ARTICLE

Evaluating odour and urinary sex preferences in the big brown bat (*Eptesicus fuscus*)

Lucas J.S. Greville, Audrey G. Tam, and Paul A. Faure

Abstract: Olfactory cues provide detailed information to mammals regarding conspecifics. Bats may identify species, colony membership, and individuals using olfaction. Big brown bats (*Eptesicus fuscus* (Palisot de Beauvois, 1796)) live in mixed-sex colonies and must differentiate between sexes to locate mates. We hypothesized that odour cues convey information about sex. In experiment 1, adult *E. fuscus* were recorded exploring a Y-maze that contained general body odours sampled from male or female conspecifics. One group of subjects was habituated to the Y-maze prior to experimental trials, whereas a second group was not. Bat exploration and the proportion of time spent near each scent were used as preference indicators for the body odour of a particular sex. Experiment 2 followed similar procedures except the odour cue tested was urine from either male or female conspecifics and without Y-maze habituation. Results found no evidence that *E. fuscus* prefer the body odours of a given sex, but females did prefer the odour of male urine. Non-habituated animals in experiment 1 were more likely to explore the Y-maze and approach a stimulus scent compared with habituated bats. These findings have important implications for courtship and mating behaviour in bats, as well for designing future behavioural studies.

Key words: big brown bat, body odour, *Eptesicus fuscus*, habituation, mate choice, olfactory cues, urine scent.

Résumé : Les signaux olfactifs fournissent aux mammifères de l'information détaillée sur leurs conspécifiques. Les chauves-souris pourraient identifier l'espèce, la colonie d'appartenance et les individus à l'aide de l'odorat. Les sérotines brunes (*Eptesicus fuscus* (Palisot de Beauvois, 1796)) vivent dans des colonies comptant des individus des deux sexes et doivent pouvoir distinguer les sexes pour trouver des partenaires. Nous avons postulé que des signaux olfactifs portent de l'information sur le sexe. Dans l'expérience 1, des sérotines brunes adultes ont été observées pendant qu'elles exploraient un labyrinthe en Y contenant des odeurs corporelles générales prélevées de conspécifiques mâles et femelles. Un groupe de sujets a été accoutumé au labyrinthe en Y préalablement aux manipulations expérimentales, alors qu'un second groupe ne l'a pas été. L'exploration des chauves-souris et la proportion de temps passé près de chaque odeur ont été utilisées comme indicateurs de préférence pour l'odeur corporelle de chacun des sexes. L'expérience 2 a suivi des procédures semblables, sauf que le signal olfactif testé était de l'urine de conspécifiques mâles ou femelles, sans accoutumance préalable au labyrinthe en Y. Les résultats n'ont relevé aucun indice d'une préférence des sérotines brunes pour les odeurs corporelles d'un des deux sexes en particulier, les femelles préférant toutefois l'odeur de l'urine de mâles. Les animaux non accoutumés dans l'expérience 1 étaient plus susceptibles d'explorer le labyrinthe en Y et de s'approcher d'un stimulus olfactif que les chauves-souris accoutumées. Ces constatations sont importantes pour la compréhension des comportements de cour et d'accouplement de chauves-souris, ainsi que pour la conception d'études sur le comportement. [Traduit par la Rédaction]

Mots-clés : sérotine brune, odeur corporelle, *Eptesicus fuscus*, accoutumance, choix des partenaires, signaux olfactifs, senteur d'urine.

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 and Maltsev 2016). This process is known as chemosensation. A critical extension of chemosensation is 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 or delineated by olfactory signaling (Eisenberg and Kleiman 1972; Caspers et al. 2008). This also holds true in bats (order Chiroptera; e.g., Gustin and McCracken 1987; Bouchard 2001; Bloss et al. 2002; Caspers et al. 2008, 2009). Olfactory signaling is effective independent of light and chemical signals persist in the environment while spanning long distances (Brown and MacDonald 1985). Like other social nocturnal animals, olfactory communication is integral in the lives of bats (e.g., Gustin and McCracken 1987; Caspers et al. 2008, 2009; Bartonička et al. 2010).

Given that odour carries a range of individualistic information, it is unsurprising that animals use chemical cues to discriminate sex. The widespread ability to assess sex by olfaction has been observed across mammalian taxa, including mice (*Mus musculus*

Linnaeus, 1758) (Bowers and Alexander 1967), beavers (*Castor fiber* Linnaeus, 1758) (Cross et al. 2014), black-tailed deer (*Odocoileus hemionus columbianus* (Richardson, 1829)) (Müller-Schwarze 1971), lions (*Panthera leo* (Linnaeus, 1758)) (Gilfillan et al. 2017), giant pandas (*Ailuropoda melanoleuca* (David, 1869)) (Swaisgood et al. 2000), and koalas (*Phascolarctos cinereus* (Goldfuss, 1817)) (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 long distances (Müller-Schwarze 1971). In temperate vespertilionid bats, fission–fusion roost dynamics are often observed (review by Patriquin and 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, there is evidence that olfactory signaling may be as important as acoustic signalling in bat social communication. For example, many bats have large olfactory bulbs (Dechmann and Safi 2005) and diverse glands and nasal structures that allow them to produce and perceive smells (Haffner 2000; Voigt et al. 2008). In social gregarious species that roost in large mixed-sex colonies, the ability to distinguish between sexes via olfaction facilitates finding suitable mates (Bouchard 2001). Consistent with this idea, the Angolan free-tailed bat (*Mops condylurus* (A. Smith, 1833)) and the little free-tailed bat (*Chaerephon pumilus* (Cretzschmar, 1826)) 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* (Palisot de Beauvois, 1796)) is found throughout the Americas (Kurta and Baker 1990). Outside of mating and hibernation, *E. fuscus* roost in colonies largely segregated by sex: adult females form maternity colonies in the spring to give birth and raise young (Kurta and Baker 1990); adult males remain solitary or form all-male roosting groups until mating season (Barbour and Davis 1969). Roosting with conspecifics also helps individual bats to thermoregulate (Willis and Brigham 2007). Past studies have shown *E. fuscus* can differentiate between sexes using auditory cues and signals, with males preferring the echolocation calls of high frequency copulatory females (Kazial and Masters 2004; Grilliot et al. 2015). 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. The role of olfaction may be unappreciated given that *E. fuscus* are sexually monomorphic except for a slight difference in body size (Kazial and Masters 2004; Mayberry and Faure 2015). The current study uses a Y-maze arena to evaluate whether male and female *E. fuscus* show an olfactory preference for either sex. Outside of psychophysical studies that routinely employ this method (for a review see Moss and Schnitzler 1995), Y-maze (or T-maze) testing is less commonly used in behavioural studies with bats, and when used, there are varied procedures (e.g., De Fanis and Jones 1995; Bartonička et al. 2010; Kilgour et al. 2013). Thus, we also examine the effect of habituation on animal exploration in behavioural testing. We hypothesize that male and female bats will prefer conspecific scents from the opposite sex. As such, we predict that females will first enter and spend more time in the stimulus arm containing male scent (i.e., general body odour or urine), and vice-versa for males with female odours. We also hypothesize that habituated animals will explore more readily than non-habituated individuals.

Materials and methods

Animal husbandry

Captive *E. fuscus* used in this study were either wild-caught in southern Ontario or were the direct descendants of wild-caught individuals. Bats were housed indoors (2.5 m long × 1.5 m wide × 2.3 m high) in a mixed-sex 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 m long × 3.8 m wide × 2.7 m high) (Skrinyer et al. 2017). Within the colony, bats had ad libitum access to water and yellow mealworms (*Tenebrio molitor* Linnaeus, 1758), as well as towels, tree bark, and a hollowed tree trunk to roost in or under. Natural tree branches and artificial hanging vines and plants served as enrichment for animals. Animals selected for experiments had been in the colony for a minimum of 6 months prior to experiments. In total, 60 females (47 wild caught, 13 captive born) and 54 males (18 wild caught, 35 captive born, 1 unknown) were used in the study.

Healthy adult bats (>1 year; unless otherwise stated) from the colony were pseudo-randomly selected from the colony the day prior to testing (~30 h prior to maze testing) and held overnight in stainless steel mesh cages (28 cm long × 22 cm wide × 18 cm high, ¼ inch (0.635 cm) mesh) in an indoor holding room. A subgroup of these bats was designated as "focal" bats and they were housed together. A second subgroup was designated as "odour" (or stimulus) bats and they were housed in two cages isolated from each other and segregated by sex to prevent the mixing of sex-specific body odours. Cages were housed apart but in the same holding room so that vocalizations and odours may have been detected by any individual. The holding room was held at room temperature (20 °C) and lights were programmed to follow the ambient light:dark cycle for Hamilton, Ontario, Canada.

The captive colony used in our study is composed primarily of wild-caught bats captured as adults from multiple roosts. Unfortunately, bats cannot be reliably aged based on morphometrics and precise aging would require molecular analysis (e.g., Wilkinson et al. 2021). As such, we classify individuals as adult (>1 year) or yearling (<1 year) in our study. Animals in our study were believed to be in reproductive condition, albeit this cannot be confirmed in females without regular vaginal swabs. All experiments were conducted during the autumn mating season when bats are frequently observed mating in the colony. In the wild, over 90% of females give birth following their first mating season as an adult, suggesting that adult females readily mate prior to hibernation (O'Shea et al. 2010). Both captive and wild *E. fuscus* mate promiscuously, suggesting that past mating status of individuals should not influence subsequent odour or urine collection or behaviour (Mendonça et al. 1996; Vonhof et al. 2006).

Ethics approval

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 Experimental Animals in Research" published by the Canadian Council on Animal Care (CCAC), Ottawa, Ontario, Canada.

Experimental design, set-up, and odour collection

Experiments were conducted during the autumn mating season and occurred in the evening between the hours of 2000–2400, coinciding with the start of the bat's natural diurnal activity period. Trials were run in a dimly lit room (4.85 m long × 3.25 m wide × 3.32 m high) where the walls were lined with sound-attenuating foam (Sonex® Classic; Pinta Acoustic, Inc., Minneapolis, Minnesota, USA). The Y-maze was located on the floor of the experimental room during experiments. At the beginning of each trial, a focal bat was placed in the start arm of a plexiglass Y-maze with a transparent cover (Fig. 1A) and it was permitted to freely move in the maze towards odours from one female bat and one male bat located at the end of the left or right stimulus (scent) arms (Fig. 1B). The dimensions of the Y-maze were based on previous behavioural studies conducted on *E. fuscus* and the Mexican free-tailed bat (*Tadarida brasiliensis* (I. Geoffroy, 1824)) (Gustin and McCracken 1987; Bloss

Fig. 1. Y-maze behavioural testing arena. (A) Side-view schematic of the Y-maze constructed with plexiglass, except for the ends of each "Y" that were covered with a ¼ inch (0.635 cm) stainless steel mesh to permit airflow through the maze arms. (B) Top-view schematic of the experimental set-up. Cotton swab bundles infused with the scent of stimulus big brown bats (*Eptesicus fuscus*) 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 farthest from the Y-junction. Broken lines represent two invisible boundaries that a test bat needed to completely cross to be scored as having entered a specific arm. Colour version online.

(A)

(B)

L - Scent Arm 1
R - Scent Arm 2
S - Start Arm
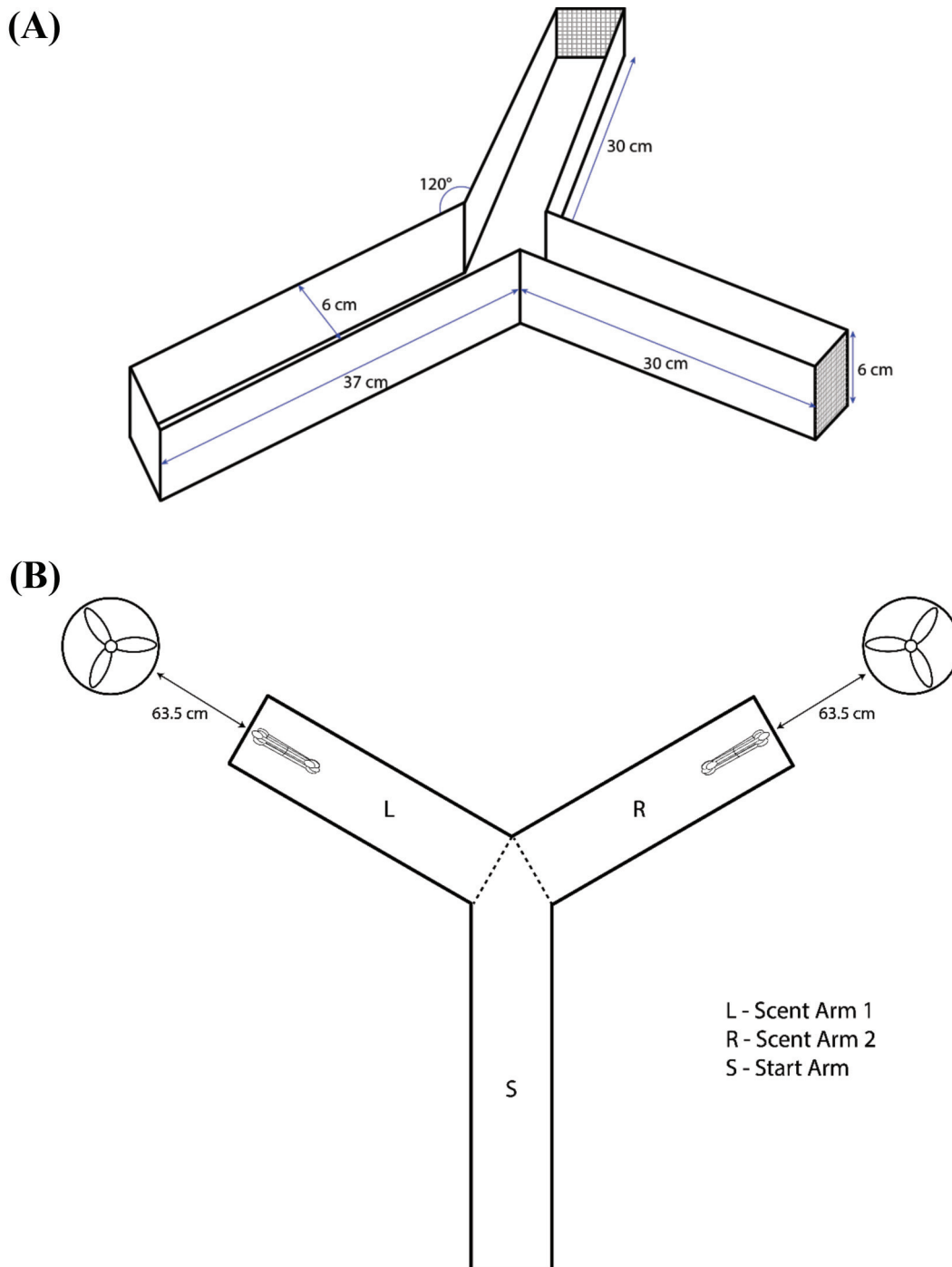
et al. 2002; Kilgour et al. 2013). In our experiments, we define odour as a chemical signal that is perceived by the olfactory system of a receiver. Individuals served as a focal bat and as an odour stimulus bat for a male or female focal bat no more than once between experiments 1A and 1B. Focal bats were never exposed to

their own odour or the odour of a known relative. At the end of testing, all animals were returned to their holding cages temporarily prior to being returned to the colony at the end of the night 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 on cotton swabs and was exclusively from adult animals. Experimenters collecting odours wore clean latex gloves that were changed each time an animal or odour was handled to avoid cross-contamination with both bat and (or) human scents. Each end of a cotton swab was manually swiped 10 times, following an antero-posterior direction, with a consistent stroke length and pressure. Odours were collected from five body areas of stimulus bats using a clean swab for each area: the muzzle (including the pararhinal region), dorsum (between shoulder blades), ventrum (chest area), plagiopatagium of the right wing, and anogenital area (both the genitals and the anus). These regions were selected due to past evidence of sexually dimorphic glands and self-anointed scents in Chiroptera (e.g., Gustin and McCracken 1987; Bouchard 2001; Muñoz-Romo et al. 2011; Flores and Page 2017). Swabs that became contaminated with saliva, urine, or feces were discarded and a new swab was used to re-collect the scent from the given area. Following scent collection, the five cotton swabs collected from 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 1 h of collection, with the majority used within 10 min.

Bats were pseudo-randomly assigned to be either a focal or a stimulus animal. Odours from stimulus bats were pseudo-randomly assigned to either the left or the 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: (i) the bundle of odour swabs was removed from its Ziploc bag; (ii) one end of the bundle was pressed and dragged across the floor of the Y-maze arm beginning from the end of the stimulus arm closest to the Y-junction; (iii) the bundle was placed at the end of the stimulus arm with the dragged end pointing toward the end of the arm; (iv) the swab bundle was 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 placed ~63.5 cm from the mesh end of each stimulus arm were turned on to their lowest setting to gently move stimulus scents down each 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 beginning of the "start" or neutral arm of the maze with the bat's head facing the Y-junction (Fig. 1B). Trials began when the experimenter released the bat; at which point, we immediately slid the clear plexiglass cover over the maze and exited the room for the remainder of the trial. Pilot testing with males and females revealed that bats explored the full Y-maze in 3 to 4 min, hence we decided upon a 5 min trial length per focal animal. 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. Trials occurred 5–10 min apart using multiple Y-mazes.

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, focal animal sex, and assignment of male and female odours in the stimulus arms. 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 stimulus arms and the start arm). A bat was deemed to have entered an arm when its head and body were completely within the arm (see broken lines in Fig. 1B). We also scored the first and last arms entered by a 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.

Experiment 1: evaluating olfactory odour preference and influence of habituation

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 (experiments 1A and 1B) to evaluate whether habituation to the test arena impacted the behavioural response.

In experiment 1A, 30 bats (n = 15 females and 15 males) were used as focal animals. Each animal was allowed to freely explore the Y-maze for 10 min 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.

Experiment 1B was designed to evaluate whether habituation influenced an animal's subsequent exploration of the Y-maze in comparison with experiment 1A. Thirty-six bats (n = 18 females and 18 males) 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. Subsequent experimental testing of odour preference in focal bats was identical to experiment 1A. None of the animals had experience with Y-maze testing prior to experiment 1B.

Experiment 2: evaluating urinary preference

Experiment 2 evaluated whether individual E. fuscus showed a preference for the odour of male and (or) female urine. Thirty-four bats (n = 15 females and 19 males) were used as focal animals. Of these, all 15 females and 13 of 19 males were adults (>1 year old), with the remaining 6 males being yearlings (~5 to 6 months old). Given that yearlings have been observed to mate within our captive colony (L.J.S. Greville, personal observation), we expected them to behave similarly as adults in response to conspecific urine. All but six 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 collection, and statistical analyses — were identical to those of experiment 1B except that we used urine as the odour stimulus instead of general body scents. We collected urine from male and female bats non-invasively following previously published procedures (Greville et al. 2017, 2020). Briefly, roosting adult bats were quickly grabbed by hand and held over a work surface lined with wax paper 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, Kent, United Kingdom) placed at the start of the 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 toward the distal end of the stimulus arm.

Data analysis

The sex of the focal and stimulus bats was decoded prior to statistical analysis. Data from each focal bat was organized by the (i) total time spent in the male-scented, female-scented, and start arms; (ii) stimulus arm the bat first entered (i.e., first choice); and (iii) stimulus arm the bat last entered when the trial ended (i.e., final choice). We also measured the proportion of male to female

focal bats that first entered a male- or female-scented arm, or never left the start arm. A focal bat's preference for the scent of a male or female stimulus bat was determined using three criteria with different time thresholds: 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 stimulus arm, 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 were excluded from the analysis. This is because a tie does not indicate an individual's inability to discriminate, but that the observer was unable to assess a preference (Siegel 1956; Gustin and McCracken 1987; Bouchard 2001). To examine the effect of habituation, a χ^2 test of independence was used to compare the total proportion of habituated and non-habituated bats that entered a stimulus arm. Multiple corrections were not necessary because the data used in statistical analyses for each question within an experiment, with the exception of the time preferences, were independent and all animals were tested as focal animals once in each experiment. If time preferences were statistically significant within an experiment, then P values would be adjusted for multiple corrections appropriately. All statistical tests were performed in Jamovi (The Jamovi Project 2020) running in the R software environment (R Core Team 2019) using an α of 0.05.

Results

General observations

Focal bats in the Y-maze exhibited a variety of behaviours. 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 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 displaying behaviour suggestive of prior assessment. Some animals remained in the initial stimulus arm that they entered, whereas others consistently explored the entire maze. Other than the "freezing" behaviour mentioned above and urination or defecation by some individuals during maze exploration, no signs of extreme stress (shaking or trembling, rapid breathing, sweating, etc.) were observed in bats during or following the experiments.

Experiment 1: evaluating olfactory odour preference and influence of habituation

In experiment 1A, a large proportion of focal bats that were habituated to the testing arena did not leave the start arm of the Y-maze, with some never leaving their initial starting position. Indeed, a majority of test bats previously habituated to the Y-maze remained in the start arm for the entire 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 1).

In experiment 1B, 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 focal animals of either sex (Table 2).

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 experiments 1A and 1B. There was a significant increase in the total number of non-habituated animals that entered a stimulus arm in comparison with habituated

Table 1. Summary of "experiment 1A: evaluating olfactory odour preference and habituation trials".

| | Scent | | | |
Variable	Female	Male	Neither	P
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

Note: Initial and last choices of focal big brown bats (*Eptesicus fuscus*) (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. Neither scent refers to animals not entering either stimulus arm for first choice, and includes animals not meeting absolute or timed preference thresholds. Reported values are the number of individuals who met specific criteria. P values calculated using a binomial test.

Table 2. Summary of "experiment 1B: evaluating olfactory odour preference and non-habituation trials".

| | Scent | | | |
Variable	Female	Male	Neither	P
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

Note: Initial and last choices of focal big brown bats (*Eptesicus fuscus*) (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. Neither scent refers to animals not entering either stimulus arm for first choice, and includes animals not meeting absolute or timed preference thresholds. Reported values are the number of individuals who met specific criteria. P values calculated using a binomial test.

animals ($\chi^2_{[1]}$ = 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 ($\chi^2_{[1]}$ = 5.66, P = 0.017) as opposed to females ($\chi^2_{[1]}$ = 2.42, P = 0.120). The proportion of animals that entered a stimulus arm along with the respective χ^2 tests, and P values are reported in Table 3.

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 initial preference for the urine of either sex (Table 4). However, female focal bats exhibited an initial preference for the stimulus arm containing male urine (binomial test, P = 0.013). 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 timed preference

Table 3. Summary of comparisons between experiments 1A and 1B summarizing results obtained by χ^2 tests of independence comparing the proportions of habituated versus non-habituated focal big brown bats (*Eptesicus fuscus*) (*n* = 15 habituated animals per sex; *n* = 18 non-habituated animals per sex) that entered either stimulus arm.

| Group | Proportion of animals entering a stimulus arm | | χ^2 | df | *P* |
	Experiment 1A: habituated	Experiment 1B: non-habituated			
Males	0.200	0.611	5.66	1	0.017
Females	0.667	0.889	2.42	1	0.120
All	0.433	0.750	6.87	1	0.009

Table 4. Summary of "experiment 2: evaluating urinary preference".

| Variable | Urine | | | *P* |
	Female	Male	Neither	
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

Note: Initial and last choices of focal big brown bats (*Eptesicus fuscus*) (*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. Neither scent refers to animals not entering either stimulus arm for first choice, and includes animals not meeting absolute or timed preference thresholds. Reported values are the number of individuals who met specific criteria. *P* values were calculated using a binomial test.

criteria, none of these comparisons reached statistical significance (Table 4).

Discussion

We found no evidence that *E. fuscus* prefer the general body odours or scents of conspecifics; however, our data show that the majority of female focal bats showed a preference by first approaching the scent arm containing male urine. Male bats did not show a preference for the scent of urine from either sex. Additionally, we demonstrated that by foregoing habituation the likelihood of individuals exploring the stimulus arms of the maze during experimental testing increased.

Across bat species, olfactory cues provide contextual information to an individual, including territory delineation, mate quality, and individual recognition (Bloss 1999; Voigt and von Helversen 1999; Keeley and Keeley 2004; Doss et al. 2016). Thus far, the ability to discriminate between sexes by scent has only been demonstrated in two bat species: *M. condylurus* and *C. pumilus* (Bouchard 2001). The interaural crest of *C. pumilus* is sexually dimorphic and only present in males, whereas 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 themselves (Bouchard 2001). In the present study, we found no evidence that *E. fuscus* distinguished between the sexes during the mating season using general body scents of conspecifics (Tables 1 and 2). Whereas 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 and 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, there is no evidence that *E. fuscus* pups differentiate the odour of their mothers from other individuals (Mayberry and Faure 2015). Altogether, studies of odour discrimination suggest that *E. fuscus* may still differentiate odours on a group level, perhaps influenced by a colony's unique microclimate or microbiota, but discrimination based on individual odour differences has not been demonstrated.

Female focal bats showed an initial preference for the urine of male conspecifics, but male focal bats showed no preference for urinary odours from either sex (experiment 2, Table 4). Females also spent more time in the stimulus arm containing male urine; however, these time preferences did not reach statistical significance. Nonetheless, these results suggest that urine may contain chemical cues that influence an animal's behaviour. 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 since chemicals present in urine and feces allow for sex discrimination in a variety of mammals, including ferrets (*Mustela furo* Linnaeus, 1758 = *Mustela putorius furo* Linnaeus, 1758) (Zhang et al. 2005), meadow voles (*Microtus pennsylvanicus* (Ord, 1815)) (Ferkin and Johnston 1995), and mice (Zhang et al. 2007). Mouse urine contains a high concentration of major urinary proteins (MUPs) that vary in composition between individuals (Hurst 2009). MUPs are often bound to pheromones to prevent their degradation and may also function independently as pheromones (reviewed by Liberles 2014). Because the types of MUP isoforms present in mouse urine is influenced by genotype and sex, this provides individual's with a unique signature that may convey sexual information to conspecifics. Additionally, pheromones in mouse urine differ between the 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 (*Rattus norvegicus* (Berkenhout, 1769)) were shown to act as attractants when they were 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 in our study demonstrated that *E. fuscus* females approach male urine. Whether this is due to urinary steroids, MUPs, or other chemical components needs further investigation, as does whether females prefer male urine or avoid 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), suggests that olfactory cues alone may not be sufficient for sex preferences to be observed. It is possible

that 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 that we tested in experiment 1 may only provide information about an individual in combination with a secondary cue, such as acoustic signals (e.g., echolocation or social 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 greater sac-winged bats (*Saccopteryx bilineata* (Temminck, 1838)), 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 and Masters 2004) similar to Mehely's horseshoe bats (*Rhinolophus mehelyi* Matschie, 1901) and Mediterranean horseshoe bats (*Rhinolophus euryale* Blasius, 1853) (Schuchmann et al. 2012). These observations support the idea that bats may require a combination of sensory cues to distinguish an individual's sex.

Olfactory cues are used in differing contexts across species including scent marking for navigation (little brown bat (*Myotis lucifugus* (Le Conte, 1831)); Buchler 1980) and territory markings (*S. bilineata*; Voigt and von Helversen 1999). Male greater short-nosed fruit bats (*Cynopterus sphinx* (Vahl, 1797)) and *T. brasiliensis* frequently mark their roosts with saliva and gular gland secretions, respectively, during the mating season to attract mates and increase mating success (Keeley and Keeley 2004; Doss et al. 2016). However, guano-laced artificial roosts failed to attract more *E. fuscus* than control roosts without guano (Brown et al. 2020). To date, the ecological implications of attraction to urinary odour and 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.

The significance of female bats initial preference of male urine in the context of mating behaviour is yet to be determined. Behaviour leading up to copulation is seldom observed and typically experimenters find animals already in copula. As such, little information regarding courting in *E. fuscus* has been documented, but observations of males approaching females and females approaching males have been observed. In captivity, male bats may crawl onto a female's back and bite her head or neck while displaying stereotypical pelvic thrusting (Mendonça et al. 1996; Mendonça and Hopkins 1997). Despite this behaviour, it remains difficult to know when or if ejaculation occurs (Mendonça et al. 1996). Females that mated would often bite back at the males before copulating, whereas others approached and nudged males before crawling under them, thus making female receptive behaviour difficult to quantify in the species (Mendonça et al. 1996). Aggression in the form of vocalization and biting is not unique to mating behaviour, as anecdotally we have observed male–male, female–female, and female–male aggression in our captive colony throughout the year. Evidence of minor aggression tends to increase during the mating season, but the nuances and cause of aggression in *E. fuscus* remain understudied. Aspects of courtship and aggression must be better understood in both captive and wild settings before the impact of the current results is known.

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 previously habituated to the arena (Table 3). 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., Gustin and McCracken 1987; Caspers et al. 2009; Kilgour et al. 2013) or (2) the bat is confined to the starting chamber within the arena prior to the trial (e.g., Bouchard 2001; Bartonička et al. 2010; Mayberry and Faure 2015; Boyer et al. 2020). 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 exploration 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", whereas Bouchard (2001) noted that wild bats 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 focal 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 and Réale 2008), songbirds (Verbeek et al. 1994), and fish (Yoshida et al. 2005). Thus far, only five studies have investigated personality (or behavioural syndromes) in bats (Kilgour and Brigham 2013; Menzies et al. 2013; Nachev and Winter 2019; Boyer et al. 2020; Webber and Willis 2020). Nonetheless, they have provided support for the existence of personality traits in Commissaris's long-tongued bat (*Glossophaga commissarisi* Gardner, 1962), *M. lucifugus*, and *E. fuscus* (Kilgour and Brigham 2013; Menzies et al. 2013; Nachev and Winter 2019; Boyer et al. 2020; Webber and Willis 2020). Tests assessing the exploratory behaviour of *E. fuscus* found that they behaved consistently across repeated trials throughout the study period (Boyer et al. 2020). The exploratory behaviour of individual *E. fuscus* was unaffected by changes in social group composition, suggesting that this behaviour is relatively stable. This may be due to genetic influences on personality and exploratory behaviour, which have been demonstrated in mice (DeFries et al. 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) reported only four individual *E. fuscus* (out of hundreds banded) being recaptured at a colony other than that of initial capture. However, *E. fuscus* display fission–fusion social dynamics where colony members alternate roosting sites within a region and subgroup composition changes frequently (e.g., Willis and Brigham 2004; Metheny et al. 2008). Given that *E. fuscus* travel multiple kilometres while foraging and frequently use night roosts that differ from day roosts (Brigham 1991), it is not unreasonable to speculate that wild bats caught at different sites may be familiar with each other. In our study, all *E. fuscus* were from a captive colony and thus had past experiences with each other. Past research in captive *E. fuscus* suggests that some individuals show preferential associations with other individuals, but the possible impact of these associations on the current results are unknown (Kilgour et al. 2013). It is possible that individuals quickly identify odours present in the maze based on past interactions and thus maze exploration is not necessary to gather information about the individuals (Hurst and Beynon 2004). 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

that the current experimental design using individuals from a single colony still provides valuable insight into olfactory sex preferences.

Given our results on the effects of maze habituation, the observed female preference for male urine must be evaluated in the context of a novel environment. It is possible that under familiar conditions, the same findings would not hold true. We must also consider that not all female bats in our study were sexually receptive. All experiments were performed during the autumn mating season of *E. fuscus* where individuals mate repeatedly over an extended period; however, physiological indicators such as vaginal smears were not collected. Thus, not all females may have been in a reproductive state during the time of experimental testing and (or) body odour or urine collection, and individual reproductive condition may influence our result. 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 (*Pipistrellus pipistrellus* (Schreber, 1774)) to reliably discriminate between the odours of individual colony members and the odours of a home versus a foreign roost using food reinforcement. Because *E. fuscus* can distinguish between individuals of their colony and strangers (Bloss et al. 2002), it is likely this 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 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 bat personality types may influence an individual's exploratory behaviour during experimental testing with Y-mazes and other arenas.

Conclusion

We found no evidence that general body scents from conspecifics were preferred by either male or female *E. fuscus*. Female bats showed an initial preference for male urine; however, male bats showed no preference for the odour of male or female urine. Animals that were not habituated to the Y-maze showed an increase in exploratory behaviour at the time of experimental testing compared with bats that were habituated.

Competing interests statement

Contributors' statement

Funding information

Data availability

Data are not currently available in an online repository. Data are available directly from authors.

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