# STEROID TRANSFER AMONG FEMALE BATS

STEROID TRANSFER AMONG COHABITATING FEMALE BIG BROWN BATS

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TITLE: Steroid Transfer Among Cohabitating Female Big Brown Bats

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## Lay Abstract

Steroid molecules are conventionally assumed to act solely within the individual that produced them; however, recent experiments have demonstrated that the sex steroid 17β-Estradiol ($E_2$) can be excreted in the urine of adult male mice and taken up into the neural, reproductive, and peripheral tissues of cohabitating females. This exogenous $E_2$ can result in changes to female physiology and behaviour. Our lab has observed $E_2$ to transfer between male and female captive big brown bats during the mating season. The current project aimed to determine whether $E_2$ transfers between captive cohabitating female bats. We also examined whether progesterone ($P_4$), an important female sex steroid involved in the preparation and maintenance of pregnancy, transfers between female bats. We determined that $P_4$ reliably transfers between female bats, but $E_2$ does not. Bioactive $E_2$ and $P_4$ were measured in the urine of non-pregnant female bats and propose urine as one likely vector of $P_4$ transfer between cohabitating individuals.

## Abstract

In addition to their conventional role as hormones, studies have shown that steroids can act as pheromones in mammals. Emphasis has been placed on evaluating the physiological and behavioural effects of male, urinary 17β-estradiol ($E_2$) exposure in pheromone phenomena including the prevention of embryo implantation and induced precocious puberty in females. Steroids have also been observed to transfer between female mice, leading to changes in the duration of their estrous cycle. Progesterone ($P_4$), a crucial female sex steroid, promotes pro-social sexual reproductive behaviour and the growth of the endometrium in preparation for ovum implantation. Few studies have investigated the effects of $P_4$ in a pheromonal context. Big brown bats (*Eptesicus fuscus*) are ideal models for pheromone research because they are evolutionarily distinct from rodents, live in highly social and sexually-competitive harems, and are regularly exposed to conspecific secretions in the close confines of their roost. Experimental analysis revealed absorption of tritium-labeled progesterone ($^3$H-$P_4$) (10 µCi) 1 h after cutaneous and intranasal application to adult females. Additionally, radioactivity was observed in mature female bats caged for 48 h with an adult female conspecific that had been intraperitoneally-injected with $^3$H-$P_4$ (50 µCi). Using the same paradigm, $^3$H-$E_2$ transfer was not observed between females. Enzyme-linked immunosorbent assays revealed measurable levels of unconjugated $P_4$ and $E_2$ present in the urine of female bats, suggesting urine as one likely vector for $P_4$ transfer. Given corroborative findings in mice, progesterone transfer during cohabitation is likely a mammalian-wide

phenomenon that could have evolved to prime conspecifics—and more specifically kin—for sexual reproduction.

**Acknowledgements**

Thank you to Dr. Paul Faure for his all of his guidance, for always encouraging me to pursue new questions, and for allowing me to integrate endocrinology research into his lab. Thank you to Dr. Denys deCatanzaro for all of his support and endocrine expertise. Many thanks to Tyler Pollock and Joseph Salter for their assistance with lab work. Committee members Joanna Wilson and Louis Schmidt are thanked for their advice and criticisms of this project. Thank you to Dr. Kathleen Delaney, Dawn Graham and the McMaster Bat Lab for all of their work in maintaining a healthy bat colony. Thanks to all my friends and family for always encouraging me to reach my goals, pushing me to be a better scientist, and for putting up with all my nerdy tendencies. Special thanks to Roberto Valdizon for always making himself available to critique my protocols and writing, challenge my way of thinking, and for helping to make my Master's degree so enjoyable. Lastly, thank you to all the bats that gave their lives for science.

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## Declaration of Academic Achievement

*Steroid Transfer Among Cohabitating Female Big Brown Bats*

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The design of this study was conceived primarily by LJG, under the supervision of PAF and DdeC. Experiments were conducted by LJG with assistance from TP and JCS. Manuscript written by LJG.

## Chapter 1: Introduction

Physiologists have historically believed that steroid hormones act solely within the individual producing them; however, studies in both mice and bats have shown that sex steroids can be excreted by individuals in bioactive form and absorbed by cohabitating conspecifics (Guzzo et al., 2010, 2012, 2013; deCatanzaro et al. 2014). Male excretions have long been implicated in changes of female sexual development and behaviour, including novel-male induced disruption of pregnancy (Bruce, 1960), precocious puberty (Vandenbergh, 1967), and alterations of the estrous cycle (Whitten, 1956). When directly administered to inseminated female mice, very low doses of exogenous 17β-estradiol ($E_2$) can mimic all of the above effects (Bronson, 1975; deCatanzaro et al., 2001, 2006; review by deCatanzaro, 2015). Multiple studies in mice (*Mus musculus*) have demonstrated that unconjugated $E_2$ is reliably present in male urine (e.g. deCatanzaro et al., 2006, 2009). Through the use of radioactive tracers, it has been shown that $E_2$ transfers from males to females at physiologically relevant levels (Guzzo et al., 2010, 2012, 2013). When placed on a mesh wire grid above female conspecifics, male urinary $E_2$ levels have been observed to rise with males displaying the behaviours of polydipsia (increased water consumption) and polyuria (increased urination pattenrs) (deCatanzaro et al., 2009). The transfer of $E_2$ and progesterone ($P_4$) between females has also been observed in mice (Guzzo et al., 2013) and deCatanzaro (2015) suggested that this transfer may explain changes in the duration of the estrous cycle when females are housed together (Lee and Boot, 1955, 1956).

Currently, very little research has investigated the hormonal regulation of reproduction in bats and the potential for conspecifics to alter breeding cycles. The big brown bat (*Eptesicus fuscus*) lives in maternal harems distributed throughout central and North America (Kurta and Baker, 1990). Temperate insectivorous bats possess a somewhat unique mating system where copulation occurs mainly during the autumn and also during intermittent arousals throughout hibernation (Oxberry, 1979). Females store sperm, and fertilization occurs alongside ovulation after arousal from hibernation in the spring (Wimsatt, 1944; Christian, 1956; Oxberry, 1979; Racey, 1979). Big brown bats are highly social, displaying promiscuous mating patterns in laboratory settings (Mendonça et al., 1996). Although many behavioural reproductive phenomena remain to be studied, recent research from our lab has demonstrated that male *E. fuscus* are able to transfer $E_2$ to the neural and peripheral tissues of cohabitating female bats during the autumn mating season (deCatanzaro et al., 2014). Specifically, the highest levels of exogenous $E_2$ derived from males were observed in the uterus and ovaries of female conspecifics, where there are high concentrations of estrogen receptors (Kuiper et al., 1997). Given the critical role of $E_2$ in female reproduction, these results suggest that that $E_2$ may act as a pheromone (deCatanzaro, 2015) and the transfer of steroid molecules between conspecifics may serve an important function in mammalian reproduction.

The presence of bioactive sex steroids has been documented in the urine of male and female mice (deCatanzaro et al., 2004, 2006, 2009; Guzzo et al., 2013). Estradiol, in tandem with progesterone ($P_4$), is crucial in regulating the

female reproductive cycle, with both steroids needed to induce estrous behaviour (Robinson, 1954). Both $E_2$ and $P_4$ are required to promote sexual development, ovulation, and endometrium preparation in female mammals. Both are small ($E_2$, 272 Da; $P_4$, 314 Da), lipophilic molecules with high chemical stability, allowing them to enter circulation via cutaneous (Goldzieher and Baker, 1960; Hueber et al., 1994; Schaefer et al., 1982; Scheuplein et al., 1969) and intranasal routes (Bawarshi-Nassar et al., 1989; Guzzo et al., 2012). The bioavailability of an intranasally-administered dose of $P_4$ in rats was found to be 100% of that of an intravenously administered (i.v.) dose, whereas the majority of intranasally-administered $E_2$ was available as bioactive $E_2$ in circulation, with the precise quantity varying with the given dose (Bawarshi-Nassar et al., 1989). Dermally applied $P_4$ is rapidly absorbed in rats, and its distribution and metabolism are comparable to those of $P_4$ administered i.v. (Waddell and O'Leary, 2002).

Given that many temperate insectivorous bats roost together in confined spaces, we hypothesized that $E_2$ and $P_4$ excreted by female bats may enter the circulatory system of cohabitating females and bind to various organs. As previous findings have shown that sexually mature female bats are capable of absorbing exogenous $E_2$ via intranasal and cutaneous routes of transmission (deCatanzaro et al., 2014), we first set out to test whether the same were true for exogenous $P_4$. This was evaluated via the administration of tritium-labeled-progesterone ($^3$H-$P_4$) to the nostrils or abdominal skin of female bats. The radioactive label allowed us to quantify $P_4$ uptake and its relative distribution in female tissues after exposure. We subsequently hypothesized that cohabitation

and contact amongst roosting female bats could be sufficient to observe $E_2$ and $P_4$ transfer between individuals. To evaluate this we injected a single female bat with tritium-labeled estradiol ($^3$H-$E_2$) or $^3$H-$P_4$ and housed them with two experimental female bats for 48 h. Our investigations focused on the ovaries and uterus due to their high concentration of steroid receptors (Couse et al., 1997; Kuiper et al., 1997). The hypothalamus was investigated because of its potential role in pheromonal effects (Hamada et al., 1996; Ichimaru et al., 1999) and because it expresses both $E_2$ and $P_4$ receptors (Kato and Onouchi, 1976; Sar and Parikh, 1986; Warembourg et al, 1989; Simerly et al., 1990). The liver and kidney were included due to their involvement in the metabolism, conjugation and excretion of steroids. Other peripheral and neural tissues were also included for comparison.

As the aforementioned experiments all involved the administration and tracking of exogenous steroids, we also quantified the presence of $P_4$ and $E_2$ in the urine of female bats. Previous studies have used blood samples to measure steroid concentrations in *Myotis lucifigus* (Buchanan and YoungLai, 1986, 1988), *Antrozous pallidus* (Oxberry, 1979), and *Eptesicus fuscus* (Mendonça, 1996), but to the best of our knowledge urinary steroid concentrations have not previously been assessed in the order Chiroptera.

## Chapter 2: Materials and Methods

### *2.1. Chemicals*

SOLVABLE solubilization cocktail, Ultima Gold scintillation cocktail, and 8 ml scintillation vials were obtained from PerkinElmer, Watham, MA, USA. Stock solution of [2,4,6,7-[$^3$H](N)]-$E_2$ (dissolved in ethanol, 1.0 µCi/µl, 81.0 Ci/mmol) and 2 stock solutions of [1,2,6,7-[$^3$H](N)]-$P_4$ (dissolved in ethanol, 1.0 µCi/µl, 101.3 and 96.0 Ci/mmol) were also attained from PerkinElmer. The $E_2$ and $P_4$ standards were obtained from Sigma-Aldrich, Oakville, ON, Canada, whereas $E_2$ and $P_4$ antibodies, and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis.

### *2.2. Animals and housing*

Wild *E. fuscus* were caught in southern Ontario and housed in a husbandry facility at McMaster University that permitted animals to fly (Faure et al., 2009). Temperature and lighting of the colony varied with ambient conditions. Bats selected for experimental use were brought to the laboratory and housed in small (28 x 22 x 18 cm) stainless steel wire mesh holding cages. All animals had access to mealworms (*Tenebrio molitor*) and water *ad libitum* unless otherwise stated. All experiments occurred during the autumn mating season of the big brown bat. Procedures were approved by the Animal Research Ethics Board of McMaster University and conform to the guidelines of the Canadian Council on Animal Care.

### *2.3. Experiment 1: Direct cutaneous exposure of female bats to $^3$H-P$_4$*

Experimental procedures closely followed the methods of deCatanzaro et al. (2014). On day 1, female bats (n=5) were randomly selected from the research colony and housed overnight in a holding cage. On day 2, each female was cutaneously administered 10 µCi of $^3$H-P$_4$ (32.8 ng exposure per bat) to the abdominal region via pipette. Animals were then housed individually in a standard polypropylene mouse cage (28 x 16 x 11 cm) with a wire grid lid, without food or water.

At 1 h after isolation, animals were anesthetized via isoflurane inhalation and a blood sample was taken via cardiac puncture. Animals were euthanized by perfusion with 20 ml of phosphate buffer solution. Tissue samples were collected and placed in pre-weighed 8 ml scintillation vials. Reproductive tissues included the whole uterus and both ovaries. Neural tissues included samples of the olfactory bulbs, cerebellum, a section of the frontal cortex, and a section of the hypothalamus taken from the ventral surface of the brain. Peripherally sampled tissues included the heart, lung, liver, external intercostal muscle, abdominal adipose tissue, and a cross section of the kidney encompassing both the cortex and medulla. Following collection, sample vials were re-weighed and wet tissue mass was recorded.

Tissue samples were solubilized by adding 1 ml of SOLVABLE to each vial. After 10 min of mechanical agitation, samples were placed into a water bath at 50° C for 2 h. Vials were re-agitated for 10 min, then allowed to sit in the water bath for a additional 2-3 h until tissue was completely dissolved. Samples were

removed from the water bath and permitted to cool before adding 5 ml of Ultima Gold scintillation cocktail to each vial. Vials were then stored in the darkness chamber of a TriCarb 2910 TR Liquid Scintillation Analyzer with a high sensitivity option (PerkinElmer, Waltham, MA) for 5 min to eliminate residual heat and luminescence. The level of radioactivity from each vial was measured for 5 min, with the final adjusted estimate quantified in disintegrations per minute (DPM) calculated by QuantaSmart software. All measures of radioactivity were adjusted for wet tissue mass and are reported as DPM/mg tissue.

Blood samples were centrifuged at 1500g for 10 min, after which 10 µl of serum was added to vials containing 5 ml of Ultima Gold. Radioactivity measurements in blood serum were quantified as described above, and reported as DPM/µl serum.

### *2.4. Experiment 2: Direct intranasal exposure of female bats to $^{3}H$-$P_4$*

As in Experiment 1, adult female bats (n=5) were randomly selected from the research colony on day 1 and housed in holding cages overnight in the lab. On day 2, animals were intranasally administered 10 µCi of $^{3}H$-$P_4$ (32.8 ng exposure per bat), with approximately 5 µCi injected into each nostril. All other procedures, including isolation of the bats, anesthesia, perfusion, tissue collection, sample processing, and scintillation counting were identical to Experiment 1.

### *2.5. Experiment 3: Direct exposure of female bats to $^{3}H$-$P_4$-injected females*

Experiment 3A was conducted in early December 2014. On day 1, adult female bats (n=3) were randomly selected from the colony and housed in two

holding cages; an isolated stimulus female ($PF_1$) and 2 subject females ($PF_A$ and $PF_B$). On day 2, $PF_1$ was injected with 50 µCi of $^3H$-$P_4$ (155.2 ng exposure) via intraperitoneal (i.p.) injection and isolated for 1 h to prevent accidental $P_4$ transfer from the injection site. Female $PF_1$ was then placed in a cage with subject females' $PF_A$ and $PF_B$ and allowed to cohabitate for 48 h before they were sacrificed and blood and tissue samples were collected. Anesthesia, perfusion, and tissue processing followed procedures described above. A subject sample size of two females per replicate was selected in order to minimalize animal usage based on minimal radioactive variation between three subject females in experiments from deCatanzaro et al. (2014).

Experiment 3B was aimed to test the generality of $^3H$-$P_4$ transfer across cohabitating female bats. In November 2015, three new adult stimulus female bats ($PF_2$, $PF_3$, and $FP_{4)}$ were each given an i.p. injection of 50 µCi $^3H$-$P_4$ (163.8 ng exposure) and after 1 h isolation each was then housed with two novel subject female bats as described in Experiment 3A ($PF_2$ paired with $PF_C$ and $PF_D$; $PF_3$ paired with $PF_E$ and $PF_F$; $PF_4$ paired with $PF_G$ and $PF_H$). Food and water dishes were sealed with Parafilm with an access hole approximately 4 cm in diameter to allowing bats to eat and drink but to minimize the possibility of contamination with bat excretions. After 48 h of cohabitation, animals were anesthetized, perfused with 20 ml of phosphate-buffer saline, and blood and tissue samples were collected using the same methods as described above in Experiment 1.

Due to our experimental design, it was impossible to measure levels of radioactivity in both experimental and control bats paired with an $^3H$-$P_4$- treated

female; however, samples taken from the water and food dishes in the holding cages were tested for radioactivity to ensure that $^{3}$H-$P_4$ from the stimulus females excretions had not contaminated the subject females' food and water. Dry swipes of the dissection table, surgical tools, and other equipment were taken between dissections to ensure that radioactivity had not contaminated the dissection station or the general experimental area.

### *2.6. Experiment 4: Direct exposure of female bats to $^{3}$H-$E_2$-injected females*

On day 1, nine adult female bats were randomly selected from the research colony and housed in holding cages in groups of three. On day 2, a female from each holding cage was randomly selected ($EF_1$, $EF_2$, and $EF_3$) and i.p. injected with 50 μCi $^{3}$H-$E_2$ (169.2 ng exposure) and isolated for 1 h, after which time the $^{3}$H-$E_2$-treated females were housed with new a pair of adult female subject bats different from the bats they were housed with the day prior ($EF_1$ paired with $EF_A$ and $EF_B$; $EF_2$ paired with $EF_C$ and $EF_D$; $EF_3$ paired with $EF_E$ and $EF_F$). After 48 h of cohabitation with subject females, all animals were anesthetized with isoflurane inhalation and perfused with 20 ml phosphate-buffer saline. Blood and tissue samples were collected and processed as described in Experiment 1.

As in Experiment 3, our experimental design did not permit experimental and control bats to be tested with the same $^{3}$H-$E_2$-treated stimulus female. Food and water samples, as well as dry swipes of the experimental area, tools, and equipment were taken to ensure contamination had not occurred.

*2.7. Experiment 5: Quantifying unconjugated steroids in female urine*

Urine was collected non-invasively from captive female bats during the non-reproductive season. Using a wax paper lined cart as a work surface, colony animals were selected and hand-held over the cart in a manner that permitted urine to fall directly on the wax paper. Urine from each animal was collected separately in 70 µl hematocrit tubes (Fisherbrand, Pittsburgh, Pennsylvania, USA) and kept frozen (-20°C) until time of analysis.

Urine analysis of unconjugated $P_4$ and $E_2$ was completed using modified ELISA protocols previously outlined (Muir et al., 2001; deCatanzaro et al., 2003; deCatanzaro et al., 2004). Approximately 50 urine samples from non-reproductive females of varying ages were pooled for the purpose of developing standard curves and validating the assay. Serial dilutions of pooled urine were used to obtain optical densities and generate standard curves. A regression line was fit to the data, and samples were interpolated into the equation to an estimate of hormone in pg/well. Data were plotted with a serially diluted standard for each steroid against logarithmically transformed doses. This test indicates whether measurable levels of steroid were present in the urine of female bats, and whether the steroid molecules react to the antibodies in a predictable manner (Kemeny, 1991).

*2.8. Data Analysis*

Radioactivity counts (DPM) exceeding background levels in the natural environment are not possible unless inadvertent contamination were to occur. All contamination swipes measured radioactive levels at or near zero. Past

experiments from our laboratory following similar experimental protocols found that animals exposed to control conditions or stimulus animals that were not treated with $^{3}$H-steroids showed radioactivity levels at or near zero, thus eliminating the need for controls in the present study (deCatanzaro et al., 2014; Guzzo et al., 2012, 2013). Given that past control animals showed radioactivity levels near zero, radioactivity measurements from bats in experimental conditions with sample size $n \geq 5$ will always reach statistical significance ($p < 0.05$) when using a Wilcoxon Rank-Sum non-parametric test. Thus, control animals were not necessary for the current experiments since previous work has shown that they did not contain radioactivity levels greater than background (deCatanzaro et al., 2014).

## Chapter 3: Results

### *3.1. Experiment 1: Direct cutaneous exposure of female bats to $^3$H-P$_4$*

Direct cutaneous exposure of $^3$H-P$_4$ resulted in measurable radioactivity 1 h later in all 5 subject bats (Fig. 1). Three subjects displayed radioactivity in all of their sampled tissues including blood serum, whereas one subject did not measure levels of radioactivity over background levels in the olfactory bulb, frontal cortex, and hypothalamus, and another lacked detectable radioactivity in the olfactory bulb and blood serum. Large ranges of radioactivity were observed in the muscle, 8.3–86.5; adipose, 6.6–613.7; uterus, 19.9–92.5; and ovary, 13.3–90.2. Measurements from peripheral tissues (heart, lung, muscle, abdominal adipose, uterus, ovary, liver, kidney) were in a higher and completely non-overlapping range from the neural tissues (olfactory bulb, cerebellum, frontal cortex, hypothalamus). This experiment demonstrates that $^3$H-P$_4$ can be directly absorbed across the ventral skin surface of female bats. In Experiment 1, 1 DPM is equivalent to 1.48 pg P$_4$ per g of tissue.

### *3.2. Experiment 2: Direct intranasal exposure of female bats to $^3$H-P$_4$*

Direct intranasal administration of $^3$H-P$_4$ to females yielded radioactivity 1 h later in all tissues sampled from all subject bats (Fig. 1). The highest values were observed in the liver, kidney, uterus, abdominal adipose, and ovaries, in that order, and values from these tissues were in completely non-overlapping ranges of all other samples from the cerebellum, frontal cortex, and hypothalamus. Large ranges of measurements among subjects were observed in

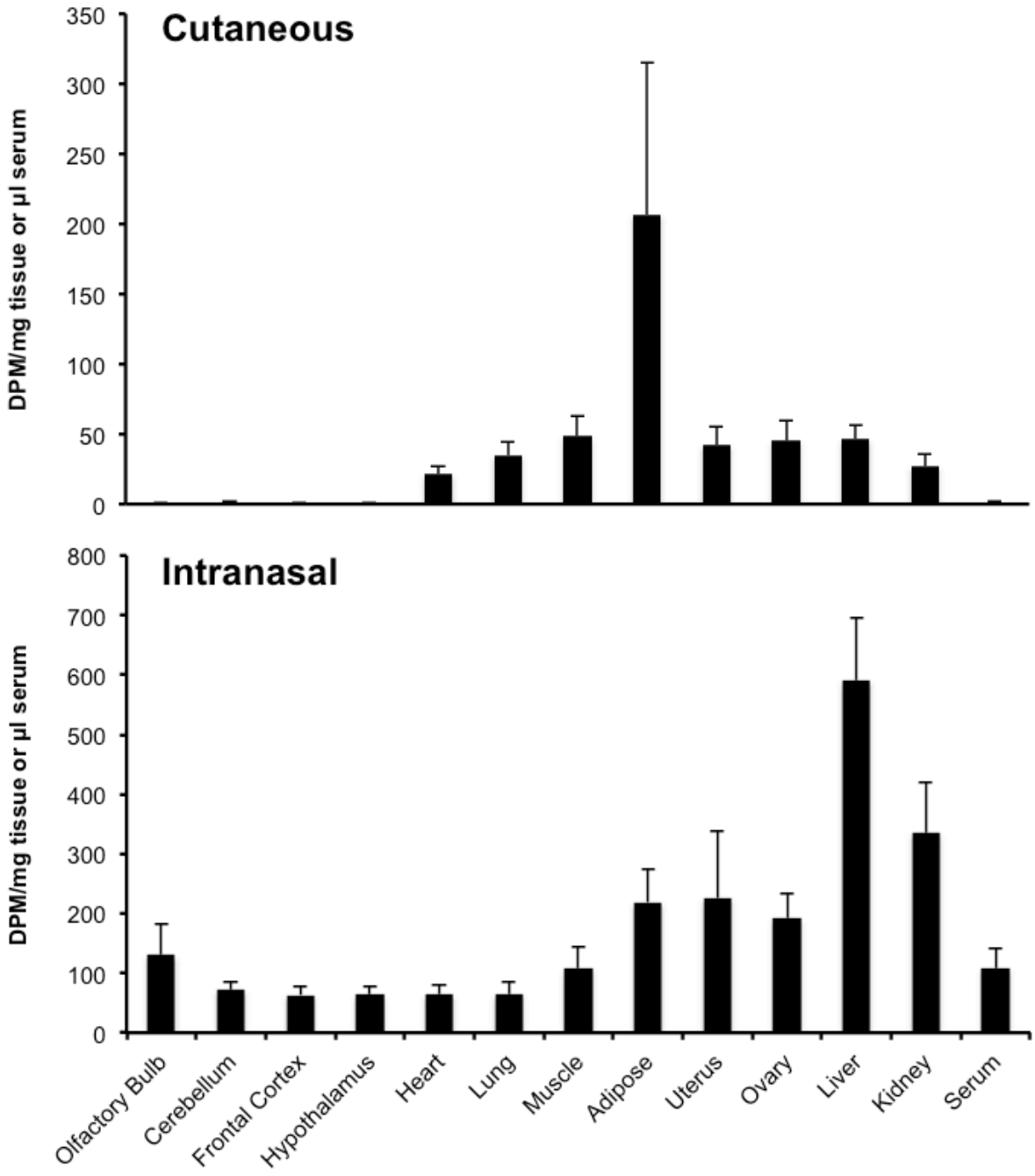
Cutaneous
DPM/mg tissue or µl serum
350
300
250
200
150
100
50
0
Intranasal
DPM/mg tissue or µl serum
800
700
600
500
400
300
200
100
0
Olfactory Bulb
Cerebellum
Frontal Cortex
Hypothalamus
Heart
Lung
Muscle
Adipose
Uterus
Ovary
Liver
Kidney
Serum

**Fig. 1. Direct intranasal and cutaneous exposure of female bats to $^{3}H$-$P_4$ .** Radioactivity (mean DPM + SEM) measured in solubilized tissues and blood serum of adult female bats 1 h after direct (*upper panel*) cutaneous administration (n=5) and (*lower panel*) intranasal administration of (n=5) of 10 µCi (32.8 ng) $^{3}H$-$P_4$ in Experiments 1 and 2. All values have been corrected for background radioactivity.

the kidney, 125.6–542.7; liver, 345.1–910.5; and uterus, 82.1–671.6. This experiment demonstrates that $^{3}$H-$P_4$ can be directly absorbed after intranasal administration in female bats. In Experiment 2, 1 DPM is equivalent to 1.48 pg $P_4$ per g of tissue.

### *3.3. Experiment 3: Direct exposure of female bats to $^{3}$H-$P_4$-injected females*

Following 48 h exposure to a $^{3}$H-$P_4$-treated stimulus female $PF_1$ in Experiment 3A, radioactivity was measured in all tissues of both subject females $PF_A$ and $PF_B$. The greatest values were measured in the olfactory bulbs, hypothalamus, and frontal cortex, in that order, with all values for each individual presented in Figure 2. Radioactive measurements (DPM/mg) of tissues from $PF_1$ taken after cohabitation were: olfactory bulb, 186.8; cerebellum, 177.6; frontal cortex, 173.9; hypothalamus, 157.6; heart, 152.6; lung, 165.4; muscle, 157.5; adipose, 510.2; uterus, 157.3; ovary, 357.0; liver, 491.8; kidney, 165.4; and serum, 368.6. In Experiment 3A, 1 DPM is equivalent to 1.40 pg $P_4$ per g of tissue.

Radioactivity counts measured in two subject females across three replicate treatments (Panel A: $PF_C$ and $PF_D$; Panel B: $PF_E$ and $PF_F$; Panel C: $PF_G$ and $PF_H$) of 48 h cohabitation with a $^{3}$H-$P_4$-treated female ($PF_2$, $PF_3$, and $PF_4$ respectively) are reported in Figure 3. Radioactivity was observed in all subject females with varying distributions. The ovaries, uterus, lung, and olfactory bulb contained the highest average levels, in that order. A larger range of values was observed in tissues of subject females exposed to $^{3}$H-$P_4$-treated females (olfactory bulbs, 0–3.7; lung, 0–4.2; uterus, 0.3–4.5; and ovaries, 0.3–5.0).

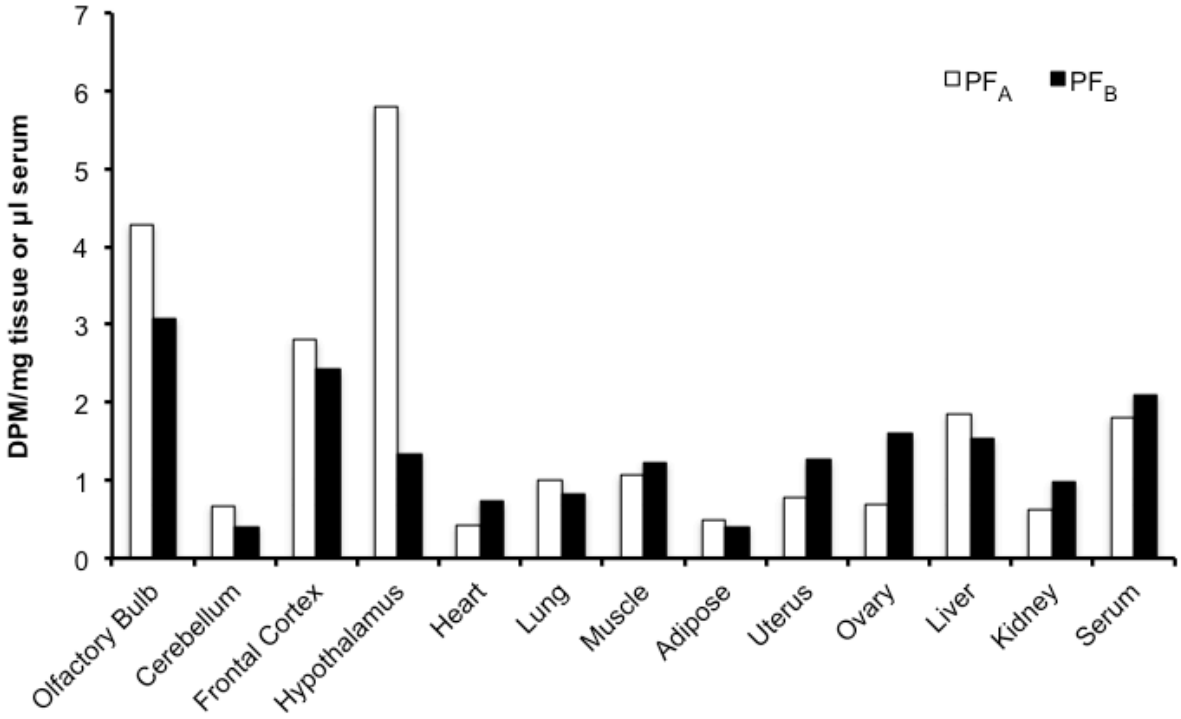


**Fig. 2. Direct exposure of two subject female bats to a single $^{3}$H-$P_4$ injected stimulus female.** Radioactivity (DPM) measured in solubilized tissues and blood serum of 2 adult female bats ($PF_A$ and $PF_B$) after 48 h of cohabitation with a $^{3}$H-$P_4$-treated adult stimulus female ($PF_1$) in Experiment 3A, conducted in 2014. Both subject females cohabited simultaneously with the stimulus female beginning 1 h post i.p. injection of 50 µCi (155.2 ng) $^{3}$H-$P_4$. All values corrected for background radioactivity.

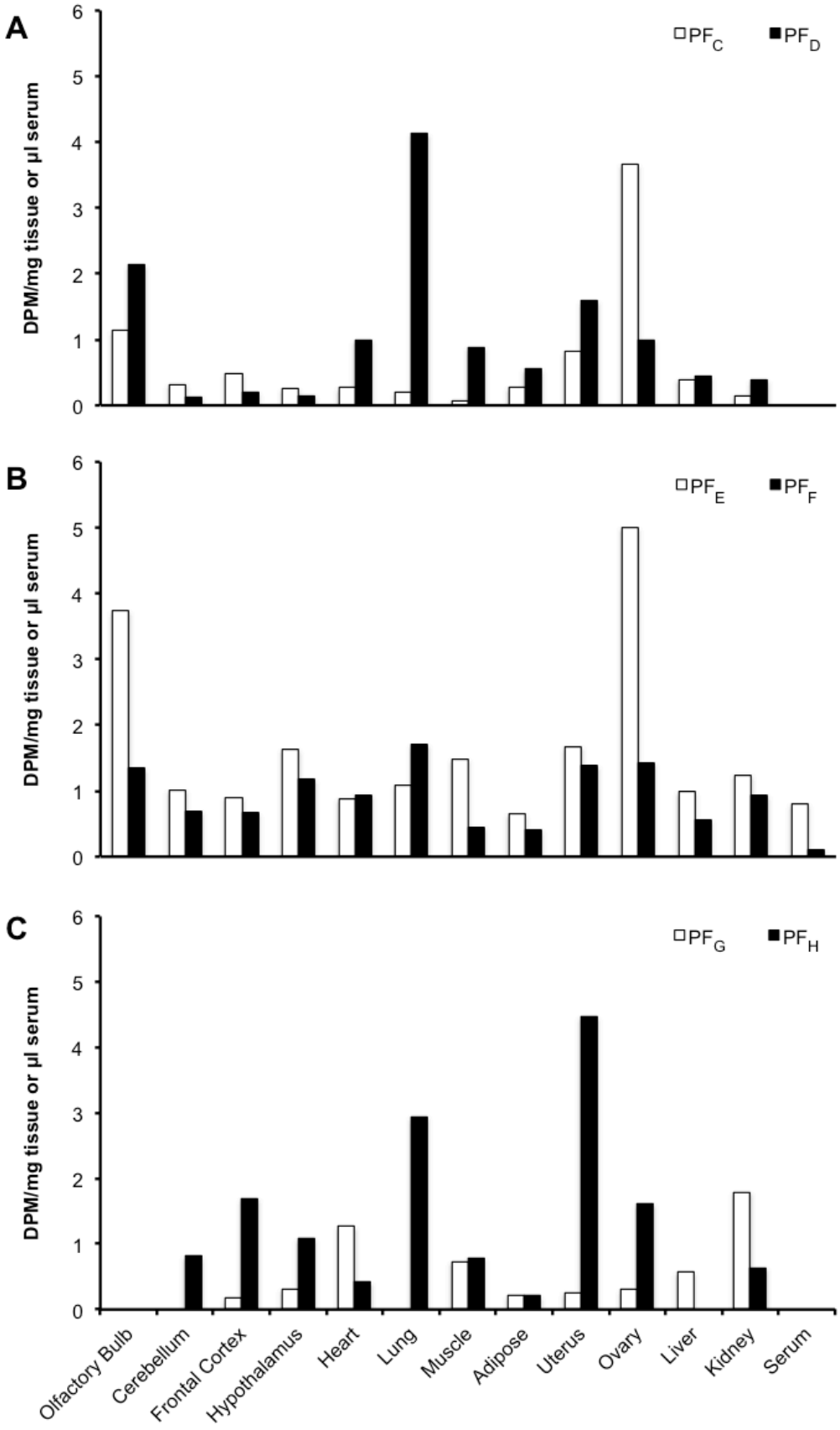
A
B
C
DPM/mg tissue or µl serum
PF_C
PF_D
PF_E
PF_F
PF_G
PF_H
0
1
2
3
4
5
6
Olfactory Bulb
Cerebellum
Frontal Cortex
Hypothalamus
Heart
Lung
Muscle
Adipose
Uterus
Ovary
Liver
Kidney
Serum

**Fig. 3. Direct exposure of a pair of subject female bats to a single $^{3}$H-P$_4$ injected stimulus female (3 replicates).** Radioactivity (DPM) measured in solubilized tissue tissues and blood serum of adult subject female bats (n=6) after 48 h cohabitation with a $^{3}$H-P$_4$-treated stimulus female in Experiment 3B, conducted in 2015. Panel A, B, and C each represent a single replicate of the simultaneous cohabitation of two adult females with a $^{3}$H-P$_4$-treated adult female beginning 1 h post i.p. injection of 50 µCi (163.8 ng) $^{3}$H-P$_4$. All values corrected for background radioactivity.

During dissection, subject $PF_G$ was observed to have an inflamed uterus. The entire uterus was taken as normal along with the white fluid inside it. This sample measured lower radioactive levels in comparison to other uteri. Mean radioactivity counts across the three replicates are reported in Figure 4. Tissue samples from $^3$H-$P_4$-treated bats $PF_2$, $PF_3$, and $PF_4$ contained the following mean DPM±SEM counts: olfactory bulb, 110.1±11.6; cerebellum, 107.2±9.6; frontal cortex, 114.7±13.0; hypothalamus, 106.1±7.0; heart, 67.3±21.8; lung, 163.1±47.9; muscle, 148.1±4.8; adipose, 2100.6±1335.6; uterus, 184.1±18.1; ovary, 214.9±15.4; liver, 439.9±62.2; kidney, 233.4±28.9; and serum, 175.4±3.1. Together, these results demonstrate that female bats are able to transfer $^3$H-$P_4$ to the tissues of cohabitating female conspecifics. In Experiment 3B, 1 DPM is equivalent to 1.48 pg $P_4$ per g of tissue.

### *3.4. Experiment 4: Direct exposure of female bats to $^3$H-$E_2$-injected females*

All three replicates investigating $^3$H-$E_2$ transfer from treated stimulus females to conspecifics yielded zero or low levels of radioactivity in subject tissues. In replicate one, radioactivity was not measured in any tissue or serum sample of either subject females $EF_A$ or $EF_B$ after correction for background radiation. In a second replicate, both subject females $EF_C$ and $EF_D$ had low levels of radioactivity measured in two tissue samples (subject 3: cerebellum, 0.06; heart, 0.03; subject 4: lung, 0.06; muscle, 0.57) but no measurable radioactivity in other tissues including the serum. Experimenters were unable to collect blood from subject female $EF_D$ and thus no corresponding serum measurement was reported. In a third replicate, radioactivity was measured in 5 sampled tissues

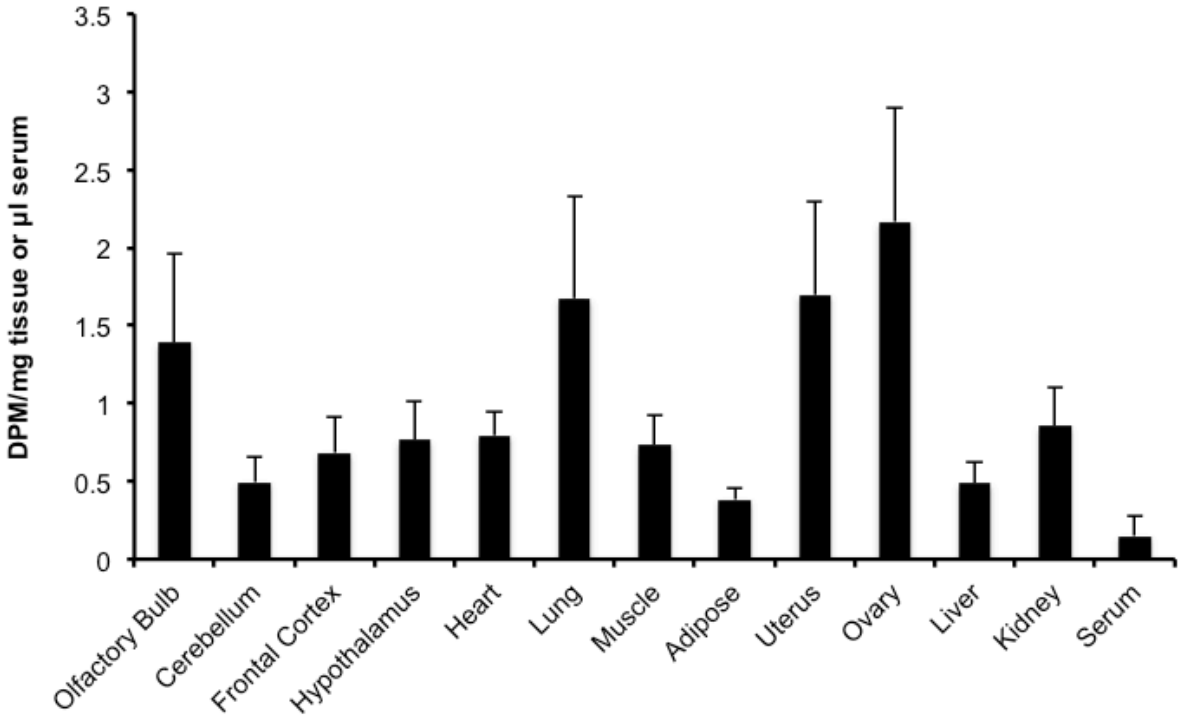


**Fig. 4. Direct exposure of a pair of subject female bats to a $^{3}$H-P$_4$ injected stimulus female (2015 mean).** Radioactivity (mean DPM + SEM) measured in solubilized tissues and blood serum of 3 replicates of 2 adult subject female bats (n=6) after 48 h of cohabitation with a single $^{3}$H-P$_4$-treated adult stimulus female in Experiment 3B, conducted in 2015. Subject females cohabited simultaneously with the stimulus female within a given replicate beginning 1 h post i.p. injection of 50 µCi (163.8 ng) $^{3}$H-P$_4$. All values corrected for background radioactivity.

(lung, 0.2; adipose, 0.04; uterus, 0.3; liver, 0.6; kidney, 0.5) of one subject female $EF_E$ and in 10 tissues of the other subject female $EF_F$ (olfactory bulb, 0.5; cerebellum, 0.4; frontal cortex, 0.6; heart, 0.1; lung, 0.3; muscle, 0.07; uterus, 0.7; liver, 1.0; kidney, 1.1; serum, 1.2). Mean radioactivity counts in subject females $EF_A$-$EF_F$ is reported in Figure 5. Tissues samples from the three $^3H$-$E_2$-treated females ($EF_1$, $EF_2$, and $EF_3$) contained the following mean DPM±SEM counts: olfactory bulb, 633.1±115.0; cerebellum, 591.2±87.7; frontal cortex, 604.3±68.0; hypothalamus, 574.7±81.1; heart, 342.7±61.4; lung, 491.6±81.3; muscle, 493.8±37.1; adipose, 327.3±139.4; uterus, 2864.6±1359.0; ovary, 1070.3±383.5; liver, 1136.7±446.2; kidney, 780.5±142.9; and serum, 1245.2±296.5. This experiment demonstrates that female bats are unable to transfer $^3H$-$E_2$ to cohabitating female bats. In Experiment 4, 1 DPM is equivalent to 1.51 pg $E_2$ per g of tissue.

### *3.5. Experiment 5: Quantifying unconjugated steroids in female urine*

Both $P_4$ and $E_2$ were present at measurable levels in female urine, as the samples were diluted in parallel with their standard curves. Figure 6 shows the dose-response curves and parallelisms for both $P_4$ and $E_2$ from urinary samples of non-reproductive female bats. This experiment demonstrates that both $P_4$ and $E_2$ are present in the urine of female bats in their bioactive unconjugated form.

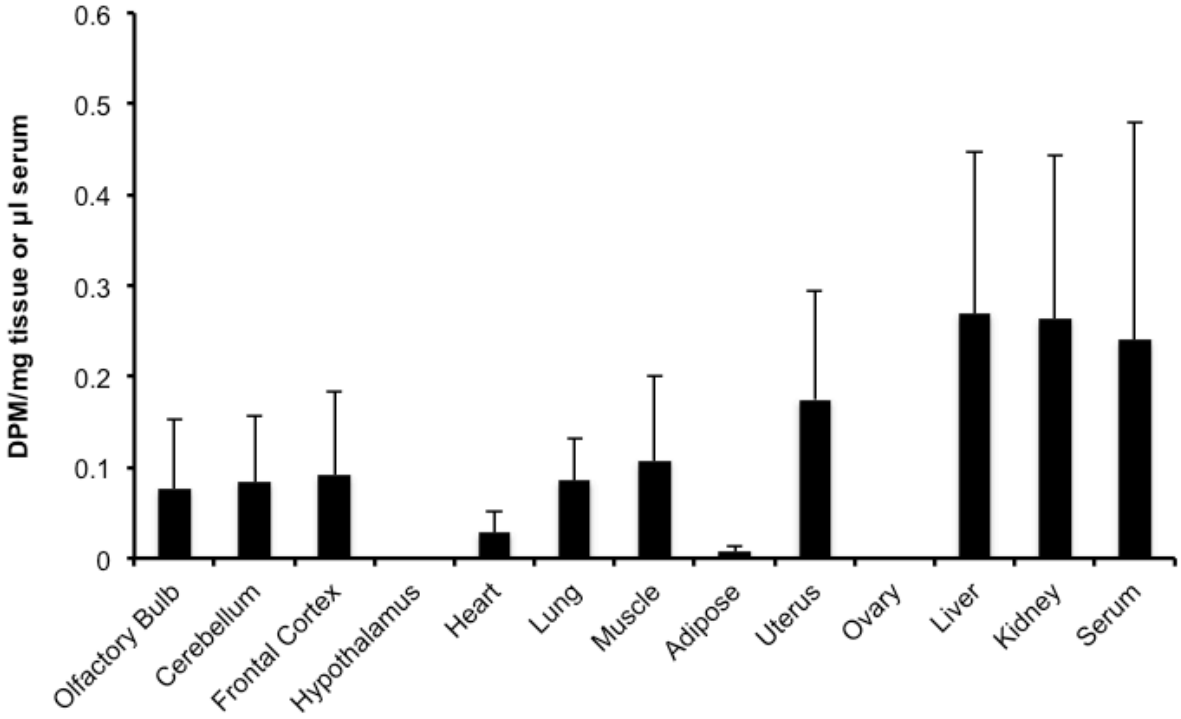


**Fig. 5. Direct exposure of a pair of subject female bats to a $^{3}$H-$E_2$ injected stimulus female (2014 mean).** Radioactivity (mean DPM + SEM) measured in solubilized tissues of 3 replicates of 2 adult subject female bats (n=6) after 48 h of cohabitation with a single $^{3}$H-$E_2$-treated adult stimulus female in Experiment 4, conducted in 2014. Both subject females cohabited simultaneously with the stimulus female within a given replicate beginning 1 h post i.p. injection of 50 µCi (169.2 ng) $^{3}$H-$E_2$. All values corrected for background radioactivity.

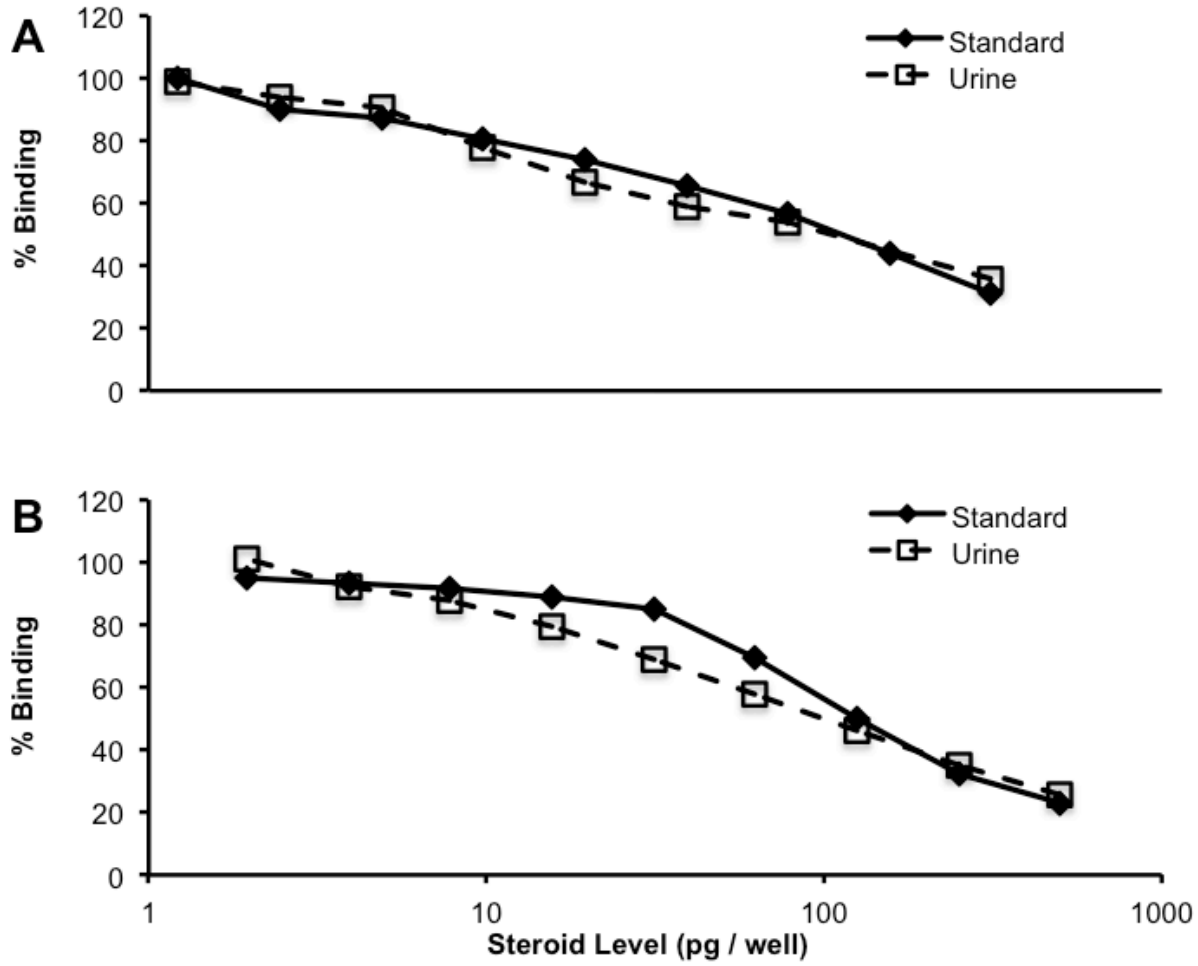


**Fig. 6. ELISA parallelisms for unconjugated urinary progesterone and 17β-Estradiol.** Serially diluted urinary samples binding to antibody in parallel with progesterone (Panel A) or estradiol (Panel B) serially diluted standards.

## Chapter 4: Discussion

Steroid molecules, produced primarily in the gonads and adrenal glands, are critical for the maintenance of the female mammalian reproductive cycle. The present data demonstrate that female big brown bats can absorb exogenous $^{3}$H-$P_4$ via percutaneous and intranasal routes. They also show that $^{3}$H-$P_4$ can transfer among female conspecifics after 48 h of cohabitation during the autumn mating season. The transfer of $^{3}$H-$P_4$ was replicated in all 8 untreated females across four replicates in which two untreated females were housed with a $^{3}$H-$P_4$-treated female. The highest levels of radioactivity were measured in the ovaries, uteri, lungs, and olfactory bulbs of untreated females, which corresponds with the highest $P_4$ receptor densities being found in female reproductive tissues (Uotinen et al., 1999). As the lungs consistently show high levels of radioactivity in steroid transfer experiments (deCatanzaro et al., 2014), we suggest that nasal exposure to the excretions of conspecifics' could lead to steroid absorption into the lungs and the vasculature via the nasal mucosa.

Given the social structure of the species, the observed progesterone transfer could have ecological implications for big brown bat. Living in harems, female *E. fuscus* occupy maternal colonies with their offspring, while the males appear to form separate bachelor colonies (Kurta and Baker, 1990). Maternal colonies mitigate the thermoregulatory costs of reproduction in bats as cool roost temperatures delay the development of prenatal offspring and the occurrence of parturition (Racey and Swift, 1981). A high $P_4$:$E_2$ ratio is critical for the success of blastocyst implantation after insemination (deCatanzaro, 2015; Gidley-Baird et

al., 1986; Ma et al., 2003). Progesterone promotes decidualization (Kurita et al, 2001; Lim et al., 1999; Ma et al., 1998; Taylor et al., 1998; reviewed by Clarke and Sutherland, 1990; Wetendorf and DeMayo, 2012), and high progesterone levels are necessary to sustain gestation (Allen and Corner, 1930; Csapo and Wiest, 1969; Meites et al, 1951; Milligan and Finn, 1997). The close confines of female conspecifics within the roost provides a suitable environment for $P_4$ transfer to naturally occur during the ovulatory period. Temperate bats spend a significant portion of their daily energy meticulously grooming their fur and wings (Burnett and August, 1981). Given our experimental findings that bioactive unconjugated $P_4$ is present in the urine of female bats, grooming behaviour provides ample opportunity for females to absorb exogenous $P_4$ from conspecific excretions via intranasal and cutaneous routes. In this context, the transfer of $P_4$ may act to prime conspecific females for implantation, or assist in the maintenance of pregnancy throughout the gestation period. In contrast, a lack of $E_2$ transfer between females (as observed in Experiment 4) during the peri-implantation or gestational period would aid in maintaining the high $P_4$:$E_2$ ratio, as exogenous $E_2$ has been shown to prevent blastocyst implantation and disrupt gestation (deCatanzaro et al., 2001, 2006; Gidley-Baird et al., 1986; Ma et al., 2003). In the wild, female bats would potentially be exposed to excretions from hundreds of other females in a similar reproductive state. The summation of absorbed $P_4$ from multiple conspecifics, in conjunction with the long acting and cumulative nature of steroid hormones, suggests that $P_4$ transfer could reach levels sufficient to influence the reproductive state of any given female bat.

Progesterone is a precursor to numerous reproductive steroids including androgens and estrogens (Hanukoglu, 1992; Payne and Hales, 2004). In the $^{3}$H-$P_4$ conditions where radioactive transfer was observed in all conditions, we assume the much of the transferred radioactivity represents $P_4$. Cutaneous absorption of $P_4$ is faster than that of $E_2$ upon dorsal cutaneous administration in the guinea pig (Goldzieher and Baker, 1960). Waddell and O'Leary (2002) found that cutaneously administered $^{3}$H-$P_4$ is rapidly absorbed into the circulatory system of the rat, with high plasma levels measured after only 15 min. Water soluble metabolites were measured after 15 min, whereas lipid soluble metabolites were not measurable until 30 min after cutaneous administration. Although both water and lipid soluble metabolites were measured in high quantities in rat urine, the radioactivity measured in numerous organs was attributed mostly to $P_4$. Tritiated-$P_4$ accounted for 93% of radioactivity measured in the uterus, 83% in the lung, and 60% in the salivary glands, but only 5% of total tritium in the urine (Waddell and O'Leary, 2002). Thus, after absorption, $^{3}$H-$P_4$ metabolites are rapidly excreted in the urine whereas $^{3}$H-$P_4$ remains bioactive in tissues.

We previously demonstrated that female bats readily absorb exogenous $E_2$ during cohabitation with stimulus males during the autumn mating season (deCatanzaro et al., 2014); however, $E_2$ transfer between female conspecifics was not observed during the same time of year in the current study. In one of three replicates where two untreated adult females cohabited with a $^{3}$H-$P_4$-treated female for 48 h, very low levels of radioactivity slightly above background

levels were measured in the tissues of the untreated females. In the other two replicates, radioactivity above background levels was not measured in untreated females. Mean radioactivity levels in females paired with male stimulus bats in deCatanzaro et al. (2014) ranged from 0.5-5 DPM/mg depending on the tissue and replicate. The current experiment saw measured minimal radioactivity in the tissues of subject females paired with a stimulus female, averaging 0-0.27 DPM/mg in various tissues. One possible reason for observing reliable $E_2$ transfer from male to females but not from female to female is that males transferred $E_2$ to females during the mating season, hence $E_2$ may have transferred via seminal fluids during mating. Saksena et al. (1978) previously demonstrated that $E_2$ levels in the seminal vesicle fluid of male rats increased after a subcutaneous dose of exogenous $E_2$. The quantity of estrogens present in semen is less than progesterone, however much of the progesterone is in the form of metabolites (Cooper and Waites, 1975). Furthermore, the presence of steroids in semen has also been observed in male dogfish (Simpson et al., 1963), rabbit (Saksena et al., 1977), bull (Reiffsteck et al., 1982), boar (Claus et al., 1983; Reiffsteck et al., 1982), stallion (Reiffsteck et al., 1982), and human (Reiffsteck et al., 1982). Although steroid levels in the seminal plasma of male bats has not yet been studied, we previously observed exceptionally high levels of radioactivity in the epididymides of male *E. fuscus* after they received $^3$H-$E_2$ injections (deCatanzaro et al., 2014). This observation suggests $E_2$ transfer from males to females may occur via mating, thus explaining why we previously observed $E_2$ transfer from male to females but not between female bats.

To the best of our knowledge, we have also presented the first evidence that bioactive unconjugated $E_2$ and $P_4$ are present in the urine of non-pregnant female bats. We have presented parallelisms for $E_2$ and $P_4$ in the urine of female big brown bats, suggesting that urinary steroids may act as a vector for steroid transmission in bats. Upon further validation, these methods could allow experimenters to reliably quantify steroid levels in bats via non-invasive methods in the laboratory and field.

These data corroborate past studies from our laboratory demonstrating the transfer of $^3$H-$E_2$ and $^3$H-$P_4$ from adult mice of either sex to adult females (Guzzo et al., 2013), as well as the transfer of $^3$H-$E_2$ from male to female mice and bats (Guzzo et al., 2013; deCatanzaro et al., 2014). Given the long evolutionary and phylogenetic separation of bats and rodents, the current data reinforce the overarching hypothesis that steroid transfer between conspecifics may occur in many mammalian species.

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