Testing the Role of Frequency-Modulated Bouts in *Eptesicus fuscus*

By

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Masters of Science

in the Department of Psychology, Neuroscience & Behaviour

Faculty of Science

McMaster University MASTERS OF SCIENCE (2025) Hamilton, Ontario

Department of Psychology, Neuroscience & Behaviour

TITLE: Testing the Role of Frequency-Modulated Bouts in *Eptesicus fuscus*

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NUMBER OF PAGES: ix, 81

Acknowledgements

I would like to thank my supervisor, Dr. Paul Faure, for graciously accepting me into his lab of wonderful colleagues and research. In this environment I have grown academically, professionally, and personally, and I am eternally grateful for my time here. Paul, thank you for all your support! Thank you to my supervisory committee, Dr. Ian Bruce, Dr. David Feinberg, and Dr. Sigal Balshine, for the fresh ideas and feedback regarding my thesis. I specifically want to acknowledge Dr. Balshine for the guidance she provided while Paul was on sabbatical.

I couldn't have completed this thesis without the members of the McMaster Bat Lab, especially Shane, Tang Tang, and Joanna. Thank you, Shane, for helping me figure out statistics, Joanna, for scoring so many videos, and Tang Tang, for being my cohort friend to the end. Thank you to Dawn Graham, Kathy Delaney, and the Central Animal Facility staff for taking excellent care of the bats. Thank you to McMaster alumni Dr. Kazuma Hase and Gary Fekete for developing my synthetic call and experimental arena.

Thank you to my family; Vineta, Indrek, and Amalie, for supporting me throughout my master's degree. I'm so grateful that I went through this process so close to home. Thank you to my cohort friends, Alyssa, Maya, Fermin, and Anmol, for making my time in Hamilton and McMaster so memorable and enjoyable and thank you to my partner Joshua for encouraging me every step of the way and making sure I never went hungry during the long nights of bat research. I will always treasure my years at McMaster.

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Declaration on Academic Achievement

Testing the Role of Frequency-Modulated Bouts in *E. fuscus*

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M. K. designed the study with assistance from P. A. F. and K. H.; K. H. developed synthetic FMB; M.K. conducted experimental trials with occasional help from McMaster Bat Lab members; J. Z. scored trial videos; M.K. analyzed data with help from S. D. I. S.; M. K. interpreted data, created figures, and wrote manuscript; P. A. F. edited manuscript.

Abstract

Social vocalizations serve multiple purposes in animal communication and are particularly common in bats (Order Chiroptera). The big brown bat (*Eptesicus fuscus*; Family Vespertilionidae) emits downward frequency modulated (FM) sweeps that are used for echolocation. Male *E. fuscus* have been reported to emit a cluster of 3-4 downward FM signals that are longer in duration than echolocation calls, known as frequency-modulated bout (FMB) signals, and have been hypothesized to play a role in food competition. Other Vespertilionid species emit social calls that share spectro-temporal properties with the FMB. These social calls have a role in food competition and incite positive phonotaxis in females, so it is conceivable that the FMB also has a function in mate attraction. My thesis focused on the role of FMB social calls and if they are attractive to females. I tested female *E. fuscus* in acoustic playback trials presenting bats with competing signals in a two-alternative forced choice paradigm. Bats were presented with combinations of three types of synthetically-generated sounds—a natural-like FMB stimulus, a time-reversed FMB stimulus, and acoustic system noise generated by a silent signal—and I measured the relative affinity or aversion of bats towards different combinations of these signals. Bats were tested first in the fall mating season and then retested in the spring after winter hibernation season. If FMBs are mainly emitted by males for mate attraction, then I predicted females would show an affinity for them, particularly during mating season. My results somewhat corroborate the hypothesis as the females showed some affinity for these calls. These results provide a foundation for further research into understanding big brown bat mate choice and social behaviour.

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Introduction

Communication in Animals

Communication between animals can be defined as the transmission of a signal from one animal to another that alters the behaviour of the receiver in a manner that is intended to increase the fitness of the sender (Kaplan, 2014). Communication spans many modalities, including visual, olfactory, and auditory. Visual signals in animal communication include mate attraction by using vibrant colouration (Hill, 1990; Sætre et al., 1994), unique patterning to warn against predators (Wang & Shaffer, 2008; Wiklund & Järvi, 1982), and presentation of dances that indicate the presence of food (von Frisch, 1967). Olfactory communication occurs via pheromones and can aid in navigating scent trails of conspecifics and prey (David Morgan, 2009; Gehlbach, 1971), act as an attractive element in courtship (Baker & Cardé, 1979; Houck & Reagan, 1990), and exacerbates or dampens male-male aggression (Chamero et al., 2007; Mugford & Nowell, 1970; Wang & Anderson, 2010).

Acoustic signaling is a diverse and effective modality of animal communication. Animal vocalizations can be separated into two categories: affiliative calls transmitted towards conspecifics that share a positive relationship with the sender, and agnostic calls emitted towards either conspecifics or heterospecifics during hostile interactions like territory defence (Kondo & Watanabe, 2009). A prevalent affiliative signal is the contact call, which is used to transmit the identity of the caller and related information to conspecifics. Many bird species use contact calls to identify their mate, including the free-ranging green-rumped parrotlet (*Forpus passerinus*), zebra finch (*Taeniopygia castanotis*), and middle spotted woodpecker (*Dendrocoptes medius*) (Berg et al., 2011;

Vignal et al., 2008; Wegrzyn et al., 2021). These calls are not limited to avian species; wild dwarf mongooses (*Helogale parvula*) use contact calls to discriminate between individuals in their group in a food competition context (Sharpe et al., 2013) and female rhesus macaques (*Macaca mulatta*) display affinity for contact calls from kin over non-kin (Rendall et al., 1996). Conversely, agnostic calls serve as a warning or call to action. One example is the mobbing call, utilized by multiple species of birds to summon conspecifics to attack a predator (Baker & Becker, 2002; Griesser, 2009).

Both affiliative and agnostic signals can incite phonotaxis, the movement of an animal towards or away from a sound source (Gerhardt, 2006). Several species use mate-advertisement calls unique to their sex to attract the opposite sex. The female túngara frog (*Engystomops pustulosus*) and Pacific treefrog (*Hyla regilla*) demonstrate significant positive phonotaxic responses towards male advertisement calls (Brenowitz & Rose, 1999; Ryan & Rand, 1993). A sex-reversed example of this phenomenon occurs in the large odorous frog (*Odorrana graminea*), where males respond to female calls with positive phonotaxis and by emitting their own antiphonal call (Shen et al., 2023). This positive phonotaxis behaviour is not exclusive to anurans; female Japanese quail (*Coturnix japonica*) show significant attraction towards speakers playing male calls during the mating season (Goodson & Adkins-Regan, 1997). In mammals, male mice have been observed producing elaborate calls in the presence of females, which elicits positive phonotaxis from the females (Hammerschmidt et al., 2009).

Social Calls in Bats

A common vocalization in bats is echolocation. Echolocation is an active sensing system where signal production is coupled with echo reception. The ability to echolocate endows bats and other organisms with the ability to orient and detect objects in their environment, using timing, frequency, and amplitude differences between outgoing emitted sound pulses and their later reflected echoes (Fenton et al., 2012; Jakobsen et al., 2013). Many species of bats possess a robust repertoire of vocalizations beyond echolocation pulses that are characterized as calls and songs. Smotherman et al. (2016) distinguishes between bat 'calls' and 'songs' with 'calls' being short, immediate vocalizations like contact and alarm calls, and 'songs' being longer, more complex sequences of syllables whose emission is dependent on time and season and is usually accompanied by courtship or territorial behaviours. 'Songs' often consist of groups of repeated syllables known as bouts. As this is simply one interpretation of the difference between bat social vocalizations, there may be overlap in the literature between what Smotherman et al. (2016) defines as a 'call' and as a 'song'.

The first social calls produced by some bat species are contact calls that develop shortly after birth. Little brown bat (*Myotis lucifungus*), big brown bat (*Eptesicus fuscus*), and evening bat (*Nycticeius humeralis*) pups all emit isolation calls (i-calls) when separated from their mother's teat until the mother facilitates a reunion (Barclay et al., 1979; Gould, 1971; Scherrer & Wilkinson, 1993; Mayberry and Faure, 2015). Bat contact calls can also play a role in groupmate recognition (Carter et al., 2008, 2009). Common vampire bats (*Desmodus rotundus*) show a preference for contact calls from conspecifics they have shared food with compared to infrequent food donors (Carter & Wilkinson, 2016).

Bat social vocalizations can result in positive phonotaxis in conspecifics. Spix's disc-winged bat (*Thyroptera tricolor*) uses unique vocalizations to attract the attention of roosting conspecifics, who in return call back, directing the original caller to the roost (Chaverri et al., 2010). Greater spear-nosed bats (*Phyllostomus hastatus*) respond to conspecific screeches broadcasted around their feeding sites and roosts by increasing passes around these areas (Wilkinson & Wenrick Boughman, 1998). Similarly, the common noctule (*Nyctalus noctula*) flies past roosts that broadcast social calls from maternity colonies significantly more often than roosts broadcasting background noise or no noise (Furmankiewicz et al., 2011).

Multi-Purpose Calls in Bats

It is also possible that a single bat song or call serves multiple social functions. The song produced by the male greater sac-winged bat (*Saccopteryx bilineata*) delineates their territory and can also lure females to new roosts (Knörnschild et al., 2017). Another well-documented example of a multipurposed call comes from the male common pipistrelle (*Pipistrellus pipistrellus*) and male soprano pipistrelle (*Pipistrellus pygmaeus*), who emit a vocal bout called 'songflight', consisting of 2-5 pulses of downward FM sweeps (Barlow & Jones, 1997a). These bats also emit 'social calls' that are indistinguishable from songflight in the case of the soprano pipistrelle. In the common pipistrelle, the main portion of songflight is visually similar to the social call in their spectrograms, although some spectro-temporal parameters differ statistically. In recent literature, the similarities in structure and function of these calls have resulted in songflight and social calls being considered as the same vocalization (Götze et al., 2020; Sachteleben & von Helversen, 2006).

Songflight aids in food competition and can elicit positive phonotaxis in females, depending on the context when sound is emitted. When songflight calls are emitted by male common pipstrelles around their roosts during mating season, visits by females increase (Lundberg & Gerell, 1986). Meanwhile, the presence of an individual hunting for prey using feeding buzzes leads to a greater likelihood of social call emission in the soprano pipistrelle (Sachteleben & von Helversen, 2006), and social calls have been observed concurrently with common pipistrelle foraging behaviour (Götze et al., 2020), indicating that they are emitted during food search. In both species, social call production significantly increased as prey density dropped, and bat activity decreased with increase of social call playback, implying that bats make this call to deter other bats when food is low (Barlow & Jones; 1997b). It is evident that vocalizations in Order Chiroptera are not limited to echolocation and serve a range of purposes, including kin recognition, conspecific attraction, and prey consumption. The prevalence of social calls can be further investigated by studying their function in a common North American species, the big brown bat. *E. fuscus* produces a relatively diverse range of social calls, mostly of unknown function. By using a common bat species, we gain insight into the extent of their ability to communicate vocally and can apply what has been learned to other bat or mammal species.

Social Calls in Big Brown Bats

The ontogenetic development of social vocalizations in big brown bats is well documented. Starting on postnatal day (PND) one, big brown bat pups emit i-calls that facilitate reunion with their mother (Gould, 1971). These i-call contact calls comprise most of the big brown bat's vocal repertoire until PND four (Mayberry & Faure, 2014; Monroy et al., 2011), after which the signals emitted by pups begin to diversify both temporally and spatially. During postnatal week two, pups deliberately produce different syllables based on the presence or absence of their mother. This difference becomes less pronounced as the pups mature, until they primarily emit FM calls regardless of the mother's presence.

The diversity of syllable types in the pup's repertoire and the pup's ability to switch between calls depending on whether the mother is present or not suggests that big brown bats are capable of complex social communication. A study conducted on adult big brown bats revealed that both sexes are capable of emitting 18 syllable types and do so in largely stationary social contexts, such as jostling for position in a roost or being manually disturbed with a cotton swab while roosting (Gadziola et al., 2012). The sounds emitted varied in duration and spectral-temporal features and could be combined as calls consisting of one syllable or as calls consisting of multiple syllables. Moreover, different syllables were emitted in different behavioural contexts. For example, short duration, downward FM sweep syllables were most common in situations where bats took part in low aggression interactions whereas short duration, broadband frequency noise bursts were most common in high aggression situations. Big brown bats also emit social vocalizations when flying. An in-flight competition for a tethered mealworm revealed seven unique syllable or call types distinct from the big brown bat's typical foraging vocalizations (Wright et al., 2013). The likelihood of each syllable and call produced in-flight depended on the competitors' age, sex, and experience with catching prey (Wright et al., 2013).

Frequency-Modulated Bouts in Big Brown Bats

A vocalization emitted during in-flight competition for food is the frequency-modulated bout (FMB) (Wright et al., 2013). The FMB consists of 3-4 syllables, each about nine ms in duration with an interpulse interval (IPI) of about 19 ms (Wright et al., 2013, 2014). Each pulse is composed of multiple downward FM sweeps with a fundamental FM element that spans in frequency from about 68 to 17 kHz. Although echolocation calls also consist of downward FM sweeps, FMB pulses tend to taper at the start and end of the syllable, somewhat resembling a helix shape in the spectrogram. This characteristic sets them apart from echolocation pulses. In a food competition context, FMBs have been emitted by male big brown bats who are experienced with catching a prey item. When two bats are in proximity and in pursuit of a tethered prey item, if the first bat emits an FMB, then the interbat distance significantly increases and the second bat distances itself from the prey, provided the second bat is less than 1.5 m from the prey during the call (Wright et al., 2014). Additionally, the bat that emitted the FMB is more likely to attack the prey item and emit a feeding buzz than the competitor. Adult female big brown bats also emit the FMB vocalization in food competition contexts (Salles et al., 2024). Crawling females emit the FMB when faced with a competitor, both approaching a dish suspected to contain food. These behaviours suggest that FMBs have a role in food competition in big brown bats.

Two-Alternative Forced Choice Experiments

Many paradigms exist to test animal behaviour decisions. A common one is the two-alternative forced choice (2AFC) test, in which a subject is presented with two stimulus presentations and must choose one of the two alternatives (Wixted & Wagenmakers, 2018). One common 2AFC testing arena is the Y-maze. The basic design of a Y-maze arena is to have one starting arm connected to two choice arms in a ‘Y-’ or ‘T-’ shape. Stimuli such as odors, sounds, or conspecifics are presented at the ends of the choice arms (Floody & Pfaff, 1977; Greville et al., 2021; Kilgour et al., 2013). Common behaviour variables recorded include the first and last arms entered, and time spent in each arm of the maze (Bartonicka et al., 2010; Greville et al., 2021; Kilgour et al., 2013). In bat research, Y-mazes have been used to measure preference between two odours (Bloss et al., 2002; Flores et al., 2019; Greville et al., 2021).

Playback Experiments

In auditory studies, playback experiments are used to gauge a subject’s response to an acoustic signal (McGregor et al., 1992). These trials involve playback of a naturally recorded or synthesized signal to the participants and observation of their behaviour. A major issue in playback experiments is pseudoreplication, where a hypothesis is tested with an insufficient range of stimuli and thus, the study measures the subjects’ responses to the few selected stimuli and not the entire class. Using a synthetic call is a promising solution, as it generates a stimulus representative to the class and removes variation in sound quality that comes from recording in the field or laboratory. A synthetic call should have parameters approximately equal to the mean values of naturally occurring signals and should elicit the same behaviour as the natural call. McGregor et al. (1992) proposed that the results of a playback experiment should not be the conclusion to the hypothesis tested, but rather data that inform the direction of future studies.

The current study looks at female preference for sounds, which has been widely tested in anurans in phonotaxic experiments (Gerhardt, 1992). In these studies, the female is placed in the center of a testing arena equidistant to two speakers that simultaneously play different stimuli (Gerhardt, 1992; Wagner, 1998). Typically, female preference in anuran studies is measured by which speaker is first approached and contacted (Gerhardt, 1974; Márquez et al., 2008; Wagner, 1998). Similar female phonotaxis experiments have been conducted in Japanese quail (*Coturnix japonica*) and black grouse (*Tetrao tetrix*), where birds are placed in a large enclosure between two stimuli-presenting speakers and response variables recorded include time spent within one metre of the focal speaker and the sum of positions relative to each speaker (Goodson & Adkins-Regan, 1997; Hovi et al., 1997).

Chiropteran playback experiments are typically conducted in the field and involve presentation of stimuli from one or more speakers located in the natural environment, with a common behavioural variable recorded being the number of bat passes (flights) in the immediate broadcast area (Arnold & Wilkinson, 2011; Furmankiewicz et al., 2011; Übernickel et al., 2013). A few 2AFC playback tests in bats have also been conducted in a laboratory setting. Grilliot et al. (2015) used this paradigm to investigate whether big brown bats prefer the echolocation calls of opposite-sex bats who copulate at high or low rates. During each playback trial, the bat was first restrained in the centre of a circular, wire mesh arena and echolocation calls from a high- or low- frequency copulator were broadcast for 30 s from one speaker, followed by 30 s of playback of the other copulator's call from another speaker. Speakers were on opposite sides of the arena, facing inwards. The bat was allowed to roam the arena while both calls were played simultaneously for 60 seconds. Parameters measured included the first side of the arena chosen by the bat and total time spent on each side. Similarly, Carter & Wilkinson (2016) studied signal choice preference by common vampire bats (*Desmodus rotundus*) listening to vocalizations emitted by conspecifics they frequently versus rarely shared food with (i.e. regurgitated blood meals). The arena was a maze of wire mesh that allowed the bat to travel to the left, right, forward, or upward. The two conspecific calls were simultaneously presented from speakers at the ends of the left and right arms. Bats were allowed to explore the maze, and variables recorded include time spent in the left and right arms and time taken to enter each arm.

The Current Study

As described above, several bat species like the common and soprano pipistrelle emit calls that serve multiple purposes (Barlow & Jones 1997b; Lundberg & Gerell, 1986). These calls share some spectro-temporal features with the big brown bat's FMB, specifically downward FM sweeps that are repeated 3 or 4 times (Barlow & Jones, 1997b; Wright et al., 2014). All three calls are produced by male vespertilionids and have a role in food competition (Barlow & Jones, 1997b; Wright et al., 2014). Thus, I predicted that in the big brown bat, the FMB also serves to attract females during the mating season. I tested this with a 2AFC paradigm, conducted during the mating (November 2024) and non-mating (April 2025) seasons (Racey & Entwistle, 2000). This repetition allowed me to measure the effects of the FMB signal on female behaviours during two stages of their reproductive cycle. Bats were placed in an arena and presented with a choice between 1) FMB calls and 2) a time-reversed FMB or one of these calls paired with 3) system noise. Our hypotheses were that female *E. fuscus* prefer FMB calls over time-reversed FMB calls and system noise, and that this preference is demonstrated more prominently in the fall mating season than the spring non-mating season.

Materials & Methods

Animals

The study was conducted on 34 adult female big brown bats in November 2024 with 33 bats re-tested in April 2025 (bat V182 died between trials; see Table 1). In April 2025, the study was also repeated on a small sample size (n=8) of male bats to qualitatively observe the effect of the FMB calls on their behaviour (Table 1). These males were a mix of adults (n=3) and juveniles (n=5) and most (n=7) had been introduced to the McMaster colony in 2024. Subjects were identified by passive integrated transponder (PIT) tags injected subcutaneously between the shoulders and visually by numbered, colored plastic split-ring forearm bands. All female bats were caught in Southern Ontario between 2018-2023 and housed in a large husbandry facility at McMaster University where the colony temperature and lighting varied according to ambient conditions (Skrinyer et al. 2017). The main colony consisted of an indoor living area (2.5 × 1. 5 × 2. 3 m; l × w × h) enclosed by concrete walls lined with ¼-inch stainless steel mesh. By passing through a narrow hole in one external wall, bats in the colony had access to a larger outdoor flying area (2.5 × 3.8 × 2.7 m). In both areas, bats were provided with a variety of roosting options and stimuli to mimic their natural environment. Bats were given *ad libitum* access to food (mealworms, *Tenebrio molitor*) and water. All procedures met the guidelines for the care and use of wild animals in research published by the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board of McMaster University.

Acoustic Stimuli

Three types of stimuli were created for acoustic playback. The first was a synthetic frequency-modulated bout (FMB) signal (Fig. 1). A FMB is a sequence (or bout) of 3-4 downward frequency-modulated (FM) sweeps emitted by male *E. fuscus* as a social call in a foraging context (Wright et al., 2013, 2014). The individual FM signals within a bout differ from typical *E. fuscus* FM echolocation calls by being longer in duration and more helical in shape. The FMB stimulus was created using custom MATLAB code (sampling rate = 250 kHz) developed by Dr. Kazuma Hase (University of Toyama). The code replicated the time-frequency structure of a natural FMB pulse based on a published spectrogram (Wright et al., 2014). Acoustic characteristics of the synthetic FMB were taken from the mean values of FMBs recorded in previous studies (ter Hofstede, unpublished; Wright et al., 2013, 2014). The FMB consisted of three FM sweeps each with two harmonics. Individual FM sweeps had a pulse duration of 10 ms and there was a 20 ms interpulse interval between pulses. The fundamental element of each FM sweep had a 55 kHz starting frequency, 33 kHz midpoint frequency, and 22 kHz ending frequency. The second playback signal was a time-reversed FMB, referred to hereon as the 'REV' stimulus, and was created by reversing the synthetic FMB along the time axis. The third stimulus was a control that contained no signal and was a playback of system noise, referred to hereon as the 'SYS' stimulus. This was created on the Audacity audio editing software by inserting 'no sound' sections in the track.

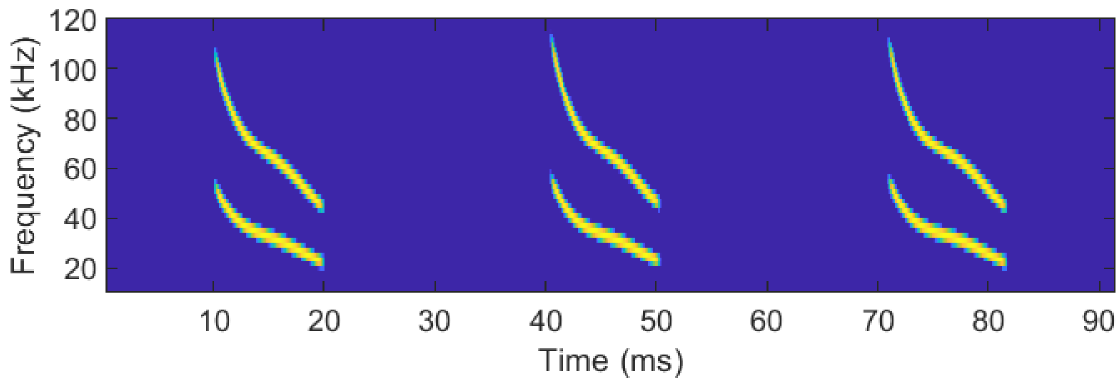

Fig. 1. Synthetic Frequency-modulated Bout. Spectrogram displaying time and frequency ranges of each pulse in the FMB.

Playback Tracks

Single-channel tracks were produced in Audacity with one of the three stimuli before being paired with another stimulus in Avisoft-SASLab Pro to create a two-channel playback track. The playback interval of the FMB and REV signals was randomly jittered in intervals ranging from 0.5 to 1.5 s to minimize habituating the bats to the repeating stimulus. Because no data exists in the literature on the repetition rate of natural FMBs, these values were chosen so the bats could hear the stimulus frequently.

Two categories of tracks were presented to every bat during a trial: a 60 s primer track and a 60 s trial track (Table 2). Primer tracks consisted of 30 s of one stimulus broadcast from one speaker followed by 30 s of another stimulus broadcast from the opposite speaker. The purpose of the primer track was to allow bats to become familiar with the trial track signal they would be immediately tested with, before exploring the arena. Trial tracks consisted of two interleaved stimuli, where tracks from both speakers played simultaneously but with seconds of separation between an acoustic bout from the first speaker and a bout from the second speaker.

A list of 34 pseudorandom track combinations was generated for each stimulus resulting in 4 playback conditions: (1) FMB vs SYS, (2) REV vs SYS, (3) FMB vs REV, (4) SYS vs SYS. The pseudorandom code dictated which speaker would play first during the primer track playback and trial playback, as well as which track would play from each speaker. For each trial, the subject bat was randomly selected as they were grabbed from their cage with no regard for identity and assigned the first available pseudorandom combination. This procedure was repeated for each bat tested in a night.

Testing Arena

Bats were presented with playback signals in a circular experimental arena composed of a plexiglass base and walls (80 × 6 cm; d × h) (Fig. 2). To prevent bats from escaping, a ¼-inch wire mesh lid was clipped to the top of the arena. Bats housed in a small cage (9 × 8 × 3.5 cm) were manually introduced to the center of the arena by lifting the lid. For sound insulation, the walls of the arena were lined with 4 cm thick acoustic foam, and the base was covered with white construction paper. A line of black electrical tape on the construction paper vertically bisected the base of the arena, demarking two sides. This line helped delineate various zones of the arena during trial scoring. A red tape square that aligned with the cage was taped to the base of the arena. The square helped the researcher place the cage during trials.

Two Avisoft Bioacoustics Vifa ultrasonic speakers (frequency range ±12 dB: 1 – 120 kHz) on retort stands were placed on opposite sides of the arena, facing inwards. The speakers were placed 10 cm from the arena wall and elevated 10 cm off the ground. A Reference Signal Generator (Avisoft Bioacoustics) was used to calibrate the output volume of each speaker. The generator played a 40 kHz tone at 80 dB SPL (re: 20 µPa) at a distance of 25 cm. The signal was recorded by a microphone and analyzed using Avisoft SASLab Pro to identify the tone's peak-to-peak amplitude and root mean square voltage. As the bats were to be presented with an 80 dB SPL sound, an appropriate loudness for their hearing range, the output volume of the FMB and REV stimuli were adjusted until the signal received by the microphone had the same peak-to-peak amplitude and root mean square voltage as the calibration signal. A third retort stand placed outside of the arena, extending to ~10 cm over the arena and standing 60 cm tall.

Was used to hold an iPhone 14 so its camera could view the entire arena to film the trials. A GoPro camera (model HERO5 Black) mounted on a tripod ~30 cm away from the arena was used to monitor the whole arena for unconventional behaviour by the bats (i.e. escape).

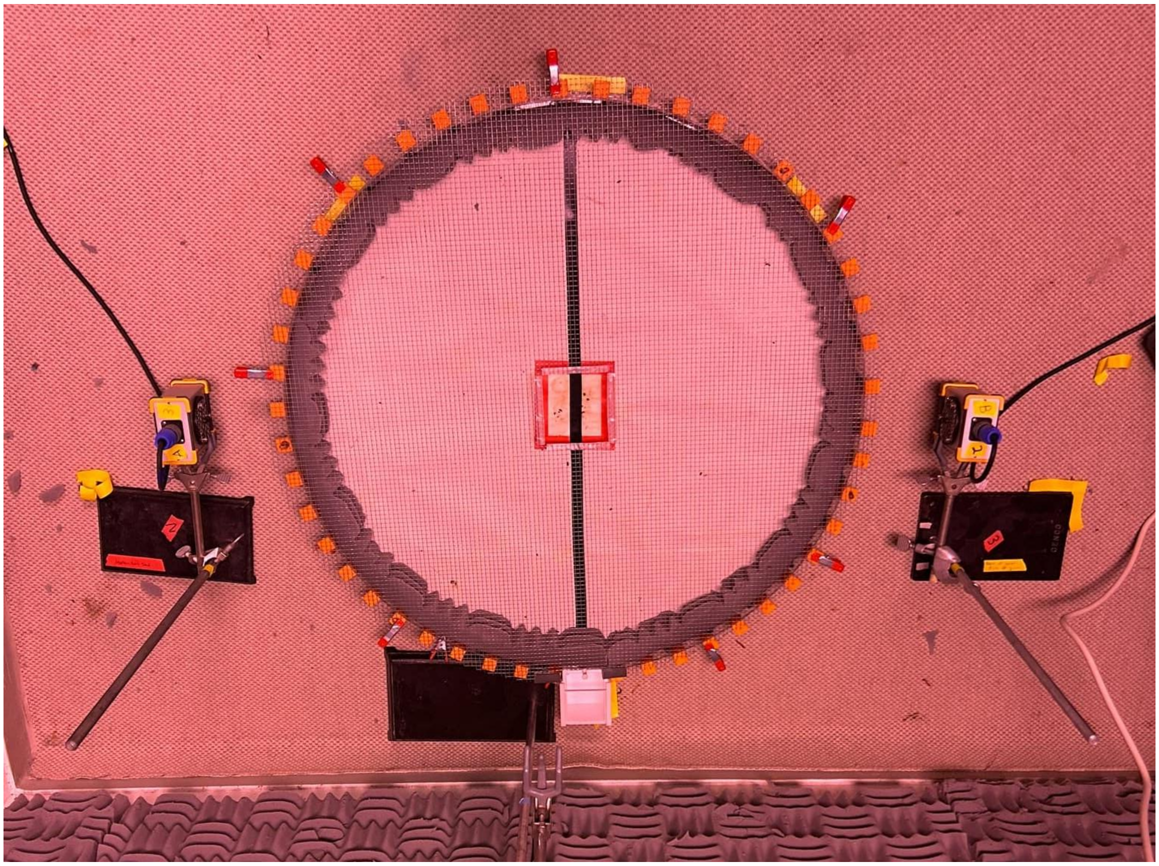

Fig. 2. Experimental arena used in trials. Arial view of the circular arena, left and right speakers, and retort stand that held the iPhone during trials. The diameter of the arena was 80 cm and each speaker was located 10 cm away from the arena and 10 cm above the floor of the playback area.

Playback Trials

Playback trials took place in an animal procedure room whose ceiling and walls were lined with 4 cm sound attenuating foam (Sonex® Classic, Pinta Acoustic, Minneapolis, MN, USA). The floor of the room was covered in a sound-absorbent carpet. The testing arena was placed in the corner of the procedure room (4.8 × 3.3 m) ~20 cm away from the back wall and ~30 cm from the side wall. Curtains hung from the ceiling ~30 cm from the arena and were used to fully enclose the arena from the rest of the room. A table placed ~1.5 m from the curtained-off area, was where researchers set up a laptop connected to an Avisoft UltraSoundGate Player 416H for stimulus playback and an iPad to view video feed from the GoPro camera. Arena trials took place in dim red lighting created by placing red cellophane over the protective diffuser of two dimmable fluorescent light ballasts set to the lowest setting.

On the day of each session, the bats to be tested were transported from the main colony to a holding room 3-4 hours prior to sunset, which fell around 1900 hr in November 2024 and 2000 hr in April 2025, and housed in stainless steel wire mesh cages (24 × 21 × 19 cm) until tested. To prepare the arena for testing, two female juvenile bats (not included in the study, see Table 1) were allowed to crawl on the construction paper and explore the arena for 5 min to deposit scent everywhere and minimize bias.

Each trial was filmed on an iPhone 14. Immediately prior to testing, the subject bat was manually held under a heat lamp for 2 minutes to warm and prepare the bat for crawling in the arena. The bat was then immediately transferred to the cage in the center of the arena, and the primer track was broadcast. The cage was then lifted and, if needed, the bat was prodded to leave the cage with a blunt probe. The cage was removed through a rectangular hole (9 × 8 cm) cut into the middle of the lid. In the November 2024 trials, in the few cases where the bat had been observed to crawl on the underside of the arena's wire lid, a wire screen was immediately placed to cover up this hole. This became common practice in the April 2025 trials, as the bats were more active in this season and were observed crawling on the lid more frequently. Once the bat was in the arena, the 60 s trial track was looped for 8 minutes. The bat was then removed from the arena and returned to its holding cage.

In November 2024, the playback stimuli were administered in ordered blocks: FMB vs REV, FMB vs SYS, REV vs SYS, and SYS vs SYS. This was done to get results least affected by habituation in the FMB vs REV condition — where bats were given the opportunity to demonstrate an initial choice. In April 2025, the order of stimuli was randomized and not administered in blocks. If a bat did not move (respond) in the arena during a trial (n = 29 in November 2024, n = 9 in April 2025), it was re-tested on another day. Trials in which the subject did not move (respond) in the arena after three times were excluded from the final dataset (n = 12 in November 2024, n = 3 in April 2025). If a trial was interrupted by a malfunction of the playback system, sudden shut-off of lights, or the escape of the subject from the arena, the subject was re-tested (n = 5 in November 2024, n = 8 in April 2025). At the conclusion of each session, bats were returned to the colony.

Trial Analysis

Trial video recordings were cropped to a duration of 8 min, starting from the release of the bat into the arena. The recordings were muted and randomly assigned an identification number. Bat behaviour in the videos was scored by a blind observer using the Behavioural Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016). To facilitate scoring, a transparency sheet was taped to the computer monitor and aligned with the arena in the videos. A dry-erase marker was used to outline two zones of interest in the arena (Fig. 3):

(1) Neutral zone. ‘G’ The edges of this zone aligned with the sides of the red tape rectangle in the centre and separated the two sides of the arena. Time spent in the neutral zone did not count toward either side.

(2) Close approach zone. This area was a semicircle that extended two big brown bat body lengths (16 cm) away from each speaker. Time spent in this area was considered a close approach to the loudspeaker.

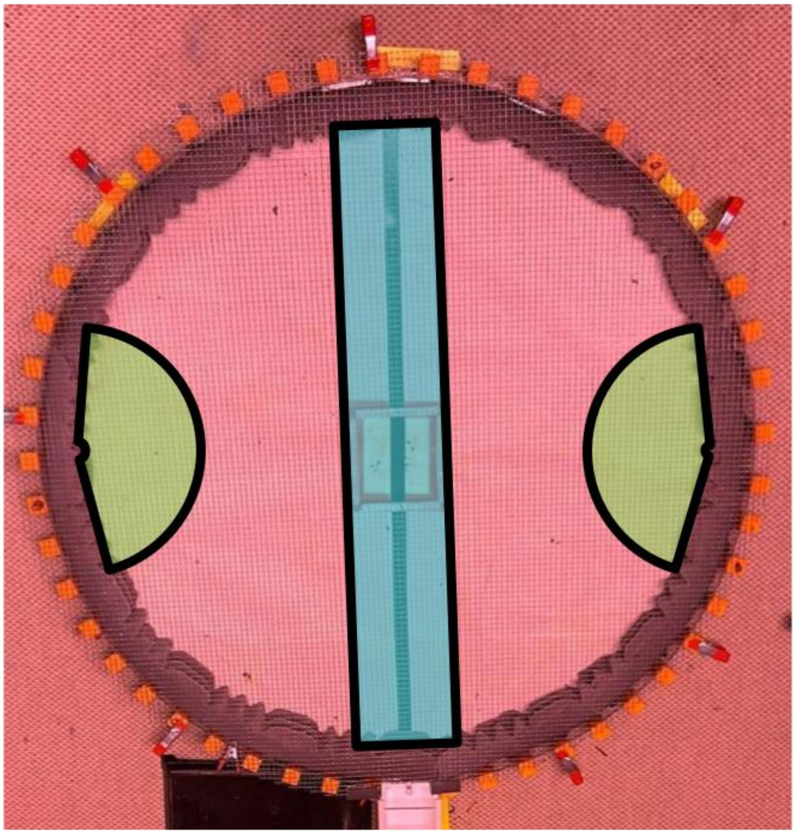

Fig. 3. Arena scoring zones. The two green zones drawn as semi-circles with a diameter of two bat body lengths represent areas of close approach to the speakers. The blue zone is drawn with a width of one bat body length (8 cm) across the diameter (80 cm) of the arena halfway between the speakers and represents a neutral zone where bats were scored as not having made a choice. The green zones are approximately 400 cm^2 each (~19.5% of total area) and the blue zone is 720 cm^2 (~17.6% of the entire area). The pink zones in between are ~1290 cm^2 each (~62.9% of the entire area).

The scorer watched the videos and scored events corresponding to the bat crossing the thresholds of each zone. A bat crossed into the next zone when its wings and legs crossed the line. This provided time stamps when a bat crossed into and out of each zone. The following dependent variables were collected for each trial: first side of the arena chosen, last side of the arena chosen, time spent on each side of the arena, number of bats that approached within two body lengths of the speakers, and time spent within two body lengths of the speakers.

Statistical Analysis

Trial videos were not included in the subset for analysis if they featured a bat who had not moved in any of their three trials or had not left the neutral zone. After these exclusions, the sample sizes for each condition tested in November 2024 were 31 for FMB vs REV, 30 for FMB vs SYS, 31 for REV vs SYS, and 31 for SYS vs SYS. The sample sizes in April 2025 were 33 for FMB vs REV, 31 for FMB vs SYS, 33 for REV vs SYS, and 31 for SYS vs SYS. RStudio (Version 2024.12.1) was used to conduct statistical analyses (Posit team, 2025).

A proportion test (R Core Team, 2023) was used to quantify the following observations: if bats initially chose one side more frequently, if bats chose the final side of their trial more frequently, if bats chose a first or last side more frequently, disregarding the stimulus type, and if bats spent more time on one side than the other. A two-proportion test was used to determine if bats who made a more ‘definitive choice’ spent more time on one side than the other, and if bats approached one speaker within two body lengths more than the other (R Core Team, 2023). A Linear Mixed-Effect Model (Bates et al., 2015) was used to determine if there was a difference between time spent within two body lengths of each speaker. The same Linear Mixed-Effect Model was used in conjunction with a post-hoc Estimated Marginal Means comparison (Lenth, 2023) to determine if this parameter changed with season. The same statistical analyses were conducted in the mating and non-mating seasons. To investigate if season had any effect on binary choices made by the bats, Generalized Linear Mixed-Effect Models (Bates et al., 2015) were run to fit data sets with binomial distributions. This included the first side of the arena chosen, last side of the arena chosen, and side of the arena spent more time on. Proportion tests were used to determine if the frequency of bats making a definitive side choice changed between seasons.

Statistical Interpretations

All proportion tests took as input the number of 'successes', in this case the number of bats choosing or spending more time on a particular side. For all alternatives presented, a 'successful' choice was the FMB side in the FMB vs REV and FMB vs SYS conditions, and the REV side in the REV vs SYS condition. As the sides of the SYS vs SYS condition were identical, half of the sides were randomly assigned to be successful. A significant proportion test indicated that a greater proportion of bats chose the successful side first, last, or chose to spend more time on the successful side.

Time thresholds were set to correlate with the inputs to the two-proportion tests for side choices to compare the proportion of bats that spent at least 120, 240, or 360 s on one side of the arena. Spending this much time was interpreted as the bat having made a 'definitive choice', compared to bats who split their time more evenly in the arena or mostly remained in the neutral zone. The two-proportion test took as input the number of bats that spent over the time threshold on the successful side and the number that spent over the threshold on the other, 'unsuccessful' side. A significant result indicated that out of the bats that made a definitive choice, a greater proportion spent time on the successful side. A different input to the two-proportion test was the number of bats that approached within two body lengths of the successful speaker compared to the number of bats that approached within two body lengths of the opposite speaker. A significant result indicated that more bats approached the successful speaker compared to the opposite speaker. Another two-proportion test took a matrix input of the number of bats that spent a minimum time threshold on the successful side and the number that spent a minimum time threshold on the opposite side, in both the mating and non-mating seasons. A significant result indicated that there was a difference in the proportion of bats to make a definitive and successful choice between seasons. A final two-proportion test took a matrix input of the number of bats who approached within two body lengths of the successful speaker and the number who did not, in both the mating and non-mating seasons. A significant result indicated that the proportion of bats that chose the successful speaker changed between seasons. The same test was run with a focus on the unsuccessful speaker, where a significant result indicated that the proportion of bats that chose the unsuccessful speaker changed between seasons.

The Linear Mixed-Effect Model took as input the time each bat spent within two body lengths of each speaker, offset by 0.1 and log-transformed. The fixed effects for the models were the successful speaker, season, and condition. The random effect was Bat ID. The output was subject to a Chi-square test where a significant result indicated that an interaction between fixed effects changed the log-transformed time spent near each speaker across at least one of those fixed effects.

The Generalized Linear Mixed-Effect Models took as input binomially distributed variables corresponding to if a bat made a successful first or last side choice or chose to spend more time on the successful side. The fixed effects were season and condition, and the random effect was Bat ID. The output was subject to a Chi-square test where a significant result indicated that season changed the proportion of successful choices.

Results

November 2024 (Mating Season)

First Side Chosen. During testing in the fall mating season, neither side was chosen first more frequently than the other (Table 3). Although the proportion of bats that chose the FMB side first in the FMB vs SYS condition was higher than the proportion that chose the FMB side in the FMB vs REV condition, the 95% confidence intervals (CIs) for these proportions overlapped (Fig. 4).

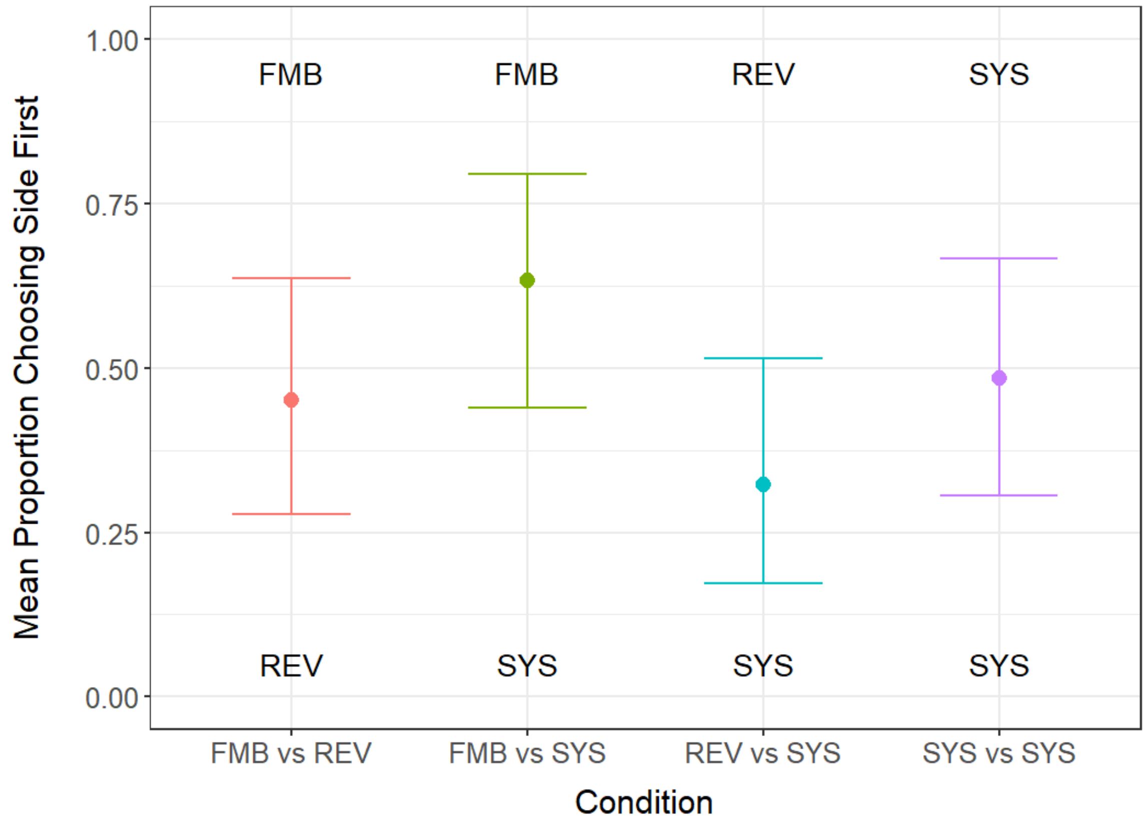

Fig. 4. Average proportion of female bats that chose a given side first in November 2024. Dots represent the proportion of bats that chose the side labelled at the top of the figure first and error bars represent the 95% confidence intervals.

Last Side Chosen. Bats favoured the FMB side as their final choice in the FMB vs SYS condition (Table 3). The proportion of bats that chose the FMB side last in the FMB vs SYS condition was higher than the proportion that chose the FMB side last in the FMB vs REV condition; however, the 95% CIs for the proportions overlapped (Fig. 5).

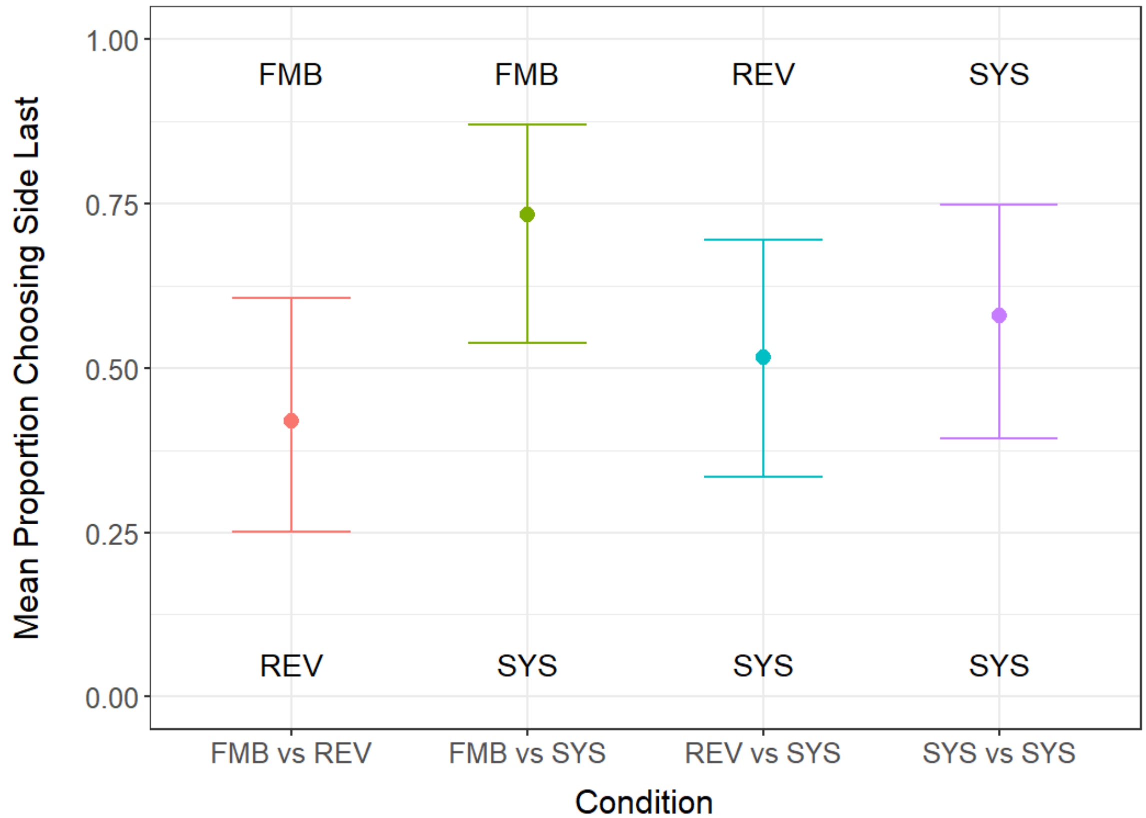

Fig. 5. Average proportion of female bats that chose a given side last in November 2024. Dots represent the proportion of bats that chose the side labelled at the top of the figure last and error bars represent the 95% confidence intervals.

Side Bias. Proportion tests were conducted on the first and last sides chosen regardless of the stimulus that was broadcast. In the REV vs SYS condition, the left side of the arena was chosen first more frequently (Table 4). Neither side was chosen last more frequently.

Time Spent on Each Side. Bats did not exhibit obvious preferences for either side of the arena across all conditions (Table 5). To visualize the times bats spent on either side of the arena, the differences in times spent on the 'successful' side and 'unsuccessful' side were plotted. This yielded a wide range of time differences; the bats between the 25^{th} and 75^{th} percentiles of time differences spent more time on the REV side in the FMB vs REV condition (Fig. 6). Similarly, the bats between the 25^{th} and 75^{th} percentiles of time differences spent more time on the FMB side in the FMB vs SYS condition.

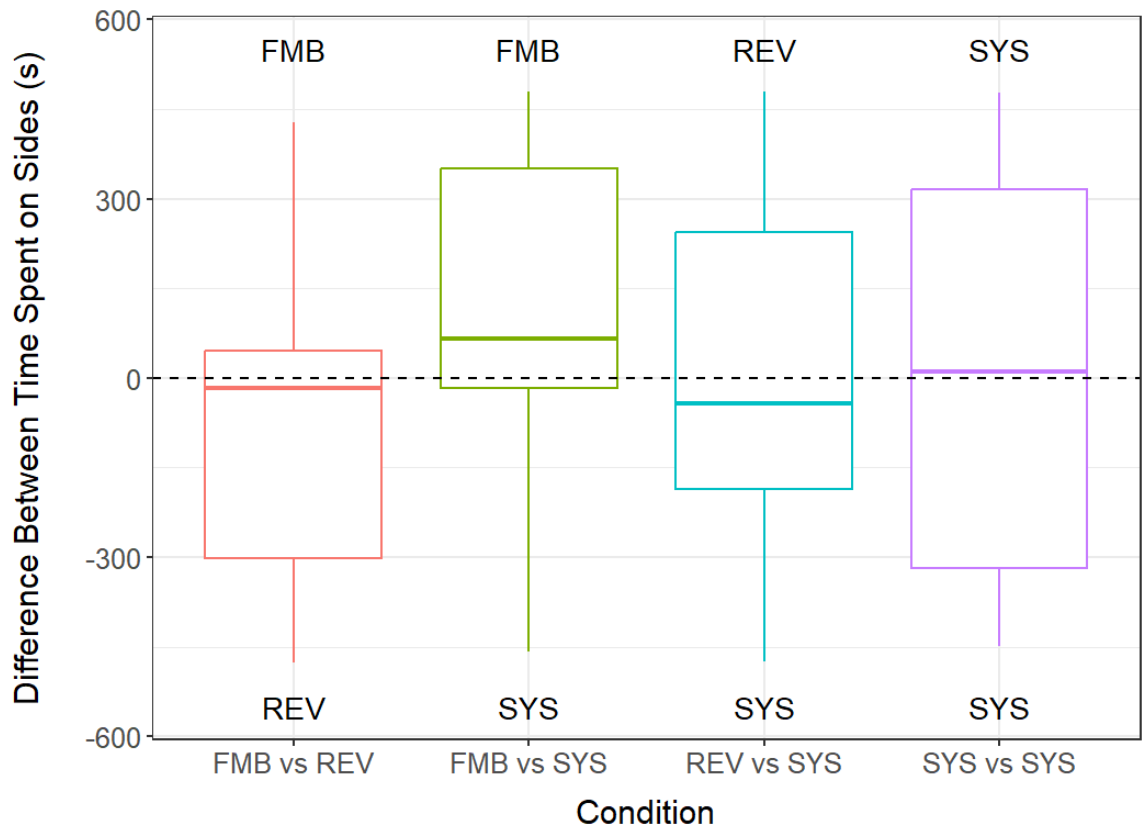

Fig. 6. Difference in time spent on each side of the arena per condition in November 2024. Data are shown as box plots where the bold centre line represents the median, the bottom and top box edges represent the 25^{th} and 75^{th} percentiles (i.e. the middle 50% of time differences), and the tips of the vertical lines represent the lowest and highest values within 1.5 times the inter-quartile range (distance between 25^{th} and 75^{th} percentiles). The horizontal dashed line represents when the difference in time spent on each side is zero.

Of the bats that made a side choice within a time threshold of at least 120 s, the proportion choosing the FMB side was greater than the proportion choosing the SYS side in the FMB vs SYS condition (Table 6). Of the bats that made a side choice and spent at least 240 s on a side, a larger proportion of bats chose the REV side in the FMB vs REV condition and the proportion choosing the FMB side was greater than the proportion choosing the SYS side in the FMB vs SYS condition.

Nearest Approach. Although time spent within two body lengths of each speaker was not affected by condition ($p = 0.360$), the proportion of bats that approached within two body lengths of the FMB speaker was greater than the proportion of bats that approached the SYS speaker in the FMB vs SYS condition (Table 7).

April 2025 (Non-Mating Season)

First Side Chosen. Investigating the first side chosen revealed that neither side was chosen first more frequently across all playback conditions (Table 8). The proportion of bats that chose the FMB side first in the FMB vs SYS condition was slightly higher than the proportion that chose the FMB side first in the FMB vs REV condition, however, the 95% CIs for the proportions overlapped (Fig. 7).

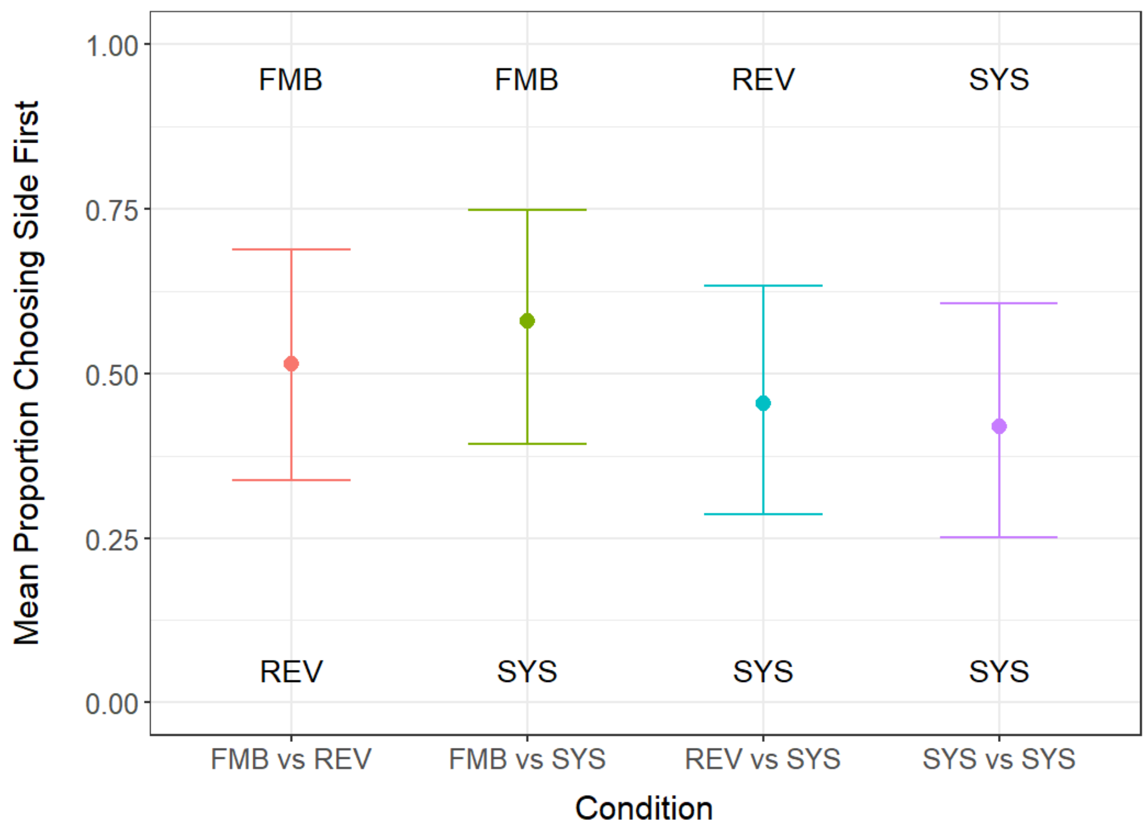

Fig. 7. Average proportion of female bats that chose a given side first in April 2025. Dots represent the proportion of bats who chose the side labelled at the top of the figure first and error bars represent the 95% confidence intervals.

Last Side Chosen. Neither side was chosen last more frequently across all conditions (Table 8). The proportion of bats who chose the FMB side last in the FMB vs SYS condition was higher than the proportion who chose the FMB side in the FMB vs REV condition; however, the 95% CIs for these proportions overlapped (Fig. 8).

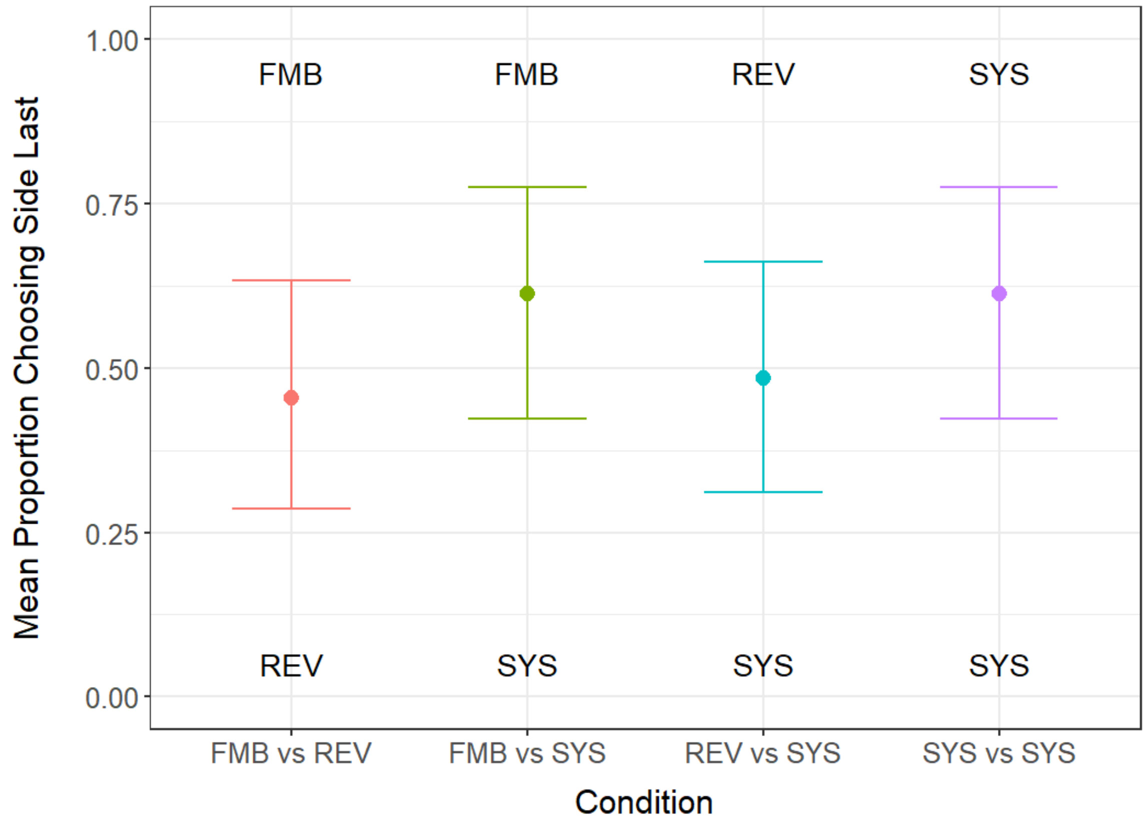

Fig. 8. Average proportion of female bats that chose a given side last in April 2025. Dots represent the mean proportion of bats who chose the side labelled at the top of the figure last and error bars represent the 95% confidence intervals.

Side Bias. Regardless of the playback stimulus, a side bias check revealed that neither side was chosen first or last more frequently (Table 9).

Time Spend on Each Side. Bats did not spend more time on either side of the arena across all conditions (Table 10). When plotting the difference in time spent on each side, it was evident that the bats between the 25^{th} and 75^{th} percentiles of time differences spent more time on the REV side in the FMB vs REV condition (Fig. 9). Similarly, bats between the 25^{th} and 75^{th} percentiles of time differences spent more time on the FMB side in the FMB vs SYS condition.

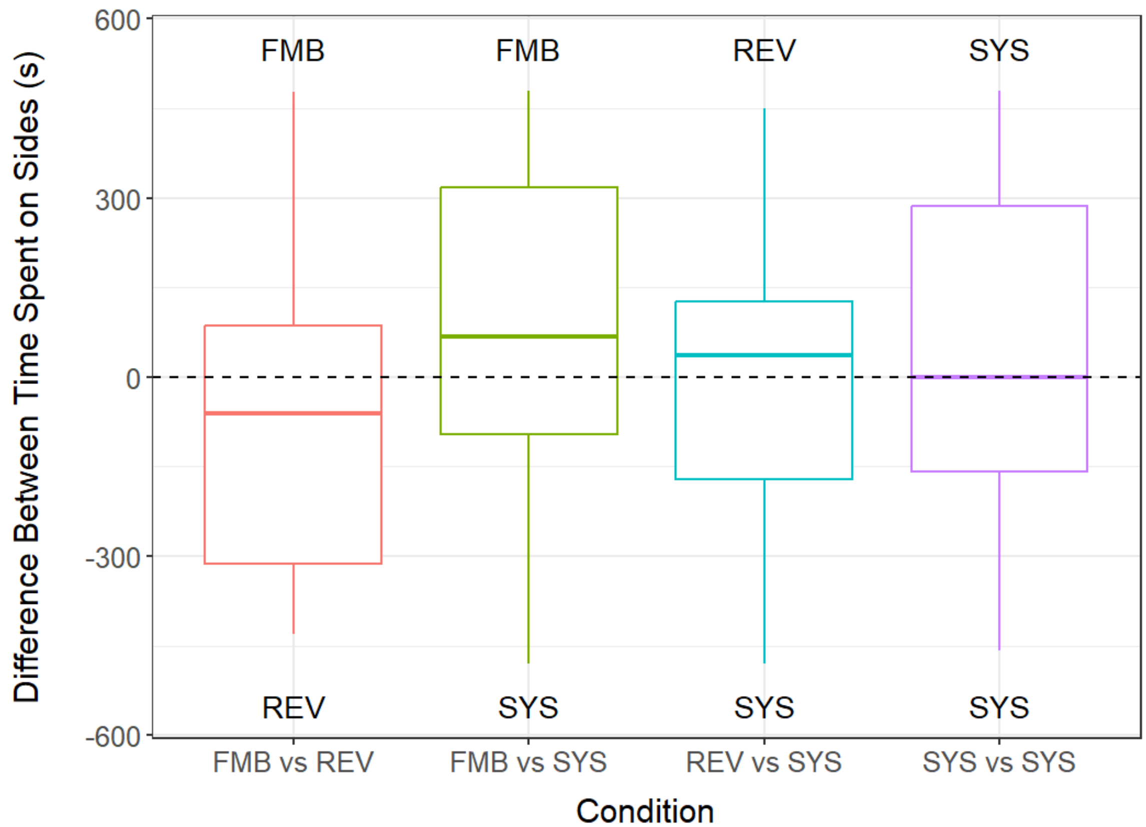

Fig. 9. Difference in time spent on each side of the arena per condition in April 2025. Data are shown as box plots where the bold centre line represents the median, the bottom and top box edges represent the 25th and 75th percentiles (i.e. the middle 50% of time differences), and the tips of the vertical lines represent the lowest and highest values within 1.5 times the inter-quartile range (distance between 25th and 75th percentiles). The horizontal dashed line represents when the difference in time spent on each side is zero.

Of the bats that made a choice at a time threshold of at least 120 s, the proportion that chose the REV side was greater than the proportion choosing the FMB side in the FMB vs REV condition (Table 11).

Nearest Approach. Time spent within two body lengths of each speaker was not modulated by condition ($p = 0.360$), and bats did not approach one speaker more frequently than the other across all conditions (Table 12).

Mating vs Non-Mating Seasons

As playback trials were conducted in both the fall mating and spring non-mating seasons, the testing season's effect on the results was investigated. In all cases, season neither affected the first and last sides chosen nor the side spent more time on (Table 13). The proportion of bats that made a choice past the time thresholds of 120 s and 240 s did not change across seasons (Table 14). Running the Linear Mixed-Effects Mode, revealed that an interaction between condition and successful speaker affected the time spent within two body lengths of a speaker ($p = 0.040$). A post-hoc Estimated Marginal Means contrast was run to investigate which of the four conditions and/or two speakers were responsible for the modulation and found that only the FMB vs SYS condition during the mating season affected time spent near the speaker (Table 15). A greater proportion of bats approached within two body lengths of the FMB speaker in the FMB vs REV condition in the mating season than in the non-mating season (Table 16).

Male Bats in Non-Mating Season

The paradigm was also run on all the colony's male bats. A sample size of eight could only indicate statistical significance if the proportions were 0/8 or 8/8, which did not occur in the data. Instead, the number of males that chose a side first or last, spent more time on a side, and approached each speaker within two body lengths were reported, as well as the results of a Wilcoxon Signed-Rank test, which investigated if there was a difference between the median time spent within two body lengths of each speaker (Tables 17-22). Significant results would have given insight into the attractive or aversive properties of the FMB to males outside of a food competition context.

Discussion

This study investigated if the FMB emitted by *E. fuscus* had an attractive or aversive effect on *E. fuscus* females, with the goal of determining if the call has behavioural effects outside of food competition. The study also investigated if any effects were modulated across the reproductive seasons.

November 2024 (Mating Season)

Trials were first run in November 2024 to quantify female *E. fuscus* response to FMB calls during the fall mating season. Combinations of three different call types were tested, and different responses were expected based on which calls were paired. If the FMB was attractive, female bats were expected to prefer this vocalization in both the FMB vs REV and FMB vs SYS conditions. More specifically, the side with the FMB playback should be chosen first and last, with bats spending more time on the FMB side, and more bats spending increased time within two body lengths of the FMB speaker compared to the opposite side and speaker. Alternatively, if the FMB call was aversive, then the converse of the previous predictions was expected. The REV vs SYS condition was tested to determine if bats were simply attracted or repelled from a noise of the same frequencies. If a prominent attraction or aversion to the REV call occurs, doubt would be cast on the relevance of the results testing the FMB. Finally, the SYS vs SYS condition was used as a baseline control to measure natural behaviour with no stimuli. There should be no difference between side chosen first and last, time spent on each side, time spent within two body lengths of each speaker, and number of bats approaching within two body lengths of each speaker during this condition.

Bats did not initially choose one side of the arena over the other in any of the four conditions, indicating that the FMB call did not immediately elicit any attractive or aversive behaviour. The proportion of bats that first chose each side in the SYS vs SYS condition was nearly even, indicating there was no side bias to the testing conditions of the arena. To corroborate this, which side was chosen first, regardless of stimulus type, was investigated. Most playback conditions had no side bias, but in the REV vs SYS condition some evidence of bias was found as more bats chose the left side of the arena over the right. There were no factors inherent to the REV vs SYS condition that could have explained this. As in all conditions, the speaker that started playback first and the speaker playing each call were randomly allocated. Therefore, this result is likely due to chance.

Results for the last side chosen aligned more closely with predictions. More bats selected the FMB side as their final choice in the FMB vs SYS condition, indicating that after being exposed to the call for eight minutes, the call may have had an attractive effect. However, this is not the case in the FMB vs REV condition, where no side was chosen last over the other. The lack of choice between the REV and SYS sides corroborates that a sound of the same frequency does not drive this choice. There was no evidence of a side bias in the SYS vs SYS case, indicating that external factors were not a consistent cause of side bias during testing. Indeed, examining the last side chosen regardless of stimulus type confirmed a lack of side bias in all conditions.

Bats did not spend more time on either side of the arena across all conditions, suggesting that the FMB call does not have an attractive or aversive effect when exposed to the call over the course of eight minutes. When only considering bats that made a definitive side choice, it was found that a greater proportion of bats spent at least 120 s and at least 240 s on the FMB side than those that spent 120 s and 240 s on the SYS side in the FMB vs SYS condition. These data suggest that the FMB call may have been attractive to bats that were scored as having made a definitive choice. Conversely, a greater proportion of bats spent over 240 s on the REV side in the FMB vs REV condition, which suggested that in that case, either the FMB call is aversive to bats that make a definitive choice or the REV call is attractive.

Playback condition did not affect the time spent within two body lengths of a speaker, indicating that the FMB stimulus did not cause approach or aversion to the speakers. A greater proportion of bats approached within two body lengths of the FMB speaker compared to the SYS speaker in the FMB vs SYS condition, suggesting that the FMB call can induce close approaches by female bats. In all other conditions, neither speaker was preferred, which again lends support to the neutrality of the REV call and the lack of bias surrounding the speakers.

Overall, the results support the prediction that the FMB call may be attractive during the fall mating season, but only when paired with silence. As the REV call has no attractive or aversive effect when paired with silence, this supports the hypothesis that the FMB can, in some situations, have positive phonotaxic properties. But when paired against a REV call, no evidence that the FMB call was more attractive was found. Indeed, some trials even suggested that the REV call could be more attractive (Table 6). A possible explanation for the inconsistency of the data is that concurrent playback sounds in the small arena may have been confusing to the bat. Although acoustic foam was used in an attempt to prevent this, playback sounds may have reflected off the arena walls and overlapped with later sound reflections.

April 2025 (Non-Mating Season)

The same experiment was repeated in April 2025 to determine if bats tested in the non-mating season showed differences in their attractiveness or aversion to the FMB call. The initial data analysis suggested that the FMB call may be attractive to female *E. fuscus*. If the call was generally attractive without a season-specific context, then the same results in April 2025 as in November 2024 should be expected. However, if the functional properties of the FMB signal related more to mate attraction, then the FMB call should be less attractive to females in April than in November.

Unlike the data collected in the fall mating season, very few tests yielded a result with a significant preference or aversion to any playback stimulus. Out of the bats who spend at least 120 s on either side of the arena in the FMS vs REV condition, a greater proportion spent time on the REV side, suggesting an aversive FMB signal or an attractive REV signal. Aside from this result, there was no evidence to suggest that the playback signals had either an attractive or aversive effect. Moreover, no evidence was found to suggest there was a side bias or inherent bias regarding either of the speakers used in the study.

Mating vs Non-Mating Seasons

Most analyses suggested that season did not influence the attractiveness or aversiveness of any of the stimuli, apart from the results of the Linear Mixed-Effects Model where there was a stark contrast between how season changes the degree to which the condition modulates the time spent near each speaker; with the modulation being much stronger during the mating season for the FMB vs SYS condition. This corroborated the prediction that the FMB call is both attractive and more attractive during the fall mating season than in the spring non-mating season. Additionally, a greater proportion of bats approached the FMB speaker in the FMB vs REV condition in the mating season than in the non-mating season, which also suggested the FMB call is more attractive during the mating season.

Results are mixed. In 4 of 8 tests conducted in the fall mating season, results supported the prediction that the FMB signal is attractive (Tables 3, 6, 7). No tests (0 of 8) supported this prediction during non-mating season. Interestingly, 2 of 8 tests comparing the seasons supported the prediction that these parameters changed across season, with these tests corroborating the theory that the FMB is more attractive during the mating season (Tables 15, 16). Given these results, further playback testing paradigms should be conducted before forming a definitive conclusion on the function(s) and phonotaxic properties of the FMB in *E. fuscus*.

Testing Male Bats

The sample size of male bats was small and never resulted in a significant result. This was not unexpected as the only way statistical significance could have been achieved was if all the bats responded similarly (e.g. chose the same side first). At present it is difficult to use the data from the study to speculate further on the functional and phonotaxic properties of the FMB when testing male bat response. Should this paradigm be used again, testing a larger sample of male bats is recommended.

Comments and Limitations

The rationale behind this study was conceived based on the available literature of the FMB signal, which was accessed mainly in 2023 and in 2024. These studies concluded that the FMB call was emitted exclusively by adult male *E. fuscus* (Wright et al., 2013, 2014). This, paired with similar findings in other Vespertilionid bats, provided the rationale that the FMB call was emitted by males to attract females. However, a very recent study—discovered after conducting the November 2024 playback trials—reported that adult female *E. fuscus* also emit FMB signals but in a food competition context (Salles et al., 2024). As food competition can occur in males and females, this removes support for the theory that the FMB signal is emitted by males to attract females.

One regret I have with my experimental design concerns the order in which I exposed bats to each condition. In November 2024, I decided to present the playback signal in a particular order. First, I tested all bats with the FMB vs REV condition, then the FMB vs SYS condition, then the REV vs SYS condition, and finally the SYS vs SYS condition. My rationale was that I wanted to observe the initial reactions of the bats before they became habituated to the arena, specifically under the condition that tested my most relevant independent variable against a non-silent control. But I soon realized that by the time bats were tested in the fourth condition, the number of times they had been exposed to the arena in that condition was higher than the number of times they had been exposed to the arena in the first condition. If I were to repeat this experiment, I would use a more balanced experimental design and randomly assign each bat to a unique order of testing conditions, as I did in April 2025

Another concern of my study was the use of a synthetic FMB stimulus. The bout consisted of three syllables, each with a slightly unique shape, as they were based off a real FMB vocalization. The playback bouts were repeated at randomly generated but limited intervals. Regardless, the bats may have become habituated to the repetition of the same bout. It is also possible that bats found the synthetic FMB to be less authentic than a natural signal. As an alternative, I could have played a recorded natural FMB signal as the experimental stimulus. I did not do this because my lab did not have pre-recorded natural FMB signals to use as a stimulus. Because the acoustic parameters of my synthetic FMB stimulus were rooted in the mean values of natural FMB calls as reported in the literature, I have high confidence that it should have been an accurate representation of FMB stimulus despite being synthetic.

Like most playback studies, the experiment was limited by the design of the testing arena. The circular design was chosen to emulate 2AFC playback experiments in which subjects could freely roam and approach speakers. With this design, bats had the ability to hear and investigate signals emitted from both speakers without being restricted by the arm of a Y-maze, for example. The relatively small diameter of the arena ensured that I could film and measure the movements of the bats. Of course, in nature bats experience less space restrictions. One alternative to my design would be to create a larger testing environment, where bats could roam freely in a flight chamber with speakers placed at opposite ends of the room. With this comes the difficulties of monitoring a small bat in a large room and ensuring easy collection at the end of each trial.

Although not formerly measured, the bats I tested seemed to be more active in April compared to November. For example, it was my impression that bats spent more time crawling upside down on the wire mesh lid and navigating the arena this way in the spring compared to the fall. While this was not necessarily an issue, as they were still traversing the arena and could be scored, it may be something to consider when designing future playback testing paradigms.

The relatively small sample size of ~30 bats tested with each condition makes the proportion statistical tests we ran, easily swayed. For example, in mating season, 19 of 30 bats chose the FMB side first in the FMB vs SYS condition, a non-significant result ($p=0.201$). However, if 21 of 30 bats chose the FMB side first, this would have yielded a significant result ($p=0.045$). Future studies should consider testing a larger sample of bats with a playback paradigm.

My results were not as straightforward as I had hoped, perhaps due to the times of year when I tested the bats. Although mating season occurs in the fall for temperate insectivorous bats (Racey & Entwistle, 2000), November is on the tail end of the mating season. Thus, it is possible that some bats had already mated earlier in the season and were not especially motivated by the playback signals. For example, if the FMB signal plays a role in mate attraction, a phonotaxic effect could be less salient after mating has occurred. If I were to repeat this study, I would test bats earlier in the fall. Testing bats in mid-summer might also be more relevant for the non-mating season, as it is possible for *E. fuscus* females to mate as late as March (Kurta & Baker, 1990).

Curiously, in May 2025, we were surprised to discover that five females from our captive colony were pregnant. All these bats were used in my study. Our captive colony does not always have successful breeding, and the last time pups were successfully born in captivity was in 2019. We can speculate that if FMB signals have a role in mate attraction, perhaps broadcasting this sound into the colony in the early fall may stimulate more bats to seek mates and copulate.

Conclusions

My study is the first to explore a functional role of the FMB signal outside of food competition, focusing on the possibility that it serves a role in mate attraction. Some of my results suggest that the FMB signal evokes phonotaxis in female *E. fuscus*, particularly during the mating season. My work provides insight into bat reproduction and acoustic communication and has possible applications in ecosystem management. For example, if a call is known to evoke positive phonotaxis, it can be used during surveys to lure bats towards researchers. Overall, the results of my thesis indicate that more experimental work is required on this topic to determine the functional role(s) of the FMB call in *Eptesicus fuscus*.

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Tables

Table 1. List of bat ID numbers, sex (M = male; F = female), age (A = adult; J = juvenile), and how/when used in this study.

Bat ID	Sex	Age	Used for	Bat ID	Sex	Age	Used for
Green 130	M	A	A	Violet 104	F	A	N, A
Grey 100	F	A	N, A	Violet 111	F	A	N, A
Grey 101	F	A	N, A	Violet 113	F	A	N, A
Orange 12	M	A	A	Violet 125	F	A	N, A
Orange 16	M	A	A	Violet 164	F	A	N, A
Pink 102	M	J	A	Violet 170	F	A	N, A
Pink 104	F	J	S	Violet 181	F	A	N, A
Pink 105	M	J	A	Violet 182†	F	A	N
Pink 106	F	J	S	Violet 200	F	A	N, A
Pink 107	F	J	S	White 167	F	A	N, A
Pink 29	F	A	N, A	White 240	F	A	N, A
Pink 59	F	J	S	White 244	F	A	N, A
Pink 62	F	J	S	Yellow 084	F	A	N, A
Pink 63	F	J	S	Yellow 127	F	A	N, A
Pink 64	M	J	A	Yellow 151	F	A	N, A
Pink 65	F	J	S	Yellow 152	F	A	N, A
Pink 67	F	J	S				
Pink 68	F	J	S				
Pink 71	F	J	S				
Pink 72	F	J	S				
Pink 73	M	J	A				
Pink 74	M	J	A				
Pink 99	F	J	S				
Sky 101	F	A	N, A				
Sky 110	F	A	N, A				
Sky 111	F	A	N, A				
Sky 130	F	A	N, A				
Sky 131	F	A	N, A				
Sky 140	F	A	N, A				
Sky 141	F	A	N, A				
Sky 144	F	A	N, A				
Sky 146	F	A	N, A				
Sky 147	F	A	N, A				
Sky 151	F	A	N, A				
Sky 154	F	A	N, A				
Sky 155	F	A	N, A				
Sky 156	F	A	N, A				
Sky 159	F	A	N, A				

N = tested in November 2024; A = tested in April 2025; S = used to scent arena
† died on March 12th, 2025

Table 2. Durations and names of primer and trial tracks (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) used in this study. Each section of audio corresponds to a different condition the bats were exposed to.

File Name	Channel A	Channel B
primer_1	FMB from 0-30s	REV from 30-60s
primer_2	REV from 30-60s	FMB from 0-30s
primer_3	REV from 0-30s	FMB from 30-60s
primer_4	FMB from 30-60s	REV from 0-30s
trial_A	FMB first	REV second
trial_B	REV second	FMB first
trial_C	FMB second	REV first
trial_D	REV first	FMB second
primer_5	FMB from 0-30s	SYS from 30-60s
primer_6	SYS from 30-60s	FMB from 0-30s
primer_7	SYS from 0-30s	FMB from 30-60s
primer_8	FMB from 30-60s	SYS from 0-30s
trial_E	FMB	SYS
trial_F	SYS	FMB
primer_9	REV from 0-30s	SYS from 30-60s
primer_10	SYS from 30-60s	REV from 0-30s
primer_11	SYS from 0-30s	REV from 30-60s
primer_12	REV from 30-60s	SYS from 0-30s
trial_G	REV	SYS
trial_H	SYS	REV
trial_I	SYS	SYS

Table 3. Summary of first side chosen and last side chosen for bats in November 2024**.** Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that chose each side first. *P*-values and 95% confidence intervals (95% CI) were calculated using a proportion test. Bolded results are significant.

First Side Choice

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	14	17	-	31	0.719	[0.278, 0.637]
FMB vs SYS	19	-	11	30	0.201	[0.439, 0.795]
REV vs SYS	-	10	21	31	0.072	[0.173, 0.515]
SYS vs SYS	-	-	15, 16	31	1	[0.306, 0.666]

Last Side Choice

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	13	18	-	31	0.473	[0.251, 0.607]
FMB vs SYS	**22**	**-**	**8**	**30**	**0.018**	**[0.538, 0.870]**
REV vs SYS	-	16	15	31	1	[0.334, 0.694]
SYS vs SYS	-	-	18, 13	31	0.473	[0.393, 0.749]

Table 4. Summary of first side chosen and last side chosen for bats in November 2024**.** Each row indicates the playback trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that chose the left side (LS) or the right side (RS) of the arena regardless of playback stimulus. *P*-values and 95% confidence intervals (95% CI) were calculated using a proportion test. Bolded results are significant.

First Side Choice

Playback Trial	LS	RS	Total	*p*	95% CI
FMB vs REV	15	16	31	1	[0.306, 0.666]
FMB vs SYS	17	13	30	0.584	[0.377, 0.740]
REV vs SYS	**22**	**9**	**31**	**0.031**	**[0.518, 0.851]**
SYS vs SYS	17	14	31	0.719	[0.363, 0.722]

Last Side Choice

Playback Trial	LS	RS	Total	*p*	95% CI
FMB vs REV	20	11	31	0.151	[0.454, 0.802]
FMB vs SYS	14	16	30	0.855	[0.288, 0.654]
REV vs SYS	14	17	31	0.720	[0.278, 0.637]
SYS vs SYS	16	15	31	1	[0.334, 0.694]

Table 5. Summary results showing number of bats that spent more time on a given side in November 2024**.** Each row indicates the playback trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that spent more time on each side. *P*-values and 95% confidence intervals (95% CI) were calculated using a proportion test.

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	14	17	-	31	0.719	[0.278, 0.637]
FMB vs SYS	20	-	10	30	0.100	[0.471, 0.821]
REV vs SYS	-	14	17	31	0.719	[0.278, 0.637]
SYS vs SYS	-	-	16, 15	31	1	[0.334, 0.694]

Table 6. Summary of time spent over a given threshold on either side of the arena in November 2024**.** Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise), the number of bats that spent time over the threshold on each side, and their sum. *P*-values and 95% confidence intervals (95% CI) were calculated using a two-proportion test. Bolded results are significant.

Spent at Least 120 s on

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	6	13	-	19	0.052	[-0.717, -0.020]
FMB vs SYS	**13**	**-**	**5**	**18**	**0.020**	**[0.096, 0.793]**
REV vs SYS	-	12	13	25	1	[-0.357, 0.277]
SYS vs SYS	-	-	14, 13	27	1	[-0.267, 0.341]

Spent at Least 240 s on

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	**4**	**11**	**-**	**15**	**0.028**	**[-0.850, -0.084]**
FMB vs SYS	**10**	**-**	**3**	**13**	**0.017**	**[0.138, 0.939]**
REV vs SYS	-	8	5	13	0.433	[-0.682, 0.220]
SYS vs SYS	-	-	11, 9	20	0.752	[-0.258, 0.458]

Spent at Least 360 s on

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	2	5	-	7	0.285	[-1, 0.188]
FMB vs SYS	6	-	2	8	0.134	[-0.049, 1]
REV vs SYS	-	5	3	8	0.617	[-0.349, 0.849]
SYS vs SYS	-	-	4, 7	11	0.394	[-0.766, 0.220]

Table 7. Summary of bats that approached each speaker within two body lengths in November 2024**.** Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that approached each speaker. *P*-values and 95% confidence intervals (95% CI) were calculated using a two-proportion test. Bolded results are significant.

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	28	25	-	31	0.471	[-0.109, 0.303]
FMB vs SYS	**25**	**-**	**15**	**30**	**0.014**	**[0.077, 0.590]**
REV vs SYS	-	19	16	31	0.609	[-0.375, 0.181]
SYS vs SYS	-	-	18, 20	31	0.794	[-0.339, 0.210]

Table 8. Summary of first side chosen and last side chosen for bats in April 2025**.** Each row indicates the playback trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that chose each side first. *P*-values and 95% confidence intervals (95% CI) were calculated using a proportion test.

First Side Choice

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	17	16	-	33	1	[0.339, 0.688]
FMB vs SYS	18	-	13	31	0.473	[0.393, 0.749]
REV vs SYS	-	15	18	33	0.723	[0.285, 0.634]
SYS vs SYS	-	-	13, 18	31	0.473	[0.251, 0.607]

Last Side Choice

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	15	18	-	33	0.728	[0.285, 0.634]
FMB vs SYS	19	-	12	31	0.281	[0.423, 0.776]
REV vs SYS	-	16	17	33	1	[0.312, 0.661]
SYS vs SYS	-	-	19, 12	31	0.281	[0.423, 0.776]

Table 9. Summary of first side chosen and last side chosen for bats in April 2025. Each row indicates the playback trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that chose the left side (LS) of the arena and the right side (RS) of the arena first. *P*-values 95% confidence intervals (95% CI) were calculated using a proportion test.

First Side Choice

Playback Trial	LS	RS	Total	*p*	95% CI
FMB vs REV	17	16	33	1	[0.339, 0.688]
FMB vs SYS	21	10	31	0.072	[0.485, 0.827]
REV vs SYS	14	19	33	0.486	[0.260, 0.606]
SYS vs SYS	17	14	31	0.719	[0.363, 0.722]

Last Side Choice

Playback Trial	LS	RS	Total	*p*	95% CI
FMB vs REV	17	16	33	1	[0.339, 0.688]
FMB vs SYS	12	19	31	0.281	[0.224, 0.577]
REV vs SYS	11	22	33	0.082	[0.186, 0.519]
SYS vs SYS	17	14	31	0.719	[0.363, 0.722]

Table 10. Summary results showing the number of bats that spent more time on a given side in April 2025. Each row indicates the playback trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise;) and the number of bats that spent more time on each side. *P*-values and 95% confidence intervals (95% CI) were calculated using a proportion test.

Playback Trial	FMB	REV	SYS	Total	p	95% CI
FMB vs REV	12	21	-	33	0.164	[0.210, 0.549]
FMB vs SYS	19	-	12	31	0.281	[0.423, 0.776]
REV vs SYS	-	19	14	33	0.486	[0.394, 0.740]
SYS vs SYS	-	-	16, 15	31	1	[0.334, 0.694]

Table 11. Summary of time spent over a given threshold on either side of the arena in April 2025. Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise), the number of bats who spent time over the threshold on each side, and their sum. *P*-values and 95% confidence intervals (95% CI) were calculated using a two-proportion test. Bolded results are significant.

Spent at least 120 s on

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	**4**	**13**	**-**	**17**	**0.006**	**[-0.873, -0.185]**
FMB vs SYS	13	-	8	21	0.217	[-0.103, 0.579]
REV vs SYS	-	9	11	20	0.752	[-0.458, 0.258]
SYS vs SYS	-	-	10, 9	19	1	[-0.318, 0.423]

Spent at least 240 s on

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	4	10	-	14	0.059	[-0.835, -0.022]
FMB vs SYS	11	-	6	17	0.170	[-0.086, 0.674]
REV vs SYS	-	6	7	13	1	[-0.537, 0.383]
SYS vs SYS	-	-	9, 6	15	0.465	[-0.217, 0.617]

Spent at least 360 s on

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	2	6	-	8	0.134	[-1, 0.049]
FMB vs SYS	5	-	5	10	1	[-0.438, 0.438]
REV vs SYS	-	6	5	11	1	[-0.416, 0.598]
SYS vs SYS	-	-	5, 2	7	0.285	[-0.188, 1]

Table 12. Summary of bats that approached each speaker within two body lengths in April 2025**.** Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that approached each speaker. *P*-values and 95% confidence intervals (95% CI) were calculated using a two-proportion test.

Playback Trial	FMB	REV	SYS	Total	*p*	95% CI
FMB vs REV	22	24	-	33	0.789	[-0.312, 0.191]
FMB vs SYS	22	-	20	31	0.786	[-0.120, 0.329]
REV vs SYS		24	24	33	1	[-0.215, 0.215]
SYS vs SYS		-	21, 19	31	0.791	[-0.205, 0.334]

Table 13. Effect of season on response variables with binomial distributions (Chi-squared = chi-square test statistic; df = degrees of freedom)**.** Each row represents sets of response variables modeled in each data set's Generalized Linear Mixed-Effects Model.

Evaluation	Chi-squared	df	*p*
First Side Choice	0.114	1	0.735
Last Side Choice	0.108	1	0.743
Side Spent More Time On	0.004	1	0.951

Table 14. Summary of time spent over a given threshold on either side of the arena across seasons. Each row indicates the playback trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise**)** and the number of bats that spent time over the threshold on each side per season. *P*-values and 95% confidence intervals (95% CI) were calculated using a two-proportion test with a matrix input.

Spent at least 120 s on

Playback Trial	November	April	*p*	95% CI
FMB vs REV	6 vs 13	4 vs 13	0.868	[-0.266, 0.427]
FMB vs SYS	13 vs 5	13 vs 8	0.733	[-0.242, 0.448]
REV vs SYS	12 vs 13	9 vs 11	1	[-0.293, 0.353]
SYS vs SYS	14 vs 13	10 vs 9	1	[-0.309, 0.293]

Spent at least 240 s on

Playback Trial	November	April	*p*	95% CI
FMB vs REV	4 vs 11	4 vs 10	1	[-0.364, 0.326]
FMB vs SYS	10 vs 3	11 vs 6	0.748	[-0.268, 0.513]
REV vs SYS	8 vs 5	6 vs 7	0.694	[-0.302, 0.609]
SYS vs SYS	11 vs 9	9 vs 6	1	[-0.430, 0.330]

Spent at least 360 s on

Playback Trial	November	April	*p*	95% CI
FMB vs REV	2 vs 5	2 vs 6	1	[-0.450, 0.521]
FMB vs SYS	6 vs 2	5 vs 5	0.552	[-0.294, 0.794]
REV vs SYS	5 vs 3	6 vs 5	1	[-0.446, 0.605]
SYS vs SYS	4 vs 7	5 vs 2	0.334	[-0.907, 0.205]

Table 15. Emmeans contrasts ran on Linear Mixed-Effect Model for the FMB vs SYS condition (SE = standard error, df = degrees of freedom)**.** Each row represents the output from the contrast. Bolded results are significant.

Season	Estimate	SE	df	t.ratio	*p*
Fall	**-2.617**	**0.745**	**453**	**-3.512**	**0.001**
Winter	-0.698	0.733	453	-0.952	0.342

Table 16. Number of bats that approached within two body lengths of a speaker in two playback testing seasons: November 2024 and April 2025. Each row shows the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and number of bats that approached the speaker (in boldface) over the total number of bats tested. *P*-values and 95% confidence intervals (95% CI) were calculated using a proportion test. Bolded results are significant.

Approached 'Successful' Speaker

Playback Trial	November	April	*p*	95% CI
FMB vs REV	**28/31**	**22/33**	**0.047**	**[0.014, 0.459]**
FMB vs SYS	25/30	22/31	0.399	[-0.117, 0.365]
REV vs SYS	19/31	24/33	0.479	[-0.375, 0.146]
SYS vs SYS	18/31	21/31	0.599	[-0.368, 0.175]

Approached 'Unsuccessful' Speaker

Playback Trial	November	April	*p*	95% CI
FMB vs **REV**	25/31	24/33	0.651	[-0.158, 0.316]
FMB vs **SYS**	15/30	20/31	0.375	[-0.424, 0.133]
REV vs **SYS**	16/31	24/33	0.137	[-0.475, 0.053]
SYS vs **SYS**	20/31	19/31	1	[-0.240, 0.305]

Table 17. Proportion of male bats making first and final side choices in April 2025. Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that chose each side first.

First Side Choice

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	4	4	-	8
FMB vs SYS	2	-	6	8
REV vs SYS	-	5	3	8
SYS vs SYS	-	-	2, 6	8

Last Side Choice

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	5	3	-	8
FMB vs SYS	4	-	4	8
REV vs SYS	-	5	3	8
SYS vs SYS	-	-	5, 3	8

Table 18. Proportion of male bats making initial and final side choices in April 2025, disregarding the track type. Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that chose the left side (LS) or the right side (RS) of the arena first or last regardless of playback stimulus.

First Side Choice

Playback Trial	LS	RS	Total
FMB vs REV	6	2	8
FMB vs SYS	4	4	8
REV vs SYS	3	5	8
SYS vs SYS	4	4	8

Last Side Choice

Playback Trial	LS	RS	Total
FMB vs REV	5	3	8
FMB vs SYS	4	4	8
REV vs SYS	3	5	8
SYS vs SYS	1	7	8

Table 19. Proportion of the sides the male bats spent more time on in April 2025. Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the number of bats that spent more time on each side.

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	5	3	-	8
FMB vs SYS	4	-	4	8
REV vs SYS	-	5	3	8
SYS vs SYS	-	-	3, 5	8

Table 20. Proportion of time spent over a given threshold on either side of the arena by male bats in April 2025. Each row represents the trial type (FMB= frequency-modulated bout; REV= time-reversed frequency-modulated bout; SYS = system noise), the number of bats that spend time over the threshold on each side, and their sum.

Spent at Least 120 s on

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	3	3	-	6
FMB vs SYS	1	-	3	4
REV vs SYS	-	2	3	5
SYS vs SYS	-	-	1, 4	5

Spent at Least 240 s on

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	3	2	-	5
FMB vs SYS	1	-	2	3
REV vs SYS	-	1	2	3
SYS vs SYS	-	-	0, 0	0

Spent at Least 360 s on

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	1	2	-	3
FMB vs SYS	1	-	2	3
REV vs SYS	-	1	1	2
SYS vs SYS	-	-	0, 0	0

Table 21. Proportion of male bats that approached within two body lengths of each speaker in April 2025. Each row indicates the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and reports the number of bats that approached the speaker.

Playback Trial	FMB	REV	SYS	Total
FMB vs REV	6	5	-	8
FMB vs SYS	4	-	4	8
REV vs SYS	-	4	5	8
SYS vs SYS	-	-	6, 6	8

Table 22. Summary of time male bats spent within two body lengths of each speaker in April 2025. Each row represents the trial type (FMB = frequency-modulated bout; REV = time-reversed frequency-modulated bout; SYS = system noise) and the maximum time spent near each speaker in seconds. *P*-values were calculated using a Wilcoxon Signed-Rank Test.

Playback Trial	FMB	REV	SYS	*p*
FMB vs REV	88	147.7	-	0.834
FMB vs SYS	339.7	-	409	1
REV vs SYS		378.2	478.8	0.675
SYS vs SYS	-	-	82.3, 190.7	0.675