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Short Communication

Seasonal transfer and quantification of urinary estradiol in the big brown bat (*Eptesicus fuscus*)

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ABSTRACT

Growing evidence shows that sex steroids not only act within the individual whose glands produce them; they can also act on proximate conspecifics. Previous studies show that exogenous 17β-estradiol (E_2) can be absorbed both nasally and percutaneously, arriving in blood, neural, reproductive, and peripheral tissues. When male bats were injected with radiolabeled E_2 (^{3}H-E_2) and housed with females during the mating season, radioactivity was reliably measured in the females' tissues. The present study was designed to compare E_2 transfer from male to female bats at three time points in the annual reproductive cycle: spring (ovulation and fertilization), summer (maternal season), and autumn (mating season). Pairs of mature female bats were housed with a mature ^{3}H-E_2-treated male (50 μCi). Following 48 h of communal housing, radioactivity was measured in the tissues of female bats. Higher levels of radioactivity were present in the uterus and other tissues during the spring and autumn seasons compared to the summer season. We also measured natural levels of bioactive, unconjugated E_2 in the urine of male bats using enzyme immunoassays, and found that it was present in all three seasons but at lower levels during the summer. Male-excreted E_2 could transfer to females within the close confines of a roost, potentially influencing their reproductive physiology and behavior. These results suggest increased E_2 transfer coincides with female reproduction, with urine as a likely vector. We suggest that sex steroid transfer among interacting individuals may explain several mammalian phenomena historically viewed as "pheromonal".

1. Introduction

Male excretions have long been known to affect the sexual development and physiology of females. They can block uterine implantation of fertilized ova (Bruce effect; Bruce, 1960), induce precocious puberty (Vanderbergh effect; Vandenbergh, 1967), and alter the estrous cycle (Whitten effect; Whitten, 1958). Very low doses of exogenous 17β-estradiol (E_2) can mimic these effects (Bronson, 1975; deCatanzaro et al., 2001, 2006; reviewed by deCatanzaro, 2015). Mouse urine reliably contains sex steroids in bioactive, unconjugated forms, with male urine containing sufficient amounts of E_2 to contribute to these effects (deCatanzaro et al., 2006, 2009; Guzzo et al., 2012). Using tritium as a radioactive tracer, the transfer of E_2 from male to female conspecifics has been observed at physiologically relevant levels in mice and bats (deCatanzaro et al., 2014; Guzzo et al., 2012, 2013).

The big brown bat (*Eptesicus fuscus*) is found throughout temperate regions of North and Central America (Kurta and Baker, 1990). This species is highly social and displays promiscuous behavior in laboratory studies (Mendonça et al., 1996), and paternity studies suggest this pattern of mating in the wild (Vonhof et al., 2006). Hibernating bats have a unique mating system in which copulation occurs primarily during the autumn, as well as during brief periods of arousal from hibernation (Oxberry, 1979). Females store sperm throughout hibernation and, pending favorable conditions upon arousal, ovulation and fertilization occur in the spring (Christian, 1956; Oxberry, 1979; Racey, 1979; Wimsatt, 1944). In the wild, parturition typically transpires in early June within maternal colonies (Christian, 1956; Schowalter and Gunson, 1979), when the majority of adult males have left to form bachelor colonies (Kurta and Baker, 1990). Maternal and bachelor colonies remerge during the autumn mating season.

We previously injected male bats during the autumn mating season with low levels of tritium-labelled estradiol (^{3}H-E_2) estimated to represent a small fraction of their endogenous E_2 levels (deCatanzaro et al., 2014). After two days of housing with untreated females, measurable levels of radioactivity were reliably detected in female tissues. The highest levels of radioactivity were in the uterus and ovaries where estrogen receptors (ER) are most abundant (Kuiper et al., 1997). We also previously examined sex steroid transfer among cohabiting female bats; although E_2 transfer did not occur among females, progesterone (P_4) transfer did occur (Greville et al., 2017). This added to much

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evidence that transfer of sex steroids among behaviorally-interacting conspecifics may explain some mammalian phenomena that have historically been characterized as "primer pheromonal effects" (reviewed by deCatanzaro, 2015).

Here we evaluated E_2 transfer from male to female bats at three time points during the annual reproductive cycle: spring (coinciding with ovulation, fertilization, and implantation), summer (coinciding with maternal care), and autumn (coinciding with mating season). A winter time point was not evaluated because bats are typically hibernating and inactive. We hypothesized increased E_2 transfer in the spring and autumn compared to the summer, because of the timing of insemination and ovulation. We focused on the uterus and ovaries due to their high concentrations of ER that may mediate the Bruce and Vandenbergh effects (Guzzo et al., 2012). We also examined the hypothalamus due to high levels of ER in the ventromedial and preoptic areas (Sar and Parikh, 1986; Simerly et al., 1990), its role in stimulating sexual receptivity (Pfaff, 1980), and its potential mediation of some pheromonal effects (deCatanzaro, 2015). We analyzed liver and kidney tissues due to their roles in steroid conjugation and excretion, as well other neural and peripheral tissues.

Estrogen concentrations have previously been quantified in the blood plasma of the North American bats, *Antrozous pallidus* (Oxberry, 1979) and *Eptesicus fuscus* (Mendonça et al., 1996). Bat fecal samples have also been used for estrogen quantification in *Saccopteryx bilineata* (Greiner et al., 2011; Voigt and Schwarzenberger, 2008) and *Carollia perspicillata* (Stukenholtz et al., 2018). Blood analyses are invasive and fecal analyses can be time-consuming. In contrast, work with mice showed that urinary steroid measurements produce temporally dynamic results that generally reflect systemic fluctuations (Thorpe et al., 2014). We have undertaken to apply these urinary methods to bat research. We recently reported the first procedural validations of urinary E_2 and P_4 analysis in female *E. fuscus* (Greville et al., 2017). The current study was designed to validate these procedures for urinary E_2 in male *E. fuscus* and to compare levels in the spring, summer, and autumn.

2. Materials and methods

2.1. Animals and housing

Wild *E. fuscus* from southern Ontario were caught and housed in a husbandry facility at McMaster University where animals were permitted to fly, temperature and lighting varied with ambient conditions, and (unless otherwise stated) had *ad libitum* access to food (mealworms) and water (Skrinyer et al., 2017). Bats selected for experiments were held in stainless steel wire mesh holding cages measuring 28 × 22 × 18 cm. Procedures were approved by the Animal Research Ethics Board of McMaster University, conforming to guidelines of the Canadian Council on Animal Care.

2.2. Chemicals

Solutions of SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, and 2 stocks of [2,4,6,7-[^{3}H](N)]-E_2 were obtained from PerkinElmer, Waltham, MA, USA. Standards for E_2 were obtained from Sigma-Aldrich, Oakville, ON, Canada, and the antibodies for E_2 and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA, USA.

2.3. Experiment 1: Seasonal ^{3}H-E_2 transfer

Transfer of ^{3}H-E_2 from stimulus male bats to conspecific females was evaluated and compared among the spring, summer, and autumn, with experimental procedures closely following those of deCatanzaro et al. (2014) and Greville et al. (2017). On day 1, adult male and female bats were randomly selected and housed in holding cages separated by sex. On day 2, each male was injected i.p. with 50 μCi of ^{3}H-E_2 and isolated for 1 h to prevent accidental transfer from the injection site. Injected males were placed in a holding cage with 2 randomly assigned females, with 3 replicates (n = 6 subject females) occurring at each time point. Following 48 h of cohabitation, animals were anesthetized via isoflurane inhalation and blood samples were collected via cardiac puncture. Anesthetized animals were euthanized by perfusion with 20 ml of phosphate-buffered saline, and tissues were collected and placed in pre-weighed 8 ml scintillation vials. Reproductive tissues included the whole uterus and ovaries in females, and the testes and epididymis in males. Neural tissues included samples from the olfactory bulbs, cerebellum, a section of the frontal cortex, and a section of the hypothalamus taken from the ventral surface of the brain. Peripheral tissues included the heart, lungs, liver, external intercostal muscle, abdominal adipose tissue, and a cross section of kidney encompassing both the cortex and medulla. All vials were re-weighed following sampling and wet tissue mass was recorded.

Tissue preparation and radioactive measurements followed procedures of Greville et al. (2017). Briefly, tissues were solubilized by adding 1 ml of SOLVABLE, 5 ml of Ultima Gold to each vial of solubilized tissue. Radioactivity was quantified in disintegrations per minute (DPM) using a TriCarb 2910 TR Liquid Scintillation Analyzer. All measures were adjusted for wet mass and reported as DPM/mg tissue or DPM/μl serum. Experimental methodology was consistent across seasons. Food and water were sampled from all cages, and dry swipes of the lab bench, dissection tools, and other lab equipment were taken between animal dissections to ensure there was no radioactive contamination.

2.4. Experiment 2: Quantifying E_2 in male urine

Urine was collected non-invasively from captive adult male bats outside of the mating season to standardize and validate the assay. Animals selected from the colony were held over a wax-paper-lined work surface. Urine expressed by each animal was collected separately in 70 μl hematocrit tubes (Fisherbrand, Pittsburgh, PA, USA) and frozen (−20 °C) until time of analysis.

Urine analysis of unconjugated E_2 was completed using modified immunoassay protocols previously established (deCatanzaro et al., 2003, 2004; Greville et al., 2017; Pollock et al., 2016, 2019). Urine samples from adult male bats of varying ages were pooled to develop standard curves and validate the assay. Standard curves were generated using optical densities obtained with serial dilutions of pooled urine. A regression line was fit to the data and samples were interpolated into the equation to obtain a concentration estimate in pg/well. Data were plotted with a serially-diluted standard against a logarithmically-transformed dose. This test indicates whether measurable levels of E_2 were present in the urine of male bats, and whether E_2 molecules react to antibodies in a predictable manner (Kemeny, 1991).

Following validation of enzyme-linked immunosorbent assay procedures for male bat urine, 10 samples of urine from randomly selected male bats in each of the spring, summer, and autumn seasons were analyzed following procedures adapted from Muir et al. (2001). Urinary E_2 measures were considered with and without adjustment for urinary creatinine, which corrects for differential hydration and urinary concentration among animals, and reported as ng E_2/mg creatinine and E_2/ml urine respectively.

2.5. Data analysis

Analyses were performed in the R software environment (R Core Team, 2019). Differences in ^{3}H-E_2 transfer among seasons were analyzed with a Kruskal–Wallis H test performed on each tissue, using false discovery rate adjustments for the number of measures (Benjamini and Hochberg, 1995). Post hoc multiple comparisons of significant tissues were conducted using the Kruskal–Conover test. For Experiment 2,

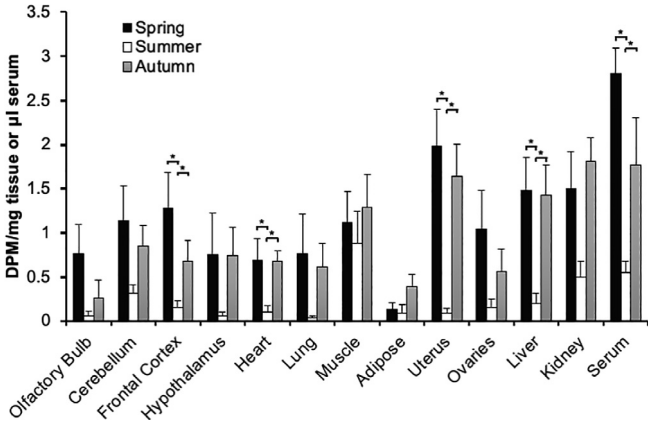

Fig. 1. Radioactivity (mean DPM + SE) measured in solubilized tissues and blood serum of adult female bats after 48 h of cohabitation with a ^{3}H-E_2-treated adult stimulus males during the spring, summer, or autumn season in Experiment 1. n = 6, except n = 5 for summer serum. *$p < 0.05$.

urinary E_2, creatinine, and creatinine-adjusted E_2 levels in the summer were compared to the spring and autumn with an ANOVA corrected for multiple comparisons using false discovery rate adjustments and a family-wise α at $p < 0.05$.

3. Results

3.1. Experiment 1: Seasonal effect of ^{3}H-E_2 transfer

We measured mean radioactivity counts in female bats following 48 h cohabitation with a ^{3}H-E_2 injected male during different seasons (Fig. 1). For the spring serum, n = 5 is reported due to an insufficient volume of one blood draw. There was a significant effect of season in the frontal cortex, $H(2) = 9.0$, $p = 0.032$; heart, $H(2) = 9.3$, $p = 0.032$; uterus, $H(2) = 11.2$, $p = 0.032$; liver, $H(2) = 9.0$, $p = 0.032$; and serum, $H(2) = 8.8$, $p = 0.032$. Multiple comparisons revealed a significant difference between the autumn and summer, as well as between the spring and summer, in each of these substrates. No differences were observed between the spring and autumn seasons in any tissues. There was no significant effect after false discovery rate adjustment in the olfactory bulb, cerebellum, hypothalamus, lung, muscle, adipose, ovaries, and kidney.

The DPM/mg for the three ^{3}H-E_2-injected male bats during each condition is reported (Table 1). Due to experimenter error, n = 2 is reported for the epididymis and kidney in the summer. Collectively,

Table 1
Radioactivity (mean DPM ± SE) measured in solubilized tissues and blood serum of ^{3}H-E_2-treated (50 µCi) adult stimulus male bats following 48 h of cohabitation with 2 adult females during the spring, summer, or autumn season in Experiment 1. n = 3 per season except n = 2 for epididymis and kidney measures collected in the summer.

	Spring	Summer	Autumn
Olfactory bulb	446 ± 167	206 ± 82	481 ± 92
Cerebellum	163 ± 71	214 ± 77	434 ± 75
Frontal cortex	226 ± 32	223 ± 75	478 ± 89
Hypothalamus	211 ± 37	187 ± 49	435 ± 72
Heart	148 ± 43	105 ± 35	296 ± 47
Lung	248 ± 51	165 ± 29	307 ± 6
Muscle	293 ± 55	231 ± 29	523 ± 131
Adipose	120 ± 29	47 ± 13	115 ± 43
Testes	457 ± 105	298 ± 94	594 ± 89
Epididymis	216 ± 80	307 ± 166	189 ± 9
Liver	347 ± 116	135 ± 73	595 ± 132
Kidney	373 ± 125	178 ± 54	793 ± 193
Serum	521 ± 68	467 ± 79	1031 ± 251

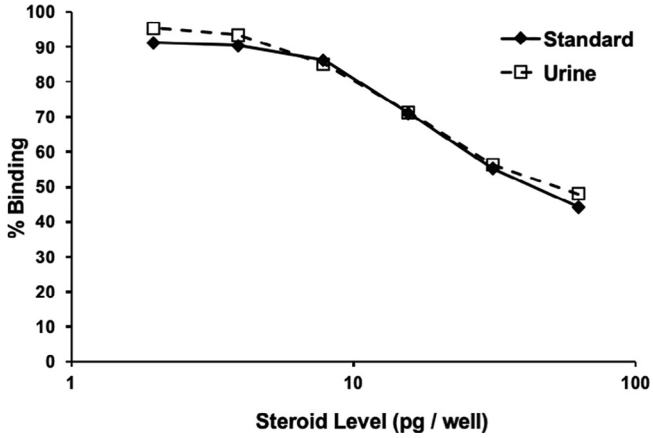

Fig. 2. Serially diluted urine samples from adult male bats bind to antibody in parallel with serially diluted samples from E_2 standards.

these results demonstrate that ^{3}H-E_2 transfers from males to the tissues of female bats more frequently and at higher levels in the spring and autumn than in the summer.

3.2. Experiment 2: Quantifying unconjugated estradiol in male urine

Unconjugated E_2 was reliably present in measurable levels in the urine of male bats. Samples were diluted in parallel with their standard curve, and both the dose–response curve and parallelisms for E_2 are reported for pooled urinary samples (Fig. 2).

Concentrations of urinary E_2 (ng E_2/ml urine), creatinine (mg creatinine/ml urine), and creatinine-adjusted E_2 (ng E_2/mg creatinine) from male bats across three seasons are reported (Fig. 3). There was no effect of season on male urinary E_2, $F(2,27) = 1.85$, $p = 0.180$, but ANOVA showed significant effects of season on creatinine, $F(2,27) = 5.02$, $p = 0.014$, and creatinine-adjusted E_2 levels, $F(2,27) = 4.68$, $p = 0.018$. Pairwise comparisons revealed increased levels of creatinine in the summer compared to the spring ($p = 0.023$), and elevated creatinine-adjusted E_2 levels in the spring ($p = 0.043$) and autumn ($p = 0.034$) compared to the summer.

4. Discussion

This study demonstrates that male big brown bats transfer E_2 to female conspecifics differentially across the annual reproductive cycle. Transfer of ^{3}H-E_2 was greatest during the autumn and spring, which respectively correspond to the mating and ovulation/fertilization periods. Transfer of ^{3}H-E_2 was significantly lower during the summer maternal period. Highest levels of radioactivity were observed in serum, liver, and kidney, suggesting that transferred E_2 moves through the circulatory system and is conjugated and excreted in a normal fashion. Sex steroids can be absorbed both nasally and percutaneously by bats (deCatanzaro et al., 2014; Greville et al., 2017). Radioactivity in the females' lungs supports the claim that steroid absorption from conspecific excretions can also occur via the vasculature of the lungs (Greville et al., 2017).

Male bats roost solo or in bachelor colonies during the summer, but rejoin females in harem roosts in late summer and remain there throughout hibernation before separating again in the spring (Kurta and Baker, 1990). The close confines and female-skewed composition of maternity roosts provide an environment where females may readily encounter and absorb steroids from conspecifics' excretions. Temperate bats are meticulous groomers and spend a significant portion of their daily energy budget combing their wings and fur (Burnett and August, 1981). Such behavior, in conjunction with allogrooming, may enhance steroid absorption within a roost. Female exposure to males promotes

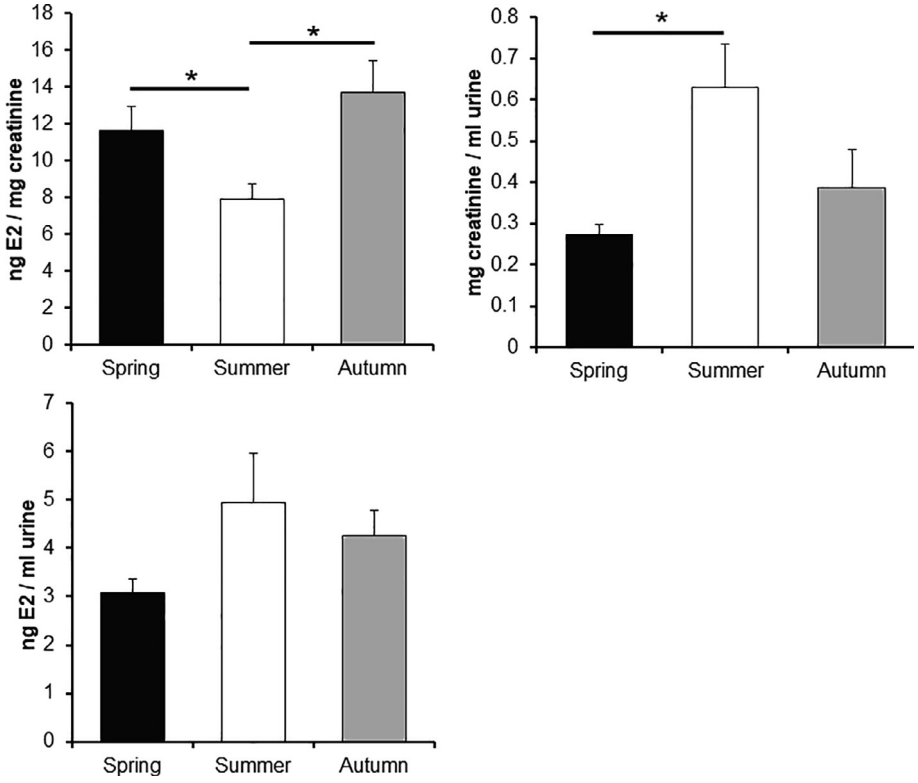

Fig. 3. Urinary concentrations (mean ± SE) of creatinine-adjusted and unadjusted urinary E_2, and urinary creatinine in male bats during the spring, summer, or autumn season in Experiment 2. n = 10 per season. $^*p < 0.05$.

regular estrous cycling in, among other species, mice (Whitten, 1958), hamsters (Dodge et al., 2002), and goats (Rivas-Munoz et al., 2007). E_2 stimulates female sexual reflexes through hypothalamic action (Pfaff, 1980), and it produces a surge of luteinizing hormone (LH) which induces ovulation (Ferin et al., 2015; Meikle et al., 2001; Robker and Richards, 1998). Indeed, ovulation can be stimulated in bats via exogenous E_2 and LH (Oxberry, 1979). Accordingly, male-to-female E_2 transfer during the autumn and spring seasons in bats is likely a trigger of sexual receptivity and ovulation.

Elevated radioactivity observed in the uteri during reproductive seasons is of interest for classical mammalian pheromonal effects. The concentration of E_2 reaching uterine ER is critical for the success or failure of blastocyst implantation. Although E_2 helps prepare the uterus for implantation, supraoptimal levels accelerate ovum transport through the oviduct promoting expulsion (Ortiz et al., 1979), decrease blastocyst survival (Valbuena et al., 2001), and render the uterine endometrium unsuitable for implantation (Ma et al., 2003). Through these mechanisms, male-sourced E_2 can account for novel-male-induced implantation failure in the Bruce effect (deCatanzaro, 2015; deCatanzaro et al., 2006; Guzzo et al., 2012). Although the Bruce effect has not been studied in bats, there is evidence of post-copulatory intermale competition (Vonhof et al., 2006). E_2 also drives maturation of the reproductive tract in juvenile female mammals (Bronson, 1975; Ogasawara et al., 1983). Accordingly, male-sourced E_2 may also help to explain male-induced precocious female puberty in the Vandenbergh effect (deCatanzaro, 2015), which has been observed in diverse mammals but has not yet been studied in bats.

In addition to actions via urine, male-sourced E_2 can also transfer to females during mating via seminal emissions. Unconjugated E_2 and other estrogens have been found in the seminal emissions of a variety of mammals, including rodents (Saksena et al., 1978), lagomorphs (Saksena et al., 1977), primates (Waites and Einer-Jensen, 1974), and ungulates (Claus et al., 1983; Eiler and Graves, 1977; Reiffsteck et al., 1982). ERα and ERβ are present in the epididymis of male mice (Couse et al., 1997). When ^{3}H-E_2-injected male mice were mated with untreated females, radioactivity was found in the females' serum and peripheral tissues, with exceptional concentrations in their uteri (deCatanzaro and Pollock, 2016). E_2 in semen and copulatory plugs can thus bind in and act directly on female reproductive tissues before entering general circulation. Steroid concentrations in seminal emissions have not been studied in *E. fuscus*; however, in a previous study (deCatanzaro et al., 2014), the epididymis of male bats contained very high levels of radioactivity following ^{3}H-E_2 injection. Although elevated in comparison to other tissues, the epididymis of ^{3}H-E_2-injected male bats in the current study (Table 1) did not contain similarly high levels, likely because males from the former study underwent multiple ^{3}H-E_2-injections, whereas males in the current study received only one.

Experiment 2 yielded the first measures of unconjugated bioactive E_2 in the urine of male bats (Figs. 2 and 3). These data show E_2 concentrations similar to those previously found in male mice (*e.g.* deCatanzaro et al., 2006, 2009). This suggests that male urinary steroids are a likely vector for the observed transfer of E_2 from male to female bats as seen in Experiment 1 and previous work (deCatanzaro et al., 2014). In wild bats, E_2 transfer would likely occur during male–female contact in seasonal roosting behavior. Our measurements show that creatinine-adjusted urinary E_2 levels in male bats differed across seasons (Fig. 3). It is presently unknown whether this observation accounts for differences in E_2 transfer between the reproductive and non-reproductive seasons or whether seasonal differences in behavior also influence the quantity of hormone transfer. The creatinine data suggest that there may be some role of seasonal differences in hydration and/or activity levels. In mice, male urinary creatinine and E_2 both change dynamically during exposure to females (deCatanzaro et al., 2009); these dynamics warrant further study in bats.

In conclusion, we have demonstrated seasonal differences in ^{3}H-E_2 transfer from male to female big brown bats. Also, this is the first study to demonstrate the presence of natural, unconjugated and bioactive E_2 in male bat urine. These data reinforce past findings that sex steroids

reliably transfer between male and female conspecifics, as demonstrated in both mice and bats, with urine being a major vector. As rodents and bats are phylogenetically distant, it is likely that inter-individual steroid transfer is a widespread phenomenon in mammals.

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