ESTRADIOL AS A POTENTIAL PHEROMONE IN BATS

EXAMINING THE PHEROMONAL POTENTIAL OF ESTRADIOL IN THE BIG BROWN BAT

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TITLE: Examining the Pheromonal Potential of Estradiol in the Big Brown Bat

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Lay Abstract

Historically, physiologists have believed steroid hormones act exclusively within the individual producing them. However, studies in mice have shown that bioactive 17β-estradiol (E_2) is excreted in male urine and absorbed by female conspecifics where it binds to estrogen receptors in reproductive and other tissues. This exogenous E_2 can result in changes to female reproductive physiology and behaviour. Our lab has previously observed E_2 to transfer between male and female captive big brown bats during the mating season. Research from this thesis provides evidence that the transfer of E_2 from male to female bats is highest during times of mating and ovulation/fertilization. I also demonstrate that E_2 naturally occurs in the urine of both male and females with age, sex, and seasonal differences in concentration. Lastly, I show that female bats are attracted to the urine of males. My research provides new evidence for the potential pheromonal actions of E_2 in bats that is consistent with how sex steroids act as pheromones in other mammals.

Abstract

Historically, physiologists have believed steroid hormones act exclusively within the individual producing them. However, studies in mice have shown that bioactive 17β-estradiol (E_2) is excreted in male urine and absorbed by female conspecifics where it binds to estrogen receptors in reproductive other tissues. This can lead to pregnancy disruption and/or cause precocious puberty in female conspecifics. In bats the transfer of tritium-labelled estradiol (^{3}H-E_2) from male to females has been shown during the mating season. I investigated the influence of season on ^{3}H-E_2 transfer and showed that females housed with ^{3}H-E_2 injected males had significantly higher levels of radioactivity in reproductive, neural, and peripheral tissues during reproductively relevant timepoints compared to the non-reproductive season. Because urine has been hypothesized as the vector of steroid hormone transmission in mice, I examined the natural patterns of sex steroids in the urine of male and female big brown bats (*Eptesicus fuscus*) across the annual reproductive cycle. I found that creatinine-adjusted E_2, which corrects for animal hydration and activity, was significantly higher in male than female urine, and in adult compared to yearling urine. Seasonal differences in urinary E_2 levels were observed within and between sexes. Finally, I designed a protocol to investigate the attractant properties of body odour and urine between bat sexes during the mating season. Using a two-alternative Y-maze arena, I found that female bats first approach the test arm containing urine of a male conspecific before exploring the arm with female urine. Females also tended to spend more time in the test-arm containing male urine and being in the male test-arm at the conclusion of the 5 min trial. My data supports the hypothesis

that E_2 has the potential to act as reproductive pheromone with urine as a likely vector. Given the close proximity of individual bats within a maternity roost, steroid hormone transfer between conspecifics quite likely occurs in nature and could have profound influences on female reproductive behaviour (e.g. receptivity) and physiology (e.g. estrus cycling). My research provides new evidence for the potential pheromonal actions of E_2 in bats that is consistent with how sex steroids act as pheromones in other mammals.

Author Biography

Lucas Greville was born and raised in Toronto, Ontario. He became interested in math and science at an early age, and began to focus on the biological sciences while in high school. Lucas moved to Hamilton, Ontario in 2010 and pursued his Honours Bachelor of Science in Biology & Psychology. During his undergrad, Lucas joined the McMaster Bat Lab where he got the first opportunity to conduct scientific research. Lucas would complete his Honours Bachelor, Master of Science, and PhD theses under the supervision of Dr. Paul Faure and Dr. Denys deCatanzaro. While Lucas' dissertation work focused on reproductive physiology and behaviour in bats, he was also involved in numerous side projects researching toxicology in mice, wound healing in bats, and bat neuroendocrinology. Lucas assisted and facilitated multiple collaborations while in the lab, and was always happy to lend a hand to his lab mates.

Lucas represented his graduate peers on departmental, faculty, and university committees, in addition to facilitating numerous connections within the bat research community. During his PhD Lucas was a sessional instructor for multiple courses including Psych 3M03 – Motivation and Emotion, LifeSci 4X03 – The BioPsychology of Sex, and Explore 3IE1 – Bat Ecology and Human Interactions: Agriculture, Disease, and Conservation; the latter of which he designed and proposed himself. Lucas was also an active member of the PNB department being a regular at department socials, as well as captaining the department volleyball and Psy Jung softball team for a number years. He was also known to make an appearance on the squash courts.

Lucas is looking forward to continue researching bat physiology as a Postdoctoral Fellow at the University of Waterloo following his PhD and hopes to obtain a career in biological research with the opportunity to continue teaching. Wherever life takes him, Lucas will always remain an advocate of bats and an active member of the bat community, while no doubt be a vocal supporter of the Toronto Maple Leafs.

This thesis is dedicated to my nephews, Brock and Cooper.

May your passions lead you on a lifetime of learning and exploration.

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As I pulled up to Bates Residence as a first-year in student September 2010, my wildest dreams could not have prepared me for the journey McMaster would have in store. How naïve I was to think that in 4 years I'd be moving on. A little over 10 years and 3 degrees later, it's hard to believe this chapter of my life is over. I'm lucky to have been able to dedicate my life thus far to education, and looked forward to going to school every day. My years at McMaster brought times of stress and exhaustion, but it's the fun, laughter, and happiness that stands out. The old cliché about a journey is that it's the people you get to experience it with that makes it special. In my case at McMaster, nothing could be truer. I would not be the academic, scientist, educator, friend, or person I am today without the support of so many. These are just a few of the people who have helped make my time at McMaster so special.

First and foremost, I need to thank my supervisor Paul Faure for years of mentorship and friendship. I applied to the lab knowing nothing about the wild world of bats, and you welcomed me with open wings. You've given me the freedom to bring reproductive endocrinology and behaviour into the lab, to start international collaborations, and to freely mentor students. You've always made sure I had the opportunity to present at international conferences, and ensured I was able to network with leaders in the field. No matter what committee I'd join, what teaching opportunity I asked permission for, or what downtime I need, I always felt supported in my decision. We have shared in lab work, publications, troubleshooting, some cleaning, animal catching, caring for sick animals, and have literally scraped shit off the colony floor

together. I always looked forward to our teaching debriefs, and stopping by the office to catch up on local news while I filled you in on the world of hockey. We always joke that your job is to pay bills and provide signatures, but you do so much more. The truth is I could not ask for a more patient, thoughtful, or caring mentor to navigate me through academia. I like to think we've put out some good research over the years, but most importantly Paul, you always made the lab a place I looked forward to going to. Because let's face it, if you can't have fun at work, where can you have fun?

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A special thanks goes out to the members of my supervisory committee — Joanna Wilson, Lauren Foster, and Ayesha Khan. It's no secret that I probably sat on one too many committees or taught a few too many units over the last couple years, but you always remained honest with your expectations of me, genuinely cared about my wellbeing and research, and always supported my academic development. I always felt that you all genuinely cared about my success and treated me as if I was in your lab.

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RN. Thank you for being by my side throughout this journey. I look forward to all the adventures that lie ahead. I love you.

Finally, thank you to all the bats who have given their life for science.

Table of Contents

List of Figures and Tables

List of Abbreviations and Symbols

Chemicals, Substances, and Receptors

17β-estradiol	E_2
Creatinine	Cre, cre
Estriol	E_3
Estrogen receptor	ER
Estrone	E_1
Horseradish peroxidase	HRP
Progesterone	P_4
Tritium/radio-labeled 17β-estradiol	^3H-E_2

Methods and Statistics

Analysis of variance	ANOVA
Degrees of freedom	DF
Enzyme-linked immunosorbent assay	ELISA
Intraperitoneal	i.p.
Linear mixed effects regression model	LMER
Mean	$\bar{x}$
Number of subjects	N, n
Post-natal day	PND
Standard error	SE

Sum of squares	SS

Units of measurement

Curie	Ci
Dalton	Da
Degrees Celsius	°C
Disintegrations per minute	DPM, dpm
Grams	g
Hours	h
Inch	”
Length	l
Litres	L, l
Metres	m
Micro	µ
Milli	m
Minutes	min
Nano	n
Pico	p
Width	w

Declaration of Academic Achievement

Chapter 1 – Introduction

Author: Lucas J. S. Greville

Chapter 2 – Seasonal transfer and quantification of urinary estradiol in the big brown bat (*Eptesicus fuscus*).

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Chapter 4 – Evaluating odour and urinary sex preferences in the big brown bat

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Chapter 5 – Discussion

Author: Lucas J. S. Greville

Chapter 1 – Introduction

1.1 – The Mammalian Endocrine System

The mammalian endocrine system functions to regulate and synchronize physiological outputs of essential organs. It is composed of a collection of glands that secrete chemical messengers, called hormones, into circulation that act on distant organs in the body (Nelson and Kriegsfeld, 2017). Essential glands for hormone synthesis include the pineal and pituitary glands of the central nervous system, the reproductive organs testes and ovaries, and peripheral tissues including the pancreas and thyroid gland. In addition to their primary functions, tissues such as the gut and bone also produce hormones. Collectively, the endocrine system is regulated by the hypothalamus which is located in the ventral medial midbrain (Nelson and Kriegsfeld, 2017).

Circulating hormones generate their effects by binding to membrane bound or intracellular receptors. The downstream effect of receptor binding can produce a range of physiological responses critical for proper development. Hormones can stimulate growth, metabolism, sexual maturation, digestion, and a range of behaviours (Nelson and Kriegsfeld, 2017). The proper balance and timing of endocrine action keeps the body in a state of homeostasis needed for proper functioning.

1.2 – Steroid Hormones

There are four main types of hormones in the mammalian endocrine system. Peptide hormones are made from long chains of amino acids and high molecular mass. In contrast, steroid hormones are small and derived through the enzymatic cleaving of a cholesterol precursor (Hanukoglu, 1992; Payne and Hales, 2004). Consisting of three 6-

carbon and one 5-carbon rings, steroids are highly stable, lipophilic, and extremely potent (Nelson and Kriegsfeld, 2017). As such, steroids can pass through phospholipid-bilayers such as cell membranes and the blood-brain barrier with relative ease whereas protein hormones require a transport protein. Eicosanoids and monoamine hormones are less common and will not be discussed here.

The subset of steroid hormones responsible for regulating reproductive behaviour and physiology are known as sex steroids. This group of hormones includes androgens, estrogens, and progestogens. Although they can be synthesized *de novo* in the central nervous system, the vast majority of mammalian sex steroids are synthesized in the ovaries and testes, with supplementation from the adrenal medulla, liver, and adipose tissue (Nelson and Kriegsfeld, 2017). Lipophilic steroids efficiently travel throughout the body to target tissues via the circulatory system by binding to a carrier protein known as sex hormone binding globulin (Anderson, 1982).

Sex steroids readily pass through cell membranes and accomplish their regulatory functions via intracellular receptor binding in the nucleus of target tissues (Walters, 1985). Each family of sex steroid has their own receptor protein. The estrogen family of steroids, including estrone (E_1), 17β-estradiol (E_2), and estriol (E_3), bind to estrogen receptors (ER). Testosterone and dihydrotestosterone are among the androgens that bind to androgen receptors. Lastly, progesterone (P_4) binds to progesterone receptors. The conventional steroid receptor resides in the nucleus in a newly synthesized, unliganded state. Upon binding of the steroid, the ligand-receptor complex acts as a transcription factor by binding to a hormone response element of a promoter gene in the DNA

(Walters, 1985). In this capacity, steroids are able to up- or down-regulate the transcription of the gene, in turn altering protein and enzyme levels that display effects on the order of hours to days. More recently, steroids have been shown to bind to G-coupled protein receptors in the cell membrane (Barton et al., 2018; Filardo and Thomas, 2005; Prossnitz et al., 2008; Thomas, 2004). In this manner, steroids initiate intracellular secondary messengers, such as adenylyl cyclase and protein kinases, that elicit cellular effects on the order of seconds to minutes (Filardo and Thomas, 2005; Prossnitz et al., 2008). Following their actions, steroids are conjugated in the liver and kidney before being excreted by the organism.

Estrogens are the smallest family of steroid molecules. The estrogens E_1 and E_2 are produced via the enzymatic cleavage of a methyl group from androgen precursors androstenedione and testosterone, respectively (Dumas and Diorio, 2011). The estrogen E_3 is produced in the liver and placenta. All forms of estrogen bind to ERs; however, the binding affinity of E_2 is much greater than E_1 and E_3 (Kuiper et al., 1997). As such, E_2 is considered the most potent form of estrogen .

1.3 – Mammalian Reproduction

The time period over which female mammals are sexually receptive and fertile is referred to as being in a state of estrus (Nelson and Kriegsfeld, 2017). In the absence of pregnancy, females of many species undergo reproductive cycling in which the individual alternates between states of fertility and infertility. In non-menstruating mammals, this cycle is referred to as the estrous cycle. The period of both the cycle and the duration of

time a female is in estrus differs drastically across species. For example, female Giant Pandas (*Ailuropoda melanoleuca*) are in a state of estrus for a few days each annual cycle (Kleiman et al., 1979). Meanwhile, laboratory rats (*Rattus norvegicus*) have an estrous cycle lasting 4 days and are in a state of estrus on the last day of each cycle (Blandau et al., 1941; Cora et al., 2015). It is also not uncommon for mammals to be reproductive only during a longer mating season and infertile otherwise.

The estrous cycle of mammals has two major segments (Nelson and Kriegsfeld, 2017). The follicular phase is characterized by the maturation of ovarian follicles prior to ovulation. The follicular phase has higher levels of E_2 than P_4 and ends when an increase in estrogen stimulates a surge of luteinizing hormone signalling ovulation. Following ovulation, the corpus luteum is active and produces high levels of progesterone which helps to prepare the endometrium for implantation and sustain pregnancy. During this time, levels of E_2 are low in comparison to P_4. This latter half of the estrous cycle is referred to as the luteal phase in primates and as pseudopregnancy in non-primate mammals—a period of physiological pregnancy preparation when cycling is suspended. If no pregnancy occurs, the corpus luteum breaks down and the body prepares for the cycle to restart. Mating often occurs around the time of ovulation, although this can differ between species. Broadly, mammalian estrous cycles have been described to follow 3 distinct patterns with subsets based on the length of the entire cycle (Conaway, 1971). Type I cycles, found in species such as humans, apes, guinea pigs, and canines, are where ovulation and pseudopregnancy occur spontaneously throughout the cycle, meaning copulation is not required to initiate the ovulatory or pseudopregnancy occurrences. Type

II cycles are characterized by copulation induced ovulation but spontaneous pseudopregnancy and are prominent in many species of lagomorphs, felines, and mustelids. Lastly, Type III cycles incur spontaneous ovulation but induced pseudopregnancy and are commonly seen in rats, mice, and hamsters (Conaway, 1971).

Levels of E_2 are critical in regulating numerous aspects of reproduction in female mammals. Endogenous E_2 stimulates DNA synthesis and cell proliferation in the uterus, resulting in uterine growth and sexual maturation of juveniles (Cooke et al., 1997; Mukku et al., 1982; Ogasawara et al., 1983; Quarmby and Korach, 1984). In tandem with P_4, E_2 can induce sexual receptivity (e.g., lordosis behaviour) and other estrus behaviour in females (Freeman, 2006; Pfaff, 1980). The ovulatory, fertilization, and implantation process is extremely sensitive to E_2 levels with small fluctuations negatively impacting reproductive output. For example, when female mice are injected with small quantities of E_2 on the day following insemination, ova undergo accelerated transport through the reproductive tract and are expelled through the vagina (Ortiz et al., 1979). Increases in E_2 also damage the blastocyst preventing uterine adhesion (Valbuena et al., 2001) and increase the E_2:P_4 ratio of the reproductive tract rendering the uterus refractory to implantation (Ma et al., 2003). Indeed, humans demonstrate the same pattern; women who became pregnant following *in vitro* fertilization and embryo transfer procedures had lower E_2:P_4 ratios than women who did not become pregnant (Gidley-baird et al., 1986).

1.4 – The Big Brown Bat and Sperm Storage

The big brown bat (*Eptesicus fuscus*) is found throughout North and Central America, and also occurs in northern regions of South America (reviewed by Kurta and Baker, 1990). Belonging to the family Vespertillionidae, *E. fuscus* are an insectivorous species that frequently roost in trees and residential buildings. Big brown bats are a highly gregarious species that live in maternally-lineated colonies with up to ~200 individuals. In colder climates, *E. fuscus* hibernates over the winter (reviewed by Kurta and Baker, 1990). Mark and recapture studies have shown that *E. fuscus* can live to at least 19 years in the wild with males typically outliving females (Hitchcock et al., 1984; Kurta and Baker, 1990; Kurta and Matson, 1980; Paradiso and Greenhall, 1967).

Female *E. fuscus* give birth annually to either a singlet or twin pups, depending on their geographical region, with a higher percentage of older adult females giving birth (Kurta and Baker, 1990). Like other temperate insectivorous bats, mating (copulation) is decoupled from gestation in *E. fuscus*: bats mate in the autumn and during brief periods of arousal during hibernation and in early spring (Oxberry, 1979). Females store sperm throughout winter hibernation, with ovulation, fertilization, and gestation occurring in the spring seasonal arousal (Christian, 1956; Oxberry, 1979; Racey, 1979; Wimsatt, 1944). Pups are born from May to June. During the summer, females form maternity colonies and care for their pups; males typically roost solo but may also be found roosting with females in low numbers or forming bachelor colonies until rejoining the colony prior to the next mating season (Christian, 1956; Davis et al., 1968; Schowalter and Gunson, 1979). In *E. fuscus*, both sexes mate with multiple partners within a captive setting

(Mendonça et al., 1996) but the same pattern is believed to occur in the wild as evidenced by multiple paternity within a litter (Vonhof et al., 2006).

In most mammals with internal fertilization and insemination preceding ovulation, the viability of sperm in the female reproductive tract is relatively short-lived. In bats (Order Chiroptera), females are able to store sperm over longer time period. Indeed, sperm storage, as observed in *E. fuscus*, is observed more frequently in Chiroptera than in other mammals (Orr and Brennan, 2015; Orr and Zuk, 2014). In mammals, sperm are stored in the lumen of the female reproductive tract where they are immersed in mucus (Holt and Fazeli, 2016). During storage, the sperm heads are aligned to face the epithelium where they often become physically bound (Holt and Fazeli, 2016). In the vespertilionid little brown bat, *Myotis lucifigus*, sperm were observed to burrow deep into the epithelial tissues (Racey et al., 1987). Since bat sperm cannot survive *in vitro*, it has been suggested this orientation may facilitate one pathway to which the sperm receive nutrients (Racey and Potts, 1970).

1.5 – Pheromones and Reproductive Phenomena

The word pheromone was coined by Karlson and Lüscher (1959) stemming from the Greek words *pherein,* "to transfer", and *hormōn* "to excite". The original definition of a pheromone read "substances that are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction" (Karlson & Lüscher, 1959). Over the past 60 years, the word pheromone has evolved to include physiological changes in conspecifics and the term is not as precise as

originally defined (deCatanzaro, 2015). Pheromones have been observed across taxa with a wide range of implications including aggregation, territorial markings, alarm signalling, trail marking, mate attraction, and stimulating reproductive behaviour (Nelson and Kriegsfeld, 2017).

Many species interact with conspecifics in their daily environment. Individual responses to the presence of a conspecific can differ, with different responses elicited depending on the individual's sex. For example, in reproductive endocrinology there are a number of well-known "effects" where male individuals can have drastic impacts on the reproductive physiology of female conspecifics. The Bruce effect, which occurs when a novel adult male is introduced to the environment of a recently inseminated female, as documented in lab mice (*Mus musculus*; Bruce, 1960a; Dominic, 1966; Parkes and Bruce, 1962), deer mice (*Peromyscus maniculatus*; Bronson and Eleftheriou, 1963), rats (Marashi and Rülicke, 2012), prairie and meadow voles (*Microtus pennsylvanicus* & *Microtus orchogaster*; Clulow and Lanford, 1971; Heske and Nelson, 1984), sheep (*Ovis aries;* Al-Gubory, 1998), wild and domestic horses (*Equus callabus* Bartoš et al., 2011; Berger, 1983), and wild geladas (*Theropithecus gelada*; Roberts et al., 2012) (reviewed by deCatanzaro 2015). In the Bruce effect scenario, the introduction of the novel male causes the implantation process to be blocked or pregnancy to be disrupted, preventing full gestation, parturition, and lactation and thus causing the female to more quickly re-enter estrous cycling. The Vandenbergh effect occurs when an adult male is introduced to the environment of juvenile females, resulting in the females undergoing precocious puberty and reach sexual maturity at a younger age (Vandenbergh, 1967). It also refers

juvenile female mice maturing later when housed wit adult females. The Vandenbergh effect has been observed across taxa (reviewed by deCatanzaro 2015), including laboratory mice (Vandenbergh, 1967), deer mice (Teague and Bradley, 1978), pine voles (*Microtus pinetorum*; Lepri and Vandenbergh, 1986), Djungarian hamsters (*Phodopus sungorus*; Reasner and Johnston, 1988), collared lemmings (*Dicrostonyx groenlandicus*; Hasler and Banks, 1975), opossums (*Monodelphis domestica*; Harder and Jackson, 2003), pigs (*Sus domesticus*; Brooks and Cole, 1970), cattle (*Bos tarus*; Izard and Vandenbergh, 1982), and geladas (Beehner and Lu, 2013). In both the Bruce and Vandenbergh Effects, the novel male gains a potential mating opportunity to females who can sire his offspring that he would not have otherwise had. Provided he is successful in mating, the ability to induce these effects holds an evolutionary advantage to the male that increases his reproductive success (i.e. fitness).

Urine has been implicated as a vector in the Bruce and Vandenbergh effects since their discoveries; however, the physiological mechanisms behind its actions were not well understood. Over the last 20 years extensive research of these effects have identified estradiol as a major driver of these reproductive phenomena (deCatanzaro, 2015). Estradiol (E_2) is a small (272 Da), lipophilic compound making it chemically stable and able to enter the general circulation of mammals via exogenous sources through cutaneous (Goldzieher and Baker, 1960; Hueber et al., 1994; Schaefer et al., 1982; Scheuplein et al., 1968) and intranasal routes (Bawarshi-Nassar et al., 1989; Guzzo et al., 2012). Following intranasal administration, the majority of E_2 is available in its unconjugated bioactive form (Bawarshi-Nassar et al., 1989). The administration of

minute quantities of E_2 to the mucus membranes of inseminated or juvenile female mice reliably replicates the Bruce and Vandenbergh effects (Bronson, 1975; deCatanzaro, 2015; deCatanzaro et al., 2006, 2001).

More extensive research has shown that male mice excrete E_2 in its unconjugated and bioactive form in their urine (deCatanzaro et al., 2009, 2006, 2004; Guzzo et al., 2013). When male mice are separated from a female by a mesh-grid for a duration of days, there is a gradual increase of E_2 in the male's urinary excretions with the male actively directing urine towards the female (deCatanzaro et al., 2009). Male urinary E_2 levels reach values high enough to induce the Bruce and Vandenbergh effects (deCatanzaro et al., 2009). Exposure to the excretions of intact males also readily produce these effects (Bruce, 1960b; deCatanzaro et al., 1999; Dominic, 1966; Parkes and Bruce, 1962), whereas castration (Bruce, 1965; Vella and deCatanzaro, 2001), the injection of antibodies that bind and inactivate E_2 (deCatanzaro et al., 1995), and aromatase inhibitors which prevent the enzymatic conversion of testosterone to E_2 (Beaton and deCatanzaro, 2005) all mitigate or eliminate these effects. In contrast, androgen and E_2 injections to castrated males can reinstate their ability to disrupt pregnancy (deCatanzaro and Storey, 1989; Rajendren and Dominic, 1988; Vella and deCatanzaro, 2001). Indeed, the transfer of male urinary E_2 to the tissues of cohabitating females has been reliably shown using radioactive tracers (Guzzo et al., 2013, 2012, 2010). Similarly, the Vandenbergh effect can be induced by application of exogenous E_2 (Bronson, 1975) and is mediated by male excretions (Colby and Vandenberg, 1974; Drickamer, 1983). As with the Bruce effect, castration eliminates a male's ability to induce the Vandenbergh effect, but treatment with

androgens or E_2 can reinstate the ability (Colby and Vandenberg, 1974; Lombardi et al., 1976; Thorpe and deCatanzaro, 2012). Cumulatively, evidence from the rodent literature suggests that male urinary E_2 is a significant contributor to natural occurrences of both the Bruce and Vandenbergh effects. Therefore, we can classify E_2 as a reproductive pheromone because it is secreted by an individual into the environment and taken up by conspecific where it elicits a change in conspecific behaviour and physiology.

1.6 – Research Overview

Our lab has previously shown that female big brown bats absorb exogenous steroids from their environment through percutaneous and intranasal exposure with tissue binding profiles similar to those observed in mice (deCatanzaro et al., 2014; Greville et al., 2017). We have documented that E_2 transfers from male to female bats during the autumn mating season, and that P_4 also transfers between cohabitating females during the same season (deCatanzaro et al., 2014; Greville et al., 2017). The transfer of these steroids suggest the possibility of steroids acting as pheromones in bats, with male sourced E_2 possibly implicated in a Bruce/Vandenbergh effect or synchronous female estrous cycling effect, with female sourced P_4 potentially facilitating the suppression of reproductive cycling in groups of cohabitating female bats such as found in maternity colonies (deCatanzaro, 2015). Reproductive pheromones and chemical signals are well studied in the greater-sac winged bat (*Saccopteryx bilineata*) where males create distinct perfumes from mixtures of their saliva, urine, and glandular secretions which they waft towards females to signal reproductive status (Caspers et al., 2008; Voigt and von

Helversen, 1999). Odours from glands and self-anointed patches in other bat species have also been postulated to act as reproductive pheromones (Flores and Page, 2017; Muñoz-Romo et al., 2011; Muñoz-Romo and Kunz, 2009).

My doctoral thesis aims to continue to evaluate the potential for E_2 to act as a reproductive pheromone in big brown bats. Because bats and rodents are phylogenetically distinct (Zhou et al., 2012), using a bat model to examine the pheromonal potential of mammalian steroids allows for broader evolutionary perspective and application of such mechanisms to include taxa in which behavioural reproductive phenomena occur but where the underlying mechanisms are still unknown or have not even been explored. Like many species of bats, *E. fuscus* is highly gregarious living in large maternity colonies within confined spaces (Kurta and Baker, 1990). Individuals roost close in proximity to facilitate thermoregulation and perform frequent allogrooming where they frequently come into contact with conspecific excretions (Burnett and August, 1981; Willis and Brigham, 2007). The maternity composition of big brown bats colonies allows colonial males to be in close contact with females, thus facilitating possible pheromone action.

The pheromonal potential of E_2 in *E. fuscus* was evaluated using an integrative approach. In **Chapter 2**, I used radio-labelled E_2 to trace steroid transfer from male to female bats during the autumn mating season, the spring ovulatory period, and summer maternity season. Additionally, a small sample of urine from male bats collected during each season was analyzed using enzyme-linked immunosorbent assays (ELISA) to determine their E_2 content. I found that radioactive levels were significantly higher in a number of tissues, including the reproductive tissues of the uterus, during the autumn and

spring in comparison to the summer. Creatinine-adjusted levels of urinary E_2—to control for differential hydration states between animals—were also higher in both the autumn and spring compared to the summer. These results suggest that seasonal differences in E_2 transfer from male to female conspecifics may be due to seasonal changes in urinary E_2 levels of male bats. The chapter concludes by examining the implications of E_2 transfer and other possible causes of the observed seasonal differences.

In **Chapter 3**, I measured urinary steroid levels in male and females bats across their annual reproductive cycle using ELISAs. I found that adult male bats had higher levels of creatinine-adjusted urinary E_2 compared to both adult females and yearling males. Urinary E_2 differed during the summer from other seasons in adult males, and many seasonal differences were observed in non-pregnant female bats. I also report trends in urinary E_2 and P_4 levels in pregnant females and seasonal urinary P_4 levels in non-pregnant females. Possible reasons for the observed results, with insights into other bat and mammalian research in general, are discussed.

Rodent research has shown that steroids can have attractant properties. **Chapters 2 and 3** of my dissertation showed the reliable presence of unconjugated bioactive E_2 in the urine of male and female bats. **Chapter 4** examines the role of body and urinary odours in attracting conspecific bats using a Y-maze testing arena. This study evaluates the ecological validity of bodily excretions, including urine which contains bioactive steroids, to act as attractants in bats prior to testing whether steroid concentrations alone act as attractants. I show that females show a preference for male urine over that of females. No preference was shown by male bats for the urine of either sex, and neither

male nor female bats showed a preference to conspecific body odours of a given sex. These results suggest that male urine may contain a pheromone which attracts female conspecifics. Whether this potential pheromone is estradiol remains to be answered.

Chapter 2 – Seasonal transfer and quantification of urinary estradiol in the big brown bat (*Eptesicus fuscus*)

Lucas J. Greville, Tyler Pollock, Paul A. Faure, and Denys deCatanzaro

General and Comparative Endocrinology, 286, 113321

2.1 – 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".

2.2 – 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 mating 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 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.

2.3 – Methods

2.3.1 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) bats 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 x 22 x 18 cm. Procedures were approved by the Animal Research Ethics Board of McMaster University, conforming to guidelines of the Canadian Council on Animal Care.

2.3.2 – 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.

2.3.3 – 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 (50μL) 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 Analyser. 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.

2.3.4 – 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 ºC) 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 (following procedures from Muir et al, 2001; Pollock et al, 2016, 2019), which

corrects for differential hydration and urinary concentration among animals, and reported as ng E_2/mg creatinine and E_2/mL urine respectively.

2.3.5 – 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, 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 $p < 0.05$.

2.4 – Results

2.4.1 – 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. 2.1). 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 all 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 2.1). Due to experimenter error, n=5 is reported for the epididymis and kidney in the summer. Collectively, 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.

2.4.2 – 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. 2.2).

Fig. 2.1 – 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. *$p < 0.05$.

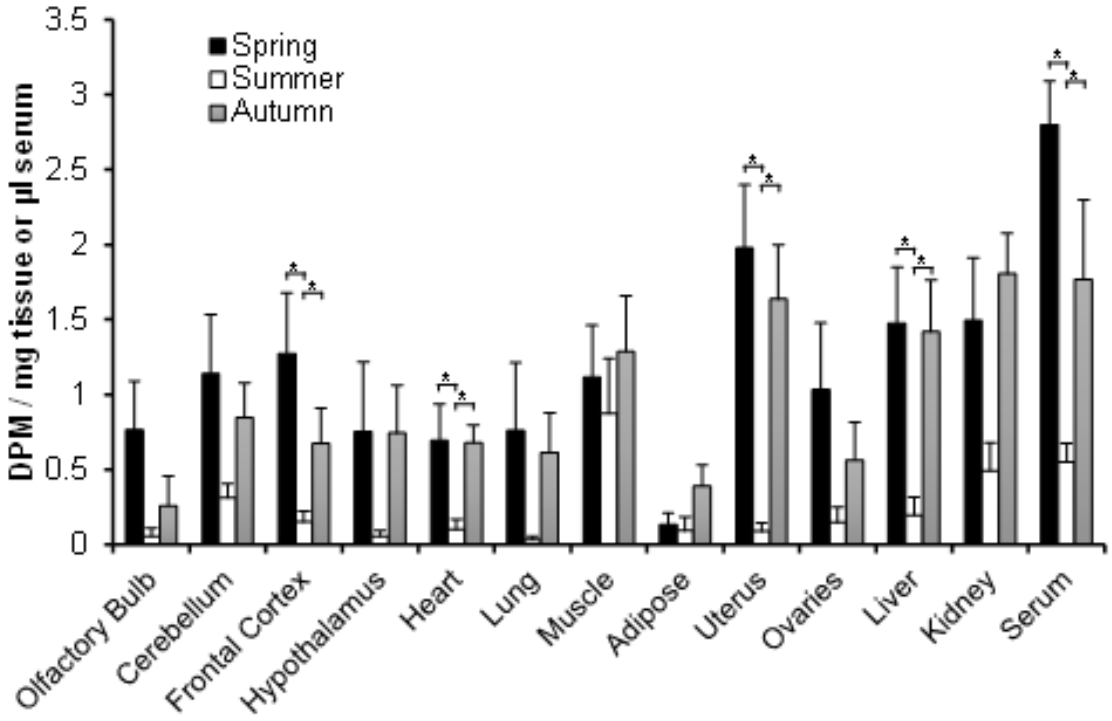
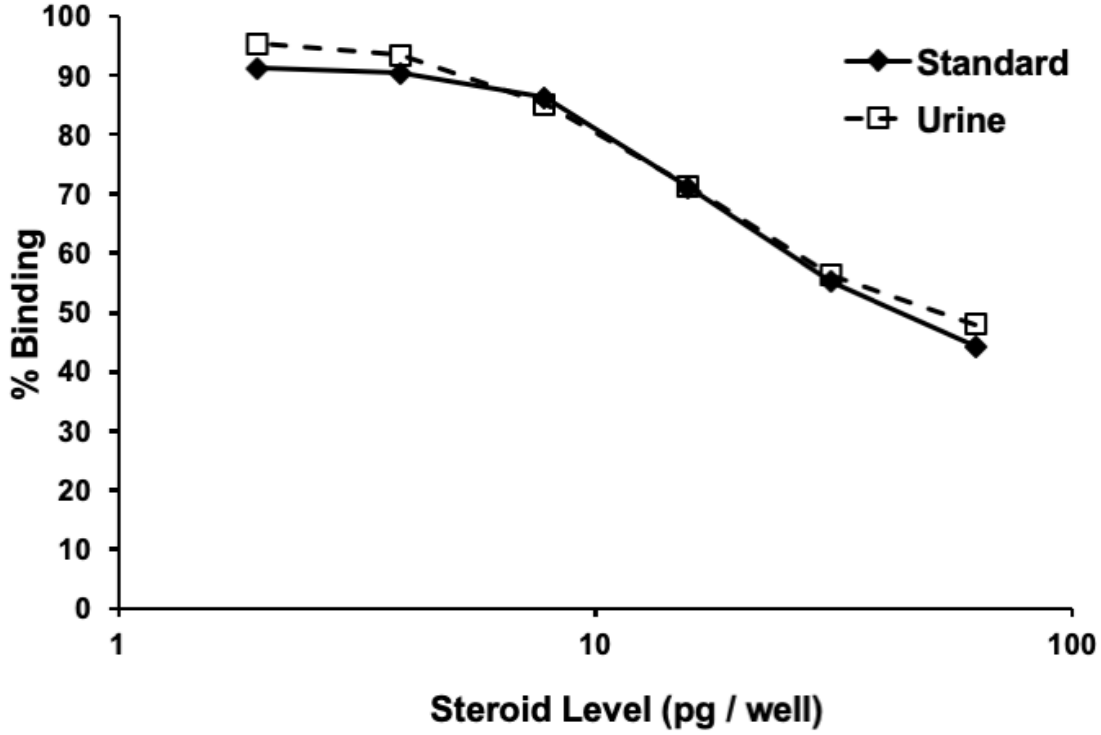


Table 2.1 – Radioactivity (mean DPM ± SE) measured in solubilized tissues and blood serum of ^{3}H-E_2-treated (50 μCi) adult stimulus male bats following 48 h of cohabitation with 2 adult females during the spring, summer, or autumn season in Experiment 1. n=3 per season except n=2 for epididymis and kidney measures collected in the summer.

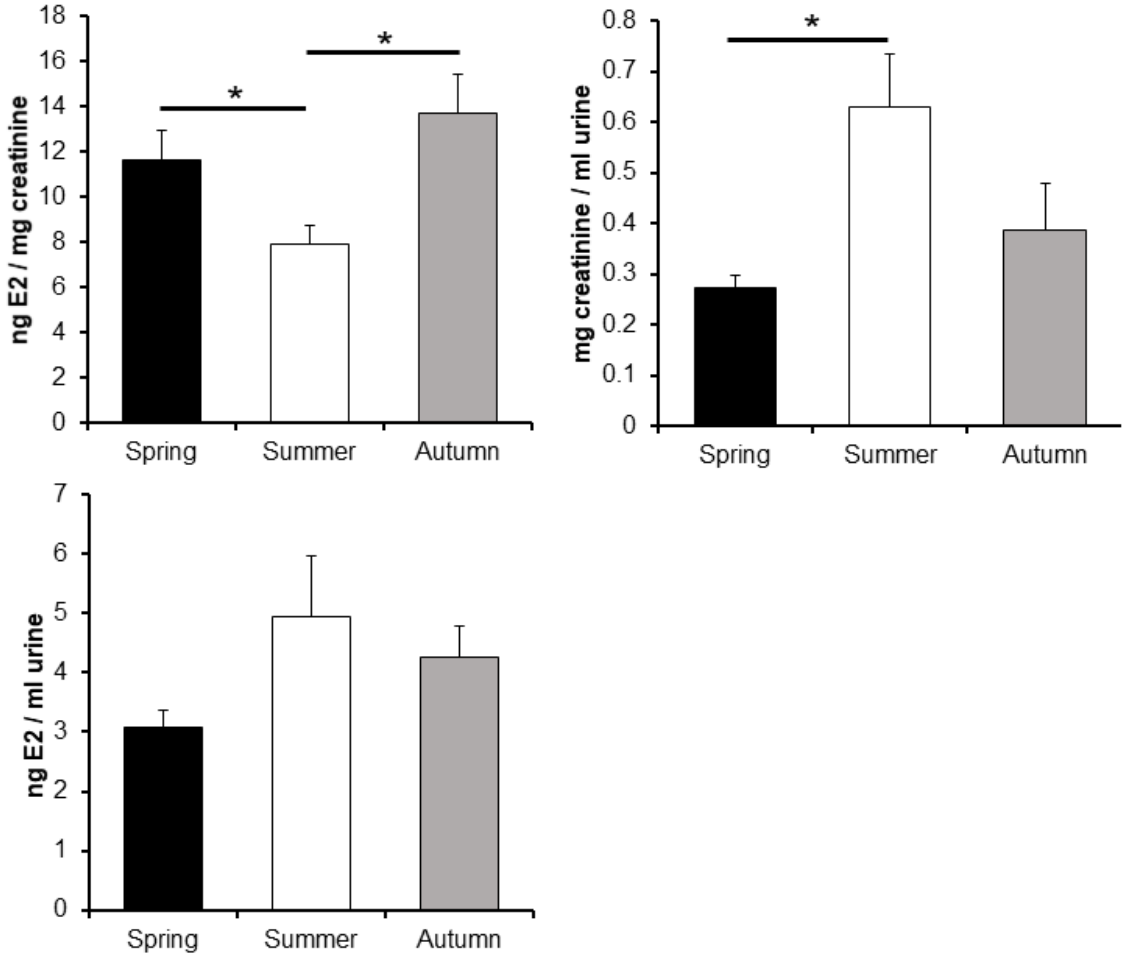
	Spring	Summer	Autumn
Olfactory Bulb	446 ± 167	206 ± 82	481 ± 92
Cerebellum	163 ± 71	214 ± 77	434 ± 75
Frontal Cortex	226 ± 32	223 ± 75	478 ± 89
Hypothalamus	211 ± 37	187 ± 49	435 ± 72
Heart	148 ± 43	105 ± 35	296 ± 47
Lung	248 ± 51	165 ± 29	307 ± 6
Muscle	293 ± 55	231 ± 29	523 ± 131
Adipose	120 ± 29	47 ± 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. 2.2 – Serially diluted urine samples from adult male bats bind to antibody in parallel with serially diluted samples from E_2 standards.



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. 2.3). 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.

Fig. 2.3 – 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.



2.5 – Discussion

This study demonstrates that male big brown bats transfer E_2 to female conspecifics differentially across the annual reproductive cycle. Transfer of ^3H-E_2 was greatest during the autumn and spring, which respectively correspond to the mating and ovulation/fertilization periods. Transfer of ^3H-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 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's 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 2.1) 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. 2.2, 2.3). 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 the wild, E_2 transfer would likely occur during male-female contact in seasonal roosting behavior of bats described above. Our measurements show that creatinine-adjusted urinary E_2 levels in male bats differed across seasons (Fig. 2.3). 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 ^3H-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.

2.6 – Acknowledgments

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2.7 – References

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

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.

Burnett, C.D., August, P. V, 1981. Time and energy budgets for dayroosting in a maternity colony of *Myotis lucifugus*. J. Mammal. 62, 758–766.

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.

Claus, R., Schopper, D., Wagner, H.-G., 1983. Seasonal effect on steroids in blood plasma and seminal plasma of boars. J. Steroid Biochem. 19, 725–729.

Couse, J.F., Lindzey, J., Grandien, K., Gustafsson, J.-Å., Korach, K.S., 1997. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 138, 4613–4621.

deCatanzaro, D., 2015. Sex steroids as pheromones in mammals : The exceptional role of estradiol. Horm. Behav. 68, 103–116. https://doi.org/10.1016/j.yhbeh.2014.08.003

deCatanzaro, D., Baptista, M.A.S., Spironello-Vella, E., 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., Beaton, E.A., Khan, A., Vella, E., 2006. Urinary oestradiol and testosterone levels from novel male mice approah values sufficient to disrupt early pregnancy in nearby inseminated females. Reproduction 132, 309–317. https://doi.org/10.1530/rep.1.00965

deCatanzaro, D., Khan, A., Berger, R.G., Lewis, E., 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. https://doi.org/10.1016/j.yhbeh.2008.10.013

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

deCatanzaro, D., Muir, C., Beaton, E.A., Jetha, M., 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69, 687–696. https://doi.org/10.1016/j.steroids.2004.07.002

deCatanzaro, D., Pollock, T., 2016. Absorption and distribution of estradiol from male seminal emissions during mating. J. Endocrinol. 231, 245–257.

deCatanzaro, D., Pollock, T., Greville, L.J., Faure, P.A., 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. https://doi.org/10.1016/j.ygcen.2014.09.011

Dodge, J.C., Kristal, M.B., Badura, L.L., 2002. Male-induced estrus synchronization in the female Siberian hamster (*Phodopus sungorus sungorus*). Physiol. Behav. 77, 227–231.

Eiler, H., Graves, C.N., 1977. Oestrogen content of semen and the effect of exogenous oestradiol-17β on the oestrogen and androgen concentration in semen and blood plasma of bulls. J. Reprod. Fertil. 50, 17–21.

Ferin, M., Tempone, A., Zimmering, P.E., Raymond, L.V.W., 2015. Effect of antibodies to 17β-Estradiol and progesterone on the estrous cycle of the rat. Endocrinology 85, 1070–1078.

Greiner, S., Schwarzenberger, F., Voigt, C.C., 2011. Predictable timing of oestrus in the tropical bat *Saccopteryx bilineata* living in a Costa Rican rain forest. J. Trop. Ecol. 27, 121–131. https://doi.org/10.1017/S0266467410000696

Greville, L.J., Pollock, T., Salter, J.C., Faure, P.A., deCatanzaro, D., 2017. Progesterone transfer among cohabitating female big brown bats (*Eptesicus fuscus*). Gen. Comp. Endocrinol. 247, 99–204. https://doi.org/10.1016/j.ygcen.2017.02.003

Guzzo, A.C., Jheon, J., Imtiaz, F., deCatanzaro, D., 2012. Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). Reproduction 143, 539–548. https://doi.org/10.1530/REP-11-0375

Guzzo, A.C., Pollock, T., deCatanzaro, D., 2013. Transfer of [3H]estradiol-17β and [3H]progesterone from conspecifics to cohabiting female mice. J. Endocrinol. 217, 1–10. https://doi.org/10.1530/JOE-12-0279

Kemeny, D.M., 1991. A Practical Guide to ELISA. Pergamon Press, Oxford, England.

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

Kurta, A., Baker, R.H., 1990. *Eptesicus fuscus*. Mamm. Species 1–10.

Ma, W., Song, H., Das, S.K., Paria, B.C., Dey, S.K., 2003. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Proc. Natl. Acad. Sci. 100, 2963–2968.

Meikle, A., Forsberg, M., Garófalo, E.G., Carlsson, M.A., Lundeheim, N., Rubianes, E., 2001. Circulating gonadotrophins and follicular dynamics in anestrous ewes after treatment with estradiol-17β. Anim. Reprod. Sci. 67, 79–90.

Mendonça, M., Chernetsky, S., Nester, K., Gardner, G., 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.

Muir, C., Spironello-Vella, E., Pisani, N., deCatanzaro, D., 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.

Ogasawara, Y., Okamoto, S., Kitamura, Y., Matsumoto, K., 1983. Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice, assayed by incorporation of [125I] iododeoxyuridine. Endocrinology 113, 582–587.

Ortiz, M.-E., Villalon, M., Croxatto, H.B., 1979. Ovum transport and fertility following postovulatory treatment with estradiol in rats. Biol. Reprod. 21, 1163–1167.

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

Pfaff, D.W., 1980. Estrogens and Brain Function. Springer-Verlag, New York, New York.

Pollock, T., Greville, L.J., Tang, B., deCatanzaro, D., 2016. Triclosan elevates estradiol levels in serum and tissues of cycling and peri-implantation female mice. Reprod. Toxicol. 65, 394–401. https://doi.org/10.1016/j.reprotox.2016.09.004

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

R Core Team. (2019). R: The R project for statistical computing. Available: https://www.r-project.org/

Racey, P.A., 1979. The prolonged storage and survival of spermatozoa in Chiroptera. J. Reprod. Fertil. 56, 391–402.

Reiffsteck, A., Dehennin, L., Scholler, R., 1982. Estrogens in seminal plasma or human and animal species: identification and quantitative estimation by gas chromatography-mass spectrometry associated with stable isotope dilution. J. Steroid Biochem. 17, 567–572.

Rivas-Munoz, R., Fitz-Rodriguez, G., Poindron, P., Malpaux, B., Delgadillo, J.A., 2007. Stimulation of estrous behavior in grazing female goats by continuous or discontinuous exposure to males 1, 2. J. Anim. Sci. 85, 1257–1263. https://doi.org/10.2527/jas.2006-115

Robker, R.L., Richards, J.S., 1998. Hormonal control of the cell cycle in ovarian cells: proliferation versus differentiation. Biol. Reprod. 59, 476–482.

Saksena, S.K., Lau, I.F., Chang, M.C., 1978. Estrogens in the seminal vesicle fluid and the effect of exogenous estradiol-17β on hormonal profile in the male rat. Biol. Reprod. 18, 521–526.

Saksena, S.K., Lau, I.F., Chang, M.C., 1977. Determination of seven unconjugated steroids in the blood and seminal plasma of the fertile male rabbit. Horm. Res. Paediatr. 8, 117–124.

Sar, M., Parikh, I., 1986. Immunohistochemical localization of estrogen receptor in rat brain, pituitary and uterus with monoclonal antibodies. J. Steroid Biochem. 24, 497–503.

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

Simerly, R.B., Chang, C., Muramatsu, M., Swanson, L.W., 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J. Comp. Neurol. 294, 76–95.

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

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

Thorpe, J.B., Gould, K.E., Borman, E.D., deCatanzaro, D., 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.

Valbuena, D., Martin, J., de Pablo, J.L., Remohi, J., Pellicer, A., Simon, C., 2001. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. Fertil. Steril. 76, 962–968.

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

Voigt, C.C., Schwarzenberger, F., 2008. Reproductive endocrinology of a small tropical bat (female *Saccopteryx bilineata*; Emballonuridae) monitored by fecal hormone metabolites. J. Mammal. 89, 50–57.

Vonhof, M.J., Barber, D., Fenton, M.B., Strobecks, C., 2006. A tale of two siblings: multiple paternity in big brown bats (*Eptesicus fuscus*) demonstrated using microsatellite markers. Mol. Ecol. 15, 241–247. https://doi.org/10.1111/j.1365-294X.2005.02801.x

Waites, G.M.H., Einer-Jensen, N., 1974. Collection and analysis of rete testis fluid from macaque monkeys. J. Reprod. Fertil. 41, 505–508.

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

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

Chapter 3 – Quantification of urinary sex steroids in the big brown bat (*Eptesicus fuscus*)

3.1 – Abstract

Hormone analysis in bats and other small mammals often occurs via blood draw and plasma analyses. Urinary steroid levels are frequently used in large mammals to track the reproductive state of individuals. Urinary steroid analysis is non-invasive and allows for repeated testing in small mammals. Here we quantify 17β-estradiol (E_2) in the urine of male and female big brown bats (*Eptesicus fuscus*) across their annual reproductive cycle in a captive breeding colony. Male bats had higher levels of urinary E_2 than female bats, and adults higher than yearlings, following creatinine adjustment for hydration levels. In non-pregnant females, a number of seasonal differences in both creatinine-adjusted and unadjusted urinary E_2 were observed. Male E_2 was higher than females in the winter in both conditions, as well as in autumn with creatinine-adjustment. Urinary progesterone (P_4) levels in non-pregnant female bats remained constant across seasons with the exception of unadjusted P_4 levels being higher in the summer compared to other seasons. In pregnant female bats we observed a peak in both urinary E_2 and P_4 beginning ~20 days prior to parturition, with both steroid hormones returning to baseline levels in the following weeks. Our findings are the first to track urinary steroid levels throughout the reproductive cycle of a bat species and are the first step towards using urinary analysis to detect pregnancy. The results also suggest a physiological delay of reproductive maturation in big brown bats. Importantly, the quantification of urinary steroid levels is critical in interpreting their roles in "primer pheromonal effects" in bats.

3.2 – Introduction

The reproductive cycle of temperate Vespertillionid bats follows an unusual de-coupling of mating prior to 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). Due to their unusual mating systems, bat reproductive physiology and endocrinology were highly studied in the mid 1900s, however there was a rapid decline in research towards 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 due to their similarities with human reproduction (Rasweiler et al., 2009; Rodrigues et al., 2019; Santiago et al., 2020).

Big brown bats (*Eptesicus fuscus*) are distributed throughout Central and North America (Kurta and Baker, 1990). This species displays promiscuous mating in the laboratory (Mendonça et al., 1996) and there is evidence of similar behaviour in the wild (Vonhof et al., 2006). Birthing patterns differ by geographical location with twins born in eastern North America and singlets born in the west (Kurta and Baker, 1990; O'Shea et al., 2010; Schowalter and Gunson, 1979). 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 favourable conditions, ovulation occurs in the spring following seasonal arousal with fertilization and

implantation soon thereafter (Christian, 1956; Oxberry, 1979; Racey, 1979; Wimsatt, 1944). Female *E. fuscus* display polyovulation releasing up to 5 ova each spring with excess embryos resorbed during early gestation (Wimsatt, 1945).

Reproductive females form summer maternity colonies in hollow trees and buildings, while males typically roost alone or in bachelor colonies (Kurta and Baker, 1990). Sex steroids have only been quantified in a handful of female bat species (reviewed in Martin and Bernard, 2000). 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, 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).

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 behaviour of conspecifics (deCatanzaro, 2015). The urine of male and female mice (*Mus musculus*) reliably contains bioactive sex steroids (deCatanzaro et al., 2009, 2006, 2004; Guzzo et al., 2013, 2012). Male urinary steroid levels are of particular interest due to their potential role in altering the sexual development and behaviour 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 17β-estradiol (E_2) directly to inseminated female mice (Bronson, 1975; deCatanzaro, 2015; deCatanzaro et al., 2006, 2001). 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). Such hormonal transfer may lead to physiological and behavioural changes in females (deCatanzaro, 2015). Together, these studies demonstrate that steroid hormones can explain phenomena traditionally labelled as primer pheromonal effects.

When adult male bats were injected with minute quantities of 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-to-female bats at higher levels during the fall mating and spring ovulatory periods in comparison to the summer non-reproductive season, and that it peaks in concentration at these same timepoints (Greville et al., 2020). In all cases, the highest levels of steroid transfer occurred in reproductive and neural tissues which have high steroid receptor densities and modulate

female reproductive physiology and behaviour (Kuiper et al., 1997). Sex steroids are critical for the proper development of female sexual behaviour and physiology, and these results suggest that they may not only act within the individuals whose glands produce them, but also potentially on proximate conspecifics.

The available evidence indicates that urine is a likely vector of E_2 transfer between bat conspecifics (deCatanzaro et al., 2014; Greville et al., 2020), as has been observed in mice (Guzzo et al., 2010, 2012). Due to its potential to induce pheromonal effects, 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 behaviour, we hypothesized females to have higher urinary E_2 levels than males. Secondary to E_2, we also measured urinary P_4 levels in the urine of female *E. fuscus* pending sample volume. This is because P_4 works in tandem with E_2 in regulating female reproductive physiology and has previously shown to have possible implications as a female pheromone (Freeman, 2006; Greville et al., 2017; Guzzo et al., 2012). We recently reported an increased level of creatinine-adjusted urinary E_2 in male *E. fuscus* during the autumn and spring compared to the summer (Greville et al., 2020). In the current experiment, we hypothesized the same seasonal pattern would be observed in 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 adult bats to 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 non-invasive repeated sample collection over an extended time period.

3.3 – Methods

3.3.1 – Animals and housing

Adult male and female *E. fuscus* were wild-caught in Southern Ontario and housed in a captive research colony at McMaster University where bats were permitted to fly (indoor and outdoor), had *ad libitum* access to food (mealworms; *Tenebrio molitor*) and water, and the indoor colony temperature and lighting varied with ambient conditions (see Skrinyer et al., 2017 for details). Juvenile bats were classified as yearlings until they reached one year old, after which they were considered adults. Newly captive animals, including pregnant females close to parturition, were kept for at least 2 weeks in an indoor holding room within an animal facility. These bats were housed in groups of 4 to 5 in stainless steel wire mesh (1/4" grid) holding cages (22 × 28 × 18 cm) prior to being introduced to a separate quarantine side of the colony. Captive breeding also 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.

3.3.2 – Chemicals

Estradiol and progesterone standards were obtained from Sigma-Aldrich, Oakville, ON, Canada. Steroid antibodies and HRP conjugates were obtained from the Department of Population Health and Reproduction at the University of California, Davis, CA, USA.

3.3.3 – Urine collection and analysis

We non-invasively 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 urinating. Urine was collected in 70µl hematocrit tubes (Fisherbrand, Pittsburgh, PA, USA) 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 non-pregnant females, n = 22 pregnant females. Note: 4 non-pregnant 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 non-pregnant female urine samples, and 47 pregnant female urine samples.

Urinary steroids were analyzed following modified enzyme immunoassay protocols previously established in the lab (deCatanzaro et al., 2004, 2003; Muir et al., 2001; Pollock et al., 2016, 2019) using dilutions validated for *E. fuscus* (Greville et al., 2020, 2017). Cross-reactivities for anti-E_2 (R2497) were: E_2 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0%, and all other measured steroids < 0.1% (Muir et al., 2001). The cross reactivity with E_2 conjugates including estradiol glucuronide and estradiol sulfate have not been reported. Using the same E_2 antibodies, Nunes et al. (2000) suggested the majority of urinary E_2 in male black tufted-eared 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, in comparison to non-hydrolyzed urine, in female hairy-nosed wombats (*Lasiorhinus latifrons*). While the ratio of conjugated to unconjugated E_2 in the urine of mammals may be species specific, hydrolysis experiments suggest the antibody used in our assay selectively measures E_2 in it's unconjugated form. However, the possibility of conjugate binding cannot be excluded.

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 non-pregnant females, n = 16 pregnant females). Cross reactivities for anti-P_4 were: progesterone 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%, estradiol < 0.001%, and estrone <0.04% (deCatanzaro et al., 2004, 2003).

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 ± standard error (SE) in both ng/mL urine and ng/mg creatinine. Briefly, 50 µL of distilled H_20, 0.75 N NaOH, and 0.4 N picric acid are added to 50 µL of diluted urine. Samples undergo the Jaffe reaction and become coloured with their optical density proportional to creatinine values. Optical density was measured 30 mins after chemical mixture. 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.

3.3.4 – Data analysis

Statistical analyses were conducted in the R software environment (R Core Team, 2020). We constructed individual linear mixed effect regression models (LMER) using the *lme4* package (Bates et al., 2015) to evaluate the dependent variables of unadjusted and creatinine-adjusted E_2, and creatinine. Given that urine was not collected from every animal on each collection date and 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 (i.e. simplest model accounting for maximum variance) was found. In all three cases, optimal models included sex by 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 progesterone analyses included season as a fixed effect and animal identity as a random effect.

F values for main effects are reported using a Type III sum of squares with Satterthwaite's method of calculating degrees of freedom. Apriori 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 familywise $\alpha = 0.05$ (Benjamini and Hochberg, 1995). Seasons were defined by months based on variations in the outdoor temperature of the colony: Spring (March-May); Summer (June-August); Autumn (September-November); and Winter (December-February).

3.4 – 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 1 male with urinary E_2 levels of 1509 ng/mL urine was discarded from the analysis because this outlier data point was so far above the highest standard that it was deemed unreliable.

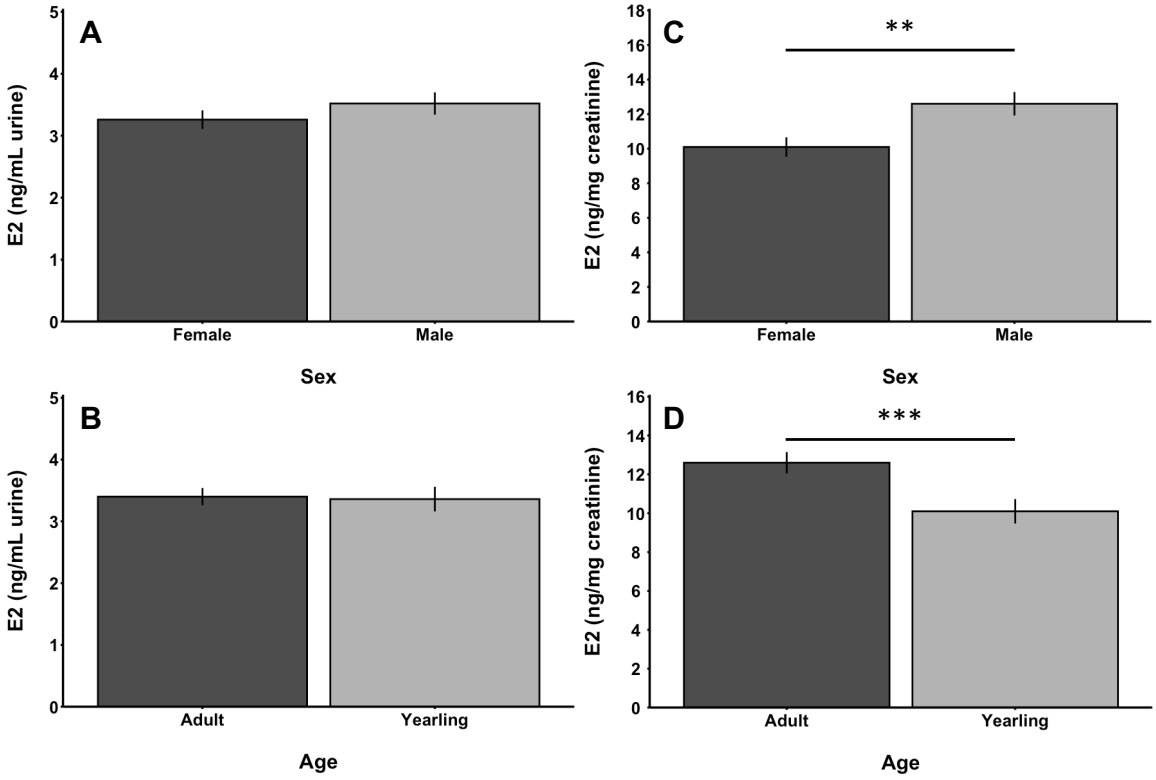
Table 3.1 summarizes the main effects from LMER analysis for unadjusted and creatinine-adjusted steroids, as well as creatinine measurements. There was no effect of sex on unadjusted E_2 levels ($F(1, 118.73) = 1.58$, $p = 0.211$) (Fig 3.1A). Significant effects of season ($F(3, 168.44) = 7.08$, $p < 0.001$) and sex by season interaction ($F(3, 1160.13) = 7.77$, $p < 0.001$) 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 season are shown in Fig 3.2. Unadjusted E_2 differed across season 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 3.2). While age was included in the initial maximal LMER model, it was not part of the optimal model and thus had no effect on unadjusted E_2 measurements (Fig. 3.1B).

Creatinine-adjusted E_2 was significantly higher in males than females ($F(1, 125.73) = 9.32$, $p = 0.003$) (Fig 3.1C) and in adults compared to yearlings ($F(1, 253.31) = 10.72$, $p = 0.001$) (Fig 3.1D). There were also an effects of season ($F(3, 180.02) = 7.01$,

Table 3.1 – Summary of variance components for different physiological measurements. Analysis of variance table with Type III sum of squares (SS) using Satterthwaite approximation for degrees of freedom. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Physiological Measurement	Source of Variation	Numerator DF	Denominator DF	Type III SS	Mean Square	F-value	*p*-value
Unadjusted E_2	Sex	1	118.73	.58	.58	1.58	0.211
	Season	3	168.44	7.83	2.61	7.08	<0.001***
	Sex × Season	3	1160.13	8.58	2.86	7.77	<0.001***
Creatinine-adjusted E_2	Age	1	253.31	4.75	4.75	10.72	0.001***
	Sex	1	125.73	4.13	4.13	9.32	0.003**
	Season	3	180.02	9.32	3.11	7.01	<0.001***
	Sex × Season	3	1164.53	17.46	5.82	13.13	<0.001***
Unadjusted P_4	Season	3	97.15	2.02	.67	4.09	0.009**
Creatinine-adjusted P_4	Season	3	98.81	.51	.17	.71	0.548
Creatinine	Age	1	132.69	3.59	3.59	8.29	0.005**
	Sex	1	103.66	1.42	1.42	3.27	0.073
	Season	3	186.98	4.85	1.62	3.73	0.012*
	Sex × Season	3	1109.18	5.25	1.75	4.05	0.007**

Fig 3.1 – Age and sex comparisons of urinary estradiol. Geometric mean ± SE concentrations of urinary estradiol, expressed as ng E_2/mL urine (*A* and *B*) and ng E_2/mg creatinine (*C* and *D*), of varying sex and age produced from the optimal linear mixed effect regression models after correction for animal id and date of urine collection. *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$



$p < 0.001$) and sex by season interaction ($F(1,1164.53) = 13.13$, $p < 0.001$). 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, $p < 0.001$; summer-winter, $p < 0.001$) and in males (autumn-winter, $p = 0.030$) (Fig 3.2). 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 non-pregnant individuals was analyzed for P_4. Seasonal differences in urinary P_4 concentrations of non-pregnant female bats are shown in Table 3.2, with main effect of season shown in Table 3.1. 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. 3.3). These data were standardized by the date of parturition (Day 0) and reported for up to 41 days prior to and 44 days after giving birth. The dataset consists of 47 urine samples from 22 pregnant female bats (1 to 7 samples per individual). Owing to the small sample size and variability in the measurements, no statistical comparisons were performed on these data. Descriptively,

Fig. 3.2 – Seasonal comparison of urinary estradiol in female and male bats. Geometric mean ± SE concentrations of urinary estradiol, expressed as ng E_2/mL urine and ng E_2/mg creatinine, of female male bats across 4 seasons. Geometric means are produced from the optimal linear mixed effect regression models after correction for animal id and date of urine collection. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

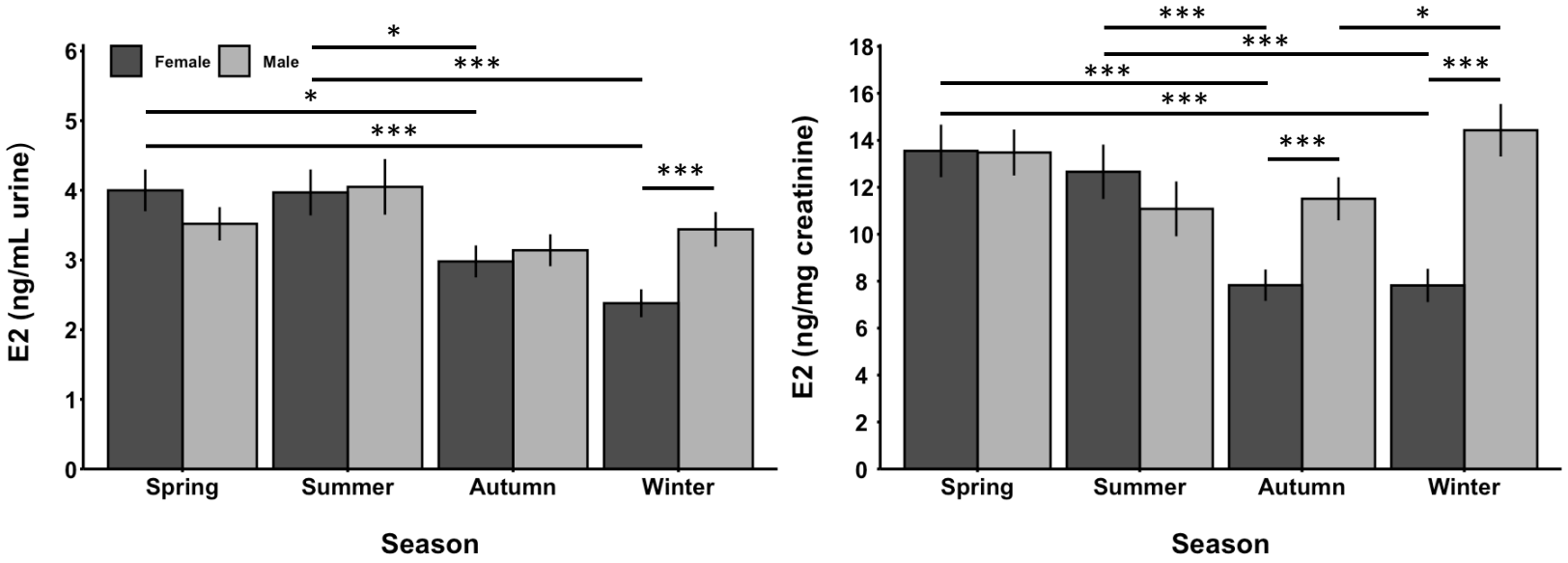
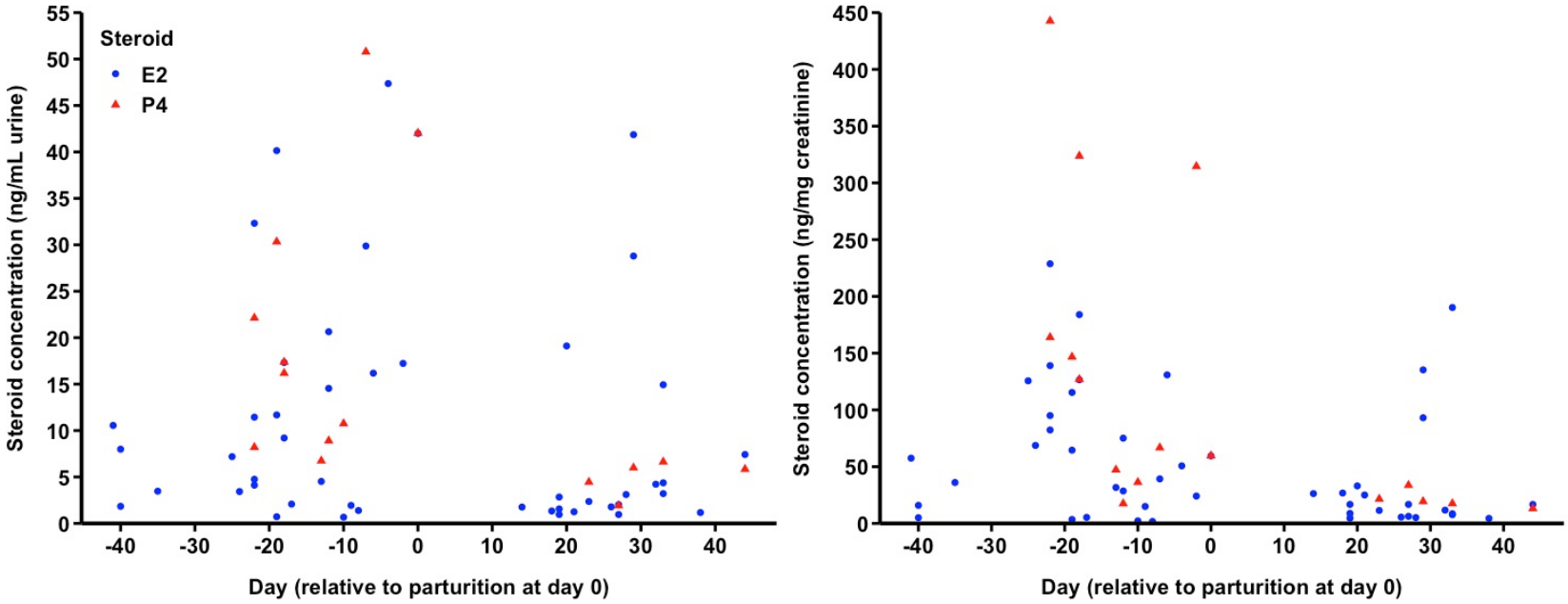


Table 3.2 – Geometric mean ± SE progesterone (P_4) levels for non-pregnant female bats in different seasons produced from the optimal linear mixed effect regression models after correction for animal id and date of urine collection. Both unadjusted and creatinine-adjusted (cre) levels are reported. Total number of animals that provided a sample for a given season and number of urine samples in each season are provided. † denotes seasons that differed statistically from summer in unadjusted P_4 ($p < 0.05$).

Season	Number of Animals	Number of Samples	Unadjusted P_4 (ng/mL)		Adjusted P_4 (ng/mg cre)	
			Mean	*SE*	Mean	*SE*
Autumn	12	21	4.98†	0.57	15.6	2.19
Spring	19	37	5.29†	0.49	17.4	1.99
Summer	13	15	7.61	0.97	20.7	3.27
Winter	14	35	4.47†	0.44	16.6	2.05

Fig. 3.3 – Steroid levels of pregnant bats. Concentrations of estradiol (E_2; circles ●) and progesterone (P_4; triangles ▲), expressed as ng steroid/mL urine and ng steroid/mg creatinine, in urine samples from pregnant mothers during the peri-parturition period. Figure does not include one unadjusted P_4 level which measured 224.98 ng/mL at -2 PND.



both creatinine-adjusted and unadjusted levels of steroids began to rise approximately 25 days prior to parturition, with creatinine-adjusted steroid levels decreasing immediately prior to birth. Following birth, both creatinine-adjusted and unadjusted steroid levels returned to the range typically measured in non-pregnant females by approximately 17 days post-partum. 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.S1 shows the geometric mean of creatinine measurements for all sex by season interactions. Bat urinary creatinine levels did not differ across sex ($F(1, 103.66) = 3.27$, $p = 0.073$; $\bar{x}_m = 0.28 \pm 0.01$, $\bar{x}_f = 0.31 \pm 0.01$), but were higher in yearlings compared to adults ($F(1, 132.69) = 8.29$, $p = 0.005$; $\bar{x}_a = 0.271 \pm 0.01$, $\bar{x}_y = 0.32 \pm 0.02$). Main effects of season ($F(3, 186.98) = 03.73$, $p = 0.012$) and sex by season interaction were also observed ($F(3, 1109.18) = 4.05$, $p = 0.007$). 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; $p = 0.025$; summer-winter, $p = 0.004$). A sex difference was seen during the autumn ($p = 0.006$).

Table 3.S1. – Geometric mean creatinine measurements (mg/mL) in female and male bats across seasons.

Sex	**Season**	**Creatinine (mg/mL)**	
		Mean	*SE*
Female	Spring	0.282	0.02
	Summer	0.306	0.03
	Autumn	0.37	0.03
	Winter	0.292	0.02
Male	Spring	0.262	0.02
	Summer	0.363	0.04
	Autumn	0.273	0.02
	Winter	0.237	0.02

3.5 – Discussion

Our study was designed to document changes in urinary E_2 levels in male and female big brown bats (*E. 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 non-invasively 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 non-pregnant females, and quantified variation in urinary steroids during the peri-parturition 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. 3.2). The highest levels of E_2 were observed in the spring for both creatinine-adjusted and unadjusted measurements which were slightly higher than the summer. The lowest levels of unadjusted-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 E_2 levels during mid-gestation (e.g. Damassa and Gustafson, 1984; Oxberry, 1979), which would be during the spring for *E. fuscus*. Parturition in wild *E. fuscus* occurs in late spring or early summer, with E_2 levels rising prior to and possibly following birth (Fig. 3.3). Surprisingly, female E_2 levels drop

in the autumn which is the time when *E. fuscus* exhibit swarming and mating behaviour (Kurta and Baker, 1990; Fig. 3.2). Average E_2 measures during the autumn were either slightly higher (unadjusted) or equal to (creatinine-adjusted) winter E_2 levels (Fig. 3.2). These findings align with previous studies showing that ovariectomy and the subsequent decrease in E_2 does not influence the mating behaviour of female *E. fuscus* (Mendonça et al., 1996).

Estrogen plasma levels for female *E. fuscus* reach as high as 250 pg/mL during October and decreased to 100 pg/mL in 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. 3.2). Mean creatinine-adjusted E_2 was equal in winter and autumn, but showed a drop prior to the winter season in Mendonça et al.'s (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 sapien*; 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 2–3 orders of magnitude higher than blood plasma which often has low or undetectable levels (Daels et al., 1991; Wasser et al., 1996). While the majority of urinary steroids are excreted in conjugated form, a vast majority of plasma steroids are bound to transport proteins such as sex hormone binding globulin (SHBG)

which render them biologically inactive and may not be quantifiable in assays. Indeed, SHBG fluctuates seasonally in both sexes of a temperate hibernating bat (Gustafson and Damassa 1985; Kwiecinski et al. 1991). As such, plasma SHBG binding results in lower assay measurements. Given that urinary E_2 reflects circulatory dynamics in lab mice (*Mus musculus*; Thorpe et al., 2014), we believe the elevation in urinary E_2 we observed in comparison to plasma levels in past studies on bats are 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 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*, 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 also were found in the spring prior to estrogen levels rising during pregnancy and reaching a peak of ~90 pg/mL at mid gestation, then fell rapidly following parturition (Oxberry, 1979). In the greater Asiatic yellow bat *Scotophilus heathii* baseline levels of E_2 were 50 pg/mL during periods of reproductive quiescence and ovulatory delay, with a peak at ~300 pg/mL during estrus and declining to ~200 pg/mL during the pre-ovulatory period (Abhilasha and Krishna, 1996). In our study the reproductive stage of female *E. fuscus* was not closely monitored during urine collection; however, post-hoc review allowed us to label urine collected from females that later gave birth. Collectively, our

data collected in captivity show similar seasonal patterns to those previously described for wild Vespertilionid bats with elevated E_2 levels in the summer prior to estrus, followed by a drop during the autumn mating season and into the winter (Fig. 3.2). Arousal from hibernation in the spring was associated with a substantial rise in E_2 that peaks in the summer in non-reproductive females. Parous females have a slight rise in E_2 starting ~40 days pre-partum and peaking ~20-25 days prior to birth (Fig. 3.3). To minimize handling stress we chose not to collect urine in peri-parous individuals immediately leading up to and following parturition; but E_2 levels had returned to baseline by ~14-20 days post-partum. 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 E_2 peaks during pregnancy followed by a decline during lactation (Burns and Wallace, 1975; Crichton et al., 1989; Crichton and Krutzsch, 1987).

We also quantified urinary P_4 levels in a subset of females to establish baseline measurements in *E. fuscus* (Table 3.2). Similarly to E_2, urinary P_4 concentrations were higher than previously reported plasma levels although only moderately (Mendonça et al., 1996). In non-reproductive females unadjusted P_4 levels remained low and did not differ in the winter, spring, and autumn (4.5 to 5.3 ng/mL), whereas summer P_4 levels (7.6 ng/mL) differed significantly from the other seasons (Table 3.1). A similar pattern was observed in creatinine-adjusted P_4 measurements, albeit without statistical differences. These P_4 urinary levels are similar to plasma levels of non-reproductive females in other Vespertilionid bats (Abhilasha and Krishna, 1996; Buchanan and Younglai, 1988; Hosken et al., 1996; Oxberry, 1979). In pregnant female *E. fuscus* unadjusted P_4 levels were at a

minimum (~8 ng/mL) 20 days before giving birth, whereas individual measurements reached ~30, 40, and 50 ng/mL immediately prior to parturition (Fig. 3.3). Moreover, adjusted P_4 levels were much higher when compared to non-pregnant 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 non-pregnant 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 hour 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 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 E_2 levels between the sexes (Fig. 3.1A). This suggests that males and females secrete E_2 in equal volumetric concentrations (i.e. per mL of urine). Because males typically had lower creatinine levels than females (Table 3.S1) this resulted in higher creatinine-adjusted urinary E_2 levels compared to non-reproductive adult females (Fig. 3.1C). This finding is in opposition to the typical convention that females have higher E_2 than males. 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 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 E_2 was significantly higher in adult *E. fuscus* compared to yearlings (Fig. 3.1D). 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 (Barclay, 2012; O'Shea et al., 2010; Sidner, 1997) 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 steroid levels across males and females differed with age, with adults having higher levels of creatinine-adjusted E_2 than yearlings (Fig. 3.1D). Since only a subset of male and female yearlings acquire a fully developed reproductive system in their first year of life (Barclay, 2012; Christian, 1956), 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 yearling and adult *E. fuscus* differ in their steroid production and/or excretion.

Our lab recently reported the first E_2 levels in male bats demonstrating that creatinine-adjusted E_2 levels in adult males were higher in the spring and autumn compared to the summer (Greville et al., 2020). These results explained additional findings that the increased transfer of radiolabelled-estradiol from male-to-female *E. fuscus* in autumn and spring compared to 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 the winter and autumn (Fig. 3.2). 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. Androgen levels in temperate male bats begin to rise in early spring following hibernation and peak in the late summer or early autumn prior to mating and the culmination of spermatogenesis (Gustafson, 1979; Racey, 1974). 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, 1979; Gustafson and Shemesh, 1976). While the unadjusted urinary E_2 follows this pattern of androgen production in males, creatinine-adjusted levels are highest in the winter (Fig. 3.2). Without work correlating urinary and plasma steroids, it cannot be said whether unadjusted or creatinine-adjusted E_2 best follows plasma steroid levels in time. Results of such

correlations may align or contradict with 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).

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 (Boeniger et al., 1993; Erb et al., 1967; Muir et al., 2001; Munro et al., 1991). 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 to the summer when they are most active. Our data clearly demonstrate creatinine to be dynamic in bats, varying with age and seasonal environmental in both sexes. Accordingly, we believe it important to present and evaluate changes in both creatinine-adjusted and unadjusted urinary steroid levels and encourage future studies to do the same.

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 typically 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 non-pregnant 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 non-reproductive. Both past studies and our data suggest circulating steroid peak in mid-pregnancy and the possibility of fetus resorption should not confound our results.

Urinary steroid analysis offers a non-invasive 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.

3.6 – Acknowledgements

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3.7 – References

Abhilasha, Krishna, A., 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., Yamada, S., Takatsu, Y., Hisanori, M., Kinoshita, M., Takase, K., Sugiura, H., Ohtaki, T., Matsumoto, H., Uenoyama, Y., Tsukarmura, H., Inoue, K., Maeda, K.-I., 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.

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

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

Boeniger, M.F., Lowry, L.K., Rosenberg, J., 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.

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

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

Burns, J.M., Wallace, E., 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., Krutzsch, P.H., 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., Seamark, R.F., Krutzsch, P.H., 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., Blake, M., Wimsatt, W., 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., Ammon, D.C., Stabenfeldt, G.H., Liue, I.K.M., Hughes, J.P., Lasley, B.L., 1991. Urinary and plasma estrogen conjugates, estradiol and estrone concentrations in nonpregnant and early pregnant mares. Theriogenology 35, 1001–1017.

Damassa, D.A., Gustafson, A.W., 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., Barbour, R.W., Hassell, M.D., 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., Baptista, M.A.S., Spironello-Vella, E., 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., Beaton, E.A., Khan, A., Vella, E., 2006. Urinary oestradiol and testosterone levels from novel male mice approah values sufficient to disrupt early pregnancy in nearby inseminated females. Reproduction 132, 309–317.

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

deCatanzaro, D., Muir, C., Beaton, E.A., Jetha, M., 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., Pollock, T., Greville, L.J., Faure, P.A., 2014b. 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., Tillson, S., Hodgen, G., Plotka, E., 1967. Urinary creatinine as an index compound for estimating rate of excretion of steroids in the domestic sow. J. Anim. Sci. 30, 79–85.

Freeman, M.E., 2006. Neuroendocrine control of the ovarian cycle of the rat, in: Neill, J.D. (Ed.), Knobil and Neill's Physiology of Reproduction. Academic Press/Elsevier, San Diego, CA, pp. 2327–2387.

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

Greville, L.J., Pollock, T., Salter, J.C., Faure, P.A., deCatanzaro, D., 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., Damassa, D.A., 1985. Annual variations in plasma sex steroid-binding protein and testosterone concentrations in adult male little brown bats: relation to the asynchronous recrudence of the testis and accessory reproductive organs. Biol Reprod. 33, 1126–1137.

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

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

Hakim, R.B., Grayb, R.H., Zacurc, H.A., 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., Leathard, H.L., Coley, J., 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., Möstl, E., Hodges, J., 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.

Hodges, J.K., Brand, H., Henderson, C., Kelly, R.W., 1983. Levels of circulating and

urinary oestrogens during pregnancy in the marmoset monkey (*Callithrix jacchus*). J. Reprod. Fertil. 67, 73–82.

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

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

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

Kwiecinski, G.G., Damassa, D.A., Gustafson, A.W., 1991. Patterns of plasma sex hormone-binding globulin, thyroxine and thyroxine-binding globulin in relation to reproductive state abd hibernation in female little brown bats. J. Endocrinol. 128, 63–70.

Martin, L., Bernard, R.T.F., 2000. Endocrine Regulation of Reproduction in Bats: the Role of Circulating Gonadal Hormones, in: Crichton, E.G., Krutzsch, P.H. (Eds.), Reproductive Biology of Bats. Academic Press, London, UK, pp. 27–64.

Mendonça, M., Chernetsky, S., Nester, K., Gardner, G., 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., Hopkins, W.A., 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., Coley, J., Gani, M.M., Perry, P.W., Talbot, D., Aspinall, L.J., 2004. Comparison between creatinine and pregnanediol adjustments in the retrospective analysis of urinary hormone profiles during the human menstrual cycle. Clin. Chmica Acta 42, 1043–1050.

Muir, C., Spironello-Vella, E., Pisani, N., deCatanzaro, D., 2001. Enzyme immunoassay of 17 β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Horm. Metab. Reserch 33, 653–658.

Munro, C.J., Stabenfeldt, G.H., Cragun, J.R., Addiego, L.A., Overstreet, J.W., Lasley, B.L., 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.

Nunes, S., Fite, J.E., French, J.A., 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., Ellison, L.E., Neubaum, D.J., Neubaum, M.A., Reynolds, C.A., Bowen, R.A., 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.

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

Pollock, T., Greville, L.J., Tang, B., deCatanzaro, D., 2016. Triclosan elevates estradiol

levels in serum and tissues of cycling and peri-implantation female mice. Reprod. Toxicol. 65, 394–401.

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

Racey, P.A., 1979. The prolonged storage and survival of spermatozoa in Chiroptera. J. Reprod. Fertil. 56, 391–402.

Racey, P.A., 1974. The Reproductive Cycle in male noctule bats, *Nyctalus Noctula*. J. Reprod. Fertil. 41, 169–182.

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

Rasweiler, John J, I., Cretekos, C.J., Behringer, R.R., 2009. The short-tailed fruit bat *Carollia perspicillata*: A Model for Studies in Reproduction and Development, in: emerging model organisms. Cold Spring Harbor, New York, pp. 519–555.

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

Schowalter, D.B., Gunson, J.R., 1979. Reproductive biology of the big brown bat

(*Eptesicus fusus*) in Alberta. Can. F. Nat. 93, 48–54.

Seki, K., 1985. determined by haemagglutination inhibition reaction and serum oestradiol levels determined by radioimmunoassay Sj. Acta Endocrinol. (Copenh). 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*. The University of Arizona.

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

Stukenholtz, E.E., Perez-Torres, J., Stevens, R.D., 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., Johnston, S.D., Janssen, T.M., Lisle, A., Keeley, T., 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.

Thorpe, J.B., Gould, K.E., Borman, E.D., deCatanzaro, D., 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.

Vonhof, M.J., Barber, D., Fenton, M.B., Strobecks, C., 2006. A tale of two siblings:

multiple paternity in big brown bats (*Eptesicus fuscus*) demonstrated using microsatellite markers. Mol. Ecol. 15, 241–247.

Wasser, S.K., Papageorge, S., Foley, C., Brown, J.L., 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.

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.

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

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

Chapter 4 – Evaluating odour and urinary sex preferences in the big brown bat

4.1 – Abstract

Effective communication is essential for the maintenance of cohesion in bat colonies, which can contain hundreds or even thousands of individuals. Several studies have shown that odour cues can provide information about species, colony membership, and identity of a bat; however, the extent to which odour cues convey information about sex remains unclear. Because big brown bats (*Eptesicus fuscus*) live in mixed-sex colonies, during the mating season the ability to differentiate between sexes is necessary to locate a mate. Experiment 1 aimed to examine whether *E. fuscus* prefer the odour of a given sex over the other. Adult *E. fuscus* were recorded moving freely within a Y-maze containing multiple chemical cues from a male and a female conspecific. One group of subjects was habituated to the maze prior to experimental trials, whereas a second group was neither introduced nor habituated to the maze prior to the experiment. The behaviour of the bats in the Y-maze and the proportion of time spent near each scent were used as preference indicators for the odour of a particular sex. Interestingly, non-habituated animals in Experiment 1 explored the maze to a greater degree and were more likely to approach a stimulus scent than bats that had been habituated to the arena. Experiment 2 followed similar procedures except the odour cue to be discriminated was the urine of male or female conspecifics and none of the subjects were habituated to the maze prior to testing. The results for Experiment 1 found no evidence that *E. fuscus* prefer the mixture of odours of a given sex. The results for Experiment 2 found that females preferred the odour of male urine. These findings have important implications for courting and mating behavior in bats, as well for designing future behavioural studies with bats.

4.2 – Introduction

The ability to perceive and respond to chemicals in the environment is widespread across taxa, including ancient bacteria. In ancestral organisms, specialized cells are responsible for the perception of chemical signals, whereas evolutionarily recent vertebrates response to chemicals is facilitated by highly organized olfactory organs that have evolved to detect important olfactory cues (Surov & Maltsev, 2016). This process is known as olfactory communication, whereby a chemical released into the environment by an individual is detected by the olfactory system of another individual. In mammals, an individual's age, health, dominance and/or reproductive status, sex, identity, and group membership can be communicated/delineated by olfactory signals (Caspers, et al., 2008; Eisenberg & Kleiman, 1972). This also holds true in bats, Order Chiroptera. Olfactory signaling is equally effective in the light and dark as chemical signals persist in the environment and can span longer distances than many auditory and visual cues. Thus, like most social nocturnal animals, olfactory communication is integral in the lives of bats.

Temperate and vespertilionid bat species often display fission-fusion dynamics within roosts (review by Patriquin & Ratcliffe, 2016). As such, effective communication is essential for the maintenance of cohesion in social groups within bat colonies (Chaverri et al., 2018). Currently, the majority of bat communication studies focus on auditory information (Chaverri et al., 2018); however, olfactory signaling may be as important as acoustic signaling in bat social communication. This is evidenced by bats having large olfactory bulbs (Dechmann & Safi, 2005) and diverse glands and nasal structures that allow them to produce and perceive smells (Haffner, 2000; Voigt et al., 2008).

Given that chemical cues carry a range of individualistic information, it is unsurprising that animals use odour to discriminate sex. The widespread ability to assess sex by olfaction has been observed in across mammalian taxa, including mice (Bowers & Alexander, 1967), beavers (Cross et al., 2014), black-tailed deer (Müller-Schwarze, 1971), lions (Gilfillan et al., 2017), giant pandas (Swaisgood et al., 2000), and koalas (Charlton, 2014). In solitary species where individuals have infrequent contact with conspecifics outside of the mating season, olfactory discrimination of sex allows animals to locate mating partners more efficiently over a long distance (Müller-Schwarze, 1971). In social bat species, which can roost in large mixed-sex colonies, the ability to distinguish between sexes via olfaction has been shown to facilitate the location of suitable mates (Bouchard, 2001). Consistent with this idea, the Angolan free-tailed bat (*Mops condylurus*) and the little free-tailed bat (*Chaerephon pumilus*) can distinguish between sexes using scents from the muzzle gland and the histologically sexually dimorphic interaural glands (Bouchard, 2001).

The temperate insectivorous big brown bat, *Eptesicus fuscus*, is found throughout the Americas (Kurta & Baker, 1990). Outside mating and hibernation, *E. fuscus* roost in colonies segregated by sex: adult females form maternity colonies in the spring to give birth and raise young (Kurta & Baker, 1990); adult males remain solitary or form all-male roosting groups until mating season (Barbour & Davis, 1969). Roosting with conspecifics also helps individual bats to thermoregulate (Willis & Brigham, 2007). Past studies have shown *E. fuscus* can differentiate between sexes using echolocation calls and males prefer the echolocation calls of high frequency copulatory females (Grilliott et al., 2015; Kazial

& Masters, 2004). It is unknown if *E. fuscus* also use odour for sex discrimination. Because *E. fuscus* live in large colonies and are most active at night when visibility is low, olfactory cues are likely important for sex discrimination. Furthermore, *E. fuscus* are sexually monomorphic except for a slight body size difference (Kazial & Masters, 2004; Mayberry & Faure, 2015).

By allowing animals to freely explore a Y-maze with male and female odours in the stimuli arms, we assessed whether individual *E. fuscus* exhibited a preference for either sex. We used both body odours and urine as olfactory cues. Except for numerous auditory and echolocation psychophysical studies that routinely employ this method (for a review, see Moss & Schnitzler 1995), Y-maze (or T-maze) behavioural testing is less commonly used in behavioural studies with bats, and when used there are varied procedures (e.g. Bartonička et al., 2010; de Fanis & Jones, 1995; Kilgour et. al., 2013). For example, not all Y-maze studies habituate bats to the arena before the start of testing trials, or do so by different methods. Thus, we also examined the effect of habituation on the exploratory behaviour of bats in a Y-maze.

4.3 – Methods

4.3.1 – Animal husbandry

Captive big brown bats (*Eptesicus fuscus*) used in this study were either wild-caught in Southern Ontario or were the direct descendants of these wild-caught individuals. Bats were housed indoors (2.5 x 1.5 x 2.3 m; l x w x h) in a captive research colony at McMaster University where the temperature and lighting varied with ambient conditions, and bats had access to an outdoor flying area (2.5 x 3.8 x 2.7 m) (Skrinyer et al., 2017). Within the colony, bats had *ad libitum* access to water and mealworms (*Tenebrio molitor*). Animals selected for experiments were removed from the colony and housed indoor in stainless steel ¼ mesh cages (28 x 22 x 18 cm) in a holding room. All experimental procedures were approved by the Animal Research Ethics Board of McMaster University, and conformed to the Guidelines for the Care and Use of Animals in Research outlined by the Canadian Council on Animal Care.

4.3.2 – Experiment 1: Evaluating Olfactory Odour Preference

Experiment 1 assessed whether male and female bats showed a preference for the body odour of conspecifics of either sex. The experiment was conducted in two parts (Experiment 1A and 1B) to evaluate whether habituation to the test arena impacted the behavioural response.

Adult bats (>1 year) from the colony were pseudo-randomly selected based on age, relation to other selected bats, and whether they were currently involved in other lab experiments. These animals were held overnight in a holding room 1 day prior to testing. A subgroup of these bats were designated as “focal” bats and were housed together. A

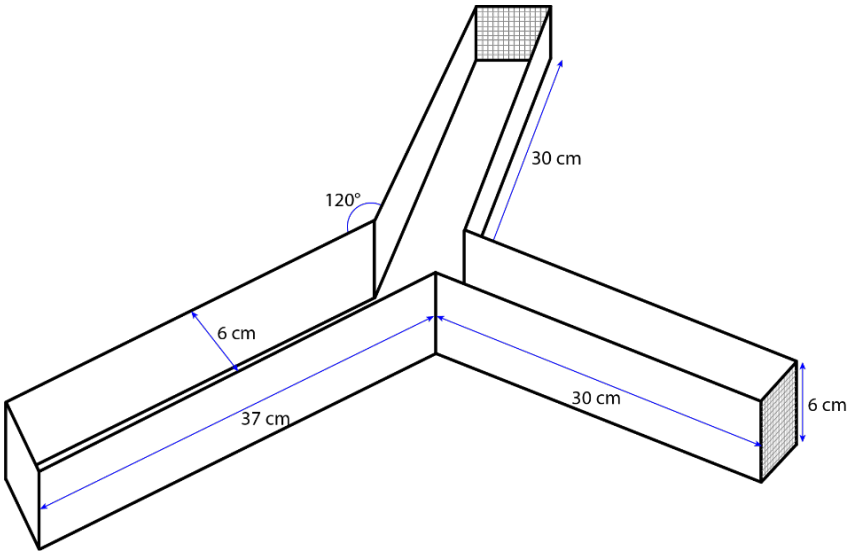
second subgroup were designated as "odour" (or stimulus) bats and were housed in two cages isolated from each other and segregated by sex to prevent the mixing of sex-specific body odours. The holding room was held at room temperature (20°C) and lights were programmed to follow the ambient light/dark cycle for Hamilton, ON. None of the animals had experience with Y-maze testing prior to the experiment.

4.3.3 – Experimental design, set-up, and odour collection

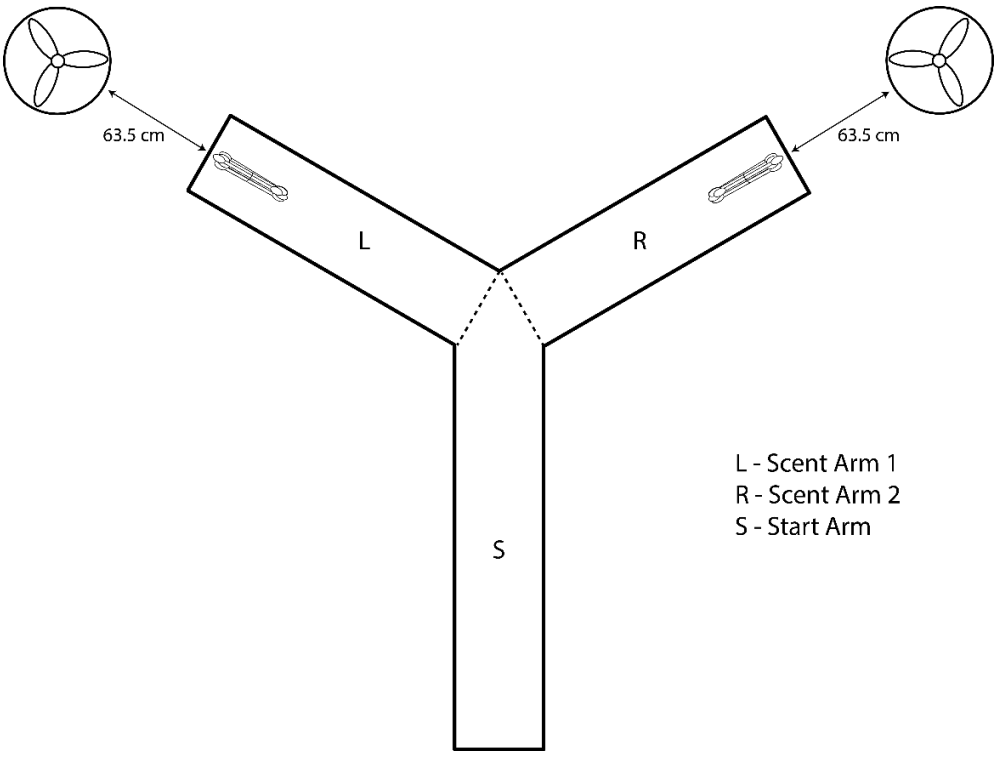
Experiments were conducted during the autumn mating season and occurred in the evening between 2000-2400 hrs, coinciding with the start of the bat's natural diurnal activity period. Trials were run in a dimly lit room (4.85 x 3.25 x 3.32 m) and walls were lined with sound-attenuating foam (Sonex® Classic; Pinta Acoustic, USA). At the start of each trial a focal bat was placed in the start arm of a plexiglass Y-maze with a transparent cover (Fig. 4.1A) and was permitted to freely move in the maze towards odours from one female and one male bat placed at the end of the left and right arms (Fig. 4.1B). The dimensions of the Y-maze were based on previous behavioural studies conducted on *E. fuscus* and *T. brasiliensis* (Bloss et al., 2002; Gustin & McCracken, 1987; Kilgour, Faure, & Brigham, 2013). Individuals served as a focal bat and as an odour stimulus bat for a male or female focal bat no more than once between experiments 1A and 1B. Focal bats were never exposed to their own odour. At the end of testing, all animals were returned to

Figure 4. 1 – Y-maze behavioural testing arena. **A** Side-view schematic of the Y-maze constructed with plexiglass, exception for the ends of each test arm which were covered with ¼-inch stainless steel mesh to permit airflow through maze arms. **B** Top-view schematic of the experimental set-up. Cotton swab bundles infused with the scent of stimulus bats were taped to the floor at each end of their assigned arms. At the beginning of a trial, the plexiglass cover of the Y-maze was lifted and the test bat was placed into the start arm furthest from the Y-junction. *Dashed lines* represent two invisible boundaries that a test bat needed to completely cross to be scored as having entered a specific arm.

A


120°
30 cm
6 cm
37 cm
30 cm
6 cm

B


63.5 cm
63.5 cm
L
R
S
L - Scent Arm 1
R - Scent Arm 2
S - Start Arm

the colony for at least 36 h before serving a different role in a separate trial. Because all bats were housed in the same captive colony prior to the experiment, individuals were assumed to have had equal opportunity to interact with each other, thus minimizing potential bias towards any scent stimulus.

Body odour was collected exclusively from adult animals. Experimenters collecting odours wore clean latex gloves which were changed each time an animal or odour were handled to avoid cross-contamination with both bat and/or human scents. Each end of a cotton swab was manually swiped ten times, in a cephalic to caudal direction, with a consistent stroke length and pressure. Odours were collected from five body areas of stimulus bats using a clean cotton swab for each area: the muzzle, dorsum (between shoulder blades), ventrum (chest area), plagiopatagium of the right wing, and anogenital area (both the genitals and anus). These regions were selected due to past evidence of sexually dimorphic glands and self-anointed scents in Chiroptera (e.g. Bouchard, 2001; Flores & Page, 2017; Gustin & McCracken, 1987; Munoz-Romo et al., 2011). Swabs that became contaminated with saliva, urine, or feces were discarded and a new swab was used to re-collect the sample from the given area. Following sample collection, the five cotton swabs for a stimulus bat were bound together with a twist-tie and immediately stored in an airtight Ziploc bag marked with the date, time, sex and ID of the stimulus animal. Odours were used within one hour of collection with majority used within 10 minutes.

Bats were pseudo-randomly assigned to be either a focal or stimulus animal. Each odour presented to stimulus bats was pseudo-randomly assigned to either the left or right

arm of a Y-maze, counterbalancing for sex. We created a scent trail for focal animals to follow by applying one scent to one arm of a Y-maze arm as follows: (1) the bundle of odour swabs was removed from its Ziploc bag; (2) one end of the bundle was pressed and dragged across the floor of the Y-maze arm beginning from the end of the test arm closest to the junction of the Y; (3) the bundle was placed at the end of the test arm with the dragged end pointing toward the end of the arm; (4) swabs were secured to the floor of the maze with clear scotch tape. This procedure was repeated for each swab bundle in its designated arm. Battery-operated fans were placed ~63.5 cm away from the mesh end of each test arm and were turned to their lowest setting to gently move stimulus bat scents down the arm and toward the junction of the Y-maze.

All focal bats were active prior to entering the maze. Focal bats were placed at the end of the "start" arm with the bat's head facing the Y-junction (Fig. 4.1B). Trials began when the experimenter released the bat, at which point we immediately slid the clear cover over the maze and exited the room for the remainder of the trial. Pilot testing with male and females revealed that bats explored the full Y-maze in 3 to 4 mins, hence we decided upon a 5 min trial length per focal animal. At the end of testing, bats were returned to their holding cage and eventually the colony. Each Y-maze and its plexiglass cover were thoroughly cleaned with 70% ethanol, rinsed with distilled water, and dried with paper towel before they were used in a subsequent trial.

4.3.4 – Experiment 1A: Evaluating olfactory odour preference – habituation trials

Thirty bats (n = 15 females) were used as focal animals in the experiment. Each animal was allowed to freely explore the Y-maze for 10 mins approximately 5 to 6 h prior to experimental testing. Habituation trials followed identical protocols as experimental trials except no odour bundles were placed in the maze. Following habituation, each focal bat was housed individually without food or water to minimize its exposure to odours. Each bat later served as a focal bat for odour preference testing in the Y-maze arena. None of the animals had experience with Y-maze testing prior to *Experiment 1A*.

4.3.5 – Experiment 1B: Evaluating olfactory odour preference – non-habituated trials

The aim of *Experiment 1B* was to investigate the effects of habituation on Y-maze behavioural testing. Thirty six bats (n = 18 females) participated as focal animals in odour preference testing but without being habituated to the Y-maze (see *Experiment 1A*). Five to 6 h prior to testing, each focal bat was housed individually without food or water to minimize its exposure to odours. These animals later acted as focal bats in experimental trials identical to those conducted in *Experiment 1A*. None of the animals had experience with Y-maze testing prior to *Experiment 1B*.

4.3.6 – Quantification of trials

Experimental trials were recorded with a GoPro HERO5 Black camera (GoPro, San Mateo, California, USA) secured to a tripod ~63.5 cm above the maze. The camera was remotely operated with an Apple iPad (Apple Inc., Cupertino, California, USA). Videos of the 5-min trials were scored by an observer blind to the experimental condition. Trials were quantified with respect to the total time that a focal bat spent in each arm of

the Y-maze (i.e. the left or right test arms, and the start arm). A bat was deemed as having entered an arm when its head and body were completely within the arm (see *dashed lines* in Fig. 4.1B). We also scored the first and last arm entered by the focal bat at the end of the 5 min trial. Qualitative observations regarding the behaviour (e.g. patterns of movement within and between maze arms) of each bat were also documented.

4.3.7 – Experiment 2: Evaluating Urinary Preference

Experiment 2 aimed to evaluate whether individual *E. fuscus* showed a preference for the odour of male and/or female urine. As in Experiment 1, we did not evaluate urine as a repellent as the implications of urinary repellent do not align with the ecology of *E. fuscus*. Thirty four bats (n = 19 males, 15 females) were used as focal animals. Of these animals, all 15 females and 13 of 19 males were adults (>1 year old), with the remaining 6 males as yearlings (~5 to 6 months old). Given that yearlings have been observed to mate within our captive colony (Greville, personal observation), we expected them to behave similarly as adults in response to conspecific urine. All but 6 of the adult males, who had participated in Experiment 1 a year prior, were naïve to Y-maze testing.

Experimental procedures—including animal housing, isolation, testing procedures, video observations, data and statistical analyses—were identical to those of Experiment 1B except we used urine as the odour stimulant as opposed to body scents. We collected urine from male and female bats non-invasively following previously published procedures (Greville et al. 2017, 2020). Briefly, adult bats were quickly grabbed and held over a wax-paper lined work surface until they urinated. Urine was collected via pipette, stored in 0.5 mL vials marked with the date, animal ID, and sex, and

frozen at -20° C until the time of the experiment. All urine was collected during the autumn mating season and used within 1 month of collection. Urine was thawed and vortexed prior to a 10 μL aliquot being pipetted onto a 5 cm diameter circular filter paper (Whatman plc, Maidstone, United Kingdom) placed at the start of a stimulus arm closest to the Y-junction. We then created a scent trail by dragging the filter paper along the floor of the plexiglass maze to the end of the stimulus arm.

4.3.8 – Data analysis

The sex of the focal and stimulus bats were decoded prior to statistical analysis. Data from each focal bat was organized into: (1) total time spent in the male-scented, female-scented, and start arms; (2) the stimulus arm the bat first entered (i.e. first choice); and (3) the stimulus arm the bat last entered when the trial ended (i.e. final choice). We also measured the proportion of male/female focal bats that first entered a male- or female-scented arm, or never left the start arm. The time spent by each focal bat in male-scented, female-scented, and neutral start arms was also recorded. A focal bat's preference for the scent of a male or female stimulus bat was determined using three threshold criteria based on time: absolute time, 30 s, and 60 s. Threshold for each criterion was met when a bat spent a greater amount of time, >30 s, and/or > 60 s in one stimulus arm *versus* the other, respectively.

A binomial test was used to analyze the preference of focal bats for the odour of conspecifics of the same or opposite sex based on first and final scent arms chosen, and by the difference in time spent in each scented arm based on the three thresholds. Trials in which the focal bat did not meet one of the timed threshold criteria or never left the start

arm were treated as a tie and excluded from the analysis (Bouchard 2001; Gustin & McCracken, 1987; Siegel, 1956). To examine the effect of habituation, a chi-squared test of independence was used to compare the total proportion of habituated and non-habituated bats that entered a stimulus arm. All statistical tests were performed in Jamovi (The Jamovi Project, 2020) running in the R software environment (R Core Team, 2020).

4.4 – Results

4.4.1 – General observations

Focal bats exhibited a variety of behaviours in the Y-maze. At the start of a trial, some animals exhibited freezing behaviour and never left the start position. Other bats examined their environment and immediately crawled and began exploring the maze. At the Y-junction point of the maze, some bats stopped and appeared to sniff or echolocate in the direction of each scent arm before crawling into one of them. Other bats did not stop and crawled quickly into a stimulus arm without showing behaviour indicating prior assessment. Some animals remained in the initial stimulus arm they entered, while others consistently explored the entire maze.

4.4.2 – Influence of habituation

The effect of habituation on Y-maze exploration by bats was evaluated by comparing the number of animals that entered at least one stimulus arm in Experiment 1A and 1B. There was a significant decrease in the total number of habituated animals that entered a stimulus arm ($X^2_1 = 6.87$, $P = 0.009$). Although a greater proportion of bats from both sexes explored at least one stimulus arm in the absence of habituation, this effect was largely driven by the behaviour of males ($X^2_1 = 5.66$, $P = 0.017$) as opposed to females ($X^2_1 = 2.42$, $P = 0.120$ for females). Table 4.1 reports the proportion of animals not entering a stimulus arm along with the respective chi-squared and p-value.

Table 4.1 – Summary of comparisons between Experiments 1A and 1B summarizing results obtained by chi-squared tests of independence comparing the proportions of habituated *versus* non-habituated focal bats (*n* = 15 habituated animals per sex; n = 18 non-habituated animals per sex) that did not enter either stimulus arm ("no choice").

Group	Proportion of no choice (habituated)	Proportion of no choice (non-habituated)	X^2 (1)	*p*-value
Males	0.800	0.389	5.66	0.017
Females	0.333	0.111	2.42	0.120
All	0.567	0.250	6.87	0.009

4.4.3 – Experiment 1A: Evaluating olfactory odour preference – habituation

A large proportion of focal bats habituated to the testing arena did not leave the starting/neutral arm of the Y-maze, with some never leaving their initial start position. Indeed, the majority of habituated test bats remained in the start arm for the entire experimental trial: 5 of 15 (33%) females and 12 of 15 (80%) males. Moreover, a majority of bats were found in the start arm at the end of a trial: 11 of 15 (73%) females and 12 of 15 (80%) males. Neither male nor female odours were preferred by focal animals of either sex (Table 4.2).

4.4.4 – Experiment 1B: Evaluating olfactory odour preference – non-habituated

The majority of non-habituated female (89%) and male (61%) focal bats actively explored the Y-maze and entered a stimulus arm. However, neither male nor female body odours were preferred by either sex (Table 4.3).

4.4.5 – Experiment 2: Evaluating urinary preference

Approximately 93% of female and 53% of male focal bats explored the Y-maze and entered a stimulus arm. Male focal bats showed no preference for the urinary odours of either sex (Table 4.4). However, female focal bats exhibited a preference for the odour of male urine (binomial test, $P = 0.013$; Table 4.4). Although more female focal bats were in the male urinary stimulus arm as opposed to the female urinary arm at the end of a trial, and females preferred the male arm using all preference criteria, none of these comparisons reached statistical significance (Table 4.4).

Table 4.2 – *Summary of Experiment 1A: Evaluating olfactory odour preference – habituation*. Initial and last choices of focal bats (n = 15 per sex) exploring a stimulus arm containing body odour from male and female conspecifics, and odour preferences for a given sex using absolute time, > 30 s, and > 60 s thresholds. Reported values are the number of individuals who met specific criteria. *p*-values were calculated using a binomial test.

Variable	Female scent	Male scent	Neither scent	*p*-value
Female focal bats				
First choice	7	3	5	0.344
Final choice	0	4	11	0.125
Absolute preference	3	7	5	0.344
> 30 s preference	1	5	9	0.212
> 60 s preference	1	4	10	0.375
Male focal bats				
First choice	1	2	12	1.00
Final choice	1	2	12	1.00
Absolute preference	1	2	12	1.00
> 30 s preference	1	2	12	1.00
> 60 s preference	1	2	12	1.00

Table 4.3 – *Summary of Experiment 1B: Evaluating olfactory odour preference – non-habituated*. Initial and last choices of focal bats (n = 18 per sex) exploring a stimulus arm containing body odour from male and female conspecifics, and odour preferences for a given sex using absolute time, > 30 s, and > 60 s thresholds. Reported values are the number of individuals who met specific criteria. *p*-values were calculated using a binomial test.

Variable	Female scent	Male scent	Neither scent	*p*-value
Female focal bats				
First choice	8	8	2	1.00
Final choice	3	3	12	1.00
Absolute preference	8	8	2	1.00
> 30 s preference	5	5	8	1.00
> 60 s preference	4	4	10	1.00
Male focal bats				
First choice	5	6	7	1.00
Final choice	1	2	15	1.00
Absolute preference	4	7	7	0.549
> 30 s preference	3	3	12	1.00
> 60 s preference	1	2	15	1.00

Table 4.4 – *Summary of Experiment 2: Evaluating urinary preference.* Initial and last choices of focal bats (n = 15 female, n = 19 male) exploring a stimulus arm containing 10 μL urine from male and female conspecifics, and odour preferences for a given sex using absolute time, > 30 s, and > 60 s thresholds. Reported values are the number of individuals who met specific criteria. *p*-values were calculated using a binomial test.

Variable	Female urine	Male urine	Neither urine	*p*-value
Female focal bats				
First choice	2	12	1	0.013
Final choice	1	7	7	0.070
Absolute preference	3	11	1	0.057
> 30 s preference	3	9	3	0.146
> 60 s preference	3	8	4	0.227
Male focal bats				
First choice	4	6	9	0.754
Final choice	3	2	14	1.00
Absolute preference	5	5	9	1.00
> 30 s preference	4	2	13	0.689
> 60 s preference	3	2	14	1.00

4.5 – Discussion

In our study we found no evidence in *E. fuscus* that general body odours/scents serve as an attractant to conspecifics; however, our data indicate that female focal bats were initially attracted to the scent of male urine. Interestingly, males did not show a preference for the scent of urine of either sex. Additionally, we showed that habituating bats prior to testing them in a plexiglass Y-maze arena decreased the likelihood of bat's exploring the maze in subsequent test trials.

Across bat species, olfactory cues provide contextual information to an individual, including territory delineation, mate quality, and individual recognition (Boss, 1999; Doss et al., 2016; Keeley & Keeley, 2004; Voigt & von Helversen, 1999). Thus far, the ability to discriminate between sexes by scent has only been demonstrated in two species of bats, *M. condylurus* and *C. pumilus* (Bouchard, 2001). The interaural crest of *C. pumilus* is sexually dimorphic and only present in males; both male and female *M. condylurus* have interaural crests. In both species, males have more sebaceous glands under the interaural epidermis than females, but no sexual dimorphism exists in the muzzle glands (Bouchard, 2001). In the present study, we found no evidence that *E. fuscus* distinguished between the sexes during the mating season using body scents of conspecifics (Tables 4.2 & 4.3). While Bouchard's (2001) study focused on muzzle scents and those from the sexually dimorphic interaural crests, our study used a combination of scent cues from distinct anatomical regions of individuals, but we did not collect scents from the interaural region. Perhaps the lack of physical and glandular sexual dimorphism in *E. fuscus* explains why they did not exhibit the ability to discriminate conspecific sex based on body scent as

observed in *M. condylurus* and *C. pumilus* (Bouchard, 2001). Previously, Bloss et al. (2002) found that *E. fuscus* could differentiate roost-mates from individuals of a different colony; however, big brown bats pups do not differentiate the odour of their mother from other individuals (Mayberry & Faure, 2015). Altogether, studies of odour discrimination suggest *E. fuscus* may still differentiate odours on a group level, perhaps influenced by a colony's unique microclimate or microbiota, but not individual odour differences.

Interestingly, female focal bats were attracted to the urine of male conspecifics, but male focal bats showed no preference for urinary odours from either sex (Experiment 2, Table 4.4). Females also spent more time in the stimulus arm containing male urine; however, this preference did not reach statistical significance. Nonetheless, these results suggest that urine may contain chemical cues that influence an animal's behaviour, and that these cues are not found in general body scents, as evidenced by the results of Experiment 1 in which body excretions were actively avoided during scent collection. The idea that urinary odours influence behaviour is not novel as chemicals present in urine and feces allow for sex discrimination in a variety of mammals, including ferrets (Zhang et al., 2005), meadow voles (Ferkin & Johnston, 1995), and mice (Zhang et al., 2007). Mouse urine contains a high concentration of communication proteins called major urinary proteins (MUPs) which vary in composition between individuals (Hurst, 2009). Because the types of MUP isoforms present in mouse urine is influenced by genotype and sex, this provides individual's with an unique signature that may convey sexual information to conspecifics. Additionally, pheromones in mouse urine differ between sexes and this may serve as an indicator of sex identity (Zhang et al., 2007). Recently,

steroids naturally found in the urine of mice and rats where shown to act as attractants when made volatile (Takács et al., 2017).

To date, urinary pheromones and MUPs have not been extensively researched in bats. Previous studies have shown bioactive steroids to be reliably present in the urine of male and female *E. fuscus* (Greville et al. 2017, 2020), and these steroids can be cutaneously absorbed by conspecifics (deCatanzaro et al., 2014; Greville et al., 2017, 2020). Experiment 2 of the current study demonstrates that *E. fuscus* females approach male urine. As to whether this is due to urinary steroids, MUPs, or other chemical components needs further investigation, as does whether females are attracted to male urine or deterred by female urine.

The lack of preference by females to remain in proximity to male urine in Experiment 2, as measured by the bat's location at the end of the trial as well as absolute time, >30 s, and >60 s timing thresholds (Table 4.4), suggests that olfactory cues alone may not be sufficient for sex discrimination. It is possible urine may act as an initial attractant but the presence of an individual or a secondary cue may be important to distinguish between sexes. Video observations showed that bats thoroughly explored the urine-laced filter paper, and this may have motivated them to continue exploring other regions of the maze in search of a conspecific. Likewise, the body scents we tested in Experiment 1 may only provide information about an individual in combination with a secondary cue, such as acoustic signals (e.g. vocalizations). Acoustic signaling is essential for successful mating in several bat species as individuals may emit social calls to attract females or advertise their presence through complex songs (Chaverri et al.,

2018; Knörnschild et al., 2012). For example, the simultaneous use of odour and mating calls has been demonstrated in male *S. bilineata*, which spread chemicals by flapping their wings while emitting a complex ultrasonic song to attract females (Voigt et al., 2008). Although the use of songs and mating calls has not been observed in *E. fuscus*, there is evidence that females can recognize sex based on variation in echolocation calls (Kazial & Masters, 2004) similar to horseshoe bats (Schumann et al., 2012). These observations support the idea that a combination of sensory cues may be required for bats to distinguish an individual's sex.

Olfactory cues are used in differing contexts across species including scent marking for navigation (*Myotis lucifugus*; Buchler, 1980) and territory markings (*Saccopteryx bilineata*; Voigt & von Helversen, 1999). Male Indian short-nosed bats (*Cynopterus sphinx*) and Mexican free-tailed bats (*Tadarida brasiliensis*) frequently mark their roosts with saliva and gular gland secretions, respectively, during the mating season to attract mates and increase mating success (Doss et al., 2016; Keeley & Keeley, 2004). To date, the ecological implications of urinary attraction as well as the urinary patterns of *E. fuscus* have not been studied. Whether male *E. fuscus* urinate in specific locations within a roost and whether they continually add urine to such locations to attract females for mating remains unknown.

A comparison between Experiments 1A and 1B showed that focal bats were significantly more likely to enter a stimulus arm if they had not been habituated to the arena prior to the experimental trial (Table 4.1). Behavioural studies with bats typically habituate subjects to the test arena to minimize the effects of stress and novel exploratory

behaviour during the experiment using one of two protocols: (1) the bat is allowed to freely explore the arena prior to the trial (e.g. Caspers et al., 2009; Gustin & McCracken, 1987; Kilgour et al., 2013) or (2) the bat is confined to the starting chamber within the arena prior to the trial (e.g. Bartonička et al., 2010; Bouchard, 2001; Boyer, 2018; Mayberry & Faure, 2015). In Experiment 1A, bats were allowed to freely explore the entire Y-maze during habituation trials and this may have decreased their motivation to re-explore the maze during experimental testing. Studies on exploratory behaviour in rats have found a decline in exploratory behaviour over repeated exposures to a testing arena (Berlyne, 1955; Ehrlich, 1959). Although not systematically quantified prior to conducting our study, similar effects have been noted in bats. Boyles and Storm (2007) noted that "bats roosted in the starting chamber if held there too long", while wild bats in Bouchard (2001) entered torpor when habituated for too long. Thus, future behavioural studies with bats should consider foregoing a habituation period prior to testing, limiting habituation time, or acclimating with a starting chamber as opposed to free exploration.

Behavioural observations during our trials revealed wide variation in both the degree to which test bats explored the Y-maze and their patterns of movement between the three arms. This variation may be attributed to individual differences in the personalities of bats. Personality, defined as individual differences in behaviour that remain consistent over time and across contexts (Menzies et al., 2013), has been quantified in several animals including rodents (Martin & Réale, 2008), songbirds (Verbeek et al., 1994), and fish (Yoshida et al., 2005). Thus far, only three studies have investigated personality (or behavioural syndromes) in bats. Nonetheless, they have

provided strong support for the existence of personality traits in *M. lucifugus* and *E. fuscus* (Boyer, 2018; Kilgour & Brigham, 2013; Menzies et al., 2013). Tests assessing the exploratory behaviour of *E. fuscus* found they behaved consistently across repeated trials throughout the study period (Boyer, 2018). The exploratory behaviour of individual *E. fuscus* was unaffected by changes in social group composition, suggesting this behaviour is relatively unchanging. This may be due to genetic influences on personality and exploratory behaviour, which has been demonstrated in mice (DeFries, Gervais, & Thomas, 1978) but not yet in bats.

Previously, Bouchard (2001) and Bloss et al. (2002) classified individuals as being from different colonies if they exited different buildings or were from different day roosts, respectively. Given *M. condylurus* and *C. pumilus* are roost-faithful species, this is a reasonable assumption (Bouchard, 2001). Bloss et al. (2002) report only 4 individual *E. fuscus* (out of hundreds banded) recaptured at a colony other than that of initial capture. However, *E. fuscus* display fission-fusion social dynamics where colony members alternate roost sites within a region with subgroup composition changing (e.g. Metheny et al., 2008; Willis & Brigham, 2004). Given that big brown bats travel multiple kilometers while foraging and frequently use night roosts that differ from day roosts (Brigham, 1991), it is not unreasonable that wild individuals caught at different sites may be familiar with each other. In our current study animals were all from the same captive colony and thus had past experiences with each other. Due to the natural sociality of *E. fuscus* across roost sites in combination with the fact that conspecifics may use olfactory cues for

identification, we believe the current experimental design using individuals from a single colony still provides valuable insight into olfactory sex discrimination.

Given our results on the effects of maze habituation, the attractant property of male urine to female bats must be evaluated in the context of a novel environment. It is possible that under familiar conditions, the same findings would not hold true. Additionally, the current experiment was designed to evaluate the innate preference of bats for a stimulus without training. de Fanis and Jones (1995) trained common pipistrelle bats (*Pippistrellus pipistrellus*) to reliably discriminate between the odours of individual colony members and the odours of a home *versus* a foreign roost using food reinforcement over repeated trials. Since *E. fuscus* can distinguish between individuals of their colony and strangers (Bloss et al., 2002), it remains likely the species could be trained to discriminate the sex of a stimulus animal from body or urine odours despite the present results. Furthermore, although the bats in both Experiments 1 and 2 explored and reacted to the Y-maze uniquely, our study was not designed to quantify such behavioural differences. Future research should systematically evaluate how personality types of bats influence an individual's exploratory behaviour during experimental testing with Y-mazes.

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4.7 – References

Barbour, R.W., Davis, W.H., 1969. *Bats of America*. Lexington: University Press of Kentucky.

Bartonička, T., Kaňuch, P., Bímová, B., Bryja, J., 2010. Olfactory discrimination between two cryptic species of bats *Pipistrellus pipistrellus* and *P. pygmaeus*. Folia. Zool. 59, 175–182.

Berlyne, D.E., 1955. The arousal and satiation of perceptual curiosity in the rat. J. Comp. Physiol. Psych. 48, 238–246.

Bloss, J., 1999. Olfaction and the use of chemical signals in bats. Acta. Chiropterol. 1, 31–45.

Bloss, J., Acree, T.E., Bloss, J.M., Hood, W.R., Kunz, T.H., 2002. Potential use of chemical cues for colony-mate recognition in the big brown bat, *Eptesicus fuscus*. J. Chem. Ecol. 28, 819–834.

Bouchard, S., 2001. Sex discrimination and roostmate recognition by olfactory cues in the African bats, *Mops condylurus* and *Chaerephon pumilus* (Chiroptera: Molossidae). J. Zool. 254, 109–117.

Bowers, J.M., Alexander, B.K., 1967. Mice: individual recognition by olfactory cues. Science. 158, 1208–1210.

Boyer, A., 2018. Personality and behavioural syndromes of the big brown bat, *Eptesicus fuscus* (Master of Science). Fargo, ND: North Dakota State University.

Boyles, J., Storm, J., 2007. Avoidance of predator chemical cues by bats: an experimental assessment. Behaviour. 144, 1019–1032.

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

Buchler, E.R., 1980. Evidence for the use of a scent post by *Myotis lucifugus*. J. Mammal. 61, 525–528.

Caspers, B.A., Schroeder, F.C., Franke, S., Streich, W.J., Voigt, C.C., 2009. Odour-based species recognition in two sympatric species of sac-winged bats (*Saccopteryx bilineata, S. leptura*): combining chemical analyses, behavioural observations and odour preference tests. Behav. Ecol. Sociobiol. 63, 741–749.

Caspers, B.A., Franke, S., Voigt, C.C., 2008. The wing-sac odour of male greater sac-winged bats *Saccopteryx bilineata* (Emballonuridae) as a composite trait: seasonal and individual differences. In J. L. Hurst, R. J. Beynon, S. C. Roberts, & T. D. Wyatt (Eds.), *Chemical Signals in Vertebrates 11* (pp. 151–160). New York: Springer.

Charlton, B.D., 2014. Discrimination of sex and reproductive state in koalas, *Phascolarctos cinereus*, using chemical cues in urine. Anim. Behav. 91, 119–125.

Chaverri, G., Ancillotto, L., Russo, D., 2018. Social communication in bats: Communication in bats. Biol. Rev. 93, 1938–1954.

Cross, H.B., Zedrosser, A., Nevin, O., Rosell, F., 2014. Sex discrimination via anal gland secretion in a territorial monogamous mammal. Ethology. 120, 1044–1052.

de Fanis, E., Jones, G., 1995. The role of odour in the discrimination of conspecifics by pipistrelle bats. Anim. Behav. 49, 835–839.

deCatanzaro, D., Pollock, T., Greville, L.J., Faure, P.A., 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.

Dechmann, D., Safi, K., 2005. Studying communication in bats. Cogn. Brain. Behav. 9, 479–496.

DeFries, J.C., Gervais, M.C., Thomas, E.A., 1978. Response to 30 generations of selection for open-field activity in laboratory mice. Behav. Genet. 8, 3–13.

Doss, D.P.S., Nagarajan-Radha, V., Kandula, S., 2016. Daily and seasonal variation in non-acoustic communicative behaviors of male greater short-nosed fruit bats (*Cynopterus sphinx*). Acta. Chiropterol. 18, 199–207.

Ehrlich, A., 1959. Effects of past experience on exploratory behaviour in rats. Can. J. Psychol. 13, 248–254.

Eisenberg, J., Kleiman, D., 1972. Olfactory communication in mammals. Annu. Rev. Ecol. Syst. 3, 1–32.

Ferkin, M.H., Johnston, R.E., 1995. Meadow voles, *Microtus pennsylvanicus*, use multiple sources of scent for sex recognition. Anim. Behav. 49, 37–44.

Flores, V., Page, R.A., 2017. Novel odorous crust on the forearm of reproductive male fringe-lipped bats (*Trachops cirrhosus*). J. Mammal. 98, 1568–1577.

Gilfillan, G.D., Vitale, J.D.T., McNutt, J.W., McComb, K., 2017. Spontaneous discrimination of urine odours in wild African lions, *Panthera leo*. Anim. Behav. 126, 177–185.

Greville, L.J., Pollock, T., Salter, J.C., Faure, P.A., deCatanzaro, D., 2017. Progesterone transfer among cohabitating female big brown bats (*Eptesicus fuscus*). Gen. Comp. Endocrinol. 247*,* 99–204.

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

Grilliot, M.E., Burnett, S.C., Mendonça, M.T., 2015. Choice experiments demonstrate that male big brown bats (*Eptesicus fuscus*) prefer echolocation calls of high high copulatory females. Acta. Chiropterol. 17, 411–417.

Gustin, M.K., McCracken, G.F., 1987. Scent recognition between females and pups in the bat *Tadarida brasiliensis mexicana*. Anim. Behav. 35, 13–19.

Haffner, M., 2000. Structure and function of pilosebaceous units on the heads of small mammals (Insectivora, Chiroptera, Rodentia): Pilosebaceous units on the heads of small mammals. Acta. Zool-Stockholm. 81, 195–203.

Hurst, J.L., 2009. Female recognition and assessment of males through scent. Behav. Brain. Res. 200, 295–303.

Kazial, K.A., Masters, W.M., 2004. Female big brown bats, *Eptesicus fuscus*, recognize sex from a caller's echolocation signals. Anim. Behav. 67, 855–863.

Keeley, A.T.H., Keeley, B.W., 2004. The mating system of *Tadarida brasiliensis* (Chiroptera: Molossidae) in a large highway bridge colony. J. Mammal. 85, 113–119.

Kilgour, R.J., Brigham, R.M., 2013. The relationships between behavioural categories and social influences in the gregarious big brown bat (*Eptesicus fuscus*). Ethology. 119, 189–198.

Kilgour, R.J., Faure, P.A., Brigham, R.M., 2013. Evidence of social preferences in big brown bats (*Eptesicus fuscus*). Can. J. Zool. 91, 756–760.

Knörnschild, M., Jung, K., Nagy, M., Metz, M., Kalko, E., 2012. Bat echolocation calls facilitate social communication. P. R. Soc. B. 279, 4827–4835.

Kurta, A., Baker, R.H., 1990. *Eptesicus fuscus*. Mamm. Species. (356), 1-10.

Martin, J.G.A., Réale, D., 2008. Temperament, risk assessment and habituation to novelty in eastern chipmunks, *Tamias striatus*. Anim. Behav. 75, 309–318.

Mayberry, H.W., Faure, P.A., 2015. Morphological, olfactory, and vocal development in big brown bats. Biol. Open. 4, 22–34.

Menzies, A.K., Timonin, M.E., McGuire, L.P., Willis, C.K.R., 2013. Personality variation in little brown bats. Plos. One. 8, e80230.

Metheny, J.D., Kalcounis-Rueppel, M.C., Willis, C.K.R., Kolar, K.A., Brigham, R.M., 2008. Genetic relationships between roost-mates in a fission-fusion society of tree-roosting big brown bats (*Eptesicus fuscus*). Behav. Ecol. Sociobiol. 62, 1043–1051.

Moss, C.F., Schnitzler, H.-U., 1995. Behavioral studies of auditory information processing. In: Popper, A.N., & Fay, R. R. (eds) *Hearing by Bats*. Springer Handbook of Auditory Research, Vol 5, pp 87-145. Springer, New York, NY.

Müller-Schwarze, D., 1971. Pheromones in black-tailed deer (*Odocoileus hemionus columbianus*). Anim. Behav. 19, 141–152.

Muñoz-Romo, M., Burgos, J.F., Kunz, T., 2011. Smearing behaviour of male Leptonycteris curasoae (Chiroptera) and female response to the odour of dorsal patches. Behaviour. 148, 461–483.

Patriquin, K.J., Ratcliffe, J.M., 2016. Should I stay or should I go? Fission-fusion dynamics in bats. In: Ortega, J. (ed) *Sociality in bats*, pp 65-103. Springer International, Switzerland.

R Core Team, 2019. R: The R project for Statistical Computing. Available: https://www.r-project.org/.

Schumann, M., Puechmaille, S., Siemers, B.J., 201). Horseshoe bats recognize the sex of conspecifics from their echolocation calls. Acta. Chiropterol. 14, 161-166.

Siegel, S., 1956. *Nonparametric Statistics for the Behavioural Sciences*. New York: McGraw-Hill.

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

Surov, A.V., Maltsev, A.N., 2016. Analysis of chemical communication in mammals: zoological and ecological aspects. Biol. Bull. 43, 1175–1183.

Swaisgood, R.R., Lindburg, D.G., Zhou, X., Owen, M.A., 2000. The effects of sex, reproductive condition and context on discrimination of conspecific odours by giant pandas. Anim. Behav. 60, 227–237.

Takács, S., Gries, R., Gries, G., 2017. Sex hormones function as sex attractant pheromones in house mice and brown rats. ChemBioChem. 18, 1391–1395.

The jamovi project, 2020. *jamovi* (Version 1.2) [Computer Software]. Retrieved from https://www.jamovi.org

Verbeek, M.E.M., Drent, P.J., Wiepkema, P.R., 1994. Consistent individual differences in early exploratory behaviour of male great tits. Anim. Behav. 48, 1113–1121.

Voigt, C.C., Behr, O., Caspers, B.A, von Helversen, O., Knörnschild, M., Mayer, F., Nagy, M., 2008. Songs, scents, and senses: Sexual selection in the greater sac-winged bat, *Saccopteryx bilineata*. J. Mammal. 89, 1401–1410.

Voigt, C.C., von Helversen, O., 1999. Storage and display of odour by male *Saccopteryx bilineata* (Chiroptera, Emballonuridae). Behav. Ecol. Sociobiol. 47, 29–40.

Willis, C.K.R., Brigham, R.M., 2007. Social thermoregulation exerts more influence than microclimate on forest roost preferences by a cavity-dwelling bat. Behav. Ecol. Sociobiol. 62: 97–108.

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

Yoshida, M., Nagamine, M., Uematsu, K., 2005. Comparison of behavioral responses to a novel environment between three teleosts, bluegill *Lepomis macrochirus*, crucian carp *Carassius langsdorfii*, and goldfish *Carassius auratus*. Fisheries. Sci. 71, 314–319.

Zhang, J.X., Soini, H.A., Bruce, K.E., Wiesler, D., Woodley, S.K., Baum, M.J., Novotny, M.V., 2005. Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. Chem. Senses. 30, 727–737.

Zhang, J.X., Rao, X.P., Sun, L., Zhao, C.H., Qin, X.W., 2007. Putative chemical signals about sex, individuality, and genetic background in the preputial gland and urine of the house mouse (*Mus musculus*). Chem. Senses. 32, 293–303.

Chapter 5 – Discussion

5.1 – Introduction

The extraordinary hearing abilities of bats has resulted in behavioural and physiological studies of bat echolocation and social communication with a major focus on their auditory systems. However, other senses, including the olfactory system, are known to be used by bats for communication (eg. Chaverri et al., 2018). Chemically transmitted information has been identified in multiple bat species. For example, pup recognition by Mexican free-tailed bat mothers (*Tadarida brasiliensis Mexicana*) relies heavily on olfaction (Gustin and McCracken, 1987; Loughry and McCracken, 1991), while the common pipistrelle bat (*Pipistrellus pipistrellus*) can learn to discriminate the scent of their home colony and individuals (de Fanis and Jones (1995). What has been less studied are chemicals excreted by individuals that can actively or passively enter into the circulation of others without activating the olfactory system.

5.2 – Contributions to the Field

To date, few studies have focused on the physiological implications of non-olfactory chemical signals. The goal of this thesis was to evaluate the potential of estradiol (E_2)—the smallest and one of the most potent mammalian sex steroid—to act as a reproductive pheromone in big brown bats (*Eptesicus fuscus*). To be classified as a pheromone, E_2 must be secreted from an individual into the environment and be taken up by a receiver conspecific, where it elicits a behavioural and/or physiological response (deCatanzaro, 2015). This more recent definition of a pheromone does not address the mechanism by which a pheromone substance acts, thus it includes non-volatiles such as

steroids. Historically, behavioural physiologists have focused on one aspect of a research question—either the physiological mechanisms or the resulting behaviour—but this approach, which lacks of integration within the field, has resulted in incomplete support for many hypotheses. My doctoral thesis has attempted to address this gap by integrating physiological and ethological studies to evaluate the pheromonal potential of estradiol from a more holistic perspective.

The results presented in this dissertation contribute key advancements to the biology of bats in the fields of reproductive endocrinology and behaviour. I have shown that E_2 transfer in big brown bats is most prominent in reproductively-relevant seasonal timepoints for the species compared to the non-reproductive season (Greville et al., 2020). Furthermore, the transferred E_2 reliably binds to estrogen receptors located in the reproductive organs and neural tissues thought responsible for eliciting reproductive behaviour. I have also created the first urinary steroid profile for any chiropteran species, showing the presence of bioactive E_2 in male urine throughout the year. These two physiological components are necessary in the further examination of E_2's potential to act as a pheromone in bats. Importantly, the creation of a large-scale profile of urinary steroids, including age, sex, and seasonal differences, demonstrates a reliable, non-invasive method of hormone analysis that is dynamic to circulating levels. This profile will serve as a critical reference for future endocrine studies in bats.

I have provided the first evidence that female *E. fuscus* are attracted to the urine of male conspecifics, albeit without elucidating the underlying chemical responsible for the behaviour. Past studies in the species have failed to show in-group olfactory

discrimination, whereas colony differences were discerned (Bloss et al., 2002; Mayberry and Faure, 2015). But the evidence of a sex-specific preference is a first step to further studying possible behavioural implications of reproductive pheromones. Collectively, my studies on the seasonality of hormone transfer, variation in naturally-excreted levels of hormone, and behavioural odour preferences provide an integrative approach to studying mammalian behavioural physiology.

5.3 – Chapter Summary

In **Chapter 2**, I evaluated the seasonal effect of estradiol transfer from male to female big brown bats. When a male bat was injected with 50 μCi of tritium-labelled estradiol (^{3}H-E_2) and housed for 48 h with two female conspecifics during the autumn mating period, radioactivity was subsequently measured in in the blood serum and all sampled reproductive, neural, and peripheral tissues. Importantly, this finding replicated a previous study by deCatanzaro et al.'s (2014) demonstrating male-to-female ^{3}H-E_2 transfer in the autumn. My work further replicated this experiment in both the spring and summer seasons. In the spring, a time where females ovulate and fertilization leads to the start of gestation (Oxberry, 1979), radioactivity was again consistently found in all sampled tissues. In fact, the average radioactivity counts in tissues during the spring were higher than the autumn in neural tissues, as well as the uterus and blood serum. However, when the experiment was replicated during the summer maternity season when mating and gestation do not occur, little to no ^{3}H-E_2 transfer was observed. In both the spring and summer seasons, radioactivity in the uterus was greater than in most other tissues, with

the exception of the liver and kidney which are involved in the metabolism and excretion of steroids. The uterine and ovarian uptake of ^{3}H-E_2 is critical because it demonstrates that male-sourced E_2 can enter the circulation of females following excretion and potentially influence reproductive tissues without necessarily activating the hypothalamic-pituitary-gonadal axis of hormone regulation. That said, the hypothalamus is a known regulator of female reproductive behaviour in mammals (Pfaff, 1980), and the consistent uptake of radioactivity in the hypothalamus suggests that male-sourced E_2 may also impact sexual behaviour.

I also described a small sample of male urinary estradiol measurements in **Chapter 2**. The urine was collected during the spring, summer, and autumn—the same seasons as the previous ^{3}H-E_2 transfer experiments. Raw comparisons (ng/mL) of urinary E_2 between the seasons showed no differences; however, when the levels were adjusted for creatinine, which accounts for animal hydration and activity level, E_2 levels were higher in the spring and autumn compared to the summer seasons. This means that the reproductively relevant seasons where I observed the highest levels of ^{3}H-E_2 transfer between male and female bats are the same seasons where male urinary E_2 is highest. Altogether, this suggests that physiological changes in male urinary E_2 may account for the seasonal differences in the levels of steroid hormone transferred.

Building on the previous urine analysis, **Chapter 3** quantifies urinary E_2 levels in male and female *E. fuscus* across their annual reproductive cycle. Given the research hypothesis that urine is the primary vector of ^{3}H-E_2 transfer between cohabitating conspecifics, I believed a large sample of urinary steroid profiles was a critical next step

in evaluating E_2's role as a potential reproductive pheromone. A urinary profile across the annual reproductive cycle allowed me to examine age, sex, and seasonal differences in E_2. Urine was collected from McMaster University's captive colony of *E. fuscus* by holding individual animals over a wax-paper lined worksurface while they excreted (Greville et al., 2017, 2020). The collected urine was frozen and later analyzed using an enzyme-linked immunosorbent assay (ELISA) for E_2 content. Creatinine was also measured in all of these samples. Linear mixed effect regression models revealed higher creatinine-adjusted E_2 levels in adults than yearlings and in males than females. However, this pattern was not found in the raw E_2 levels unadjusted for creatinine. There were a number of seasonal differences within and between sexes in our dataset. Male creatinine-adjusted E_2 did not differ across the spring, summer, and autumn seasons. In contrast to the data from Chapter 2, this result suggests that perhaps behavioural differences may be responsible for seasonal differences in ^{3}H-E_2 transfer. Nonetheless, E_2 was present in male urine, which is a required component if it is to act as a pheromone.

In **Chapter 4** I used Y-maze arenas to evaluate whether bats preferred the body odour or urine from individuals of the opposite sex. In doing so, I designed a Y-maze apparatus and use behavioural protocols based on prior publications in bats. In evaluating the role of body odour as an attractant, cotton swabs were swiped on animals to collect scents from the muzzle, dorsal and ventral surfaces, wing, and anogenital region. Each focal animal was tested once with the general body scent of a male and female bat placed pseudo-randomly at the ends of the test arms of the Y-maze. Focal animals were recorded exploring the maze for 5 min and the videos were later analyzed. In a second set of

experiments, I evaluated the preference of conspecific urine with similar behavioural testing procedures with the exception that focal bats were given a choice between male or female urine as test stimuli instead of body odour. I found that female bats had a strong, initial preference and approached the urine of males before females. Albeit not significant, there was a trend suggesting female bats spent more overall time in the test-arm containing male urine and were more likely to be in that arm at the end of the 5-min trial. While these experiments do not directly test the attractant properties of steroids or their metabolites, they provide a first step in suggesting that a naturally excreted substance in male urine may serve to attract female conspecifics. Clearly, additional research is needed to further explore this idea.

5.4 – Future Directions

I believe that science progresses in two main ways. The first is to slowly build upon past studies, one by one, in a logical progression. Alternatively, one can more quickly integrate concepts into hypotheses without all the fundamental studies having already taken place. I believe the experiments in my dissertation follow the latter approach. I have brought theories and results that are well established in rodents into the field of bat biology. As such, I believe my research opens the door to a number of avenues of future work.

First, there is a need to better understand the presence of reproductive phenomena in bats and the possible implication of E_2 transfer between conspecifics. Determining an assay to detect fertilization or early gestation is critical in evaluating if the Bruce effect

exists in bats. In *E. fuscus*, the gestation period is believed to be ~60 days (Kurta and Baker, 1990), but without ultrasound imaging or more invasive (e.g. blood analyses) procedures a bat's pregnancy cannot be detected until the fetus is large enough to be palpated. By this time, gestation has progressed beyond the point of pregnancy disruption and the time when E_2 transfer would render the endometrium unsuitable for implantation of the developing embryo. Additional research is also required to evaluate whether the Vandenbergh effect exists in bats. deCatanzaro et al (2014) showed that ^3H-E_2 transfers from adult males to yearling females during the mating season. Studies have also shown that females are less likely to reproduce when they are yearlings (Barclay, 2012; O'Shea et al., 2010; Sidner, 1997). Future studies should examine whether the administration of E_2 to juvenile female bats about to enter their first mating season leads to an increase in uterine mass, or if cohabitation with an adult male can lead to an accelerated maturation of the female reproductive system.

Urinary analyses are often used in large mammals but are less common in small mammals. I have shown urine analysis to be a reliable method of evaluating steroid dynamics in bats. Blood sampling is intrusive, and for small mammals such as bats the quantity of blood needed for reliable analyses can make repeated sampling dangerous over a short period of time. Many aspects of bat urinary steroid concentrations remain uninvestigated and should be prioritized. Of course, a broad analysis of unquantified bioactive urinary steroids and steroid metabolites is an essential starting point. Correlational studies between plasma and urinary steroid levels are also needed. In mice, the presence of female conspecifics can lead to polyuria and elevated urinary E_2 levels in

males (deCatanzaro et al., 2009). Before investigating the effects of conspecifics on urinary patterns and concentrations in bats, natural patterns of excretion volume and patterns need to be systematically evaluated. For example, urination is a social response in mice in which males urinate in many more small droplets (~300-350) than females do (~25-95) in the presence of conspecifics (Maruniak et al., 1975, 1974). This is yet to be quantified in bats. In the context of natural urinary steroid transfer, the roosting behaviour and position of male and female bats in a maternity colony over the annual reproductive cycle would be essential to understand.

Male E_2 dynamics is another interesting avenue for future work. Based on extensive studies in mice (reviewed by deCatanzaro, 2015), urine is believed to be the primary vector of E_2 transfer in bats. However, deCatanzaro and Pollock (2016) noted the presence of ^{3}H-E2 in the seminal emissions of male mice, with the level of male-to-female ^{3}H-E_2 transfer increasing with the duration of mating. My data from **Chapter 2** supports the finding of high radioactivity measurements in the epididymis of treated-males (deCatanzaro et al., 2014). Critical questions worthy of further investigation include whether E_2 is stored and progressively accumulates in the epididymis, and if E_2 is present in natural seminal emissions of male bats.

These future studies are essential in strengthening the current understanding of bat reproductive biology and will serve as new pillars in the foundation of reproductive endocrinology within the Order Chiroptera.

5.5 – Conclusion

The data presented in this thesis centers around the first exploration of the pheromonal potential of sex steroid hormones in Chiroptera and have provided novel insights into the natural ability of steroid transfer between *E. fuscus* conspecifics. The persistent presence of E_2 in male urine across the seasons in conjunction with seasonal variation in E_2 transfer male to female conspecifics may profoundly impact the reproductive physiology and behaviour of bats (Greville et al., 2020). The continued investigation of hormone transfer, exogenous steroids, and their effect on the reproductive success of a species or colony may further aid in critical conservation efforts. While the current studies are merely an entry into the exploration of reproductive behavioural endocrinology in bats, I sincerely hope that my thesis will ignite future physiologists to continue integrated studies of bat behavioural, neural, and reproductive endocrinology by combining physiological, behavioural, and ecological perspectives.

5.6 – General References (Intro and Conclusion)

Al-Gubory, K.H., 1998. Effects of the presence of rams during pregnancy on lambing performance in ewes. Anim. Reprod. Sci. 52, 205–211. https://doi.org/10.1016/S0378-4320(98)00095-5

Anderson, D.C., 1982. Sex-hormone-binding globulin. Clin. Endocrinol. 3, 69–96. https://doi.org/10.1016/S0140-6736(82)90560-8

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., Filardo, E.J., Lolait, S.J., Thomas, P., Maggiolini, M., Prossnitz, E.R., 2018. Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. J. Steroid Biochem. Mol. Biol. 176, 4–15. https://doi.org/10.1016/j.jsbmb.20 17.03.021

Bartoš, L., Bartošová, J., Pluháček, J., Šindelářová, J., 2011. Promiscuous behaviour disrupts pregnancy block in domestic horse mares. Behav. Ecol. Sociobiol. 65, 1567–1572. https://doi.org/10.1007/s00265-011-1166-6

Bawarshi-Nassar, R.N., Hussain, Hussain, A.A., Crooks, P.A., 1989. Nasal absorption and metabolism of progesterone and 17B-estradiol in the rat. Drug Metab. Dispos. 17, 248–254.

Beaton, E.A., deCatanzaro, D., 2005. Novel males' capacity to disrupt early pregnancy in mice (*Mus musculus*) is attenuated via a chronic reduction of males' urinary 17β-estradiol. Psychoneuroendocrinology 30, 688–697. https://doi.org/10.1016/j.psyneuen.2005.03.001

Beehner, J.C., Lu, A., 2013. Reproductive suppression in female primates: A review. Evol. Anthropol. 22, 226–238. https://doi.org/10.1002/evan.21369

Berger, J., 1983. Induced abortion and social factors in wild horses. Nature 303, 59–61. https://doi.org/10.1038/303059a0

Blandau, R.J., Boling, J.L., Young, W.C., 1941. The length of heat in the albino rat as determined by the copulatory response. Anat. Rec. 79, 453–463.

Bloss, J., Acree, T.E., Bloss, J.M., Hood, W.R., Kunz, T.H., 2002. Potential use of chemical cues for colony-mate recognition in the big brown bat, *Eptesicus fuscus*. J. Chem. Ecol. 28, 819–834.

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

Bronson, F.H., Eleftheriou, B.E., 1963. Influence of strange males on implantation in the deermouse. Gen. Comp. Endocrinol. 3, 515–518. https://doi.org/10.1016/0016-6480(63)90084-4

Brooks, P.H., Cole, D.J.A., 1970. The effect of the presence of a boar on the attainment of puberty in gilts. J. Reprod. Fertil. 23, 435–440. https://doi.org/10.1530/jrf.0.0230435

Bruce, H.M., 1965. Effect of castration on the reproductive pheromones of male mice. J. Reprod. Fertil. 10, 141–143. https://doi.org/10.1530/jrf.0.0100141

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

Bruce, H.M., 1960b. Further observations on pregnancy block in mice caused by the proximity of strange males. J. Reprod. Fertil. 1, 311–312.

Burnett, C.D., August, P. V, 1981. Time and energy budgets for dayroosting in a maternity colony of *Myotis lucifugus*. J. Mammal. 62, 758–766.

Caspers, B., Franke, S., Voigt, C.C., 2008. The wing-sac odour of male greater sac-winged bats *Saccopteryx bilineata* (Emballonuridae) as a composite trait: seasonal and individual differences, in: Chemical Signals in Vertebrates. Springer, New York, New York, pp. 151–160.

Chaverri, G., Ancillotto, L., Russo, D., 2018. Social communication in bats. Biol. Rev. https://doi.org/10.1111/brv.12427

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

Clulow, F. V, Lanford, P.E., 1971. Pregnancy-block in the meadow vole (*Microtus pennsylvanicus*). J. Reprod. Dev. 24, 275–277. https://doi.org/10.2307/3503976

Colby, D.R., Vandenberg, J.G., 1974. Regulatory effects of urinary pheromones on puberty in the mouse. Biol. Reprod. 11, 268–279. https://doi.org/10.1095/biolreprod11.3.268

Conaway, C.H., 1971. Ecological adaptation and mammalian reproduction. Biol. Reprod. 4, 239–247. https://doi.org/10.1093/biolreprod/4.3.239

Cooke, P.S., Buchanan, D.L., Young, P., Setiawan, T., Brody, J., Korach, K.S., Taylor, J., Lubahn, D.B., Cunha, G.R., 1997. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc. Natl. Acad. Sci. U. S. A. 94, 6535–6540. https://doi.org/10.1073/pnas.94.12.6535

Cora, M.C., Kooistra, L., Travlos, G., 2015. Vaginal cytology of the laboratory rat and

mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. Toxicol. Pathol. 43, 776–793. https://doi.org/10.1177/0192623315570339

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

de Fanis, E., Jones, G., 1995. The role of odour in the discrimination of conspecifics by pipistrelle bats. Anim. Behav. 49, 835–839. https://doi.org/10.1016/0003-3472(95)80215-0

deCatanzaro, D., Storey, A.E., 1989. Partial mediation of strange-male-induced pregnancy blocks by sexual activity in mice (*Mus musculus*). J. Comp. Psychol. 103, 381–388. https://doi.org/10.1037/0735-7036.103.4.381

deCatanzaro, D., 2015. Sex steroids as pheromones in mammals: the exceptional role of estradiol. Horm. Behav. 68, 103–116. https://doi.org/10.1016/j.yhbeh.2014.08.003

deCatanzaro, D., Baptista, M.A.S., Spironello-Vella, E., 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., Beaton, E.A., Khan, A., Vella, E., 2006. Urinary oestradiol and testosterone levels from novel male mice approah values sufficient to disrupt early pregnancy in nearby inseminated females. Reproduction 132, 309–317. https://doi.org/10.1530/rep.1.00965

deCatanzaro, D., Khan, A., Berger, R.G., Lewis, E., 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. https://doi.org/10.1016/j.yhbeh.2008.10.013

deCatanzaro, D., Muir, C., Beaton, E.A., Jetha, M., 2004. Non-invasive repeated measurement of urinary progesterone, 17β-estradiol, and testosterone in developing, cycling, pregnant, and postpartum female mice. Steroids 69, 687–696. https://doi.org/10.1016/j.steroids.2004.07. 0 02

deCatanzaro, D., Muir, C., O'Brien, J., Williams, S., 1995. Strange-male-induced pregnancy disruption in mice: reduction of vulnerability by 17β-estradiol antibodies. Physiol. Behav. 58, 401–404. https://doi.org/10.1016/0031-9384(95)00063-O

deCatanzaro, D., Muir, C., Sullivan, C., Boissy, A., 1999. Pheromones and novel male-induced pregnancy disruptions in mice: exposure to conspecifics is necessary for urine alone to induce an effect. Physiol. Behav. 66, 153–157. https://doi.org/10.1016/S0031-9384(98)002 77-7

deCatanzaro, D., Pollock, T., 2016. Absorption and distribution of estradiol from male seminal emissions during mating. J. Endocrinol. 231, 245–257. https://doi.org/10.1530/JOE-16-0247

deCatanzaro, D., Pollock, T., Greville, L.J., Faure, P.A., 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. https://doi.org/10.1016/j.ygc en.2014. 09.011

Dominic, C.J., 1966. Observations on the reproductive pheromones of mice. J. Reprod. Fertil. 11, 407–414. https://doi.org/10.1530/jrf.0.0110407

Drickamer, L.C., 1983. Male acceleration of puberty in female mice (*Mus musculus*). J.

Comp. Psychol. 97, 191–200. https://doi.org/10.1037/0735-7036.97.3.191

Dumas, I., Diorio, C., 2011. Estrogen pathway polymorphisms and mammographic density. Anticancer Res. 31, 4369–4386.

Filardo, E.J., Thomas, P., 2005. GPR30: A seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol. Metab. 16, 362–367. https://doi.org/10.1016/j.tem.2005.08.005

Flores, V., Page, R.A., 2017. Novel odorous crust on the forearm of reproductive male fringe-lipped bats (*Trachops cirrhosus*). J. Mammal. 98, 1568–1577. https://doi.org/10.1093/jmammal/gyx137

Freeman, M.E., 2006. Neuroendocrine control of the ovarian cycle of the rat, in: Neill, J.D. (Ed.), Knobil and Neill's Physiology of Reproduction. Academic Press/Elsevier, San Diego, CA, pp. 2327–2387.

Gidley-baird, A.A., O'Neill, C., Sinosich, M.J., Porter, R.N., Pike, I.L., Saunders, D.M., 1986. Failure of implantation in human in vitro fertilization and embryo transfer patients: the effects of altered progesterone/estrogen ratios in humans and mice. Fertil. Steril. 45, 69–74. https://doi.org/10.1016/S0015-0282(16)49099-0

Goldzieher, J.W., Baker, R.E., 1960. The percutaneous absorption of estradiol-17B and progesterone. J. Invest. Dermatol. 35, 215–218. https://doi.org/10.1038/jid.1960.106

Greville, L.J., Pollock, T., Faure, P.A., deCatanzaro, D., 2020. Seasonal transfer and quantification of urinary estradiol in the big brown bat (*Eptesicus fuscus*). Gen. Comp. Endocrinol. 286, 113321. https://doi.org/10.1016/j.ygcen.2019.113321

Greville, L.J., Pollock, T., Salter, J.C., Faure, P.A., deCatanzaro, D., 2017. Progesterone

transfer among cohabitating female big brown bats (*Eptesicus fuscus*). Gen. Comp. Endocrinol. 247, 199–204.

Gustin, M.K., McCracken, G.F., 1987. Scent recognition between females and pups in the bat *Tadarida brasiliensis mexicana*. Anim. Behav. 35, 13–19. https://doi.org/10.1016/S0003-3472(87)80205-1

Guzzo, A.C., Berger, R.G., deCatanzaro, D., 2010. Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): implications for the Bruce effect. Reproduction 139, 255–263. https://doi.org/10.1530/REP-09-0382

Guzzo, A.C., Jheon, J., Imtiaz, F., deCatanzaro, D., 2012. Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). Reproduction 143, 539–548. https://doi.org/10.1530/REP-11-0375

Guzzo, A.C., Pollock, T., deCatanzaro, D., 2013. Transfer of [3H]estradiol-17β and [3H]progesterone from conspecifics to cohabiting female mice. J. Endocrinol. 217, 1–10. https://doi.org/10.1530/JOE-12-0279

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

Harder, J.D., Jackson, L.M., 2003. Male pheromone stimulates ovarian follicular development and body growth in juvenile female opossums (*Monodelphis domestica*). Reprod. Biol. Endocrinol. 1, 1–9. https://doi.org/10.1186/1477-7827-1-21

Hasler, J.F., Banks, E.M., 1975. The influence of mature males on sexual maturation in female collared lemmings (*Dicrostonyx groenlandicus*). J. Reprod. Fertil. 42, 583–

586.

Heske, E.J., Nelson, R.J., 1984. Pregnancy in *Microtus ochrogaster*: laboratory artifact of field phenomenon? Biol. Reprod. 31, 97–103.

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

Holt, W. V., Fazeli, A., 2016. Sperm storage in the female reproductive tract. Annu. Rev. Anim. Biosci. 4, 291–310. https://doi.org/10.1146/annurev-animal-021815-111350

Hueber, F., Bernard, M., Schaefer, H., Wepierre, J., 1994. Percutaneous absorption of estradiol and progesterone in normal and appendage-free skin of the hairless rat: lack of importance of nutritional blood flow. Skin Pharmacol. Physiol. 7, 245–256.

Izard, M.K., Vandenbergh, J.G., 1982. The effects of bull urine on puberty and calving date in crossbred beef heifers. J. Anim. Sci. 55, 1160–1168.

Karlson, P., Lüscher, M., 1959. "Pheromones": a new term for a class of biologically active substances. Nature 183, 55–56. https://doi.org/10.1038/183055a0

Kleiman, D.G., Karesh, W.B., Chu, P.R., 1979. Behavioural changes associated with oestrus in the Giant panda (*Ailuropoda melanoleuca*) with comments on female proceptive behaviour. Int. Zoo Yearb. 19, 217–224. https://doi.org/10.1111/j.1748-1090.1979.tb00568.x

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

Kurta, A., Baker, R.H., 1990. *Eptesicus fuscus*. Mamm. Species 1–10.

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

Lepri, J.J., Vandenbergh, J.G., 1986. Puberty in Pine Voles, Microtus pinetorum, and the influence of chemosignals on female reproduction. Biol. Reprod. 34, 370–377.

Lombardi, J.R., Vandenbergh, J., Whitsett, J.M., 1976. Androgen control of the sexual maturation pheromone in house mouse urine. Biol. Reprod. 15, 179–186.

Loughry, W.J., McCracken, G.F., 1991. Factors Influenicing Female-Pup Scent Recogntion in Mexican Free-Tailed Bats. J. Mamm 72, 624–626.

Ma, W., Song, H., Das, S.K., Paria, B.C., Dey, S.K., 2003. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Proc. Natl. Acad. Sci. 100, 2963–2968.

Marashi, V., Rülicke, T., 2012. The bruce effect in Norway rats. Biol. Reprod. 86, 1–5. https://doi.org/10.1095/biolreprod.111.093104

Maruniak, J.A., Owen, K., Bronson, F.H., Desjardins, C., 1975. Urinary marking in female house mice: effects of ovarian steroids, sex experience, and type of stimulus. Behav. Biol. 13, 211–217. https://doi.org/10.1016/S0091-6773(75)91920-3

Maruniak, J.A., Owen, K., Bronson, F.H., Desjardins, C., 1974. Urinary marking in male house mice: responses to novel environmental and social stimuli. Physiol. Behav. 12, 1035–1039. https://doi.org/10.1016/0031-9384(74)90151-6

Mayberry, H.W., Faure, P.A., 2015. Morphological, olfactory, and vocal development in big brown bats. Biol. Open 4, 1–13. https://doi.org/10.1242/bio.201410181

Mendonça, M., Chernetsky, S., Nester, K., Gardner, G., 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.

Mukku, V.R., Kirkland, J.L., Hardy, M., Stancei, G.M., 1982. Hormonal control of uterine growth: Temporal relationships between estrogen administration and deoxyribonucleic acid synthesis. Endocrinology 111, 480–487. https://doi.org/10.1210/endo-111-2-480

Muñoz-Romo, A.M., Burgos, J.F., Kunz, T.H., Behaviour, S., Munoz-romo, M., Burgos, J.F., Kunz, T.H., 2011. Smearing behaviour of male *Leptonycteris curasoae* (Chiroptera) and female responses to the odour of dorsal patches. Behaviour 148, 461–483.

Muñoz-Romo, M., Kunz, T.H., 2009. Dorsal patch and chemical signaling in males of the long-nosed bat, *Leptonycteris curasoae* (Chiroptera: Phyllostomidae). J. Mammal. 90, 1139–1147. https://doi.org/10.1644/08-MAMM-A-324.1

Nelson, R.J., Kriegsfeld, L.J., 2017. An introduction to behavioural endocrinology, 5th ed. Sinauer Associates Inc., Sunderland, Massacusetts.

O'Shea, T.J., Ellison, L.E., Neubaum, D.J., Neubaum, M.A., Reynolds, C.A., Bowen, R.A., 2010. Recruitment in a Colorado population of big brown bats: breeding probabilities, litter size, and first-year survival. J. Mammal. 91, 418–428. https://doi.org/10.1644/08-MAMM-A-295.1.

Ogasawara, Y., Kitamura, Y., Matsumoto, K., 1983. Proliferative pattern of uterine cells from birth to adulthood in intact, neonatally castrated, and/or adrenalectomized mice, assayed by incorporation of [125I]Iododeoxyuridine. Endocrinology 113, 582–

587.

Orr, T.J., Brennan, P.L.R., 2015. Sperm storage: Distinguishing selective processes and evaluating criteria. Trends Ecol. Evol. 30, 261–272. https://doi.org/10.1016/j.tree.2015.03.006

Orr, T.J., Zuk, M., 2014. Reproductive delays in mammals: An unexplored avenue for post-copulatory sexual selection. Biol. Rev. 89, 889–912. https://doi.org/10.1111/brv.12085

Ortiz, M.-E., Villalon, M., Croxatto, H.B., 1979. Ovum transport and fertility following postovulatory treatment with estradiol in rats. Biol. Reprod. 21, 1163–1167.

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

Paradiso, J.L., Greenhall, A.M., 1967. Longevity records for american bats. Am. Midl. Nat. 78, 251–252.

Parkes, A.S., Bruce, H.M., 1962. Pregnancy-block in female mice placed in boxes soiled by males. J. Reprod. Fertil. 4, 303–308. https://doi.org/10.1530/jrf.0.0040303

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25, 947–970. https://doi.org/10.1210/er.2003-0030

Pfaff, D.W., 1980. Estrogens and Brain Function. Springer-Verlag, New York, New York.

Prossnitz, E.R., Arterburn, J.B., Smith, H.O., Oprea, T.I., Sklar, L.A., Hathaway, H.J., 2008. Estrogen signaling through the transmembrane G protein-coupled receptor

GPR30. Annu. Rev. Physiol. 70, 165–190. https://doi.org/10.1146/annurev.physiol.70.113006.100518

Quarmby, V.E., Korach, K.S., 1984. The influence of 17β-estradiol on patterns of cell division in the uterus. Endocrinology 114, 694–702. https://doi.org/10.1210/endo-114-3-694

Racey, P.A., 1979. The prolonged storage and survival of spermatozoa in Chiroptera. J. Reprod. Fertil. 56, 391–402.

Racey, P.A., Potts, D.M., 1970. Relationship between stored spermatozoa and the uterine epithelium in the pipstrelle bat (*Pippistrellus pippistrellus*). J. Reprod. Fertil. 22, 57–63.

Racey, P.A., Uchida, T.A., Mōri, T., Avery, M.I., Fenton, M.B., 1987. Sperm-epithelium relationships in relation to the time of insemination in little brown bats (*Myotis lucifugus*). J. Reprod. Fertil. 80, 445–454.

Rajendren, G., Dominic, C.J., 1988. Effect of cyproterone acetate on the pregnancy-blocking ability of male mice and the possible chemical nature of the pheromone. J. Reprod. Fertil. 84, 387–392. https://doi.org/10.1530/jrf.0.0840387

Reasner, D.S., Johnston, R.E., 1988. Acceleration of reproductive development in female Djungarian hamsters by adult males. Physiol. Behav. 43, 57–64. https://doi.org/10.1016/0031-9384(88)90098-4

Roberts, E.K., Lu, A., Bergman, T.J., Beehner, J.C., 2012. A Bruce effect in wild geladas. Science. 335, 1222–1225. https://doi.org/10.1126/science.1213600

Schaefer, H., Zesch, A., Stüttgen, G., 1982. Skin Permeability. Springer-Verlag, Berlin,

Germany.

Scheuplein, H.J., Blank, I.H., Brauner, G.J., MacFarlane, D.J., 1968. Percutaneous absorption of steroids. J. Invest. Dermatol. 52, 63–70. https://doi.org/10.1038/jid.1969.9

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

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

Teague, L.G., Bradley, E.L., 1978. The existence of a puberty accelerating pheromone in the urine of the male prairie deermouse (*Peromyscus maniculatus bairdii*). Biol. Reprod. 19, 314–317.

Thomas, P., 2004. Discovery of a new family of membrane progesterone receptors in vertebrates and detection of the alpha and beta subtypes in mouse brain, testis and uterus. Med. Chem. Res. 13, 202–209. https://doi.org/10.1007/s00044-004-0026-6

Thorpe, J.B., deCatanzaro, D., 2012. Oestradiol treatment restores the capacity of castrated males to induce both the Vandenbergh and the Bruce effects in mice (*Mus musculus*). Reproduction 143, 123–132. https://doi.org/10.1530/REP-11-0251

Valbuena, D., Martin, J., de Pablo, J.L., Remohi, J., Pellicer, A., Simon, C., 2001. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. Fertil. Steril. 76, 962–968.

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

Vella, E.S., deCatanzaro, D., 2001. Novel male mice show gradual decline in the capacity to disrupt early pregnancy and in urinary excretion of testosterone and 17β-estradiol during the weeks immediately following castration. Horm. Metab. Res. 33, 681–686. https://doi.org/10.1055/s-2001-18690

Voigt, C.C., von Helversen, O., 1999. Storage and display of odor in male *Saccopteryx billineata* Emballonuridae. Behav. Ecol. Sociobiol. 47, 29–40. https://doi.org/10.1007/s002650050646

Vonhof, M.J., Barber, D., Fenton, M.B., Strobecks, C., 2006. A tale of two siblings: multiple paternity in big brown bats (*Eptesicus fuscus*) demonstrated using microsatellite markers. Mol. Ecol. 15, 241–247. https://doi.org/10.1111/j.1365-294X.2005.02801.x

Walters, M.R., 1985. Steroid hormone receptors and the nucleus. Endocr. Rev. 6, 512–543. https://doi.org/10.1210/edrv-6-4-512

Willis, C.K.R., Brigham, R.M., 2007. Social thermoregulation exerts more influence than microclimate on forest roost preferences by a cavity-dwelling bat. Behav. Ecol. Sociobiol. 62, 97–108. https://doi.org/10.1007/s00265-007-0442-y

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

Zhou, X., Xu, S., Xu, J., Chen, B., Zhou, K., Yang, G., 2012. Phylogenomic analysis resolves the interordinal relationships and rapid diversification of the laurasiatherian mammals. Syst. Biol. 61, 150–164. https://doi.org/10.1093/sysbio/syr089