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Quantification of Urinary Sex Steroids in the Big Brown Bat (*Eptesicus fuscus*)

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ABSTRACT

Bats (order Chiroptera) are the second largest group of mammals, diverging ~52.5 million years ago. Many species exhibit an unusual reproductive cycle and extreme longevity without reproductive senescence, yet steroid profiles exist for few bats. Big brown bats (*Eptesicus fuscus*) are temperate insectivores found throughout North America. They mate promiscuously in fall, store sperm during winter hibernation, and have delayed ovulation and fertilization in spring. Here, we report the first urinary steroid profile in bats by quantifying 17β-estradiol (E_2) in captive male and female *E. fuscus* across their reproductive cycle. Male bats had higher urinary E_2 levels than females, and adults had higher levels than yearlings following creatinine adjustment for hydration. In nonpregnant females, several seasonal differences in creatinine-adjusted and unadjusted urinary E_2 levels were observed. Urinary E_2 was higher in males than females in winter for both conditions and in autumn for creatinine-adjusted levels. We quantified progesterone (P_4) in a subset of females. In nonpregnant females, urinary P_4 was constant across seasons except for unadjusted levels, which were highest in the summer. In pregnant females, urinary E_2 and P_4 levels peaked beginning ~20 d before parturition, with both steroids returning to baseline in the following weeks. Knowing how urinary steroid levels fluctuate with age and sex and across the annual season is key to understanding reproductive cycling in bats. Our research furthers the potential for bats as a model for medical reproductive research. Moreover, it complements previous studies on the potential role of steroids in primer pheromonal effects in bats.

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Keywords: Chiroptera, estradiol, hormones, pheromone, progesterone, reproduction, sex steroids, urine analysis.

Introduction

The reproductive cycle of temperate Vespertillionid bats follows an unusual decoupling of mating before hibernation and subsequent fertilization after hibernation. Female bats hibernate following the autumn mating season, storing viable sperm in the reproductive tract until ovulation in the spring (Kurta and Baker 1990). Because of their unusual mating systems, bat reproductive physiology and endocrinology were highly studied in the mid-1900s; however, there was a rapid decline in research toward the end of the century. Past reproductive and endocrinological research in bats focused on a small subset of species, leaving many unanswered questions regarding the monitoring and endocrine control of reproduction. Today, biologists monitor reproduction in a wider range of bat species (e.g., Stukenholtz et al. 2018), some of which have been proposed as model organisms for reproductive research because of their similarities with human reproduction (Rasweiler et al. 2009; Rodrigues et al. 2019; Santiago et al. 2020).

Modern genetics suggests that the superorder Laurasiatheria, of which bats (order Chiroptera) are a part, diverged from the rodent superorder Euarchontoglires during the mid-Cretaceous period (Murphy et al. 2004; Springer et al. 2004; Zhou et al. 2012). The earliest fossils representative of modern bats have been dated to 52.5 million years ago (Simmons et al. 2008; Veselka et al. 2010). Today, the big brown bat (*Eptesicus fuscus*) is one of the most commonly distributed Vespertillionid bats in Central and North America (Kurta and Baker 1990). They display fission-fusion roost dynamics where individuals frequently rotate between different roosting sites within a region, and day roosts often differ from those used during the night (e.g., Brigham 1991; Willis and Brigham 2004). In colder climates, *E. fuscus* hibernate over the winter (reviewed by Kurta and Baker 1990). Bats have extreme longevity, with mark-recapture studies showing that big brown bats can live at least 19 yr in the wild, and males typically outlive females (Paradiso and Greenhall 1967; Kurta and Matson 1980; Hitchcock et al. 1984; Wilkinson et al. 2021).

Big brown bats display promiscuous mating in the laboratory (Mendonça et al. 1996), and there is evidence of similar behavior in the wild (Vonhof et al. 2006). Birthing patterns differ by geographical location, with twins born in eastern North America

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and singlets born in western North America (Schowalter and Gunson 1979; Kurta and Baker 1990; O'Shea et al. 2010). Copulation occurs in the autumn before hibernation and also intermittently during periods of arousal throughout hibernation (Oxberry 1979). Females store sperm during hibernation, and given favorable conditions, ovulation occurs in the spring following seasonal arousal, with fertilization and implantation occurring soon thereafter (Wimsatt 1944; Christian 1956; Oxberry 1979; Racey 1979). Female *E. fuscus* display polyovulation, releasing up to five ova each spring with excess embryos resorbed during early gestation (Wimsatt 1945). Typically, females become more parous with age during the first years of life (e.g., Barclay 2012).

Sex steroids have been quantified only in a handful of female bat species (reviewed in Martin and Bernard 2000). Reproductive female big brown bats form summer maternity colonies in hollow trees and buildings, while males typically roost alone or in bachelor colonies (Kurta and Baker 1990). While ecological patterns of reproduction are relatively well-known in *E. fuscus*, the endocrine aspects are not. Plasma concentrations of estradiol (E_2) and progesterone (P_4) in females and androgens in males have been reported for intact and gonadectomized individuals during the autumn and winter (Mendonça et al. 1996; Mendonça and Hopkins 1997). Currently, a lack of sufficient knowledge of their endocrinology impedes reproductive research in this species. To date, there have been no long-term studies of steroid quantification in *E. fuscus* and only one report of seasonal fluctuations in urinary E_2 levels in males (Greville et al. 2020).

When adult male bats were injected with radiolabeled E_2 and housed with adult or juvenile females during the mating season, radioactivity was consistently found in the neural, reproductive, and peripheral tissues of cohabitating female bats (deCatanzaro et al. 2014). When untreated females were housed with females given radiolabeled P_4, radioactivity was similarly found in the untreated females (Greville et al. 2017). Recently, we showed that E_2 transfers from male bats to female bats at higher levels during the fall mating and spring ovulatory periods in comparison to the summer nonreproductive season and that it peaks in concentration at these same time points (Greville et al. 2020). In all cases, the highest levels of steroid transfer occurred in reproductive tissues that have high steroid receptor densities and modulate female reproductive physiology and behavior (Kuiper et al. 1997; deCatanzaro et al. 2014; Greville et al. 2017, 2020). Sex steroids are critical for the proper development of female sexual behavior and physiology, and these results suggest that they may act not only within the individuals whose glands produce them but also potentially on proximate conspecifics.

The available evidence indicates that urine, as a main excretion route of E_2, is a likely vector of E_2 transfer between bat conspecifics (deCatanzaro et al. 2014; Greville et al. 2017, 2020), as has been observed in mice (Guzzo et al. 2010, 2012). The vast majority of mammalian sex steroids are synthesized in the mammalian gonads (Nelson and Kriegsfeld 2017) and efficiently travel throughout the body to target tissues via the circulatory system by binding to carrier proteins (Anderson 1974). Free steroids readily pass through cell membranes and accomplish their regulatory functions via intracellular receptor binding in the nucleus of target tissues, where they influence gene transcription (Walters 1985). More recently, steroids have been shown to bind to G-coupled protein receptors in the cell membrane, where they initiate intracellular messengers and pathways (reviewed by Filardo and Thomas 2005; Barton et al. 2018). Following their actions, steroids are conjugated, primarily in the liver, to inactive hydrophilic metabolites through glucuronidation and sulfation pathways allowing for urinary excretion (reviewed by Zhu and Conney 1998). As estrogens are near the end of the steroid biosynthesis pathway, circulating E_2 levels may rise as precursor steroids undergo catabolic processes. One example of this is the aromatization of testosterone into E_2. As such, individuals with elevated testosterone levels may have higher concentrations of E_2, E_2 conjugates, and other metabolites excreted in their urine as testosterone is aromatized. E_2 itself may not be excreted as E_2-glucuronide or E_2-sulfate, but it can be converted to a weaker estrogen known as estrone and remain in the body or excreted as an estrone conjugate. Enzymes such as estrogen sulfatase and β-glucuronidase are able to convert conjugated E_2 back into its bioactive unconjugated form (reviewed by Zhu and Conney 1998). As such, steroid metabolism and urinary excretion can become complex and influenced by the dynamics of many other hormones and enzymes. Despite typically being excreted as a metabolite or in a conjugated form, unconjugated E_2 has broadly been measured in mammalian urine (e.g., Kjeld et al. 1977; Ramsay et al. 1981; Hodges et al. 1983; Thorpe et al. 2014; Greville et al. 2020).

Owing to its role in facilitating the transfer of steroids between conspecifics, we were interested in quantifying E_2 levels in the urine of male and female *E. fuscus*. Because of its critical importance in female reproductive physiology and behavior, we hypothesized that females have higher urinary E_2 levels than males. Secondary to E_2, urinary P_4 levels were measured in the urine of female *E. fuscus* depending on sample volume. This is because P_4 works in tandem with E_2 in regulating female reproductive physiology and has previously been shown to have possible implications as a female pheromone (Freeman 2006; Guzzo et al. 2012; Greville et al. 2017). We recently reported an increased level of creatinine-adjusted urinary E_2 in male *E. fuscus* during the autumn and spring compared with the summer (Greville et al. 2020). In the current study, we hypothesized that the same seasonal pattern would be replicated in a larger sample of males. We also hypothesized that females would have the highest urinary E_2 levels during the fall and spring, when mating and ovulation/gestation occur, respectively. Since bats have low rates of reproduction in their first mating season (Sidner 1997; O'Shea et al. 2010; Barclay 2012), we hypothesized that adult bats have higher urinary E_2 than yearlings. We conclude by describing a preliminary urinary steroid profile for pregnant females. We use urinary steroid measurements in this study because they reflect systemic steroid fluctuations in mammals (Thorpe et al. 2014) and allow for noninvasive repeated sample collection over an extended time period.

Methods

Animals and Housing

Adult male and female *Eptesicus fuscus* were wild caught in southern Ontario and housed in a captive research colony at McMaster

University, where bats were permitted to fly (indoors and outdoors) and had ad lib. access to food (mealworms; *Tenebrio molitor*) and water. The indoor colony temperature and lighting varied with ambient conditions (for details, see Skrinyer et al. 2017). Juvenile bats were classified as yearlings until they reached 1 yr old, after which they were considered adults. Newly captive animals, including pregnant females close to parturition, were kept for at least 2 wk in an indoor holding room within an animal facility. These bats were housed in groups of four or five in stainless steel wire-mesh (1/4-inch grid) holding cages (22 cm × 28 cm × 18 cm) before being introduced to a separate quarantine side of the colony. Captive breeding occurred within the colony, and these individuals were also housed in holding cages near the end of gestation. All procedures were approved by the Animal Research Ethics Board of McMaster University and were in accordance with the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care.

Chemicals

E_2 and P_4 standards were obtained from Sigma-Aldrich, Oakville, Ontario. Steroid antibodies and horseradish peroxidase (HRP) conjugates were provided by Coralie Munro of the Department of Population Health and Reproduction at the University of California, Davis. That laboratory provided data on cross-reactivities for anti-E_2 (R4972) as follows: E_2 100%, estrone 3.3%, P_4 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids including estrone conjugates <0.1%. Cross-reactivities for anti-P_4 were as follows: P_4 100%, 11α-hydroxyprogesterone 45.2%, 5α-pregnen-3,20-dione 18.6%, 17α-hydroxyprogesterone 0.38%, 20α-hydroxyprogesterone 0.13%, 20ß-hydroxyprogesterone 0.13%, pregnanediol <0.001%, pregnenolone 0.12%, E_2 <0.001%, and estrone <0.04%.

Urine Collection and Analysis

We noninvasively collected urine from male and female *E. fuscus* following published protocols (Greville et al. 2017, 2020). Bats were randomly selected from the colony and held over a wax-paper-lined work surface while they urinated. Urine was collected in 70-μL hematocrit tubes (Fisherbrand, Pittsburgh, PA) and kept frozen (−20°C) until time of steroid analysis. Urine samples collected from 219 bats were tested for E_2 (n = 48 adult and 36 yearling males; n = 98 adult and 19 yearling nonpregnant females; n = 22 pregnant females; four nonpregnant females later became pregnant, so they have samples in both groups). We collected 409 adult and 308 yearling male urine samples, 440 adult and 82 yearling nonpregnant female urine samples, and 47 pregnant female urine samples.

Urinary steroids were analyzed following methodology first described by Munro et al. (1991) for steroid quantification in humans using a competitive enzyme-linked immunosorbent assay (ELISA). These methods were previously adapted for use in small mammals (Muir et al. 2001; deCatanzaro et al. 2003, 2004; Pollock et al. 2016, 2019) and later validated for *E. fuscus* using parallelisms to ensure that the standard curve captured the physiological range of steroids in bats (Greville et al. 2017, 2020). Briefly, plates were coated with stock antibodies diluted in coating buffer and left overnight. The following morning, plates were washed to remove unbound antibody, EIA buffer was added to each well, and plates were left to incubate at room temperature for 2–5 h. For E_2 analysis, samples were diluted 1:4 with EIA buffer. Samples and serially diluted standards were added to wells with HRP and allowed to sit for 2 h, after which time plates were washed and ABTS was added to each well. Plates were read at 405 nm approximately 2–4 h later. We also measured P_4 levels in a subset of female urine samples with a sufficient volume of urine remaining after E_2 analysis (n = 84 adult and 24 yearling nonpregnant females; n = 16 pregnant females). Procedures for P_4 were similar to E_2 except that urine samples were diluted 1:8. A regression line was fitted to the standards, and samples were interpolated into the equation to determine hormone concentration per well. Standard curves allowed for reliable assay measurement between 3.9–250 pg/well for E_2 (equivalent to 0.98–62.5 ng/mL urine) and 9.8–625 pg/well for P_4 (equivalent to 1.8–112.2 ng/mL urine). E_2 and P_4 were quantified in all analyzed urine samples.

We considered urinary steroid values with and without adjustment for urinary creatinine, which corrects for differences in hydration and urine concentration at the time of sample collection. As such, all steroid levels are reported as mean ± SE in both nanograms per milliliter urine and nanograms per milligram creatinine. Briefly, 50 μL of distilled H_20, 0.75 N NaOH, and 0.4 N picric acid were added to 50 μL of diluted urine. Urine samples used for creatinine analyses had a final dilution of 1:38. Samples underwent the Jaffe reaction and became colored with their optical density proportional to creatinine values. Optical density was measured 30 min after chemical mixture. Creatinine standards ranged from 0 to 100 pg/well (0–3.8 mg/mL urine). Extremely low or negative creatinine measurements were adjusted to a minimal value of 0.05 mg/mL urine to prevent artificial inflation of creatinine-adjusted urinary E_2 values.

Data Analysis

Statistical analyses were conducted in the R software environment (R Development Core Team 2020). We constructed individual linear mixed effects regression (LMER) models using the lme4 package (Bates et al. 2015) to evaluate the dependent variables of unadjusted E_2, creatinine-adjusted E_2, and creatinine. Given that urine was not collected from every animal on each collection date and that every animal does not have a sample in each season, LMER was selected as it is robust to missing data points. Maximum models included age, sex, season, and their interactions as fixed effects. Random effects included animal identity and day of urine sample collection. Following the simplification of models to eliminate singular fits, models were further backfitted and compared via likelihood ratio tests until the optimal fit was found. In all three cases, optimal models included sex × season interaction as a fixed effect and both animal identity and urine collection date as random effects. Animal age, but not its interactions, was also included as a fixed effect in creatinine-adjusted E_2 and creatinine analyses.

Separate LMERs were constructed to analyze unadjusted and creatinine-adjusted P_4 in female animals with maximum models including age, season, and their interaction as fixed effects and animal identity and date of urine collection as random effects. Models were backfitted as described above. Final models for both unadjusted and creatinine-adjusted P_4 analyses included season as a fixed effect and animal identity as a random effect.

The *F* values for main effects are reported using a type III sum of squares with Satterthwaite's method of calculating degrees of freedom. Both the Satterthwaite and Kenward-Roger approximations are suitable for use in mixed models and produce extremely similar results (Luke 2017; Kuznetsova et al. 2017). However, the Satterthwaite approximation is less computationally intensive than the Kenward-Roger method and is the default setting in R (Kuznetsova et al. 2017). A priori multiple comparisons of seasonal differences within and between sexes were conducted using the emmeans package (Lenth 2020) followed by false discovery rate adjustment with a family-wise $\alpha = 0.05$ (Benjamini and Hochberg 1995). Seasons were defined by months according to variations in the outdoor temperature of the colony: spring (March–May), summer (June–August), autumn (September–November), and winter (December–February).

Results

We aimed to evaluate sex, age, and seasonal differences in urinary E_2 and P_4 concentrations of big brown bats. Both E_2 and P_4 were present in all analyzed urine samples. Data from one male with urinary E_2 levels of 1,509 ng/mL urine were discarded from the analysis because this outlier data point was so far above the highest standard that it was deemed unreliable.

Table 1 summarizes the main effects from LMER analysis for unadjusted and creatinine-adjusted steroids, as well as creatinine measurements. There was no effect of sex on unadjusted E_2 levels ($F_{1, 118.73} = 1.58$, $P = 0.211$; fig. 1*A*). Significant effects of season ($F_{3, 168.44} = 7.08$, $P < 0.001$) and sex × season interaction ($F_{3, 1,160.13} = 7.77$, $P < 0.001$) were observed for unadjusted E_2 measurements. Post hoc comparisons using false discovery rate adjustment evaluating seasonal differences within the sexes and sex differences within seasons are shown in figure 2. Unadjusted E_2 differed across seasons in females (autumn-spring: $P = 0.013$; autumn-summer: $P = 0.027$; spring-winter: $P < 0.001$; summer-winter: $P < 0.001$), as well as between sexes during the winter ($P < 0.001$; fig. 2). While age was included in the maximal LMER model, it was not part of the optimal model and thus had no effect on unadjusted E_2 measurements (fig. 1*B*).

Creatinine-adjusted E_2 was significantly higher in males than females ($F_{1, 125.73} = 9.32$, $P = 0.003$; fig. 1*C*) and in adults compared with yearlings ($F_{1, 253.31} = 10.72$, $P = 0.001$; fig. 1*D*). There were also effects of season ($F_{3, 180.02} = 7.01$, $P < 0.001$) and sex × season interaction ($F_{1, 1,164.53} = 13.13$, $P < 0.001$). Post hoc comparisons using false discovery rate adjustment showed seasonal creatinine-adjusted E_2 differences in females (autumn-spring: $P < 0.001$; autumn-summer: $P < 0.001$; spring-winter:

Table 1: Summary of variance components for different physiological measurements

Physiological measurement, source of variation	ndf	ddf	Type III SS	Mean square	*F*	*P*
Unadjusted E_2:						
Sex	1	118.73	.58	.58	1.58	.211
Season	3	168.44	7.83	2.61	7.08	<.001***
Sex × season	3	1,160.13	8.58	2.86	7.77	<.001***
Creatinine-adjusted E_2:						
Age	1	253.31	4.75	4.75	10.72	.001***
Sex	1	125.73	4.13	4.13	9.32	.003**
Season	3	180.02	9.32	3.11	7.01	<.001***
Sex × season	3	1,164.53	17.46	5.82	13.13	<.001***
Unadjusted P_4:						
Season	3	97.15	2.02	.67	4.09	.009**
Creatinine-adjusted P_4:						
Season	3	98.81	.51	.17	.71	.548
Creatinine:						
Age	1	132.69	3.59	3.59	8.29	.005**
Sex	1	103.66	1.42	1.42	3.27	.073
Season	3	186.98	4.85	1.62	3.73	.012*
Sex × season	3	1,109.18	5.25	1.75	4.05	.007**

Note. Analysis of variance table with type III sum of squares (SS) using the Satterthwaite approximation for degrees of freedom. E_2 = estradiol; P_4 = progesterone; ndf = numerator degrees of freedom; ddf = denominator degrees of freedom.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

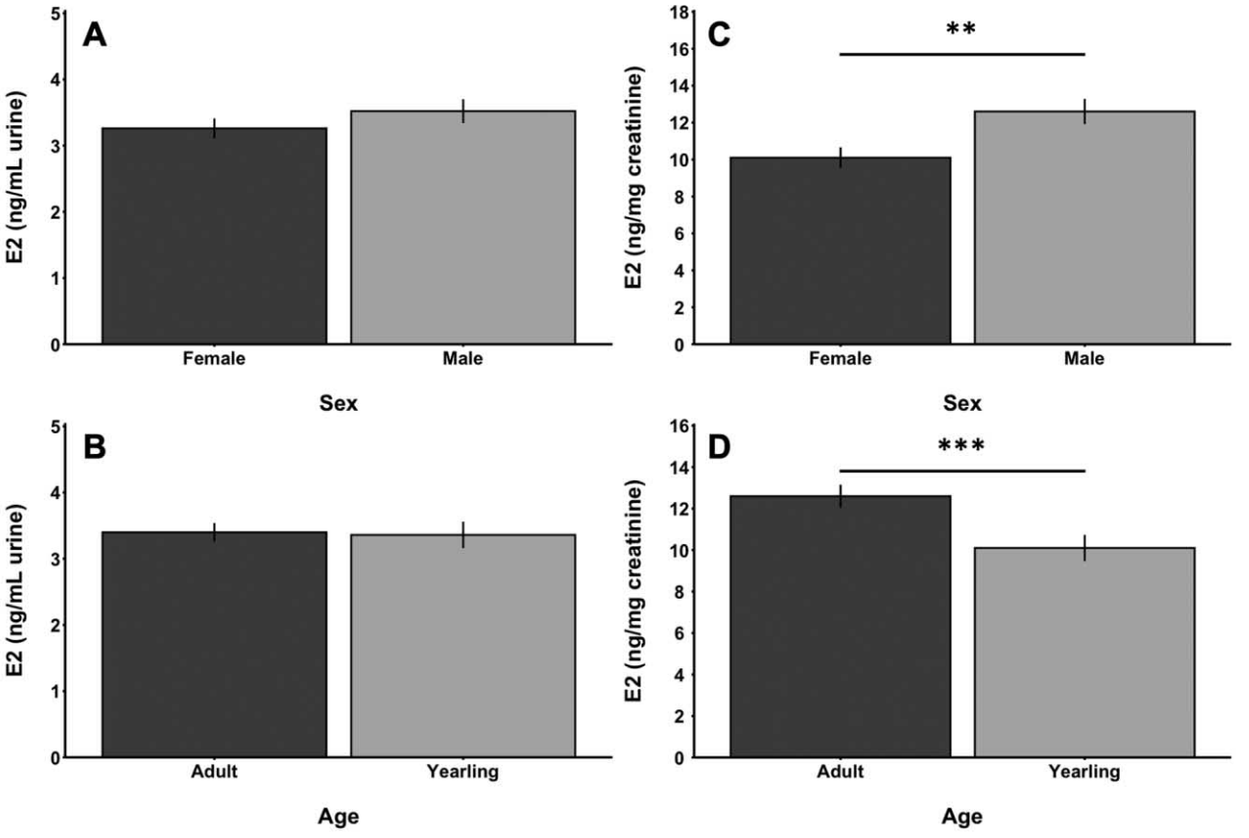

Figure 1. Age and sex comparisons of urinary estradiol (E_2). Geometric mean ± SE concentrations of urinary E_2, expressed as nanograms of E_2 per milliliter of urine (*A*, *B*) and nanograms of E_2 per milligram of creatinine (*C*, *D*), of bats of varying sex and age. Geometric means are produced from the optimal linear mixed effects regression models after correction for animal identity and date of urine collection. There were significant differences in creatinine-corrected E_2 between sexes at a given age and age within sexes. $^{**}P < 0.01$; $^{***}P < 0.001$.

$P < 0.001$; summer-winter: $P < 0.001$) and in males (autumn-winter: $P = 0.030$; fig. 2). Sex differences during the autumn ($P < 0.001$) and winter ($P < 0.001$) were also observed.

Following E_2 analysis, a subset of urine from nonpregnant individuals was analyzed for P_4. Seasonal differences in urinary P_4 concentrations of nonpregnant female bats are shown in table 2, with main effect of season shown in table 1. There was a significant effect of season on unadjusted P_4 levels ($F_{3, 97.15} = 4.09$, $P = 0.009$) but not on creatinine-adjusted P_4 ($F_{3, 98.81} = 0.71$, $P = 0.548$). Post hoc comparisons using false discovery rate adjustment showed unadjusted P_4 was higher in the summer than in the spring ($P = 0.034$), autumn ($P = 0.033$), and winter ($P = 0.005$). Age was included in the maximal LMER model for both unadjusted and creatinine-adjusted P_4 but was not part of the optimal model for either and thus had no effect on measurements.

Following quantification of urinary E_2 and P_4 concentrations, we separately analyzed a subset of data from pregnant females (fig. 3). These data were standardized by the date of parturition (day 0) and reported for up to 41 d before and 44 d after the females gave birth. The data set consists of 47 urine samples from 22 pregnant female bats (one to seven samples per individual). Owing to the small sample size and variability in the measurements, no statistical comparisons were performed on these data. Descriptively, both creatinine-adjusted and unadjusted levels of steroids began to rise approximately 20 d before parturition, with creatinine-adjusted steroid levels decreasing immediately before birth. Following birth, both creatinine-adjusted and unadjusted steroid levels returned to the range typically measured in nonpregnant females by approximately 17 d postpartum. Notably, the highest steroid levels we measured were from pregnant females (E_2: 47.36 ng/mL and 228.74 ng/mg creatinine; P_4: 224.98 ng/mL and 442.69 ng/mg creatinine).

Table 3 shows the geometric mean and standard error—which provides a more accurate measurement of skewed data—of creatinine measurements for all sex × season interactions. Bat urinary creatinine levels did not differ across sex ($F_{1, 103.66} = 3.27$, $P = 0.073$; $\overline{X}_m = 0.28 \pm 0.01$, $\overline{X}_f = 0.31 \pm 0.01$), but they were higher in yearlings compared with adults ($F_{1, 132.69} = 8.29$, $P = 0.005$; $\overline{X}_a = 0.27 \pm 0.01$, $\overline{X}_y = 0.32 \pm 0.02$). Main effects of season ($F_{3, 186.98} = 03.73$, $P = 0.012$) and sex × season interaction ($F_{3, 1,109.18} = 4.05$, $P = 0.007$) were also observed. Post hoc analyses with false discovery rate adjustment showed creatinine differences across seasons in females (autumn-spring: $P = 0.031$) and males (autumn-summer: $P = 0.048$; spring-summer:

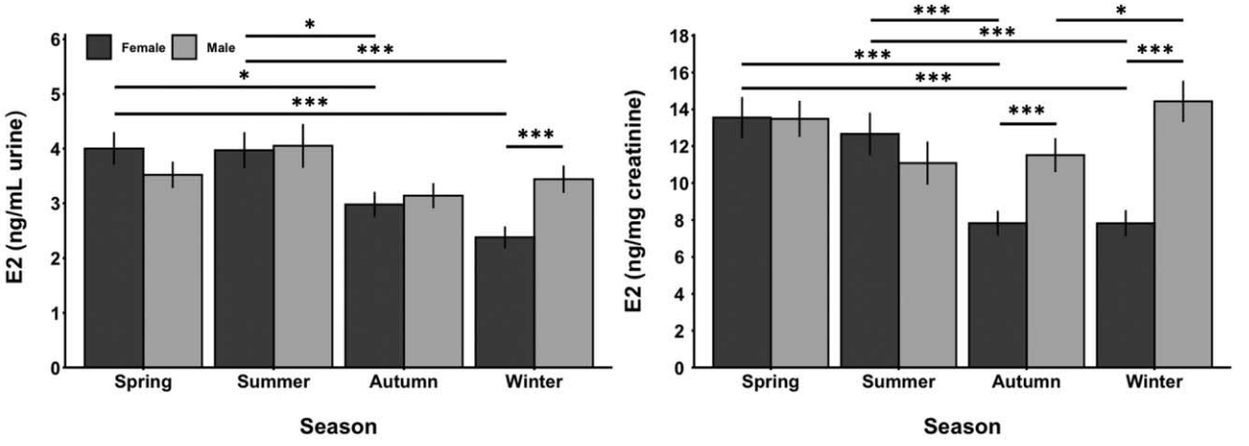

Figure 2. Seasonal comparison of urinary estradiol (E_2) in female and male bats. Geometric mean ± SE concentrations of urinary E_2, expressed as nanograms of E_2 per milliliter of urine and nanograms of E_2 per milligram of creatinine, of female and male bats across four seasons. Geometric means are produced from the optimal linear mixed effects regression models after correction for animal identity and date of urine collection. $^*P < 0.05$; $^{***}P < 0.001$.

$P = 0.025$; summer-winter: $P = 0.004$). A sex difference was seen during the autumn ($P = 0.006$).

Discussion

Our study was designed to document changes in urinary E_2 levels in male and female big brown bats (*Eptesicus fuscus*) across the annual reproductive cycle. Urinary E_2 often reflects systemic dynamics of this hormone's actions, is believed to be a vector for pheromonal steroid transfer among individuals, and can be collected noninvasively from laboratory animals. Our results showed age, sex, and seasonal differences in urinary E_2 measurements from a captive research colony of *E. fuscus*. We also documented urinary P_4 levels in a subset of nonpregnant females and quantified variation in urinary steroids during the periparturition period in reproductive females.

Although previously validated (Greville et al. 2017), the current data are the first to describe E_2 measurements in the urine of female *E. fuscus*. Female urine showed seasonal variation in E_2 levels (fig. 2). The highest levels of urinary E_2 were observed in the spring for both creatinine-adjusted and unadjusted measurements, which were slightly higher than in the summer. The lowest levels of unadjusted urinary E_2 were observed in the winter, and intermediate levels were seen in autumn; levels of creatinine-adjusted E_2 were roughly equal in the autumn and winter. This pattern is not unexpected given the reproductive cycle of *E. fuscus*. Ovulation occurs in the spring, and although hormonally cued by a surge in luteinizing hormone, it is preceded and signaled by a rise in E_2 (Adachi et al. 2007). Other bat studies have observed elevated plasma E_2 levels during midgestation (e.g., Oxberry 1979; Damassa and Gustafson 1984), which would be during the spring for *E. fuscus*. Parturition in wild *E. fuscus* occurs in late spring or early summer, with urinary E_2 levels rising before and possibly following birth (fig. 3). Surprisingly, female urinary E_2 levels drop in the autumn, which is the time when male *E. fuscus* rejoin maternity colonies and exhibit mating behavior (Kurta and Baker 1990; fig. 2). Average urinary E_2 measures during the autumn were either slightly higher (unadjusted) or equal to (creatinine-adjusted) winter urinary E_2 levels (fig. 2). These findings align with previous studies showing that ovariectomy and the subsequent decrease in plasma E_2 do not influence the mating behavior of female *E. fuscus* (Mendonça et al. 1996).

Estrogen plasma levels for female *E. fuscus* reached as high as 250 pg/mL during October and decreased to 100 pg/mL in

Table 2: Geometric mean ± SE progesterone (P_4) levels for nonpregnant female bats across seasons

			Unadjusted P_4 (ng/mL)		Adjusted P_4 (ng/mg creatinine)	
Season	No. animals	No. samples	Mean	SE	Mean	SE
Autumn	12	21	4.98*	.57	15.6	2.19
Spring	19	37	5.29*	.49	17.4	1.99
Summer	13	15	7.61	.97	20.7	3.27
Winter	14	35	4.47*	.44	16.6	2.05

Note. Data values produced from the optimal linear mixed effects regression models after correction for animal identity and date of urine collection. Both unadjusted and creatinine-adjusted levels are reported. Total number of animals that provided a sample for a given season and number of urine samples in each season are provided.

*Differed statistically from summer at $P < 0.05$.

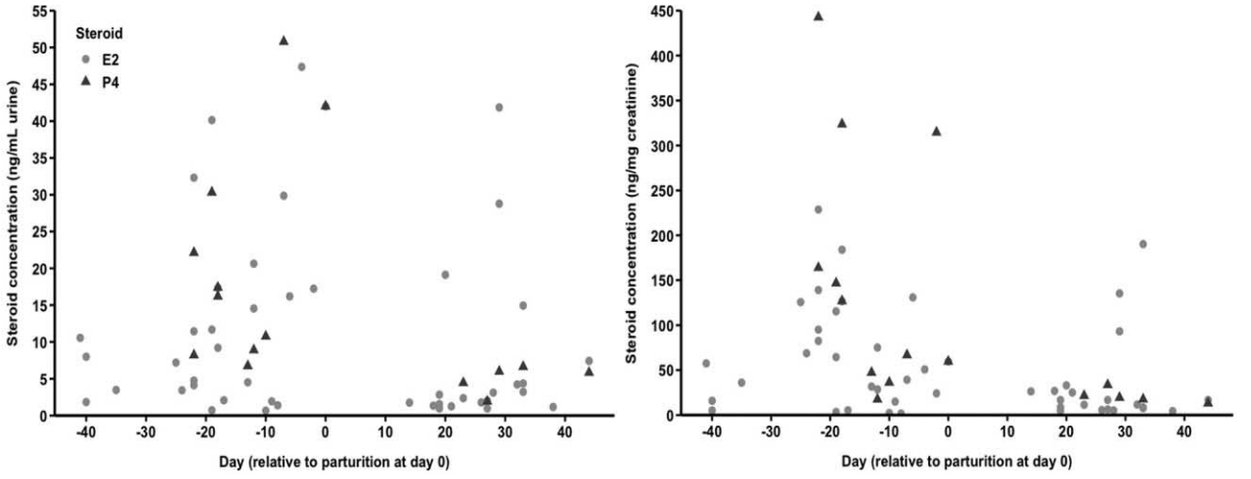

Figure 3. Steroid levels of pregnant bats. Concentrations of estradiol (E_2; circles) and progesterone (P_4; triangles), expressed as nanograms of steroid per milliliter of urine and nanograms of steroid per milligram of creatinine, in urine samples from pregnant mothers during the periparturition period. One unadjusted P_4 level, which measured 224.98 ng/mL 2 d before parturition, is not included.

November and December, when bats enter hibernation (Mendonça et al. 1996). In our study, female unadjusted urinary E_2 averaged ~2.5–4 ng/mL across all seasons, with the lowest levels in winter (fig. 2). Mean creatinine-adjusted urinary E_2 was equal in winter and autumn in our study but plasma E_2 showed a drop before the winter season in the Mendonça et al. (1996) study. The discrepancy may be due in part to interindividual variation in hydration levels and sample size differences. Our urinary E_2 levels were much higher than those reported from *E. fuscus* plasma (Mendonça et al. 1996). Studies from larger mammals, including common marmosets (*Callithrix jacchus*; Hodges et al. 1983), humans (*Homo sapiens*; Seki 1985), horses (*Equus caballus*; Daels et al. 1991), African elephants (*Loxodonta africana*; Wasser et al. 1996), and northern white rhinoceros (*Ceratotherium simum*; Hindle et al. 1992) all show that urinary steroid levels can reach more than two or three orders of magnitude higher than blood plasma, which often has low or undetectable levels (Buchanan and Younglai 1988; Daels et al. 1991; Wasser et al. 1996). Given that urinary E_2 reflects circulatory dynamics in lab mice (Thorpe et al. 2014), we believe that the elevation in urinary E_2 that we observed in comparison to plasma levels in past studies on bats is standard in the mammalian endocrine literature.

Our data contribute to the small literature evaluating female E_2 levels across the reproductive cycle in bats of the Vespertilionidae family. While past studies were unable to detect circulating plasma E_2 in the little brown bat (*Myotis lucifugus*; e.g., Buchanan and Younglai 1988), Damassa and Gustafson (1984) reported E_2 concentrations of 80 pg/mL in pooled plasma samples from *M. lucifugus* during late pregnancy. In the pallid bat (*Antrozous pallidus*), plasma estrogen rose to ~55 pg/mL at the onset of estrus in late August before dropping to a baseline of ~20 pg/mL throughout hibernation. Small fluctuations were also found in the spring before plasma estrogen levels rose during pregnancy and reached a peak of ~90 pg/mL at midgestation, then fell rapidly following parturition (Oxberry 1979). In the greater Asiatic yellow bat (*Scotophilus heathii*), baseline levels of plasma E_2 were 50 pg/mL during periods of reproductive quiescence and ovulatory delay, with a peak of ~300 pg/mL during estrus and declining to ~200 pg/mL during the preovulatory period (Abhilasha and Krishna 1996). Collectively, our urinary E_2 measurements collected in captive animals show seasonal patterns similar to plasma concentrations previously described for wild Vespertilionid bats with elevated E_2 levels in the summer before estrus followed by a drop during the autumn mating season and into the winter (fig. 2). Arousal from hibernation in the spring was associated with a substantial rise in urinary E_2 that continues into the summer in nonreproductive females. In our study, the reproductive stage of female *E. fuscus* was not closely monitored during urine collection. Further studies examining changes in vaginal cytology across the estrous cycle are needed in order to use urinary E_2 as a firm predictor of reproductive state (e.g., Stukenholtz et al. 2018). However, post hoc review allowed us to label urine collected from females that later gave birth. Parous females have a slight rise in urinary E_2 starting ~40 d prepartum and peaking ~20–25 d before birth (fig. 3). To minimize handling stress we chose not to collect urine in periparous

Table 3: Geometric mean ± SE creatinine (mg/mL) measurements in female and male bats across seasons

Sex, season	Mean	SE
Female:		
Spring	.282	.021
Summer	.306	.026
Autumn	.369	.029
Winter	.292	.024
Male:		
Spring	.262	.017
Summer	.363	.036
Autumn	.273	.020
Winter	.237	.017

individuals immediately leading up to and following parturition, but urinary E_2 levels had returned to baseline by ~14–20 d postpartum. The fluctuating patterns we observed are not limited to Vespertilionid bats but also occur in other species of the suborder Yangochiroptera (or Vespertilioniformes), showing similar plasma E_2 peaks during pregnancy followed by a decline during lactation (Burns and Wallace 1975; Crichton and Krutzsch 1987; Crichton et al. 1989).

We also quantified urinary P_4 levels in a subset of females to establish baseline measurements in *E. fuscus* (table 2). Similar to E_2, urinary P_4 concentrations were higher than previously reported plasma levels, although only moderately (Mendonça et al. 1996). In nonreproductive females, unadjusted urinary P_4 levels remained low and did not differ in the winter, spring, and autumn (4.5–5.3 ng/mL), whereas summer P_4 levels (7.6 ng/mL) differed significantly from the other seasons (table 1). A similar pattern was observed in creatinine-adjusted urinary P_4 measurements, albeit without statistical differences. In contrast to E_2, P_4 urinary levels are similar to plasma levels of nonreproductive females in other Vespertilionid bats (Oxberry 1979; Buchanan and Younglai 1988; Abhilasha and Krishna 1996; Hosken et al. 1996). A lower ratio of urinary to plasma P_4 in comparison to the E_2 ratio may be due to a number of factors. Plasma E_2 levels are often lower than P_4 levels (e.g., Oxberry 1979; Mendonça et al. 1996), which may be due to the high receptor affinity and potency of E_2 compared with P_4. Lower rates of P_4 excretion in mammalian urine versus feces (e.g., Ziegler et al. 1989; Wasser et al. 1996) and high antibody cross-reactivities with P_4 metabolites in our study may also impact these ratios, which all require further experimental evaluation.

In pregnant female *E. fuscus*, unadjusted urinary P_4 levels were at a minimum (~8 ng/mL) 20 d before giving birth, whereas individual measurements reached ~30, 40, and 50 ng/mL in the days before parturition (fig. 3). Moreover, adjusted urinary P_4 levels were much higher when compared with nonpregnant females, with many samples in the 50–150 ng/mg creatinine range and some measurements as high as ~300 and 443 ng/mg creatinine. Notably, both adjusted and unadjusted P_4 levels were in the range of nonpregnant females in all samples following birth. Such urinary levels are also reflective of plasma P_4 concentrations previously observed for pregnant *M. lucifigus* (Buchanan and Younglai 1986; Currie et al. 1988). We note that peripheral P_4 levels are known to increase in response to stress (Vermeulen 1976). In female mice, urinary P_4 rises sharply within 1 h of a stress exposure, whereas E_2 levels did not change unless mice were exposed to a major stressor (Thorpe et al. 2014). Our daily urine collection times and methods were consistent throughout the experiment, but the exact timing of individual sample collections relative to first entering the colony were not recorded; however, because bats in the colony experience daily human interactions (e.g., handling and care), we do not believe this was a stressor responsible for the observed changes in P_4.

Surprisingly, there were no differences in unadjusted urinary E_2 levels between the sexes (fig. 1*A*). This suggests that males and females secrete E_2 in equal volumetric concentrations. Because males typically had lower creatinine levels than females (table 3), this resulted in higher creatinine-adjusted urinary E_2 levels compared with nonreproductive adult females (fig. 1*C*). This finding is in opposition to the typical convention that females have higher E_2 than males. Androgen levels in temperate male bats begin to rise in early spring following hibernation and peak in the late summer or early autumn before mating and the culmination of spermatogenesis (Racey 1974; Gustafson 1979). Biochemically, testosterone aromatizes to produce E_2 (Hanukoglu 1992; Payne and Hales 2004); hence, male urinary E_2 peaks in temperate bats during the late summer may be a by-product of increased testosterone during spermatogenesis (Gustafson and Shemesh 1976; Gustafson 1979). While the unadjusted urinary E_2 follows this pattern of androgen production in males, creatinine-adjusted levels are highest in the winter (fig. 2). It is also well documented that male mice display fluctuating steroid and creatinine levels when paired with females (deCatanzaro et al. 2009). In the wild, male *E. fuscus* often roost individually or in small bachelor colonies from late spring to early autumn (Kurta and Baker 1990), whereas in captivity males must share the enclosure with females year-round but often roost in separate locations. This differs from a typical summer maternity colony observed in the wild, where the colony consists of 80%–90% females (Davis et al. 1968). In a mixed-sex captive colony, males may experience a sustained increase in steroid levels that otherwise would not be observed naturally. Further studies should investigate these hypotheses.

Creatinine-adjusted urinary E_2 was significantly higher in adult *E. fuscus* compared with yearlings (fig. 1*D*). This result may provide important insight into the reproductive physiology of temperate insectivorous bats. Multiple studies have shown that wild female *E. fuscus* are less likely to be parous in the first year of life (Sidner 1997; O'Shea et al. 2010; Barclay 2012), and a similar pattern has been observed from known-aged individuals in our captive colony. Both wild and captive *E. fuscus* display promiscuous mating patterns (Mendonça et al. 1996; Vonhof et al. 2006). Maternal lineages within the colony are easily documented from births, but assigning paternity requires genetic testing for each individual. We found that urinary steroid levels across males and females differed with age, with adults having higher levels of creatinine-adjusted E_2 than yearlings (fig. 1*D*). Since only a subset of male and female yearlings acquire a fully developed reproductive system in their first year of life (Christian 1956; Barclay 2012), it is possible that differences in a developing endocrine system may impact whether individuals reach sexual maturity in their first breeding season; however, the current study did not further explore this hypothesis. While the exact dynamics of male urinary E_2 remain to be understood, our current results suggest that yearling and adult *E. fuscus* differ in their steroid production and/or excretion.

Our lab recently reported the first urinary E_2 levels in male bats demonstrating that creatinine-adjusted urinary E_2 levels in adult males were higher in the spring and autumn compared with the summer (Greville et al. 2020). These results explained additional findings that the transfer of radiolabeled E_2 from male to female *E. fuscus* increased in spring and autumn compared with summer (Greville et al. 2020). The present study used a much larger sample size and failed to replicate the seasonal differences in male urinary E_2 levels found by Greville et al. (2020). In the current study, the only

seasonal difference observed in males was between winter and autumn (fig. 2). Interestingly, in both the current data and Greville et al. (2020) the mean unadjusted urinary E_2 is highest in adult males during the summer, but this trend is reversed when corrected for creatinine. Without work correlating urinary and plasma steroids, it cannot be said whether unadjusted or creatinine-adjusted urinary E_2 best follows plasma steroid levels in time. Results of such correlations may align with or contradict past work showing that male temperate bats will actively mate during periods of arousal from hibernation and in the early spring even though their androgen levels remain low (Racey 1974; Racey and Tam 1974).

Using the same assay as the current study, urinary E_2 has been found at similar concentrations in male big brown bats (Greville et al. 2020) and mice (e.g., deCatanzaro et al. 2003, 2004, 2006, 2009; Thorpe et al. 2014). Unconjugated E_2 has also been measured in the urine of numerous mammals using both ELISA and radioimmunoassay techniques. Briefly, these species include wombats (Swinbourne et al. 2017), Asian elephants (*Elephas maximus*; Ramsay et al. 1981), marmosets (Hodges et al. 1983), koalas (*Phascolarctos cinereus*; Takahashi et al. 2009), naked mole rats (*Heterocephalus glaber*; Westlin et al. 1994), horses (Daels et al. 1991), and humans (Kjeld et al. 1977). Despite its hydrophobic chemical nature, unconjugated E_2 is reliably measured in the urine of mammals, although concentrations differ among species. The cross-reactivity with E_2 conjugates, including E_2-glucuronide and E_2-sulfate, has not been reported for our E_2 antibody (R4972). Using the same E_2 antibody, Nunes et al. (2000) suggested that the majority of urinary E_2 in male black-tufted-ear marmosets (*Callithrix kuhlii*) is conjugated given that a 2.2-fold increase in measured E_2 occurred following experimental hydrolysis. Similarly, Swinbourne et al. (2017) used the same E_2 antibodies and reported a 1.14-fold increase in creatinine-corrected urinary E_2 following enzyme hydrolysis compared with nonhydrolyzed urine in female hairy-nosed wombats (*Lasiorhinus latifrons*). Ultimately, future studies must use more advanced techniques, such as chromatography and mass spectrometry, to properly evaluate the ratio of unconjugated to conjugated steroids in the urine of bats.

We have shown seasonal and sex differences in creatinine-adjusted urinary steroid levels. Creatinine adjustments are commonly used to account for variations in animal fluid intake and activity based on the assumption of constant creatinine excretion (Erb et al. 1967; Munro et al. 1991; Boeniger et al. 1993; Muir et al. 2001). However, several researchers have suggested that this correction may be misleading (Boeniger et al. 1993; Hakim et al. 1994; Hall Moran et al. 2001; Miro et al. 2004; deCatanzaro et al. 2009). In our study, creatinine did not differ between sexes but was higher in yearlings than adults. Creatinine was also low during the winter when bats are largely inactive and have a reduced metabolism compared with the summer when they are most active. Our data clearly demonstrate creatinine to be dynamic in bats, varying with age and seasonal environment in both sexes. Accordingly, we believe it is important to present and evaluate changes in both creatinine-adjusted and unadjusted urinary steroid levels and encourage future studies to do the same.

The reliable and dynamic presence of urinary steroids in male bats suggests that steroids may act as reproductive pheromones in the species. A modern, inclusive definition of "pheromones"—and one that does not presume specific mechanisms of action—defines them simply as substances excreted by an individual that can alter the physiology and/or behavior of conspecifics (deCatanzaro 2015). The urine of male and female mice (*Mus musculus*) reliably contains sex steroids (deCatanzaro et al. 2004, 2006, 2009; Guzzo et al. 2012, 2013). Male urinary steroid levels are of particular interest because of their potential role in altering the sexual development and behavior of females. For example, the introduction of a novel male mouse into a female's enclosure can induce changes to her estrous cycle (Whitten 1958), cause precocious puberty (Vandenbergh 1967), and/or disrupt ova implantation in inseminated females (Bruce 1960). These same effects are also observed upon administration of low doses of exogenous E_2 directly to inseminated female mice (Bronson 1975; deCatanzaro et al. 2001, 2006; deCatanzaro 2015). When male mice were each housed across a wire-mesh grid from a female, there was a progressive increase in male urinary E_2 levels over time, and males progressively directed urine at females (deCatanzaro et al. 2009). Through radioisotope tracking it has been shown that male urinary E_2 transfers to female mice at physiologically relevant levels (Guzzo et al. 2010, 2012, 2013). When females were injected with unlabeled E_2 before being exposed to a conspecific treated with radiolabeled E_2, the subsequent radioactivity measured in the female tissues was lower than controls, which is suggestive of competitive tissue uptake (Guzzo et al. 2013). Radiolabeled E_2 transfer between bats also occurs reliably (deCatanzaro et al. 2014; Greville et al. 2017, 2020). Such hormonal transfer may lead to physiological and behavioral changes in females (deCatanzaro 2015). Together, these studies suggest that urinary steroids may play a critical role in explaining phenomena traditionally labeled as primer pheromonal effects.

Our results of urinary steroid levels in *E. fuscus* can serve as a baseline for future comparisons considering possible differences between captive and wild bat populations. For example, wild female *E. fuscus* give birth in early to mid-June, whereas in captivity pups have been born as early as February but are typically born in mid-April and May. The causes of premature ovulation and birthing relative to wild populations has not been studied in *E. fuscus* but are likely due to higher seasonal ambient temperatures within the colony and the continued presence of food (Racey 1973). We have also described patterns of urinary hormones in pregnant and nonpregnant female bats. Because females can resorb fetuses in early pregnancy (Wimsatt 1945), the possibility remains that our spring samples contain data from females that began gestation but resorbed the fetus and thus were visually identified as nonreproductive. Both past studies and our data suggest that circulating steroids peak in midpregnancy, and the possibility of fetus resorption should not confound our results.

Urinary steroid analysis offers a noninvasive alternative to both classical blood plasma and fecal approaches. Urinary steroid measures have frequently been used to assess large mammals but are now used in smaller mammals, such as mice. Urine analysis has the potential to become the primary method for assessing hormones in bats, but first, we must become more knowledgeable about their urinary dynamics. We have described trends in urinary steroid levels from a captive colony of *E. fuscus*. Future

researchers should compare daily steroid fluctuations in wild and captive bats and investigate the effects of isolation versus housing with conspecifics. Also needed are valid correlations between plasma, urinary, and fecal steroid concentrations. Topics such as the urinary patterns of the sexes, onset of polydipsia or polyuria due to the presence of conspecifics, and influence of steroids on sperm storage are all worthy of further exploration to evaluate how steroids may act as sex pheromones in bats. Addressing these research questions, as well as establishing baseline urinary steroid levels for a wider range of bat species, will serve as a critical starting point for this exciting field of research.

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Literature Cited

Abhilasha and A. Krishna. 1996. High androgen production by ovarian thecal interstitial cells: a mechanism for delayed ovulation in a tropical Vespertilionid bat, *Scotophilus heathi*. J Reprod Fertil 106:207–211.

Adachi S., S. Yamada, Y. Takatsu, M. Hisanori, M. Kinoshita, K. Takase, H. Sugiura, et al. 2007. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 53:367–378.

Anderson D.C. 1974. Sex-hormone-binding globulin. Clin Endocrinol 3:69–96.

Barclay R.M.R. 2012. Variable variation: annual and seasonal changes in offspring sex ratio in a bat. PLoS ONE 7:e36344. https://doi.org/10.1371/journal.pone.0036344.

Barton M., E.J. Filardo, S.J. Lolait, P. Thomas, M. Maggiolini, and E.R. Prossnitz. 2018. Twenty years of the G protein-coupled estrogen receptor GPER: historical and personal perspectives. J Steroid Biochem Mol Bio 176:4–15.

Bates D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J Stat Soft 67:1–48.

Benjamini Y. and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 57:289–300.

Boeniger M.F., L.K. Lowry, and J. Rosenberg. 1993. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. Am Ind Hyg Assoc J 54: 615–627.

Brigham R.M. 1991. Flexibility in foraging and roosting behaviour by the big brown bat (*Eptesicus fuscus*). Can J Zool 69:117–121.

Bronson F.H. 1975. Male-induced precocial puberty in female mice: confirmation of the role of estrogen. Endocrinology 96: 511–514.

Bruce H.M. 1960. A block to pregnancy in the mouse caused by proximity of strange males. J Reprod Fertil 1:96–103.

Buchanan G.D. and E.V. Younglai. 1986. Plasma progesterone levels during pregnancy in the little brown bat *Myotis lucifigus* (Vespertilionidae). Biol Reprod 34:878–884.

———. 1988. Plasma progesterone concentrations in female little brown bats (*Myotis lucifugus*) during hibernation. J Reprod Fertil 83:59–65.

Burns J.M. and E. Wallace. 1975. Hormonal control of delayed development in *Macrotus waterhousii*. II. Radioimmunoassay of plasma estrone and estradiol 17-β during pregnancy. Gen Comp Endocrinol 25:529–533.

Christian J.J. 1956. The natural history of a summer aggregation of the big brown bat, *Eptesicus fuscus fuscus*. Am Midl Nat 55:66–95.

Crichton E.G. and P.H. Krutzsch. 1987. Reproductive biology of the female little mastiff bat, *Mormopterus planiceps* (Chiroptera: Molossidae) in southeast Australia. Am J Anat 178:369–386.

Crichton E.G., R.F. Seamark, and P.H. Krutzsch. 1989. The status of the corpus luteum during pregnancy in *Miniopterus schreibersii* (Chiroptera: Vespertilionidae) with emphasis on its role in developmental delay. Cell Tissue Res 258:183–201.

Currie W., M. Blake, and W. Wimsatt. 1988. Fetal development, and placental and maternal plasma concentrations of progesterone in the little brown bat (*Myotis lucifugus*). J Reprod Fertil 82:401–407.

Daels P.F., D.C. Ammon, G.H. Stabenfeldt, I.K.M. Liue, J.P. Hughes, and B.L. Lasley. 1991. Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in nonpregnant and early pregnant mares. Theriogenology 35:1001–1017.

Damassa D.A. and A.W. Gustafson. 1984. Control of plasma sex steroid-binding protein (SBP) in the little brown bat: effects of photoperiod and orchiectomy on the induction of SBP in immature males. Endocrinology 115:2355–2361.

Davis W.H., R.W. Barbour, and M.D. Hassell. 1968. Colonial behavior of *Eptesicus fuscus*. J Mammal 49:44–50.

deCatanzaro D. 2015. Sex steroids as pheromones in mammals: the exceptional role of estradiol. Horm Behav 68:103–116.

deCatanzaro D., M.A.S. Baptista, and E. Spironello-Vella. 2001. Administration of minute quantities of 17β-estradiol on the nasal area terminates early pregnancy in inseminated female mice. Pharmacol Biochem Behav 69:503–509.

deCatanzaro D., E.A. Beaton, A. Khan, and E. Spironello-Vella. 2006. Urinary oestradiol and testosterone levels from novel male mice approach values sufficient to disrupt early pregnancy in nearby inseminated females. Reproduction 132:309–317.

deCatanzaro D., A. Khan, R.G. Berger, and E. Lewis. 2009. Exposure to developing females induces polyuria, polydipsia,

and altered urinary levels of creatinine, 17β-estradiol, and testosterone in adult male mice (*Mus musculus*). Horm Behav 55:240–247.

deCatanzaro D., C. Muir, E.A. Beaton, and M. Jetha. 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69:687–696.

deCatanzaro D., C. Muir, E. Beaton, M. Jetha, and K. Nadella. 2003. Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 126:407–414.

deCatanzaro D., T. Pollock, L.J. Greville, and P.A. Faure. 2014. Estradiol transfer from male big brown bats (*Eptesicus fuscus*) to the reproductive and brain tissues of cohabiting females, and its action as a pheromone. Gen Comp Endocrinol 208:126–133.

Erb R., S. Tillson, G. Hodgen, and E. Plotka. 1967. Urinary creatinine as an index compound for estimating rate of excretion of steroids in the domestic sow. J Anim Sci 30:79–85.

Filardo E.J. and P. Thomas. 2005. GPR30: a seven-transmembrance-spanning estrogen receptor that triggers EGF release. Trends Endocrinol Metab 16:362–367.

Freeman M.E. 2006. Neuroendocrine control of the ovarian cycle of the rat. Pp. 2327–2387 in J.D. Neill, ed. Knobil and Neill's physiology of reproduction. Academic Press, San Diego, CA.

Greville L.J., T. Pollock, P.A. Faure, and D. deCatanzaro. 2020. Seasonal transfer and quantification of urinary estradiol in the big brown bat (*Eptesicus fuscus*). Gen Comp Endocrinol 286:113321.

Greville L.J., T. Pollock, J.C. Salter, P.A. Faure, and D. deCatanzaro. 2017. Progesterone transfer among cohabitating female big brown bats (*Eptesicus fuscus*). Gen Comp Endocrinol 247:199–204.

Gustafson A.W. 1979. Male reproductive patterns in hibernating bats. Reproduction 56:317–331.

Gustafson A.W. and M. Shemesh. 1976. Changes in plasma testosterone levels during the annual reproductive cycle of the hibernating bat, *Myotis lucifugus lucifigus* with a survey of plasma testosterone levels in adult male vertebrates. Biol Reprod 15:9–24.

Guzzo A.C., R.G. Berger, and D. deCatanzaro. 2010. Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): implications for the Bruce effect. Reproduction 139:255–263.

Guzzo A.C., J. Jheon, F. Imtiaz, and D. deCatanzaro. 2012. Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). Reproduction 143:539–548.

Guzzo A.C., T. Pollock, and D. deCatanzaro. 2013. Transfer of [^{3}H]estradiol-17β and [^{3}H]progesterone from conspecifics to cohabiting female mice. J Endocrinol 217:1–10.

Hakim R.B., R.H. Grayb, and H.A. Zacurc. 1994. Is there a need for creatinine adjustment of urinary steroid hormone levels in studies of early fetal loss? Clin Chim Acta 230:209–214.

Hall Moran V., H.L. Leathard, and J. Coley. 2001. Urinary hormone levels during the natural menstrual cycle: the effect of age. J Endocrinol 170:157–164.

Hanukoglu I. 1992. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J Steroid Biochem Mol Biol 43:779–804.

Hindle J., E. Möstl, and J. Hodges. 1992. Measurement of urinary oestrogens and 20α-hydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. J Reprod Fertil 94:237–249.

Hitchcock H.B., R. Keen, and A. Kurta. 1984. Survival rates of *Myotis leibii* and *Eptesicus fuscus* in southeastern Ontario. J Mammal 65:126–130.

Hodges J.K., H. Brand, C. Henderson, and R.W. Kelly. 1983. Levels of circulating and urinary oestrogens during pregnancy in the marmoset monkey (*Callithrix jacchus*). J Reprod Fertil 67:73–82.

Hosken D.J., J.E. O'Shea, and M.A. Blackberry. 1996. Blood plasma concentrations of progesterone, sperm storage and sperm viability and fertility in Gould's wattled bat (*Chalinolobus gouldi*). J Reprod Fertil 108:171–177.

Kjeld J.M., C.M. Puah, and G.F. Joplin. 1977. Measurement of unconjugated testosterone, 5α-dihydrotestosterone, and oestradiol in human urine. Clin Chim Acta 80:271–284.

Kuiper G.G.J.M., B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, and J-Å Gustafsson. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138:863–870.

Kurta A. and R.H. Baker. 1990. *Eptesicus fuscus*. Mamm Species 356:1–10.

Kurta A. and J.O. Matson. 1980. Disproportionate sex ratio in the big brown bat (*Eptesicus fuscus*). Am Midl Nat 104:367–369.

Kuznetsova A., P.B. Brockhoff, and R.H.B. Christensen. 2017. lmerTest package: tests in linear mixed effects models. J Stat Softw 82:1–26.

Lenth R. 2020. emmeans: estimated marginal means, aka least-squares means. R package version 1.4.7. https://CRAN.R-project.org/package=emmeans.

Luke S.G. 2017. Evaluating significance in linear mixed-effect models in R. Behav Res 49:1494–1502.

Martin L. and R.T.F. Bernard. 2000. Endocrine regulation of reproduction in bats: the role of circulating gonadal hormones. Pp. 27–64 in E.G. Crichton and P.H. Krutzsch, eds. Reproductive biology of bats. Academic Press, London.

Mendonça M., S. Chernetsky, K. Nester, and G. Gardner. 1996. Effects of gonadal sex steroids on sexual behavior in the big brown bat, *Eptesicus fuscus*, upon arousal from hibernation. Horm Behav 30:153–161.

Mendonça M.T. and W.A. Hopkins. 1997. Effects of arousal from hibernation and plasma androgen levels on mating behavior in the male big brown bat, *Eptesicus fuscus*. Physiol Zool 70:556–562.

Miro F., J. Coley, M.M. Gani, P.W. Perry, D. Talbot, and L.J. Aspinall. 2004. Comparison between creatinine and pregnanediol adjustments in the retrospective analysis of urinary hormone profiles during the human menstrual cycle. Clin Chim Acta 42:1043–1050.

Muir C., E. Spironello-Vella, N. Pisani, and D. deCatanzaro. 2001. Enzyme immunoassay of 17 β-estradiol, estrone conjugates,

and testosterone in urinary and fecal samples from male and female mice. Horm Metab Res 33:653–658.

Munro C.J., G.H. Stabenfeldt, J.R. Cragun, L.A. Addiego, J.W. Overstreet, and B.L. Lasley. 1991. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. Clin Chem 37:838–844.

Murphy W.J., P.A. Pevzner, and S.J. O'Brien. 2004. Mammalian phylogenomics comes of age. Trends Genet 20:631–639.

Nelson R.J. and L.J. Kriegsfeld. 2017. An introduction to behavioural endocrinology. 5th ed. Sinauer, Sunderland, MA.

Nunes S., J.E. Fite, and J.A. French. 2000. Variation in steroid hormones associated with infant care behaviour and experience in male marmosets (*Callithrix kuhlii*). Anim Behav 60:857–865.

O'Shea T.J., L.E. Ellison, D.J. Neubaum, M.A. Neubaum, C.A. Reynolds, and R.A. Bowen. 2010. Recruitment in a Colorado population of big brown bats: breeding probabilities, litter size, and first-year survival. J Mammal 91:418–428.

Oxberry B.A. 1979. Female reproductive patterns in hibernating bats. J Reprod Fertil 56:359–367.

Paradiso J.L. and A.M. Greenhall. 1967. Longevity records for American bats. Am Midl Nat 78:251–252.

Payne A.H. and D.B. Hales. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25:947–970.

Pollock T., L.J. Greville, B. Tang, and D. deCatanzaro. 2016. Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice. Reprod Toxicol 65:394–401.

Pollock T., L.J. Greville, R.E. Weaver, M. Radenovic, and D. deCatanzaro. 2019. Bisphenol S modulates concentrations of bisphenol A and oestradiol in female and male mice. Xenobiotica 49:540–548.

Racey P.A. 1973. Environmental factors affecting the length of gestation in heterothermic bats. J Reprod Fertil 19(suppl.): 175–189.

———. 1974. The reproductive cycle in male noctule bats, *Nyctalus noctula*. J Reprod Fertil 41:169–182.

———. 1979. The prolonged storage and survival of spermatozoa in Chiroptera. J Reprod Fertil 56:391–402.

Racey P.A. and H.W. Tam. 1974. Reproduction in male *Pipistrellus pipistrellus* (Mammalia: Chiroptera). J Zool 172:101–122.

Ramsay E.C., B.L. Lasley, and G.H. Stabenfeldt. 1981. Monitoring the estrous cycle of the Asian elephant (*Elephas maximus*), using urinary estrogens. Am J Vet Res 42:256–260.

Rasweiler J.J., IV, C.J. Cretekos, and R.R. Behringer. 2009. The short-tailed fruit bat *Carollia perspicillata*: a model for studies in reproduction and development. Pp. 519–555 in Emerging model organisms: a laboratory manual. Cold Spring Harbor, New York.

R Development Core Team. 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.r-project.org/.

Rodrigues A.F., C.S. Santiago, and S.R. Taboga. 2019. Morphological variation of the female reproductive organs of the bat *Artibeus lituratus* during its different reproductive phases. J Morphol 280:1141–1155.

Santiago C.S., E.S.S. Albernaz, R.T.S. Santos, L.H.A. Guerra, F.C.A. Santos, R.M. Góes, and E.M. Sebasti. 2020. Evaluation of the uterine hormonal control of the bat *Artibeus lituratus* during the different phases of its reproductive cycle. J Morphol 281:302–315.

Schowalter D.B. and J.R. Gunson. 1979. Reproductive biology of the big brown bat (*Eptesicus fuscus*) in Alberta. Can F Nat 93:48–54.

Seki K. 1985. Correlation between urinary oestrogen levels determined by haemagglutination inhibition reaction and serum oestradiol levels determined by radioimmunoassay. Acta Endocrinol 110:130–134.

Sidner R.M. 1997. Studies of bats in southeastern Arizona with emphasis on aspects of life history of *Antrozous pallidus* and *Eptesicus fuscus*. PhD diss. University of Arizona, Tuscon.

Simmons N.B., K.L. Seymour, J. Habersetzer, and G.F. Gunnell. 2008. Primitive early Eocene bat from Wyoming and the evolution of flight and echolocation. Nature 451:818–821.

Skrinyer A.J., P.A. Faure, S. Dannemiller, H.C. Ball, K.H. Delaney, R. Orman, M. Stewart, and L.N. Cooper. 2017. Care and husbandry of the world's only flying mammals. Lab Anim Sci 5:24–27.

Springer M.S., M.J. Stanhope, O. Madsen, and W.W. de Jong. 2004. Molecules consolidate the placental tree. Trends Ecol Evol 19:430–438.

Stukenholtz E.E., J. Perez-Torres, and R.D. Stevens. 2018. Variation of vaginal cyology, progesterone and estradiol metabolites in Seba's short-tailed fruit bat during the estrous cycle and gestation. Mastozool Neotrop 25:151–162.

Swinbourne A.M., S.D. Johnston, T.M. Janssen, A. Lisle, and T. Keeley. 2017. Validation of techniques to measure reproductive hormones in the urine of female southern hairy-nosed wombats (*Lasiorhinus latifrons*). Gen Comp Endocrinol 252:130–141.

Takahashi M., J.R. Tobey, C.B. Pisacane, and C.H. Andrus. 2009. Evaluating the utility of an accelerometer and urinary hormone analysis as indicators of estrus in a zoo-housed koala (*Phascolarctos cinereus*). Zoo Biol 28:59–68.

Thorpe J.B., K.E. Gould, E.D. Borman, and D. deCatanzaro. 2014. Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*). Horm Metab Res 46:211–218.

Vandenbergh J.G. 1967. Effect of the presence of a male on the sexual maturation of female mice. Endocrinology 81:345–349.

Vermeulen A. 1976. Postmenopausal ovarian function. Pp. 237–244 in V.H.T. James, M. Serio, and G. Guisti, eds. The endocrine function of the human ovary. Academic Press, London.

Veselka N., D.D. McErlain, D.W. Holdsworth, J.L. Eger, R.K. Chhem, M.J. Mason, K.L. Brain, P.A. Faure, and M.B. Fenton. 2010. A bony connection signals laryngealecholocation in bats. Nature 463:939–942.

Vonhof M.J., D. Barber, M.B. Fenton, and C. Strobecks. 2006. A tale of two siblings: multiple paternity in big brown bats (*Eptesicus fuscus*) demonstrated using microsatellite markers. Mol Ecol 15:241–247.

Walters M.R. 1985. Steroid hormone receptors and the nucleus. Endocr Rev 6:512–543.

Wasser S.K., S. Papageorge, C. Foley, and J.L. Brown. 1996. Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. Gen Comp Endocrinol 102:255–262.

Westlin L.M., N.C. Bennett, and J.U.M. Jarvis. 1994. Relaxation of reproductive suppression in non-breeding female naked-mole rats, *Heterocephalus glaber*. J Zool Lond 234:177–188.

Whitten B.W.K. 1958. Modification of the oestrus cycle of the mouse by external stimuli associated with the male. J Endocrinol 13:307–313.

Wilkinson G.S., D.A. Adams, A. Haghani, A.T. Lu, J. Zoller, C. Breeze, B.D. Arnold, et al. 2021. DNA methylation predicts age and provides insight into exceptional longevity of bats. Nat Commun 12:1–3.

Willis C.K.R. and R.M. Brigham. 2004. Roost switching, roost sharing and social cohesion: forest-dwelling big brown bats, *Eptesicus fuscus*, conform to the fission-fusion model. Anim Behav 68:495–505.

Wimsatt W.A. 1944. Further studies on the survival of spermatozoa in the female reproductive tract of the bat. Anat Rec 88:193–204.

———. 1945. Notes on breeding behavior, pregnancy, and parturition in some Vespertilionid bats of the eastern United States. J Mammal 26:23–33.

Zhou X., S. Xu, J. Xu, B. Chen, K. Zhou, and G. Yang. 2012. Phylogenomic analysis resolves the interordinal relationships and rapid diversification of the Laurasiatherian mammals. Syst Biol 61:150–164.

Zhu B.T. and A.H. Conney. 1998. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 19:1–27.

Ziegler T.E., S.A. Sholl, G. Scheffler, M.A. Haggerty, and B.L. Lasley. 1989. Excretion of estrone, estradiol, and progesterone in the urine and feces of the female cotton-top tamarin (*Saguinus oedipus oedipus*). Am J Primatol 17:185–195.