrus, may explain why estradiol is high at this point (Rasweiler & Badwaik 1997). Estradiol concentrations are rarely studied in bats, but the trend for *C. perspicillata* is similar to plasma concentration in *Macrotus waterhousii* (Burns & Wallace 1975) and *Sus scrofa domesticus* (Robertson & King 1974).

Progesterone concentrations decreased throughout pregnancy and into early lactation. Just like estradiol, progesterone is crucial for implantation and cell proliferation in rodents (Dey et al. 2004) and has been found to peak during implantation in other bats, such as *Miniopterus schreibersii natalensis* (Van der Merwe & Van Aarde 1989) and hibernating *Antrozous pallidus* (Oxberry 1979). Prior radioimmuno assays of *Macrotus californicus* indicated two peaks in progesterone concentrations, one during placentation and the second coinciding with accelerated fetal development (Burns & Easley 1977). In other mammals such as domesticated pigs (*S. scrofa domesticus*), plasma progesterone concentrations were also found to be high during implantation and to steadily decrease into parturition (Robertson & King 1974). Progesterone concentrations

analyzed in this study are similar to those found in other free-ranging bats (Buchanan & Younglai 1986).

While estradiol and progesterone concentrations were not effective for differentiating metestrus from pregnancy in our study, repeated measures of hormone concentrations from a number of individuals over time may help determine reproductive status of *C. perspicillata* or other species (Voigt & Schwarzenberger 2008).

Hormone levels stimulate changes in the vaginal epithelium, and the proportion of specific cell types may be a better predictor of reproductive status due to subtle changes from one reproductive stage to the next (Bekyürek et al. 2002). In a study conducted on *Diphylla ecaudata*, the estrus phase was identified by high frequencies of enucleated superficial cells whereas metestrus was identified based on high levels of intermediate cells (Elizalde-Arellano et al. 2008). For *C. perspicillata*, intermediate cells were the most abundant cell type within all six reproductive groups and four of the eight females characterized to be in estrus had high levels of superficial cells relative to intermediate cells. According to Crichton & Krutzch (2000), vaginal cytology remains mostly unchanged during both the breeding season and pregnancy. This may explain why intermediate cells are highly abundant during estrus and why examining superficial cell abundance is a more nuanced way to evaluate reproductive states. A previous study done by Stone et al. (1975) on humans indicated that even with estradiol supplements intermediate cells were most abundant, with superficial cells only being moderately abundant.

To our knowledge, this is the first study to examine all cell types present at different reproductive stages and to try to differentiate metestrus and pregnancy in free-ranging bats. During metestrus and pregnancy, intermediate cells were highly abundant, but superficial cells were more abundant during metestrus than during pregnancy. Our logistic regression indicated good predictive power, but could be improved by including more females. Our AUC is also within the range of acceptable discrimination for the model (Hosmer et al. 2013). Because of a developing fetus, time post-implantation is more energetically expensive than time pre-implantation (Gittleman & Thompson 1988). By being able to predict these two stages, we may better answer ecological questions (i.e. how environmental characteristics and fetal developmental demands influence pregnancy), understand reproductive characteristics of free-ranging bats or assist with conservation efforts by increasing the odds of reproductive success.

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