Target strength of bat prey in relation to pulse frequency and vegetation density in bat species, *Rhinolophus fumigatus* (Rüppell's horseshoe bat)

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DECLARATION

I, Micaela Freeks, know the meaning of plagiarism and hereby declare that the work on which this these is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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ABSTRACT

The relationship between echolocation and morphology is evident in several insectivorous bat species where a negative correlation between peak echolocation frequency and body size is observed. However, there are various exceptions to the general allometric relationship observed between body size and echolocation pulse frequency of both low-duty cycle and high-duty cycle bats. One such example in high duty-cycle bats is *Rhinolophus fumigatus*, where east African populations echolocate at a frequency of 55.1 ± 1.5 kHz, lower than that predicted by its body size (12.7 ± 0.9 g). The foraging habitat hypothesis states that a deviation from the allometric relationship between pulse frequency and body size is related to the foraging habitat and foraging style of the species and predicts a negative relationship between peak echolocation frequency and wing loading. Lower echolocation frequency penetrates dense vegetation more effectively than higher frequency pulses, resulting in greater energy for the generation of audible target strengths from the insect prey. Furthermore, a small body size allows manoeuvrable flight which is required for foraging in dense vegetation. The combination of low echolocation frequency and small body size, which represents a deviation from allometry between frequency and body size may be an adaptation for detecting and capturing, respectively, insect prey in dense vegetation. The target strengths produced by *Rhinolophus fumigatus_East*, *Rhinolophus fumigatus_West* (a sister lineage with a larger body size (18.8 ± 1.5 g) and similar echolocation frequency (55.1 ± 1.5 kHz)), and *Rhinolophus capensis* (a species of similar body size (12 ± 1.7 g) but higher echolocation frequency (84.8 ± 3.6 kHz) were measured in three relative vegetation densities and compared to determine if the deviation of *Rhinolophus fumigatus_East* from the general allometric relationship can be explained by the foraging habitat hypothesis. Moths were ensonified with semi-synthesized echolocation calls of the three bats in sparse, moderate, and

dense vegetation densities and the returning echoes measured using Avisoft SASLab Pro. Target strengths were then calculated after accounting for atmospheric attenuation and non-parametric tests were conducted as the data did not meet the requirements for parametric tests, even after normalisation techniques were applied. Within lineage and species analysis showed no significant difference in target strength between the three vegetation densities. Between lineage and species analysis showed a significant difference between *Rhinolophus fumigatus_East* and *Rhinolophus capensis* in all three vegetation densities, for both high (HDC) and low duty cycles (LDC). However, within the series of various tests (where each lineage and species pulses were played consecutively) a significant difference exists between both *R. fumigatus* lineages and with *R. capensis* for both HDC and LDC pulses. In the series of natural pulses, a significant differences was found to exists between *R. fumigatus_West* and *R. capensis* for HDC pulses, and *R. fumigatus_East* and *R. capensis* for LDC pulses. When combining all the HDC and LDC data, a significant difference was found between *R. fumigatus_East* and *R. capensis*, and *R. fumigatus_West* and *R. capensis*. The results of the study do not support the foraging habitat hypothesis, and this may be due to Rhinolophidae being clutter forage specialists. Their echolocation pulses are already suited for clutter foraging and any slight deviations are unlikely to confer any additional benefit in prey detection. Allopatric divergence may explain *R. fumigatus_East's* deviation in echolocation frequency where extended periods of geographic isolation lead to natural and sexual selection on signalling systems (the sensory drive hypothesis) which allowed speciation to occur. Alternatively, *R. fumigatus_East's* deviation may also be caused by phenotypic plasticity as well as genetic differences. Additionally, this may have important implication for intraspecific communication, where studies have shown the role that echolocation plays in communication in bats. Other morphological traits may be better predictors

of echolocation frequency (i.e., nose-leaf width, pinna size, and cochlea size) and although other studies have produced varied results, this provides avenues for further research.

Keywords: *Rhinolophus fumigatus*, echolocation frequency, target strength, vegetation density, foraging habitat hypothesis

INTRODUCTION

Bats (order Chiroptera) occur in every biome (Metzner, 1991) and on every continent except Antarctica (Jacobs, 2016), exploiting a wide variety of food, ranging from insects and other arthropods, blood meals and small vertebrates including frogs and fish to fruit, pollen, and nectar (Neuweiler, 1989; Schnitzler & Kalko, 2001; Jacobs, 2016). The evolution of powered flight and echolocation (in all bats except flying foxes (Pteropodidae)) have allowed bats to exploit diverse niches and contributed to their global success (Jones & Teeling, 2006). Echolocation is the ability to locate objects using echoes, where sound is generated by the vocal cord and larynx and is emitted either through the mouth (mouth echolocators) or the nostrils (nasal echolocators). The pulse travels through the air as sound waves, reflecting off surrounding objects or prey (Metzner, 1991), where the returning echo contains auditory cues about the direction, timing, and composition of surrounding objects (Collen, 2012). This allows bats to distinguish, locate and recognise obstacles and potential prey (Pollak & Casseday, 1989; Siemers & Schnitzler, 2004).

Echolocation pulses can be characterised by several components such as the bandwidth and the duty cycle. Bandwidth is obtained by subtracting the minimum frequency from the maximum frequency, resulting in either a narrowband (small range of frequencies i.e., CF pulses) or a broadband (high range of frequencies i.e., FM pulses) signal (Jacobs, 2016). Each signal has its own strength: where narrowband signals are useful for target detection but less so for precise target localisation, which requires the measurement of range and both horizontal and vertical angles (Schnitzler & Kalko, 2001). The reduced accuracy in range measurement is due to narrowband signals persisting in the neuronal filter for an extended period. Furthermore, the

reduced accuracy of angle determination is due to information on different angles being encoded in different cues (i.e., information about horizontal angles is encoded in binaural echo cues (information from both ears), whereas monaural echo cues (information from one ear) encode information about vertical angles) (Schnitzler & Kalko, 2001). Alternatively, broadband signals are useful for target localisation and not so much for target detection (Barclay & Brigham, 1991). These signals only activate the neuronal filters for a short time (Schnitzler & Kalko, 2001) but stimulate more neuronal filters, which improve the accuracy of range and angle determination (Moss & Schnitzler, 1995). Therefore, most bats use a combination of narrowband and broadband pulses to optimise both target localisation and detection range (Schnitzler & Kalko, 2001).

The proportion of time a bat spends emitting a signal in a set period is known as the duty cycle (Lazure & Fenton, 2011), which can be altered by changing either the pulse duration (the time between the initiation and termination of a pulse) or the period (the time between the initiation of successive pulses) (Fenton, *et al*., 2012). The pulse duty cycle can be divided into two categories: high duty cycles (HDC) and low duty cycles (LDC), characterised by long pulse durations and short pulse durations (respectively) relative to the pulse period (Fenton, *et al*., 2012). High duty cycle echolocators make use of (FM)-CF-FM pulses, where the wider bandwidths of the FM components at the beginning and end of the pulses are proposed to increase the ranging acuity of the signal (Neuweiler, *et al*., 1987; Schnitzler & Denzinger, 2011). Apart from the difference in the ratio of pulse duration to pulse period, LDC bats emit a pulse and then listen for the returning echo rather than emitting and listening concurrently (Holderied & von Helversen, 2003), mainly attributed to the contraction of middle-ear muscles during signal

emission (Henson, 1965). Alternatively, HDC bats can emit a signal and simultaneously listen to the returning echo. High duty cycle bats compensate for Doppler effects caused by the bats' flight speed (Habersetzer, *et al*., 1984), where the frequency of the preceding signal is lowered to compensate for the increase in frequency of the returning echo of the previous pulse (known as Doppler shift compensation) (Schuller, *et al*., 1974). This is done to ensure that the returning echo falls within the range of the acoustic fovea- the region of the cochlea which is sensitive to a narrow range of frequencies due to an over-representation of neurons (Schuller & Pollak, 1979; Jacobs, 2016). This frequency range is known as the reference frequency and is higher than the frequency produced when the HDC echolocators are stationary (known as the resting frequency) (Neuweiler, 1980; 1989). HDC echolocation allows bats to detect echoes reflected off flying insects in high clutter environments (i.e. in environments with dense vegetation) through the generation of acoustic glints (distinct peaks of amplitude and frequency generated by flapping insect wings (Neuweiler, 1989)) which overcome the masking effects of high clutter environments (Jacobs & Bastian, 2018). Doppler shift compensation keeps the frequency of the returning echo within the sensitivity range of the acoustic fovea, allowing bats to analyse the glints produced (Neuweiler, 1989; Schnitzler & Denzinger, 2011; Jacobs & Bastian, 2018). The intensity of the returning echo is dependent on the angle between the wing and the bat, where maximum echo intensity is produced by a perpendicular angle (Metzner, 1991). Weak echoes are produced when the wing of the insect is parallel to the direction of the emitted pulse, as only the edge of the wing reflects the pulse. There is a decrease in the frequency of the returning echo when the wing moves away from the bat (i.e., towards the top or the bottom of the wing beat cycle) and an increase when moving toward the bat (i.e., either by the downward or upward wing stroke) due to Doppler shifting (Jacobs & Bastian, 2018). Furthermore, acoustic glints produced

are species-specific, containing information regarding wing beat frequency, wing length, and wing beat type, therefore bats can also use the glints to identify prey (Kober, 1986).

The intensity of the emitted pulse directly influences the strength of the returning echo reflected off the target, i.e., the target strength. Thus, the target strength influences the distance at which an echo is audible to the bat. Target strength is defined as the logarithmic ratio of the acoustic energy emitted by the bat relative to the energy reflected off the target. This is measured along an acoustic axis at a specific distance from the target (Møhl, 1988). The target strength is dependent on the ratio of the wavelength of the sound, and the size and shape of the target (Waters, *et al*., 1995). High frequency pulses have a limited range due to atmospheric attenuation (Lawrence & Simmons, 1982). Alternatively, it is theorized that low frequency pulses may be limited due to Rayleigh scattering, where most of the energy is diffracted around the target when the wavelength of the emitted pulses is larger than the target size (i.e., prey having weaker target strengths) (Stilz & Schnitzler, 2012). Therefore, target strength is an important component in determining the operational range of echolocation pulses. However, the target strength of most insects is unavailable, and studies use categorized target strengths (i.e., small being 40dB, medium being 50dB, and large being 65dB (Stilz & Schnitzler, 2012)). It is expected that with a fixed echolocation frequency, the detection range decreases with decreasing target size. Therefore, at a large enough distance it may be more difficult for larger bats (who use lower echolocation frequencies following the general allometric relationship) to detect smaller insects (Barclay & Brigham, 1991).

The relationship between echolocation and morphology is evident in several insectivorous bat species from families such as Rhinolophidae and Hipposideridae (Heller & von Helversen, 1989), where a negative correlation between peak echolocation frequency and body size is observed (Jones, 1996; Jacobs, *et al*., 2007). This correlation has been attributed to various factors including the physics of sound production, where larger bats produce lower frequency pulses due to having thicker vocal cords and larger resonant chambers (Jacobs, *et al*., 2007), and ecological factors, where the lower echolocation frequency enables larger bats to increase the range for target detection. Larger bats need to fly faster to remain aloft as a result of the disproportionate increase of wing area relative to body size, resulting in increased wing loading (Norberg & Rayner, 1987; Jacobs, *et al*., 2007). This decreases the manoeuvrability of bats, whilst larger body size also contributes to the decrease in the agility of bats (Norberg & Rayner, 1987; Barclay & Brigham, 1991). Therefore, using lower echolocation frequency, bats can increase the detection range and compensate for faster flight and reduced manoeuvrability (Barclay & Brigham, 1991). However, there are various exceptions to the general allometric relationship observed between body size and echolocation pulse frequency of both low-duty cycle and high-duty cycle bats. For example, among high-duty cycle bats, east African populations of the rhinolophid species, *Rhinolophus fumigatus* (body size:12.7 ± 0.9 g) echolocates at a frequency of 55.1 ± 1.5 kHz, lower than that predicted by its body (Jacobs, *et al*., 2007). In contrast, *R. capensis* (a species of similar body size: 12 ± 1.7 g) echolocates at a frequency of 84.8 ± 3.6 kHz (Jacobs & Bastian, 2018). *Rhinolophus mehelyi* (echolocation frequency: 109 kHz; forearm length: 53 mm) has a relatively large body size but has an echolocation frequency similar to *Rhinolophus hipposideross* (echolocation frequency: 114 kHz; forearm length: 37 mm), one of the smallest rhinolophids found in Asia and Europe (Heller &

von Helversen, 1989; Jones, *et al*., 1992). Similarly, *R. clivosus* has a relatively larger body size than most rhinolophids in Africa (i.e., *R. darlingi*, *R. blasii*, *R. simulator*, and *R. capensis*), but echolocates at higher frequencies (Taylor, 2000; Jacobs, *et al*., 2007).

The foraging habitat hypothesis (FHH) states that a deviation from the allometric relationship between pulse frequency and body size is related to the foraging habitat and foraging style of the species (Jones & Barlow, 2004). This hypothesis predicts that high clutter environments should select for higher frequency pulses, which provide greater target resolution of smaller prey against background clutter. These pulses are therefore suited for short distance prey detection due to the great degree of atmospheric attenuation experienced by higher frequencies (Jones & Barlow, 2004). Furthermore, a negative relationship between peak echolocation frequency and wing loading is predicted (Jacobs, *et al*., 2007), where lower wing loading combined with higher peak frequency pulses may allow optimal foraging within or close to dense vegetation (Jones, 1996). Alternatively, lower frequency pulses are selected for by low clutter habitats. These pulses are suitable for long distance prey detection as they are less susceptible to atmospheric attenuation (Jones & Barlow, 2004). Additionally, it is predicted that species with lower frequency pulses and higher wing loading may forage faster in open spaces (Jacobs, *et al*., 2007). Both foraging habitat and behaviour can be linked to divergence of aspects of echolocation pulses (i.e. pulse frequency) and phenotypic traits associated with echolocation (wing morphology (Aldridge & Rautenbach, 1987) and body size (Jacobs, *et al*., 2007)), where closely related species or even different populations of the same species have diverged in their echolocation systems (Aldridge & Rautenbach, 1987; Schnitzler, *et al*., 2003). For example, rhinolophids display adaptations for slow, manoeuvrable flight, including, low wing loading (less

body weight relative to wing area (Poole, 1936)), low aspect ratios (calculated as the wingspan squared, divided by the area of the wings (Farney & Fleharty, 1969)), and rounded tips (Norberg & Rayner, 1987), which allow greater manoeuvrability in dense vegetation (where dense vegetation is defined as narrow space cluttered habitats where bats experience more obstacles both perceptually and mechanically). Lower echolocation frequency penetrates dense vegetation more effectively than higher frequency pulses as the longer wavelengths of lower frequencies are reflected, scattered, and absorbed less by the vegetation, resulting in greater energy for the generation of audible target strengths from the insect prey. Furthermore, a small body size allows manoeuvrable flight which is required for foraging in dense vegetation. Thus, the combination of low echolocation frequency and small body size, which represents a deviation from allometry between frequency and body size (Jacobs, *et al*., 2007; Jacobs & Bastian, 2018) may be an adaptation for detecting and capturing insect prey in dense vegetation. Support for the FHH has been found through inter-specific comparisons (Barclay, 1986; Aldridge & Rautenbach, 1987; Barclay, *et al*., 1999) and for some LDC bat species such as *Myotis lucifugus* (Wund, 2006) and *Macrophyllum macrophyllum* (Brinkløv, *et al*., 2010), where higher echolocation frequencies are used in highly cluttered habitats. However, as the echolocation system of HDC species is already well suited to detect prey in highly cluttered habitats (i.e., high frequencies and an acoustic fovea, combined with Doppler Shift Compensation) (Neuweiler, 1989; Waters, *et al*., 1995), they do not need to increase pulse frequencies for greater image resolution. Furthermore, due to the extreme degree of atmospheric attenuation experienced by the already high frequency pulses used by HDC species, any further increase in pulse frequency would not only result in insignificant differences in target strength (Jacobs, *et al*., 2007), but also decrease prey detection distances (Finger, 2021). Previous studies have proposed that the evolution of lower frequency

pulses in HDC species may be the result for the need of increase prey detection distance in open habitats (Xu, *et al*., 2008; Odendaal, *et al*., 2014). Here, the FHH can still be applied as the frequency of the CF portion of echolocation pulses are still affected by the transmission properties of the habitats used by these HDC echolocators (Odendaal, 2015). For example, *Rhinolophus ferrumequinum's* distribution in China (Jilin Province) spans a variety of habitats with varying levels of clutter, where populations in low clutter environments utilize lower pulse frequencies (Xu, *et al*., 2008).While intra-specific variation of echolocation pulses has been documented (i.e., variation of calls depending on amount of clutter in the habitat and the foraging task (Obrist, 1995)) it is not clear whether geographic variation in pulse frequency between populations of the same species (Thomas, *et al*., 1987) is the result of differences in body size or rather due to differences in habitat structure between locations (Barclay, *et al*., 1999). To date, very few studies relating to the significance of deviations in pulse frequency in relation to the FHH have been conducted (Finger, 2021).

This study aimed to determine why east African populations of the rhinolophid species, *R. fumigatus_East*, have a lower echolocation frequency (55.1 ± 1.5 kHz) relative to their body size (12.7 ± 0.9 g), thus deviating from the general allometric pattern observed in rhinolophid species (Jacobs & Bastian, 2018). This was achieved by measuring the target strengths of insect prey in relation to pulse frequency and vegetation density by ensonifying insects with the echolocation pulses of *R. fumigatus* and *R. capensis* in various degrees of vegetation density (i.e., sparse, moderate, and dense vegetation). Based on the foraging habitat hypothesis, it is predicted that the combination of low echolocation frequency and small body size of east African populations of *R. fumigatus_East* enables this species to better detect prey in dense vegetation

and subsequently enter dense vegetation to catch the detected prey than, for example, *R. capensis* (a species of similar body size (12 ± 1.7 g) but higher echolocation frequency (84.8 ± 3.6 kHz), or *R. fumigatus_West* (a lineage with similar echolocation frequencies but a larger body size) (Jacobs & Bastian, 2018). If so, *R. fumigatus_East* and *R. fumigatus_West* should have similar target strengths but stronger than *R. capensis*.

METHODS AND MATERIALS

Ethical Statement

No ethical clearance was required as the study did not require the capture or handling of bat species in the lab or field. Furthermore, the use of insects does not require ethical clearance (confirmed by the UCT Science Faculty Animal Ethics Committee, in compliance with the South African National Standard SANS 10386:2008 (The care and use of animals for scientific purposes) (South African Bureau of Standards, 2008)).

Brief overview of experimental approach

Bat prey was ensonified with the echolocation pulses of three rhinolophid lineages to determine the target strengths of prey in varying degrees of vegetation density (sparse, moderate, and dense vegetation density). This was done under constant environmental conditions (i.e., temperature and humidity) that the three lineages experience in natural conditions (as described later).

Study species

The study included two horseshoe bat species, *R. fumigatus* and *R. capensis* (Figure 1). There are at least two geographically isolated *R. fumigatus* lineages: *R. fumigatus* from the eastern part of southern Africa (Figure 1a) and *R. fumigatus* from the western part of southern Africa (Figure 1b). These lineages are distinct sister lineages (Dool, *et al*., 2016) differing not only genetically but also in body size while having similar echolocation frequencies (Jacobs & Bastian, 2018). The western lineage has a body mass of 18.8 g ± 1.5 g and an echolocation frequency of 55.1 kHz ± 1.5 kHz (Jacobs & Bastian, 2018).

Figure 1: Two of the *Rhinolophus* species used in this study, a) *Rhinolophus fumigatus*_East (Rüppell's Horseshoe Bat), b) *Rhinolophus fumigatus*_West (Rüppell's Horseshoe Bat) and c) *Rhinolophus capensis* (Cape horseshoe bat).

This lineage is distributed widely in the northern and central parts of Namibia and south-western Angola (Figure 2) (Monadjem, *et al*., 2010). In contrast, the eastern lineage has a smaller body mass of 12.7 g ± 0.9 g and a similar echolocation frequency of 54.7 kHz ± 1.1 kHz (Jacobs & Bastian, 2018). This lineages' distribution ranges from northern South Africa through Zimbabwe, Mozambique, Malawi, Southern and eastern Zambia, and the southern Democratic Republic of Congo (Figure 2). Both the eastern and western lineages are clutter foragers and are associated with arid savanna and savanna woodland biomes respectively (Monadjem, *et al*., 2010). *Rhinolophus capensis* (Figure 1c) is distributed along the coastal belt of South Africa's Cape (Monadjem, *et al*., 2010), extending throughout the Cape Floristic Region in the western cape of South Africa, up to the border between South Africa and Namibia (Figure 2) (Raw, *et al*., 2018). This species is associated with fynbos and succulent karoo biomes (Monadjem, *et al*., 2010), and forages predominantly in or near cluttered habitats (Jacobs, *et al*., 2007; Odendaal, *et*

al., 2014). *Rhinolophus capensis* has a body mass of 11.1 g ± 1.5 g (Jacobs, *et al*., 2007) and an echolocation frequency of 84.2 kHz (Fawcett, *et al*., 2015). The *R. capensis* population used in this study is the Heidehof population (34°36'33.4" S, 19°30'32.4" E), which is characterised as a fynbos shrubland ecoregion with an average yearly rainfall of 576 mm and an average range of monthly air temperature of 12 ˚C and 20 ˚C (Hersbach, *et al*., 2020; Lobelia, 2020). From this point forward the eastern *R. fumigatus* lineage will be referred to as *R. fumigatus_East*, and the western lineage will be referred to as *R. fumigatus_West.*

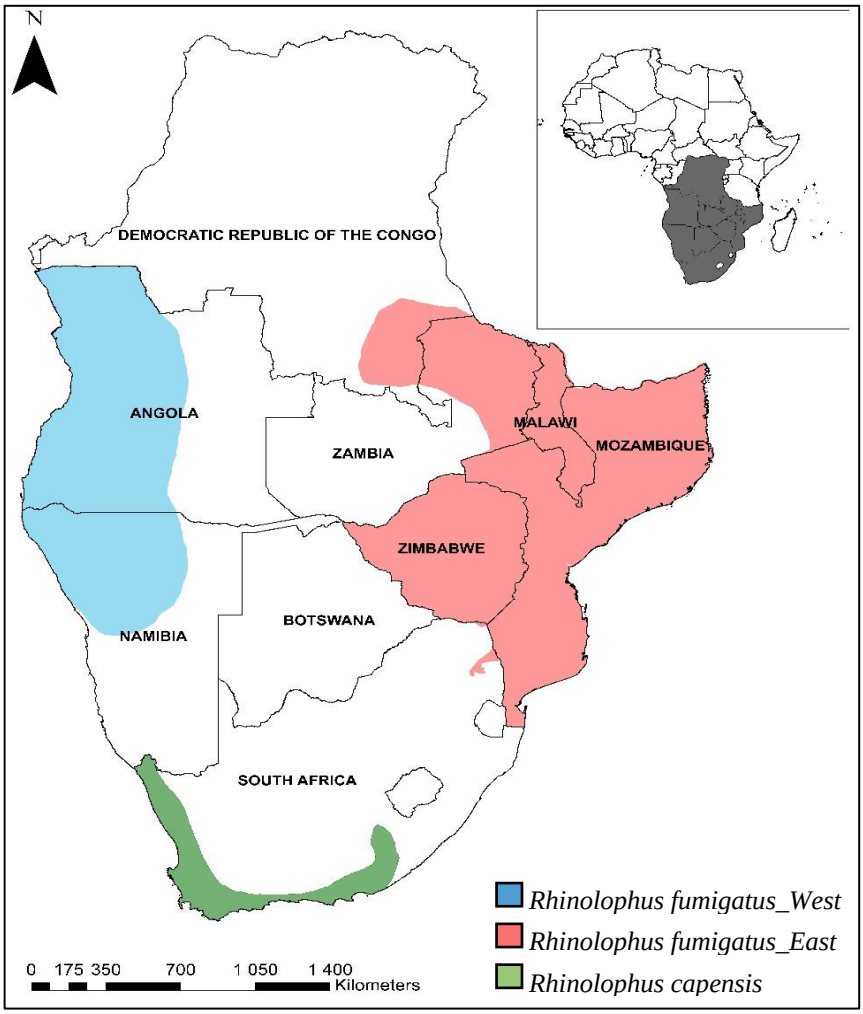

Figure 2: Map of southern Africa illustrating the distributions of three horseshoe bat specis, *Rhinolophus capensis* in southern South Africa (green), *Rhinolophus fumigatus*_West (blue) in northern and central Namibia and south-western Angola, and *Rhinolophus fumigatus*_East (red) in northern South Africa, Zimbabwe, Mozambique, Malawi, Southern and eastern Zambia, and southern Democratic Republic of Congo. Distribution adapted from Monadjem et al. (2010).

Microphone calibration and speaker test

An ultrasonic microphone was calibrated using the Calibrated 40 kHz Reference Signal Generator (Avisoft Bioacoustics (2022)). This was done to ensure the microphone detects and records absolute sound level measurements. The reference signal (i.e. semi-synthesized pulses) was recorded using the microphone (positioned 25 cm from the speaker) and a subsection of the recording was selected. Under the heading "Tools/Calibration" in Avisoft-SASLab Pro (v5.2, Avisoft Bioacoustics, Glienicke, Germany), the method "SPL with reference sound" was selected and the dB SPL sound level was entered into the "level" field (this value was printed on the reference sound generator- 84 dB SPL at a distance of 25 cm). This was done to ensure that the target strength values obtained were not the result of the microphone recording different echolocation frequencies at different sensitivities.

A speaker test was conducted to determine the intensity that the speaker plays the echolocation pulses. This was done by placing the microphone 2m away from the speaker (distance between the speaker and the target under the experimental setup) under the same environmental conditions (temperature (23°C) and relative humidity (40%)) under which the experiments were conducted. For each echolocation frequency, 10 echolocation pulses were played over a speaker and recorded to determine the intensity of the pulses when they reached the microphone. The results showed that the speaker played higher frequencies (i.e., *Rhinolophus capensis*) at lower intensities. Therefore, the intensities of *R. fumigatus_East* and *R. fumigatus_West* pulses were decreased (both set to 15%) relative to that of *R. capensis* (set to 90%) for both HDC and LDC pulses, so that the intensities of all three *Rhinolophus* lineages

were the same when arriving at the target. The change in intensity was achieved using the automated normalisation function in AviSoft-SASLab Pro. This allowed for the control of variation in target strength differences due to pulse intensity. Equipment settings that could influence recordings (i.e., frequency gain of the bat detector, and playback volume) were also kept constant (frequency gain on the lowest setting and the playback volume set to 0 dB). The playback volume of the speaker was set to 0 dB to avoid the noise produced by the sudden onset of sound.

Echolocation recordings and preparation of playback files for ensonification

Playback files were prepared using previously recorded echolocation pulses of *R. fumigatus_East*, *R. fumigatus_West*, and *R. capensis* and used to ensonify moths in varying degrees of vegetation density. The pulses for both species were already available from previous studies (Odendaal, *et al*., 2014; Wechuli, 2020; Finger, 2021) conducted in the Animal Evolution & Systematics Group (AES), University of Cape Town. Echolocation calls were recorded from handheld bats located 30 cm in front of a microphone (see Finger 2021 for details). Echolocation recordings were obtained at various sites by D. Jacobs as follows: *R. capensis*: Heidehof Farm, Western Cape, South Africa (Recordings taken in February of 2010), R. fumigatus_East: Jiri Estate, Harare, Zimbabwe (Recordings taken in June of 2013), *R. fumigatus_West*: Ludwig caves near the Otavi region, Namibia (Recordings taken in April of 2010).

Playback files were prepared using AviSoft SASLab Pro (v5.2, Avisoft Bioacoustics, Glienicke, Germany). The natural recordings were uploaded to the software, and good-quality

pulses were selected, clipped, and saved as a new file. Good quality pulses are those with a high signal to noise ratio, calculated as:

$$SNR\ dB = 20\ LOG_{10}\left(\frac{A_{signal}}{A_{noise}}\right)$$ (Houston, *et al*., 2004; Lazure & Fenton, 2011)

Where A_{signal} = peak amplitudes of the signal

A_{noise} = peak amplitude of the noise

A ratio greater than 1:1 indicates there is more signal than noise, where any value greater than 5 was considered a good quality call. These natural pulses served as a template to generate semi-synthetic pulses to eliminate noise or recording artifacts from the playback files. The first five pulses of each recording were avoided as horseshoe bats tune into their peak resting frequency after a period of silence (Siemers, *et al*., 2005). The second harmonic was used in the synthesis of the duplicates because rhinolophids emit pulses dominated by the second harmonic, which contains the most acoustic energy (Pye & Roberts, 1970) and has the best signal-to-noise ratio (Finger, 2021). Only calls emitted by males were used for ensonification due to the difference in resting frequency between sexes for *R. capensis* (Odendaal & Jacobs, 2011). For each echolocation frequency, 10 pulses were selected for both HDC and LDC sequences, where pulses of similar amplitudes were selected for each echolocation frequency and duty cycle. Pulses with clear FM components displayed on the spectogram were used in the synthesis of playback files to ensure all pulse information would be transferred to the next step of semi-synthesized pulse generation. A Time-Domain Filter (FIR) filter was applied to remove any noise (type: high pass frequency, with the FCO set to 5 kHz). Spectral characteristics were set to detect the peaks of the pulse using the following one-dimensional functions setting: Function set to Power spectrum (logarithmic), Evaluation window set to FlatTop, and zero padding set to disabled. In the Power spectrum window, the axis scales were changed from the defaults to

display a greater range, to ensure all peaks were detected correctly (Y-axis: Ymax = 0, Ymin = -150; X-axis: Xmin = 0, Xmin = 150). The spectral characteristic was then measured using the following settings: Peak set to auto (with the maximum and at frequency boxes selected), Bandwidth threshold set to 0 dB, peak detection threshold set to -60 dB, and hysteresis set to 65 dB. The values were then copied into an excel sheet and the difference between the amplitudes of the harmonics was calculated (by subtracting the amplitude of the 2nd (prominent peak) from the amplitudes of the other harmonics. These values were then used to create a semi-synthesized pulse. Using the spectrogram parameters window with the following settings: Frequency resolution: FFT length set to 256, Frame size set to 100%, window set to FlatTop, temporal resolution: overlap set to 87.5%, and the enable waveform editing box selected. Within the spectrogram window, any visible noise is removed using the eraser cursor. The frequency and amplitude information was then scanned using the following settings: Element separation set to automatic (single threshold) with a threshold value of -30 dB and a hold time of 20 ms; the scan amplitude envelope, scan entire spectrogram, and automatic update boxes all selected; the regular intervals (relative) box was selected and the duration set to 40 (this represents the temporal properties of the frequency contour, where 40 points at fixed time intervals will be displayed in the graphic synthesizer window). The pulses were then synthesized in the graphic synthesizer window. Within the graphic synthesizer window, the algorithm detected the prominent harmonic as the fundamental, therefore the contours were re-scaled into the true fundamental. The fundamental frequency was multiplied by a factor of 0.5 as the frequency was too high (factor 2). This resulted in the frequency and amplitude contour switching to the true fundamental without changing the sample point values. The other harmonics were then added using the dB values calculated in the excel sheet, using the graphic synthesizer parameters

window with the following settings: The fundamental frequency and overall amplitude boxes were selected and set to 1000Hz and 1V respectively, under the relative amplitude of harmonics (dB) heading, the dB values calculated in the excel sheet are entered, and each block with an input value was selected (this enables the editing of the FM components of all the harmonics when re-drawing the contours). The fade-in and fade-out setting "t" was set to 0.1 ms and the average time for parameter smoothing was also set to 0.1 ms. The contour was then redrawn to match the spectrogram shown in the graphic synthesizer window. The first and last amplitude point was moved down to the 0dB line to avoid the short bursts of sound emitted by the loudspeaker when pulses start with high amplitudes. The newly synthesized pulse was then saved as a WAV file. To ensure that no information was lost during the process of synthesization, various measurements were taken of the original and semi-synthesized pulses (including: call duration (ms), initial and terminal FM duration (ms), CF duration (ms), the minimum frequency of the initial and terminal FM component (kHz), and peak frequency of the CF component (kHz). As previously described, the intensities of *R. fumigatus_East* and *R. fumigatus_West* pulses were both decreased to 15% relative to that of *R. capensis* (set to 90%) for both HDC and LDC pulses, to ensure the intensities of all three Rhinolophus lineages were the same when arriving at the target.

The synthesized pulses were used to create various sequences (Figure 3) that were compiled into a single playback file. These sequences consisted of a high duty cycle (HDC) test (Figure 3a) which uses the mean maximum pulse duration and inter-pulse-interval (IPI; defined as the silent section between two pulses (Novick & Vaisnys, 1964) or time between the end of one signal and

the beginning of the subsequent signal (Ho, *et al*., 2013)) for each lineage, and a low duty cycle (LDC) test (Figure 3b), which uses the mean minimum pulse duration and IPI for each lineage .

Table 1: Measurements of additional parameters taken to ensure the semi-synthesized pulses produced contain all the information of the original pulse.

	Measurements of additional parameters					
	High Duty Cycle					
	R. fumigatus_East		*R. fumigatus_West*		*R. capensis_Heidehof*	
	Original	semi-synthesized	Original	semi-synthesized	Original	semi-synthesized
Call duration (ms) [o]	44,99	44,48	43,45	43,291	31,75	31,8
FM_i duration (ms) [s]	2,18	2,15	0,88	0,88	1,33	1,35
CF duration (ms) [s]	41,27	41,18	40,93	40,91	28,96	28,72
FM_t duration (ms) [o]	1,21	1,21	1,76	1,79	1,7	1,76
FM_i min frequency (kHz) [p]	39	39	42,9	42,9	70,3	70,3
CF peak frequency (kHz) [p]	53,727	53,507	53,65	53,64	84,133	84,143
FM_t min frequency (kHz) [p]	44,9	44,9	41,016	41	68,3	68,3
	Low Duty Cycle					
	R. fumigatus_East		*R. fumigatus_West*		*R. capensis_Heidehof*	
	Original	semi-synthesized	Original	semi-synthesized	Original	semi-synthesized
Call duration (ms) [o]	39,6	39,6	34,84	34,813	26,92	26,91
FM_i duration (ms) [s]	1,98	1,98	1,29	1,61	1,01	1
CF duration (ms) [s]	36,16	36,16	31,85	31,24	24,2	24,2
FM_t duration (ms) [o]	1,45	1,45	1,65	1,65	1,58	1,58
FM_i min frequency (kHz) [p]	40	40	43,9	43,9	74,2	74,2
CF peak frequency (kHz) [p]	53,54	53,54	55,0023	55,0075	84,22	84,22
FM_t min frequency (kHz) [p]	44,434	44,7	39	39	66,4	66,4

The mean maximum and mean minimum IPI was calculated by measuring the maximum and minimum silent sections respectively between two consecutive pulses, using 10 pulses. The order in which the pulses of each echolocation frequency is played was compiled to cover all possible combinations, therefore both the HDC and LDC tests consist of 18 pulses each.

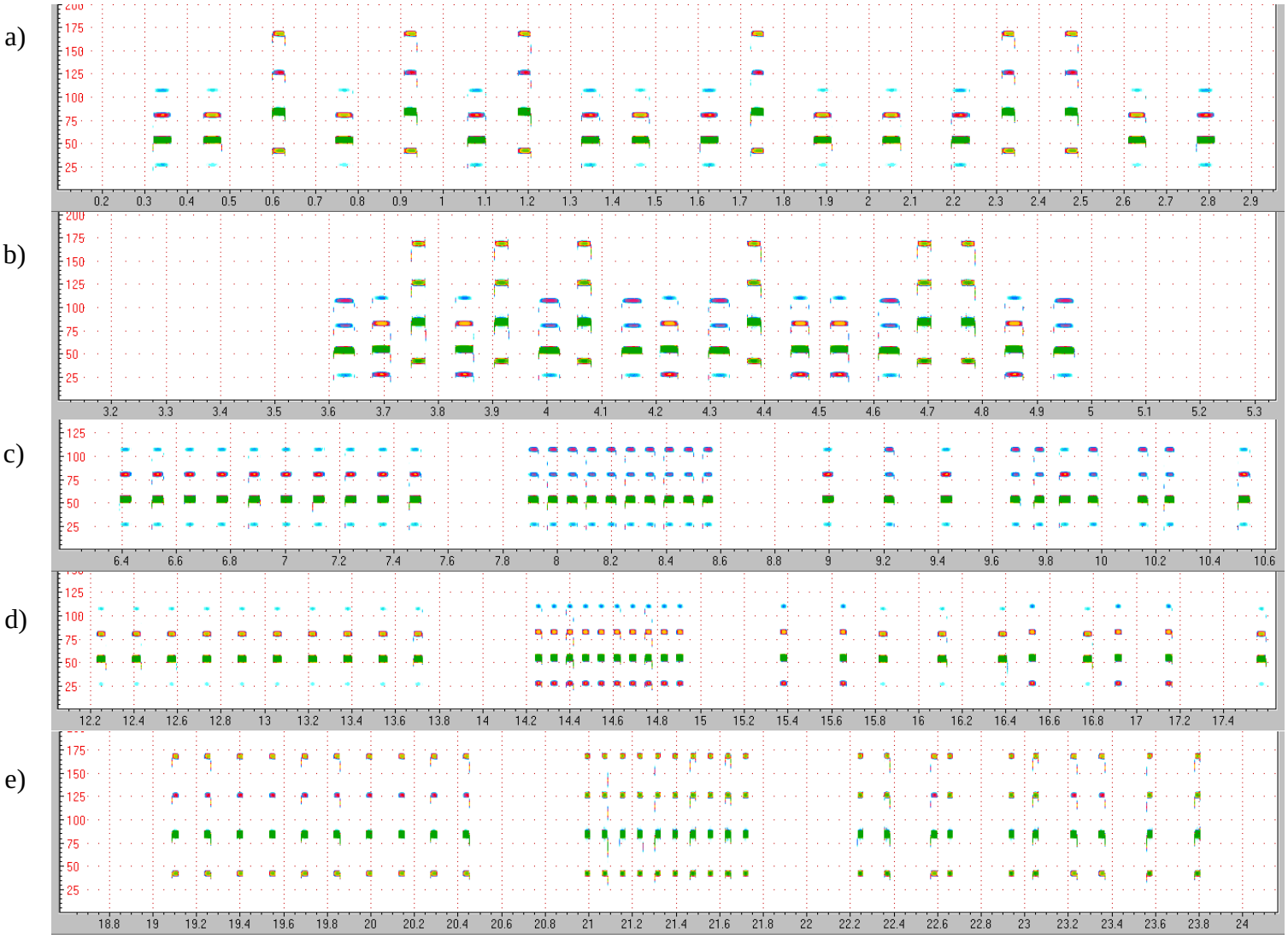

Figure 3: The various sequences of *R. fumigatus*_East, *R. fumigatus*_West and *R. capensis* echolocation pulses used in the ensonification trials. a) High duty cycle comprises of three stimuli classes that are similar in pulse duration. The inter-pulse-interval (IPI) used is the mean maximum value for each stimuli class and the order of the pulses cover all possible combinations. b) Low duty cycle comprises of three stimuli classes that are similar in pulse duration. IPI used is the mean minimum value for each stimuli class and the order of the pulses cover all possible combinations. c - e) Variation of tests consist of three sections: 1) a high duty cycle sequence using the maximum mean duty cycle for the species, 2) a low duty cycle sequence using the minimum mean duty cycle for the lineage, and 3) a sequence of variable pulses with the natural durations and IPI's for the stimuli class.

The combination of the HDC and LDC (Figure 3 a & b) pulses are: *R. fum_East, R. fum_West, R. capensis; R. fum_West, R. capensis, R. fum_East; R. capensis, R. fum_East, R. fum_West; R. fum_East, R. capensis, R. fum_West; R. fum_West, R. fum_East, R. capensis; R. capensis, R. fum_West, R. fum_East.* The inclusion of both HDC and LDC pulses and IPI's is due to the effect of clutter, where Schnitzler and Kalko (2001) found that HDC echolocators update the acoustic image by increasing echolocation call emission rate more frequently when foraging in dense vegetation. The final three sequences (Figure 3c-e) consist of a variety of tests for each lineage, to ensure that any differences in target strength are not attributed to the duty cycle nor the order of the pulses of each lineage. These tests consist of three sections: 1) an HDC sequence where the highest mean duty cycle for the lineage is used; 2) an LDC sequence where the lowest mean duty cycle for the lineage is used, and 3) a sequence of variable pulses in which the lineages' natural durations and IPI's are used. All three subsections of the latter three sequences (i.e. sections 1, 2, and 3 of sequences c, d, and e were composed of 10 pulses. The sequences (a-e) were compiled into one playback file so that a single moth preparation was exposed to all the tests and each trial in succession. A period of silence lasting 2 seconds was inserted between each test, to avoid echoes from the preceding test overlapping the proceeding test, as well as for simpler analysis of the recordings.

Moth collection

Moths were obtained from breeders in South Africa, therefore species used include those that were available from breeders, but still closely match the morphology of the known diet of *R. capensis* (Jacobs, *et al.*, 2008) as the diet of *R. fumigatus_East* and *R. fumigatus_West* is currently unknown. *Rhinolophus capensis'* diet includes noctuid moth species such as

Helicoverpa armigera (wingspan 350 - 400 mm, 14-18 mm in length), a cathemeral species (organisms that have sporadic and irregular intervals of activity and are neither nocturnal or diurnal), *Desmocreara griseiviridis*, a nocturnal species, and *Phyllalia* spp. (Euperotidae). Both *H. armigera* and *D. griseiviridis* are eared species where *H. armigera* has a sensitivity to frequencies between the ranges of 5 – 20 kHz and 85 – 95 kHz, and *D. griseiviridis* between the range of 85 – 95 kHz (Jacobs, *et al*., 2007). The moth species used in this study is the greater wax moth (13-19 mm in length), a tympanate species which has a sensitivity to frequencies in the range of 30 – 300 kHz (Bloudoff-Indelicato, 2013). There is a significant body of evidence that supports the conclusion that the detection of bats is the primary purpose that ears exist in moths, where moths either stop flapping their wings to avoid detection (Bell & Fenton, 1984), or employ evasive flying manoeuvres (i.e. erratic turns and dives) to avoid predation (Roeder & Treat, 1961).

Moth preparation

Moths were placed on an ice brick covered in a thin layer of cloth to decrease the body temperature of the moth enough so that flight is not initiated (thus enabling easy handling). Once the moth was immobilized, the fine hairs on the ventral part of the thorax were removed using a fine, soft-bristled paintbrush. A small amount of adhesive putty was moulded to the rounded tip of an entomological insect pin (BioQuip products, No.3 Stainless steel insect pins) to increase the surface area of the attachment point. A small amount of non-toxic medical skin glue (Osto Bond Skin Bond Adhesive) was applied to the rounded area on an entomological pin and given 10-20 seconds to dry slightly. This was then gently applied to the ventral part of the thorax (after removal of the hairs as mentioned above) and held in place until the glue was completely dry.

After ensonification trials, the moth was removed with a soft entomological tweezer (Entomology tweezers, soft, blunt-r), and was placed in the freezer to ensure ethical discarding of the moth.

Experimental set-up and Target strength

The temperature in the anechoic room was set to 23 °C and humidity levels were set to 40% humidity. The temperature and humidity levels used are based on the conditions both moths and bats are likely to experience on summer nights under natural conditions (Churchill, *et al*., 1997; Kelly, 2008). The temperature (°C) and humidity levels (%) in the anechoic room were continuously observed using a weather station (Professional Weather Centre, Model WMR200A, Oregon Scientific INC., Tualatin, Oregon, USA) to allow for the calculation of atmospheric attenuation. The interior of the room is lined with ultrasound absorbing acoustic foam (porous acoustic panels), specifically designed to absorb any noise produced by echoes reflecting off objects and surfaces in the room (the pores of the foam fill with sound wave vibrations which are converted to kinetic energy. The conversion of energy results in fewer sound waves being reflected from the surface of the foam (Anon., 2021)), thus providing clearer echoes from the ensonified target. At one end of the room, a thin wooden rod (10 mm) was inserted into the acoustic foam, jutting from the wall approximately 30 cm in length and 1m from the ground (Figure 4). The wooden rod that extends from the wall was also covered in acoustic foam to decrease the production of background noise produced by the rod. This serves as the attachment point of the moth. At the opposite end of the room (2m from the moth), a loudspeaker (from which the recorded echolocation pulses were played (Avisoft UltraSoundGate player BL Light, Avisoft Bioacoustics, Glienicke, Germany)), together with an ultrasound detector (Pettersson D-

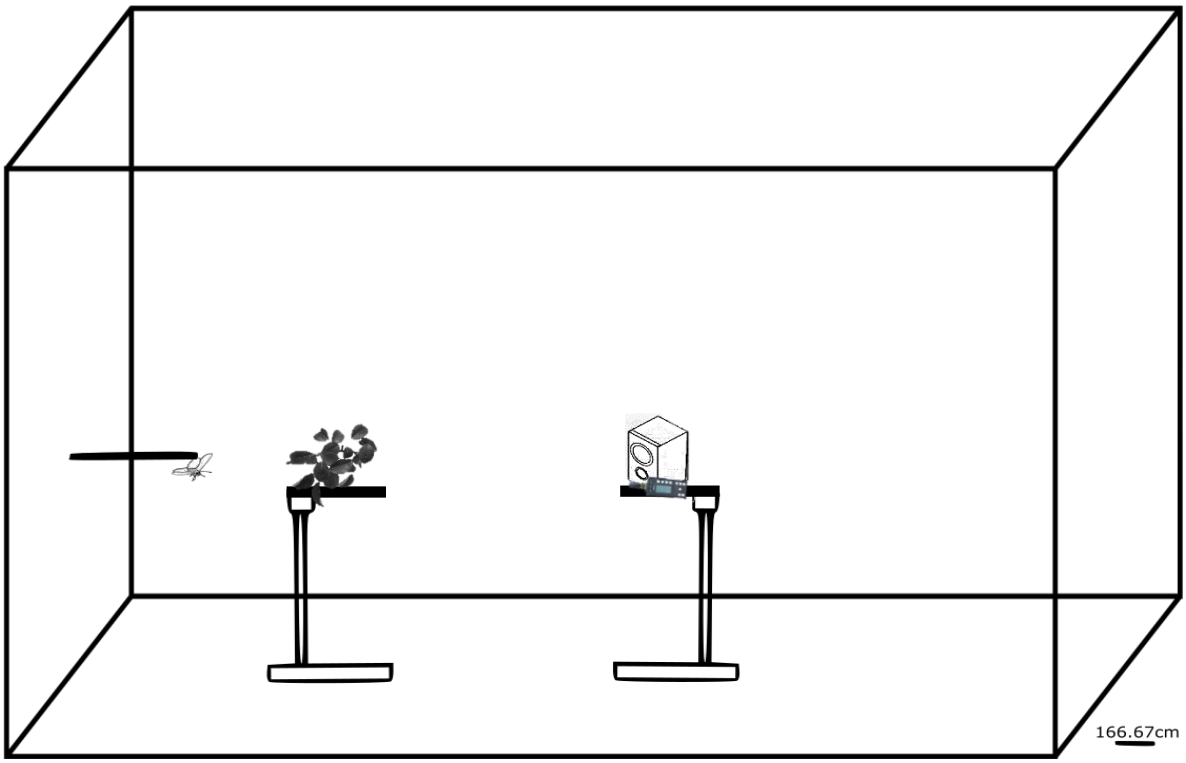

Figure 4: Illustration of the setup of the moth (target), microphone and speaker within the anechoic room at the University of Cape Town.

1000X bat detector) were placed on a modified retort stand covered in acoustic foam. Modifications to the retort stand include removing the clamps and replacing the rod of the retort stand with a thin piece of PVC pipe to increase the height of the rod to 1m. Additionally, a flat surface was created to hold the experimental equipment, achieved by attaching a square piece of plywood perpendicular to the rod of the retort stand. A hole was drilled through one end of the plywood and the rod was inserted through the hole, so that the plywood rests on the upper-most part of the rod. The platform was not fixed and was adjustable by ± 10cm to ensure the height of the equipment resting on the platform is exactly 1m above ground. The stand was then covered in acoustic foam to avoid any echoes coming from the modified retort stand. The speaker and the microphone were placed on the platform at an inward angle (using a laser pointer) to ensure a

direct line-of-sight propagation of sound to and from the moth (Figure 5 a & b). Vegetation was placed 0.5m from the moth on a second modified foam-covered retort stand.

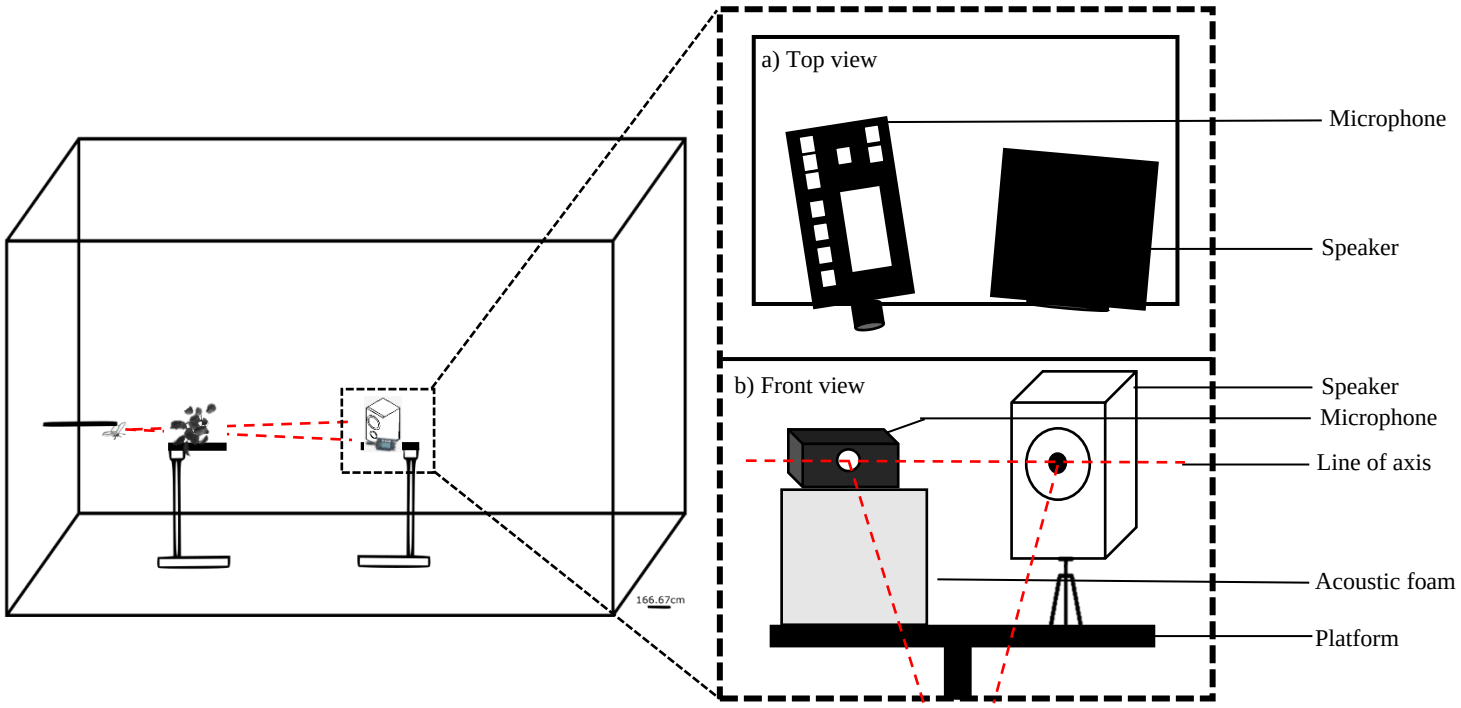

Figure 5: Diagram illustrating the setup of the playback experiment. a) & b) Speaker and ultrasound recorder on the retort stand. A platform at the upper end of a retort stand held the experimental equipment. A) Top view of the placement of the ultrasound recorder and the speaker. Both microphone and speaker are angled inward and directed at the moth to create a direct line-of sight propagation of sound from the speaker to the moth, and from the moth to the microphone. b) Front view of the placement of the recorder and speaker on the retort stand. The recorder was placed on top of a piece of acoustic foam so that the central point of the microphone capsule is in line with the central point of the speak (illustrated by red line).

Five ensonification trials were conducted:

Trial 1 (control 1) - Measuring the target strength of the experimental equipment without the moth: To determine the target strength produced by the experimental equipment, the equipment was set up (as shown in Figures 4 and 5) without the moth and vegetation, and ensonified with the compiled playback file (as illustrated in Figure 2).

Trial 2 (control 2) – Measuring the target strength of the moth: To determine the target strength produced by moths, the moth was suspended (without vegetation) and ensonified with the compiled playback file. To determine the target strength of moths through varying degrees of vegetation, the relative vegetation density was varied between the three proceeding ensonification trials, where all ensonification occurred with the same relative density for each echolocation frequency. The three vegetation density categories used were: Sparse, moderate, and dense vegetation density. *Searsia crenata* (previously known as *Rhus crenata*) was the plant species used in the ensonification trials. *S. crenata* is a multibranched, dense evergreen shrub with small trifoliolate leaves.

Trial 3 – Measuring the penetration of echolocation pulses through sparse vegetation: The moth was suspended, and sparse vegetation was placed on the retort stand. Sparse vegetation is defined as a single branch with three twigs extending from the branch, where vegetation on the twigs covers approximately 25% of the twig surface area. The moth was then ensonified with the compiled playback file. The penetration of echolocation pulses was measured by subtracting target strength differences between control one and two (i.e. control two – control one), from the target strengths measured in Trial 3. As the pulses and retuning echoes of HDC echolocators are not separated in time, this was done in trials 3 – 5 in order to separate the echoes produced by the moths and those produced by the surrounding vegetation.

Trial 4 – Measuring the penetration of echolocation pulses through moderately dense vegetation: Following trial 3, the moth was suspended, and vegetation was placed on the retort stand. The relative density of the vegetation increased to mimic moderately dense vegetation. Moderately

dense vegetation is defined as a single branch with six twigs extending from the branch, where the vegetation on the twigs covers approximately 50% of the twig surface area. The moth was then ensonified with the compiled playback file. The penetration of echolocation pulses was measured by subtracting target strength differences between control one and two, from the target strengths in Trial 4.

Trial 5 – Measuring the penetration of echolocation pulses through dense vegetation: Following trials 3 and 4, the moth was suspended, and vegetation was placed on the retort stand. The relative density of the vegetation increased to mimic dense vegetation. Dense vegetation is defined as two branches with six twigs extending from each branch, where vegetation on the twigs covers 40% of the twig surface area on each branch (thus 80% of vegetation cover relative to trials 3 and 4). The moth was ensonified with the complied playback file. The penetration of echolocation pulses was measured by subtracting target strength differences between control one and two, from the target strengths in Trial 5.

For each trial, all other variables were kept constant, including pulse intensity between trials and the frequency gain on the microphone (set to the lowest setting). To be able to compensate for atmospheric attenuation in each individual case, room temperature (23°C) and humidity (40%) were kept constant as these variables greatly affect the atmospheric attenuation of sound. Audio recordings were done with 16 bits depths and a 500 kHz sample rate. The files were saved onto a compact flash (CF) card in a WAV format and then analysed using the Avisoft software.

Statistical analyses

Voltages recorded from the returning echo were measured using Avisoft SASLab Pro (v5.2, Avisoft Bioacoustics, Glienicke, Germany and converted to absolute dB per SPL (sound pressure levels) values, to calculate target strength (dB) at a standardized distance of 2 m. This was done by measuring the peak-to-peak voltage of the echo, and the target strength being calculated using the formula:

$$20 \times log_{10}\left(\frac{incident\ SPL}{echo\ SPL}\right)$$ (Waters, *et al*., 1995; Houston, *et al*., 2004; Lazure & Fenton, 2011)

Incident SPL was measured from the semi-synthesized pulses played during the experiment and echo SPL was measured from the recorded echoes. The echo SPL values were then corrected to account for atmospheric attenuation according to the equations given by the International Organization for Standardization (1996), where the attenuation due to atmospheric absorption (A_{atm}) in decibels is given by:

$$A_{atm} = \alpha d / 1000$$

Where: A_{atm} = attenuation due to atmospheric absorption during propagation through a distance, in decibels

α = atmospheric attenuation coefficient, in decibels per kilometer

d = distance, in meters

The atmospheric attenuation was calculated using an online calculator (National Physical Laboratory (Anon., 2021)) (Figure 6) which is based on and uses the formula described by the International Organization for Standardization. The atmospheric attenuation coefficient (dB/km) is dependent on the relative humidity of the air (% RH), the temperature (°C), the atmospheric pressure (kPa), and the frequency of the sound (Hz). This was done for each echolocation frequency and each vegetation type, to compare the target strengths. The target strength values were obtained by subtracting the values obtained in trial one from trial two, to obtain the target strength of the echo produced by the moth. This value was then subtracted from the target strength values in trials three, four, and five to determine the target strength of the moths through sparse, moderate, and dense vegetation.

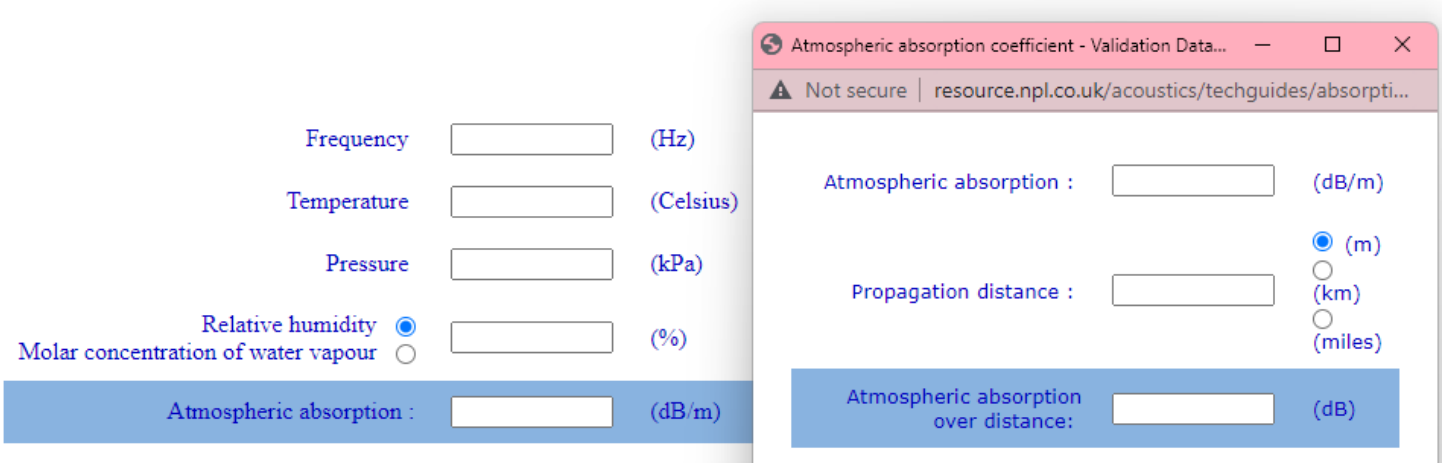

Figure 6: Screenshot of the inputs of the online atmospheric attenuation calculator. The left side shows in input windows of frequency (Hz), temperature (°C), atmospheric pressure (kPa) and relative humidity (%RH), all of which are used to calculate the atmospheric attenuation coefficient (dB/m). The atmospheric attenuation coefficient value calculated was then inserted on the right-side window with the propagation distance of 2m, producing the total atmospheric absorption (dB) over 2m.

All statistical testing was conducted using R version 4.1.1, R core team, 2021)) with a significance level of $\alpha = 0.05$ (data given as mean ± SD). Multiple statistical analyses were run to determine if various factors influence the target strength values obtained during experiments.

The data was separated into various groups for testing: a) Data was grouped by each vegetation density and further separated by duty cycle (i.e. HDC recordings for each lineage taken under sparse vegetation density were grouped and the target strengths were compared between each lineage (also done to LDC and natural variation recordings in all three vegetation densities)). This separation of pulses by duty cycle was done to determine if any variation in target strength results within various vegetation densities were due to duty cycle or the order in which each lineage's echolocation pulse was presented; b) Data was grouped based on echolocation frequency (i.e. HDC recordings for *R. fumigatus_East* in all three vegetation densities (also done for *R. fumigatus_West* and *R. capensis*) were grouped and target strengths compared to determine if vegetation density influences the target strength within each echolocation frequency; c) The difference between duty cycles was determined by comparing HDC and LDC target strength data for both echolocation frequency and vegetation density.

For all grouped data, the distributions (Shapiro-Wilks normality test) and variances were analysed (using the var() function for normally distributed data and the Fligner-Killeen test homogeneity of variances on data with a skewed distribution). The normally distributed with equal variances was analysed using a one-way ANOVA. Pairwise Tukey posthoc tests were then conducted to determine where the significant differences in target strength lie. For the data that did not follow a normal distribution or had unequal variances, a log transformation was applied to the data in an attempt to normalize the data distribution. After the log transformation, the data remained skewed, thus non-parametric data analysis was used to analyse these groups of data. A non-parametric Kruskal-Wallis rank sum test was used to determine if significant differences

exist between the means of the various groups, and a Kruskal-Wallis Multiple Comparison Post-hoc test was conducted to determine where the differences lie.

RESULTS

A total of 126 semi-synthesized echolocation pulses (42 for each lineage) were played in trials one to five. The recorded echoes were analysed, and the SPL values were extracted to calculate the target strength values for equipment only (Control 1), equipment and moth (Control 2), sparse vegetation (Test 1), moderate vegetation (Test 2), and dense vegetation (Test 3), after accounting for atmospheric attenuation (Table 2).

Table 2: Target Strength data (dB) of High Duty Cycle, Low Duty Cycle, and the natural sequence of echolocation pulses for tests run on equipment only (Control 1), equipment and moth (Control 2), Sparse vegetation (Test 1), Moderate vegetation (Test 2), and Dense vegetation (Test 3).

			Target Strength Data				
		Species	**Equipment only**	**Equipment + Moth**	**Sparse vegetation**	**Moderate Vegetation**	**Dense Vegetation**
***R. fumigatus*_East**	**High Duty Cycle**	1	29,25	29,28	29,30	29,34	29,32
		2	29,26	29,28	29,34	29,29	29,35
		3	29,30	29,26	29,29	29,28	29,32
		4	29,31	29,23	29,33	29,18	29,31
		5	29,31	29,27	29,32	29,31	29,35
		6	29,25	29,35	29,35	29,29	29,31
		7	29,30	29,35	29,33	29,34	29,32
		8	29,26	29,29	29,28	29,27	29,32
		9	29,32	29,28	29,31	29,33	29,28
		10	29,16	29,21	29,21	29,18	29,19
	Low Duty Cycle	1	28,55	28,65	28,65	28,60	28,63
		2	28,33	28,34	28,32	28,25	28,34
		3	28,42	28,38	28,41	28,40	28,33
		4	28,28	28,24	28,26	28,25	28,27
		5	28,18	28,16	28,20	28,17	28,17
		6	28,52	28,52	28,54	28,50	28,56
		7	28,01	28,08	28,05	28,08	28,03

		8	28,22	28,31	28,28	28,29	28,27
		9	28,122	28,12	28,17	28,155	28,12
		10	28,42	28,38	28,39	28,34	28,37
	Natural Variation	1	29,19	29,22	29,21	29,22	29,23
		2	28,49	28,54	28,58	28,46	28,58
		3	29,25	29,26	29,28	29,28	29,23
		4	28,49	28,47	28,46	28,45	28,44
		5	15,82	28,39	28,38	28,32	28,38
		6	29,25	29,25	29,31	29,245	29,23
		7	28,49	28,44	28,44	28,47	28,41
		8	28,45	28,49	28,51	28,52	28,52
		9	28,30	28,32	28,28	28,249	28,24
		10	29,28	29,26	29,27	29,24	29,25
***R. fumigatus*_West**	**High Duty Cycle**	1	28,97	28,97	28,98	28,97	29,02
		2	29,01	29,06	29,04	29,00	29,06
		3	29,01	29,01	28,99	28,97	28,95
		4	29,04	29,06	29,04	29,01	29,02
		5	29,02	29,03	28,99	28,98	29,03
		6	29,04	29,03	29,01	29,01	29,03
		7	29,02	28,96	28,96	29,02	28,98
		8	29,04	28,96	29,00	29,00	28,98
		9	29,086	29,08	29,03	29,03	29,06
		10	29,03	29,05	29,08	29,08	29,04
	Low Duty Cycle	1	27,53	27,60	27,60	27,58	27,56
		2	27,59	27,64	27,65	27,66	27,63
		3	27,66	27,69	27,71	27,65	27,60
		4	26,96	27,11	27,09	27,06	27,04
		5	25,98	26,07	26,03	26,01	26,01
		6	26,96	27,04	27,00	26,99	27,04
		7	27,38	27,36	27,32	27,38	27,36
		8	26,70	26,81	26,74	26,69	26,73
		9	27,61	27,62	27,66	27,63	27,62
		10	27,11	27,14	27,08	27,00	27,15
	Natural Variation	1	27,41	27,44	27,41	27,41	27,39
		2	25,80	25,80	25,79	25,83	25,81
		3	28,97	28,96	29,00	29,02	28,97
		4	29,10	29,16	29,07	29,16	29,12
		5	28,98	28,96	28,93	28,96	28,945
		6	27,10	27,11	27,02	27,09	27,00
		7	28,99	28,98	28,97	28,97	28,96
		8	26,86	26,87	26,90	26,86	26,88
		9	26,89	26,93	26,91	26,95	26,98

		10	28,99	29,03	29,06	29,02	29,05
R. capensis	**High Duty Cycle**	1	27,2	27,23	27,16	27,16	27,17
		2	26,84	26,86	26,79	26,79	26,86
		3	27,45	27,46	27,41	27,48	27,41
		4	27,17	27,27	27,22	27,25	27,229
		5	27,44	27,42	27,52	27,44	27,42
		6	27,34	27,32	27,33	27,38	27,39
		7	26,89	27,01	26,98	26,99	26,99
		8	26,8	26,88	26,86	26,85	26,90
		9	27,31	27,36	27,28	27,32	27,32
		10	25,39	25,34	25,33	25,33	25,24
	Low Duty Cycle	1	19,26	19,29	19,47	19,45	19,51
		2	23,07	23,09	23,10	23,05	23,04
		3	20,87	20,89	20,91	20,90	21,00
		4	17,76	17,82	17,87	17,68	17,72
		5	18,19	18,08	18,09	17,99	18,13
		6	15,99	16,19	16,13	16,10	16,24
		7	13,58	13,92	13,58	13,52	13,86
		8	7,97	8,76	7,97	8,78	8,85
		9	16,54	16,72	16,69	16,77	16,54
		10	20,51	20,37	20,37	20,45	20,44
	Natural Variation	1	16,37	16,34	16,45	16,49	16,24
		2	11,79	12,00	11,72	11,93	12,12
		3	26,89	26,96	26,97	26,93	26,96
		4	19,13	19,20	19,23	19,30	19,09
		5	21,01	21,19	21,21	21,20	21,11
		6	23,36	23,24	23,24	23,46	23,27
		7	27,23	27,27	27,26	27,23	27,22
		8	26,87	26,94	26,98	26,90	26,88
		9	16,37	16,22	16,15	15,75	16,16
		10	21,58	21,60	21,73	21,68	21,67

Vegetation density

To test the influence of vegetation density on target strength within each Rhinolophus lineage, the data was grouped by lineage for each duty cycle (High Duty cycle, low duty cycle, and natural variation of echolocation pulses). The target strengths of high duty cycle pulses for *R. fumigatus_East* (29.26 dB ± 0.06 dB) and *R. fumigatus_West* (29.09 dB ± 0.06 dB) were

normally distributed with unequal variances. A non-parametric Kruskal-Wallis rank sum test showed insufficient evidence of a significant difference in the target strengths of *R. fumigatus_East* ($\chi 2 = 0.011696$, df = 2, p = 0.9942), and *R. fumigatus_West* ($\chi 2 = 0.24561$, df = 2, p = 0.8844) in the three degrees of vegetation density (Figure 7). The target strength data of *R. capensis* (26.61 dB ± 0.86 dB) followed a normal distribution with equal variances, where a one-way ANOVA showed that there was no significant difference in target strength (F-value: 0.001, df =2, Pr(>F) = 0.999) in sparse, moderate, and dense vegetation density. For low duty cycle pulses, the target strengths of *R. fumigatus_East* (27.64 dB ± 1.26 dB), *R. fumigatus_West* (26.71 dB ± 1.36 dB,) and *R. capensis* (19.68 dB ± 2.79 dB) had skewed distributions with equal variances (Figure 7). A Kruskal-Wallis rank sum test showed that under the three vegetation densities there is insufficient evidence to show a significant difference in the target strengths of

R. fumigatus_East ($\chi 2 = 0.14035$, df = 2, p = 0.9322), *R. fumigatus_West* ($\chi 2 = 0.081871$, df = 2, p = 0.9599) and *R. capensis* ($\chi 2 = 0.31579$, df = 2, p = 0.8539).

The target strength of the variation of echolocation pulses of *R. fumigatus_East* (29.29 dB ± 0.07 dB) and *R. fumigatus_West* (29.02 dB ± 0.04 dB) had a normal distribution, where *R. fumigatus_East* had unequal variances and *R. fumigatus_West* equal variances for HDC pulses. *Rhinolophus capensis* (26.96 dB ± 0.6 dB) had a skewed distribution and equal variances. Non-parametric tests show no significant difference in target strength of *R. fumigatus_East* ($\chi 2 = 0.21677$, df = 2, p = 0.8973) and *R. capensis* ($\chi 2 = 0.095484$, df = 2, p = 0.9534) in the three vegetation densities. A one-way ANOVA showed insufficient evidence of a significant difference in target strength in the varying degrees of vegetation density for *R. fumigatus_West* (F-value: 0.119, df =2, Pr(>F) = 0.889) (Figure 8).

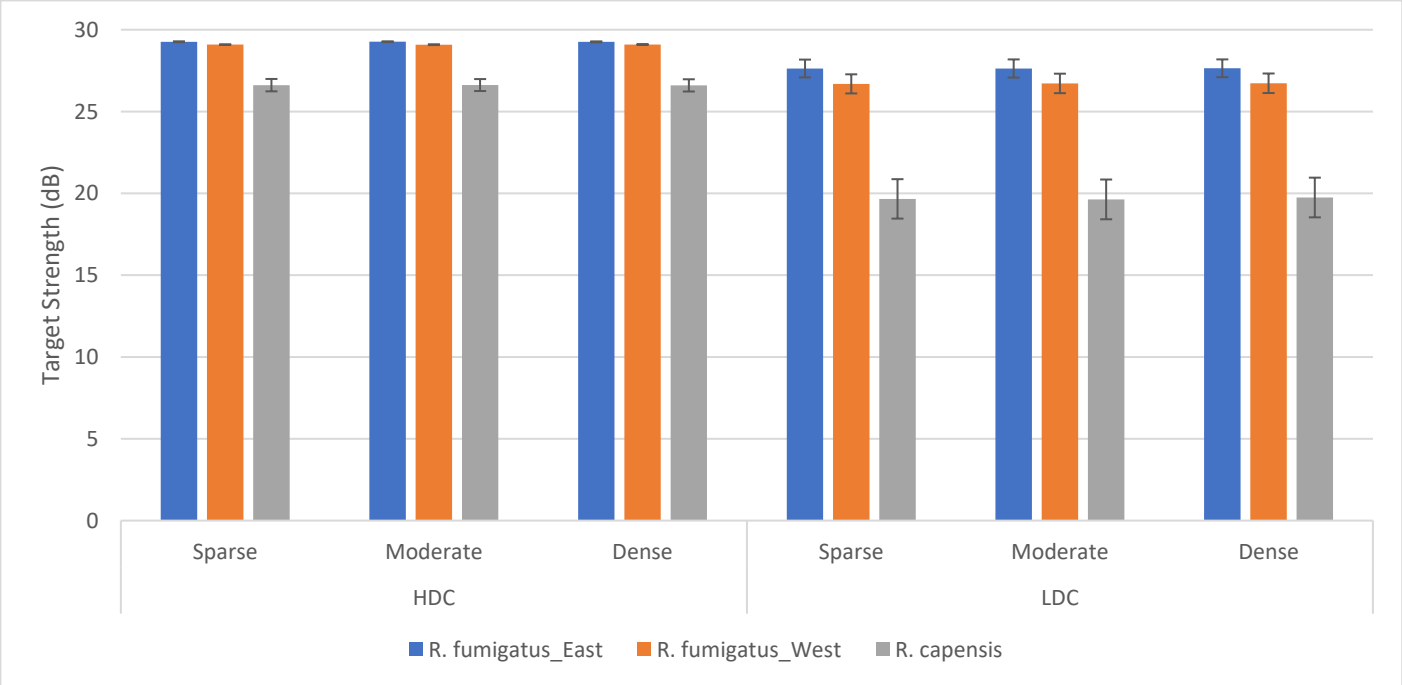

Figure 7: Average target strength of high duty cycle and low duty cycle echolocation pulses of *R. fumigtaus_East*, *R. fumgatus_West* and *R. capensis* in sparse, moderate, and dense vegetation.

The target strength of LDC echolocation pulses for the variation of echolocation pulses of *R. fumigatus_East* (28.30 dB ± 0.17 dB) followed a normal distribution with unequal variances, whereas *R. fumigatus_West* (27.11 dB ± 0.53 dB) and *R. capensis* (17.34 dB ± 4.22 dB) had a skewed distribution with equal variances. There was insufficient evidence to show a significant difference in target strength in sparse, moderate, and dense vegetation for *R. fumigatus_East* ($\chi 2$ = 0.13419, df = 2, p = 0.9351), *R. fumigatus_West* ($\chi 2$ = 0.20903, df = 2, p = 0.9008), and *R. capensis* ($\chi 2$ = 0.04129, df = 2, p = 0.9796) (Figure 8).

For the natural sequence of echolocation pulses, the HDC and LDC target strength data was separated and analysed. The HDC target strength data of *R. fumigatus_East* (26.74 dB ± 5.05 dB) followed a skewed distribution with equal variances, *R. fumigatus_West* (29.00 dB ±

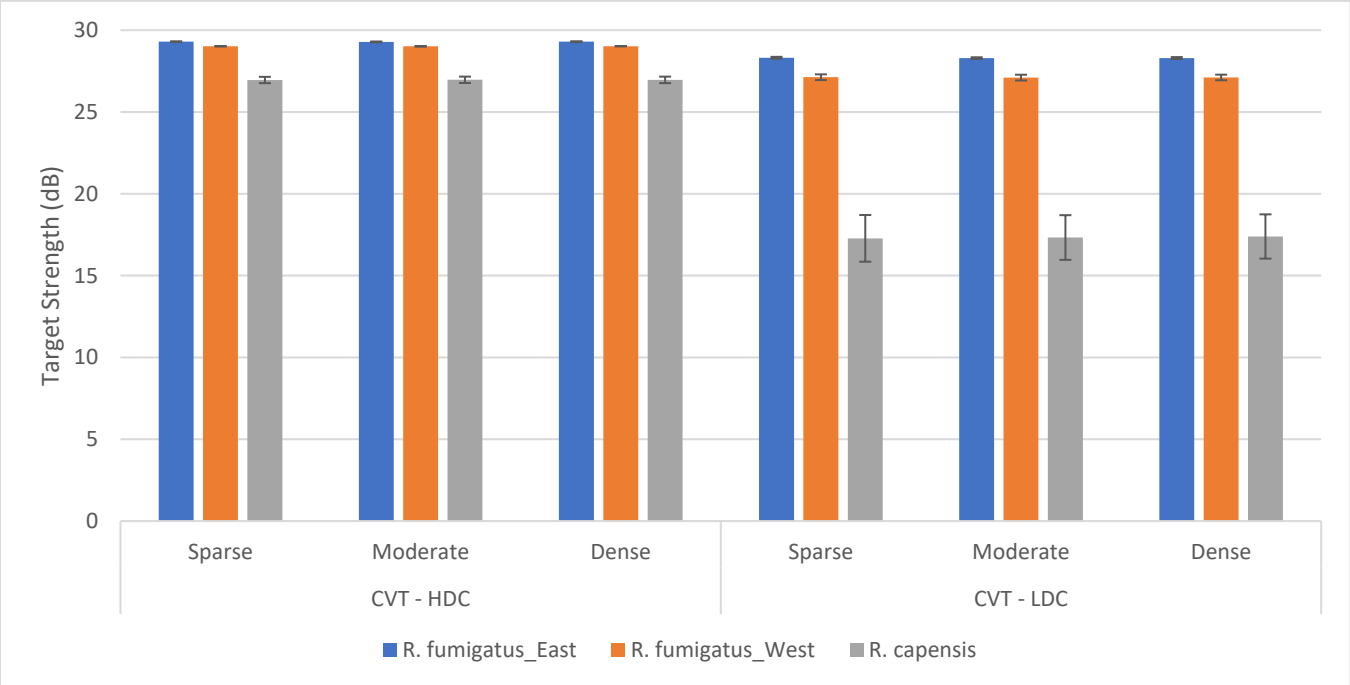

Figure 8: The average target strength of high duty and low duty cycles of the variation of natural pulses in sparse, moderate, and dense vegetation densities, of three Rhinolophus lineages.

0.04 dB) followed a normal distribution with unequal variances, and *R. capensis* (24.51 dB ± 2.65 dB) followed a skewed distribution with equal variances. Non-parametric tests show insufficient evidence that the target strength means of *R. fumigatus_East* ($\chi 2 = 0.046784$, df = 2, p =0.9769), *R. fumigatus_West* ($\chi 2 = 0.98$, df = 2, p = 0.6126) and *R. capensis* ($\chi 2 = 0.36257$, df = 2, p = 0.8342) differ significantly in sparse, moderate, and dense vegetation (Figure 9). The LDC target strength data for natural variation of echolocation pulses of *R. fumigatus_East* (28.62 dB ± 0.41 dB), *R. fumigatus_West* (26.80 dB ± 0.54 dB), and *R. capensis* (15.86 dB ± 2.78 dB) followed a skewed distribution with equal variances. There was no significant difference between the target strength means of *R. fumigatus_East* ($\chi 2 = 0.038462$, df = 2, p = 0.981), *R. fumigatus_West* ($\chi 2 = 0.06$, df = 2, p = 0.9704), and *R. capensis* ($\chi 2 = 0.06$, df = 2, p = 0.9704) (Figure 9).

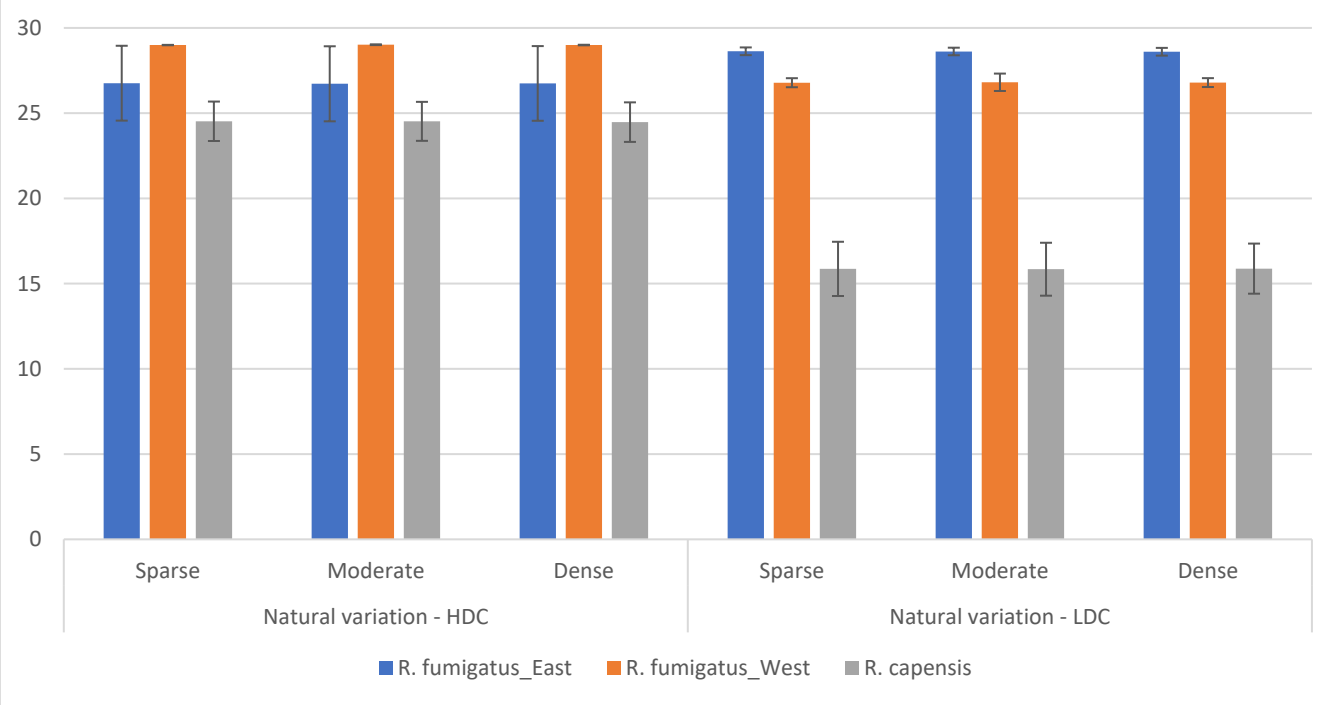

Figure 9: The average target strength of high duty and low duty cycle pulses of the sequence of natural pulses in sparse, moderate, and dense vegetation densities within each Rhinolophus lineage.

Duty cycle

The target strength data of three echolocation frequencies of two *Rhinolophus* species was separated and grouped by vegetation density (i.e. sparse, moderate, and dense vegetation) to investigate the influence of echolocation frequency on the target strength of insect prey. The target strength data was analysed based on the duty cycle to account for any differences that may be due to the duty cycle.

The HDC target strength data of all three *Rhinolophus* lineages in sparse (28.32 dB ± 1.34 dB), moderate (28.32 dB ± 1.34 dB) and dense vegetation (28.32 dB ± 1.35 dB) followed a skewed distribution with unequal variances. Non-parametric tests show a significant difference exists between *R. fumigatus_East* and *R. capensis* in sparse (observable difference = 12, critical difference = 7.378741), moderate (observable difference = 12, critical difference = 7.378741)

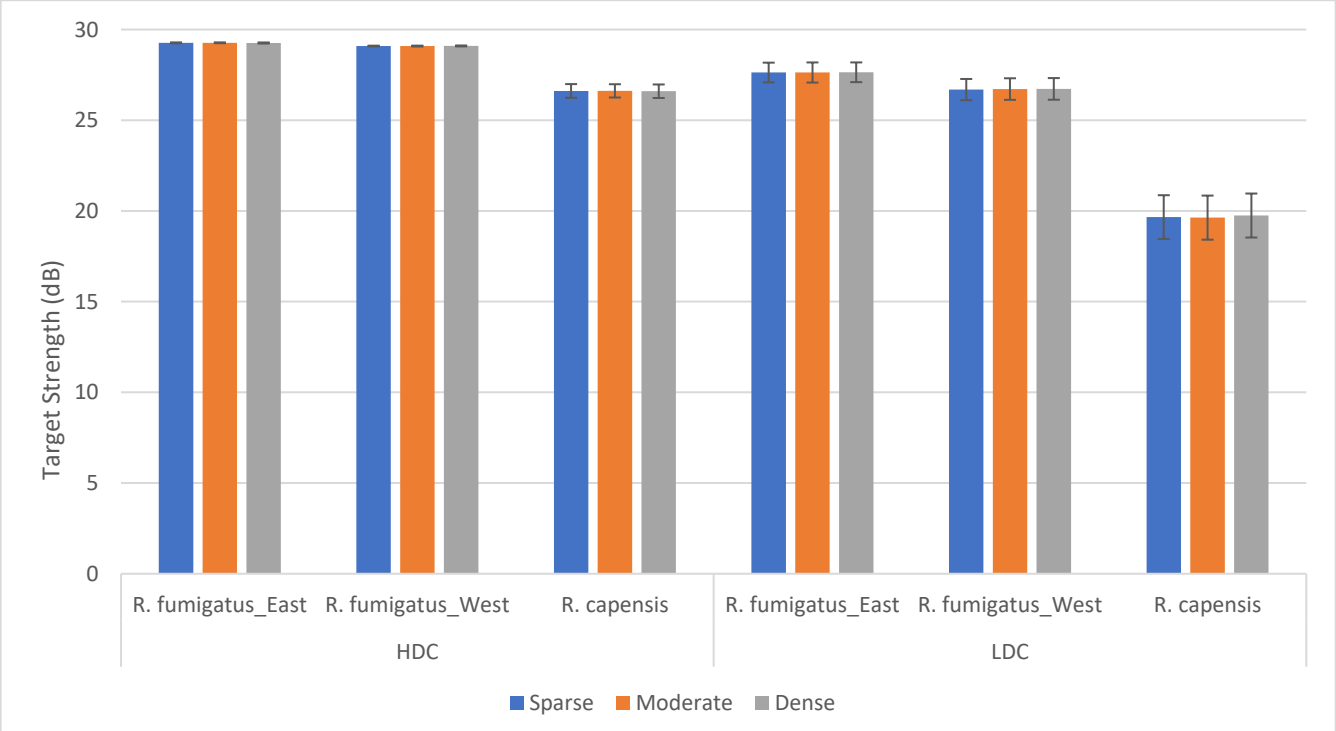

Figure 10: The average target strength of high duty and low duty cycle pulses of *Rhinolophus fumigatus_East*, *Rhinolophus fumigatus_West*, and *Rhinolophus capensis* in sparse, moderate, and dense vegetation densities, illustrating the differences between lineages.

and dense vegetation (observable difference = 11.666667, critical difference = 7.378741). However, there is insufficient evidence that a significant difference exists in sparse, moderate and dense vegetation between *R. fumigatus_West* and *R. capensis* (sparse: observable difference = 6, critical difference = 7.378741; moderate: observable difference = 6, critical difference = 7.378741; Dense: observable difference = 6.333333, critical difference = 7.378741) and *R. fumigatus_East* and *R. fumigatus_West* (sparse: observable difference = 6, critical difference = 7.378741; moderate: observable difference = 6, critical difference = 7.378741; dense: observable difference = 5.333333, critical difference = 7.378741) (Figure 10).

The LDC target strength data in sparse (24.66 dB ± 4.13 dB), moderate (24.66 ± 4.16 dB) and dense vegetation (24.71 dB ± 4.11 dB) followed a skewed distribution with equal variances. Non-parametric tests show a significant difference exists between *R. fumigatus_East* and *R. capensis* in sparse (observable difference = 11.166667, critical difference = 7.378741), moderate (observable difference = 11.166667, critical difference = 7.378741) and dense vegetation (observable difference = 11.1666667, critical difference = 7.378741). However, no significant difference exists between *R. fumigatus_West* and *R. capensis* in sparse (observable difference = 6.833333, critical difference = 7.378741), moderate (observable difference = 6.833333, critical difference = 7.378741) and dense vegetation (observable difference = 6.833333, critical difference = 7.378741); and *R. fumigatus_East* and *R. fumigatus_West* in sparse (observable difference = 4.333333, critical difference = 7.378741), moderate (observable difference = 4.333333, critical difference = 7.378741) and dense vegetation (observable difference = 4.333333, critical difference = 7.378741) (Figure 10).

Within the sequences c-e, the HDC target strength data in sparse vegetation (28.43 dB ± 1.12 dB), moderate (28.43 dB ± 1.11 dB), and dense vegetation (28.43 dB ± 1.12 dB) followed a skewed distribution with unequal variances. A significant difference exists in all three vegetation densities between *R. fumigatus_East* and R. *capensis* (Sparse: observable difference = 20, critical difference = 9.425108; moderate: observable difference = 20, critical difference = 9.425108; dense: observable difference = 20, critical difference = 9.425108), *R. fumigatus_West* and R. *capensis* (sparse: observable difference = 10, critical difference = 9.425108; moderate: observable difference = 10, critical difference = 9.425108; dense: observable difference = 10, critical difference = 9.425108), and *R. fumigatus_East* and *R. fumigatus_West* (sparse: observable difference = 10, critical difference = 9.425108; moderate: observable difference = 10, critical difference = 9.425108; dense: observable difference = 10, critical difference = 9.425108) (Figure 11).

The LDC target strength data in sparse vegetation (24.24 dB ± 5.63 dB), moderate (24.24 dB ± 5.55 dB) and dense vegetation (24.27 dB ± 5.52 dB) followed a skewed distribution (Sparse: $W = 0.76$, $p < 0.001$; Moderate: $W = 0.76$, $p < 0.001$; Dense: $W = 0.76$, $p < 0.001$), with unequal variances (Sparse: $\chi 2 = 16.708$, $df = 2$, $p < 0.001$; Moderate: : $\chi 2 = 17.851$, $df = 2$, $p < 0.001$; Dense: $\chi 2 = 17.482$, $df = 2$, $p < 0.001$). Non-parametric tests show significant differences in target strength exists in sparse, moderate, and dense vegetation between *R. fumigatus_East* and *R. capensis* (Sparse: observable difference = 20, critical difference = 9.425108: Moderate: observable difference = 20, critical difference = 9.425108; Dense: observable difference = 20, critical difference = 9.425108), *R. fumigatus_West* and R. *capensis* (Sparse: observable difference = 10, critical difference = 9.425108: Moderate: observable difference = 10, critical

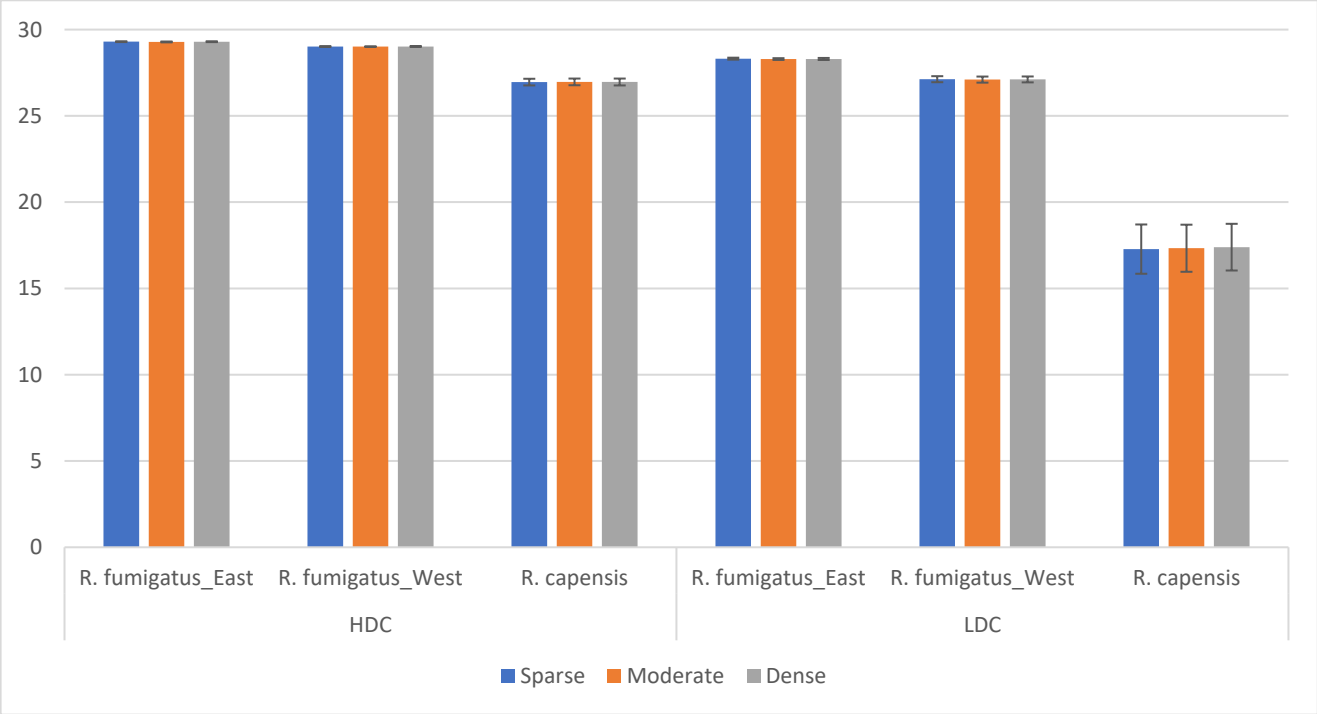

Figure 11: The average target strength of high duty and low duty cycle pulses under the variation of natural pulses, of *Rhinolophus fumigatus_East*, *Rhinolophus fumigatus_West*, and *Rhinolophus capensis* in sparse, moderate, and dense vegetation densities, illustrating the differences between lineages.

difference = 9.425108; Dense: observable difference = 10, critical difference = 9.425108), and *R. fumigatus_East* and *R. fumigatus_West* (Sparse: observable difference = 10, critical difference = 9.425108; Moderate: observable difference = 10, critical difference = 9.425108; Dense: observable difference = 10, critical difference = 9.425108) (Figure 11).

Within the sequence of natural variation of pulses, the HDC pulses in sparse (26.63 dB ± 3.87 dB), moderate (26.62 dB ± 3.87 dB) and dense vegetation (26.61 dB ± 3.88 dB) (Figure 12) all followed a skewed distribution with unequal variances. A significant difference exists between *R. fumigatus_West* and *R. capensis* in sparse, moderate, and dense vegetation (Sparse: observable difference = 7.5, critical difference = 7.320256: Moderate: difference = 7.5, critical difference = 7.320256; Dense: difference = 7.5, critical difference = 7.320256). However, there

was insufficient evidence that a significant difference exists between *R. fumigatus_East* and *R. capensis* (Sparse: observable difference = 6.5, critical difference = 6.979591; Moderate: observable difference = 6.5, critical difference = 6.979591; Dense: observable difference = 6.5, critical difference = 6.979591), and *R. fumigatus_East* and *R. fumigatus_West* in the three vegetation densities (Sparse: observable difference = 1, critical difference = 7.320256; Moderate: observable difference = 1, critical difference = 7.320256; Dense: observable difference = 1, critical difference = 7.320256) (Table 3).

Table 3: The results of the Kruskal-Wallis rank sum test and the posthoc tests run show significant and insignificant results in the HDC target strengths in the natural variation of echolocation pulse sequences.

	χ^2	df	p-value		Observable difference	Critical difference	Significant Difference
Sparse	7.47	2	P = 0.02387	*R. fum_East* and *R. capensis*	6.5	6.979591	False
				R. fum_East and *R. fum_West*	*1.0*	7.320256	False
				R. fum_West and *R. cap*	*7.5*	*7.320256*	True
Moderate	7.47	2	P = 0.02387	*R. fum_East* and *R. cap*	6.5	6.979591	False
				R. fum_East and *R. fum_West*	1.0	7.320256	False

Dense				*R. fum_West* and *R. cap*	7.5	7.320256	True
				R. fum_East and *R. cap*	6.5	6.979591	False
	7.47	2	P = 0.02387	*R. fum_East* and *R. fum_West*	1.0	7.320256	False
				R. fum_West and *R. cap*	7.5	7.320256	True

The LDC data of the natural variation of echolocation pulses in sparse (23.99 dB ± 5.93 dB), moderate (23.99 dB ± 5.93 dB) and dense vegetation (23.99 dB ± 5.88 dB) (Figure 12) follows a skewed distribution with equal variances. In all three vegetation densities, a significant

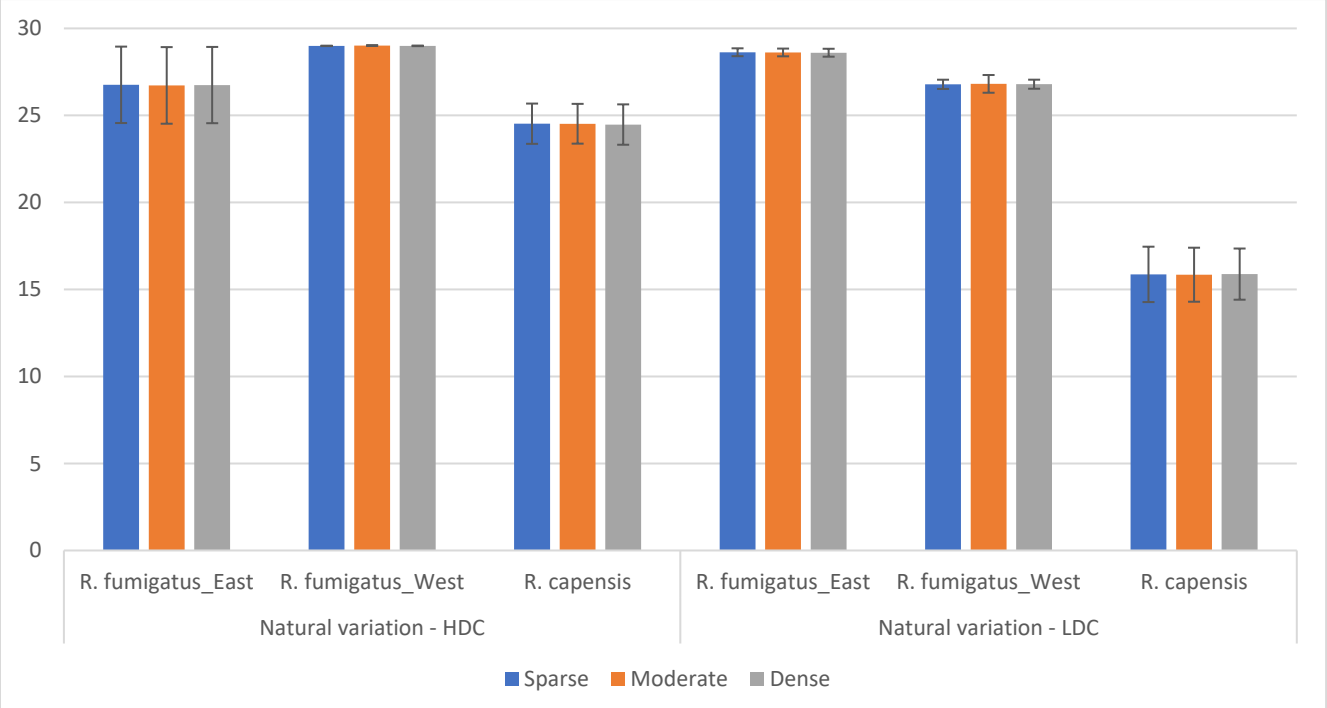

Figure 12: Average (mean ± SE) target strength of the natural sequence of echolocation pulses of three Rhinolophus lineages in sparse, moderate, and dense vegetation densities.

difference was found between *R. fumigatus_East* and *R. capensis* (Sparse: observable difference = 9, critical difference = 6.592506; Moderate: observable difference = 9, critical difference = 6.592506; Dense: observable difference = 9, critical difference = 6.592506) (Table 4). However, no significant difference was found to exist between the target strength of *R. fumigatus_West* and *R. capensis*, and *R. fumigatus_East* and *R. fumigatus_West* in sparse, moderate, and dense vegetation densities (Table 4).

Table 4: The results of the Kruskal-Wallis rank sum test and the posthoc tests run show significant and insignificant results in the LDC target strengths in the natural variation of echolocation pulse sequences.

	χ^2	df	p-value		Observable difference	Critical difference	Difference
Sparse	10.681	2	P = 0.004793	*R. fum_East* and *R. capensis*	9	6.592506	True
				R. fum_East and *R. fum_West*	*4.5*	6.254201	False
				R. fum_West and *R. capensis*	*4.5*	6.254201	False
Moderate	10.681	2	P = 0.004793	*R. fum_East* and *R. capensis*	9	6.592506	True
				R. fum_East and *R. fum_West*	4.5	6.254201	False
				R. fum_West and *R. capensis*	4.5	6.254201	False
De	10.681	2	P < 0.05	*R. fum_East* and *R. capensis*	9	6.592506	True

	R. fum_East and *R. fum_West*	4.5	6.254201	False
	R. fum_West and *R. capensis*	4.5	6.254201	False

Comparing the target strengths of HDC and LDC echolocation pulses in varying degrees of vegetation density, the target strength data in sparse vegetation (26.49 dB ± 3.55 dB), moderate (26.49 dB ± 3.57 dB), and dense vegetation (26.51 dB ± 3.53 dB) follows a skewed distribution with unequal variances. The was a significant difference in target strengths in all three vegetation densities between *R. fumigatus_East* and *R. capensis* (Sparse: observable difference = 18.416667, critical difference = 10.2969; moderate: observable difference = 18.416667, critical difference = 10.2969; dense: observable difference = 18.333333 critical difference = 10.2969), and *R. fumigatus_West* and *R. capensis* (Sparse: observable difference = 12.583333, critical difference = 10.2969; moderate: observable difference = 12.583333, critical difference = 10.2969; dense: observable difference = 12.916667, critical difference = 10.2969). However, there was no significant difference between the target strengths of *R. fumigatus_East* and *R. fumigatus_West* in sparse (observable difference = 5.833333, critical difference = 10.2969), moderate (observable difference = 5.833333, critical difference = 10.2969) and dense vegetation density (observable difference = 5.416667, critical difference = 10.2969).

DISCUSSION

In this study, semi-synthesized echolocation pulses were used to ensonify bat prey to investigate the influence of echolocation frequency on the target strengths of bat prey in varying relative degrees of vegetation density. Specifically, whether the foraging habitat hypothesis can explain why the *Rhinolophus* lineage *Rhinolophus fumigatus_East* falls outside of the 95% confidence interval for the general allometric relationship observed between body size and echolocation frequency in bats. By analysing the data by lineage (i.e., echolocation frequency), thus investigating the target strength produced in varying degrees of vegetation density within each lineage, I found no evidence in support of the foraging habitat hypothesis within each lineage in the form of a significant relationship between echolocation frequency and the target strengths produced within each vegetation density. The expected significant difference in the target strengths produced by the lower echolocation frequencies used by *Rhinolophus fumigatus* as opposed to that produced by *Rhinolophus capensis* was not evident in denser vegetation. Furthermore, higher duty cycles and longer pulse durations also did not produce stronger target strengths in all three vegetation densities. Previous laboratory and field studies show that both higher duty cycles and longer pulse durations resulted in higher flutter detection in cluttered habitats (Roverud, *et al*., 1991; Lazure & Fenton, 2011). Lazure and Fenton (2011) suggest that the shorter silent period between pulses in higher duty cycles make it easier to detect, track and lock onto a fluttering target for capture due to the increased rate of feedback (and thus tracking) the bat receives from the target. Furthermore, due to the uninterrupted modulation in echolocation frequency and amplitude that characterise longer echolocation pulses, these pulses are better for encoding target movements (Schnitzler, *et al*., 2003). The absence of a significant relationship between target strengths in relative vegetation densities within each lineage may be

the result of the scale of the experiment, which may be too small to explain the patterns relating to target strength between vegetation densities within each lineage, as well as the deviation from the general allometric relationship between echolocation frequency as predicted by body size. A larger-scale experiment, where live bats are flown in flight rooms and larger scale relative vegetation densities are used (e.g., varying the degrees of vegetation density of bushes instead of branches) and consequent field experiments, may provide better information regarding the relationship between echolocation frequency and vegetation density and the effect on target strength within each lineage and species. The inverse relationship between frequency and wing loading (as predicted by the foraging habitat hypothesis) that allows bats to successfully forage in their particular habitat has been shown to exist for South African rhinolophids (Jacobs, *et al*., 2007), but not for other rhinolophids (Stoffberg, 2007). While it has been shown that all rhinolophids are adapted for clutter foraging (Heller & von Helversen, 1989; Schnitzler & Kalko, 1998; Stoffberg, *et al*., 2011) differences in the micro-habitats used for foraging by each species may be the result of differences in echolocation frequencies and/or structure and wing morphology (Davidsonn-Watts, *et al*., 2006). Stoffberg *et al*. (2011) suggests that finer scale analysis on how each species uses their habitat within different biomes is needed to test the foraging habitat hypothesis as the definition of clutter may be species specific and depend on the species and how it utilizes its habitat.

By analysing the data by relative vegetation density (thus analysing the target strength data between each lineage), the results show significant differences in target strength between each *Rhinolophus* lineage, where the order in which the pulses were played influence the strength of the target strengths produced. For both the HDC and LDC part of the duty cycle test

(where pulse order covers all possible combinations of both the *R. fumigatus* lineages and *R. capensis*), a significant difference exists between the target strengths of *R. fumigatus_East* and *R. capensis* in sparse, moderate, and dense vegetation. Due to the similar echolocation frequencies used by the two *R. fumigatus* lineages, it was expected that a significant difference would also exist between *R. fumigatus_West* and *R. capensis* in all three vegetation densities, however no such significant difference was found. Alternatively, within the series of various tests (where each lineage's HDC, LDC, and natural sequence of pulses were played consecutively), a significant difference exists between all lineages for both HDC and LDC pulses in sparse, moderate, and dense vegetation. Again, this does not follow the assumptions of the foraging habitat hypothesis with the prediction that a significant difference in target strengths would exist between *R. fumigatus_East* and *R. capensis*, and *R. fumigatus_West* and *R. capensis*. However, within the natural sequence of echolocation pulses (where both HDC and LDC pulses and IPI's that follow that natural echolocation calls for each lineage was used), a significant difference was found between *R. fumigatus_West* and *R. capensis* for HDC pulses, and *R. fumigatus_East* and *R. capensis* for LDC pulses in all three relative vegetation densities. Alternatively, when combining all the HDC and LDC target strength data of all three lineages, the results revealed a significant difference between *R. fumigatus_East* and *R. capensis*, and *R. fumigatus_West* and *R. capensis*. The pulse rate and duration of echolocation pulses vary with each foraging phase, where the search phase is dominated by narrow bandwidth and long calls with long IPI's, the approach phase is dominated by shorter broadband calls and shorter intervals, and the terminal stage is dominated by very narrow bandwidths with significantly shorter calls and intervals (Jones & Siemers, 2010). This may suggest that the HDC and/or LDC pulses and inter-pulse intervals alone are not enough to maximize target strength, but rather a combination of the two

are required for maximum efficiency. This may explain why the two *R. fumigatus* lineages produced varying target strengths in the various tests run, and why only when all the HDC and LDC data were combined, did the data show a significant difference between the two *R. fumigatus* lineages and *R. capensis* as predicted. Finger (2021) found that in *R. capensis* populations inhabiting two different biomes with different levels of environmental clutter (one population inhabiting fynbos and the other population in the desert), both the acoustic properties of the echolocation pulses (i.e., the bandwidth of FM components) and the components relating to their emission rates (i.e., duty cycle, call duration, and IPI) were locally adapted to their relative degree of clutter and displayed a degree of behavioural flexibility. In areas of higher clutter, bats from both biomes used echolocation pulses of shorter durations, IPIs and distomax (the time from the start of an echolocation pulse to its maximum amplitude (ms)). Furthermore, acoustic properties during flight versus when stationary also differed, where bats increased the duration and bandwidth of initial and terminal FM components and shortened the IPIs and pulse durations (thus higher duty cycles) during flight. Similarly, Neuweiler *et al*. (1987) showed that *R. rouxi* increased the percentage of FM components when flying compared to when stationary. This suggests that the use of the semi-synthesized calls and lab run experiments are not sufficient to test the FHH in this manner, but rather live bats flown on flight rooms would provide more insight as to why *R. fumigatus_East* has a lower echolocation frequency than predicted by its body size.

Although support for the FHH has been found for some LDC echolocating species, such as *Myotis lucifugus* (Wund, 2006) and *Macrophyllum macrophyllum* (Brinkløv, *et al*., 2010), it has been less well established for HDC echolocating species (Finger, 2021). Russo *et al.*, (2007)

concluded that differences in echolocation frequency in *R. hipposideros* and *R. euryale* may not be due to the different foraging habitats these species utilise as no significant relationship between echolocation frequency and wing morphology was found. Similarly, in three *R. capensis* populations, two of the three populations did not follow the general allometric relationship (these to were larger with greater skull dimensions than the third population) (Odendaal, 2009). Odendaal (2009) concluded that echolocation frequency evolved independently of wing morphology and did not evolve based on which habitants were being exploited by each population. Furthermore, Jacobs *et al.* (2007) found that the foraging habitat hypothesis could not explain *R. clivosis* using echolocation frequencies higher than expected for their body size. In LDC species, an increase in echolocation frequency affords an increase in resolution of prey against background clutter in habitats of dense vegetation (Norberg & Rayner, 1987; Jones & Barlow, 2004). As HDC echolocating species are clutter foraging specialists (Neuweiler, 1980; Fenton, *et al*., 2012), they already possess the ability to acquire greater resolution images and distinguish fluttering targets from background vegetation (Schuller & Pollak, 1979; Neuweiler, 1989), through the use of high echolocation frequencies, acoustic fovea and doppler shift compensation (Neuweiler, 1989; Waters, *et al*., 1995). Therefore, due to the already high echolocation frequencies (and thus resolution) used by HDC echolocators, a shift in echolocation frequency (whether higher or lower than predicted by body size) would not produce a significant difference in target strengths and thus detection of prey (Jacobs, *et al*., 2007). Furthermore, previous studies have proposed that HDC echolocators in open habitats may have evolved to use lower frequency pulses for increased detection distance in environments of lower levels of clutter, as higher frequencies experience greater atmospheric attenuation and thus confers a disadvantage in open habitats (Xu, *et al*., 2008; Odendaal, *et al*., 2014). Although Finger (2021)

found evidence that the use of lower echolocation frequency pulses by *R. capensis* in desert (i.e., open) habitats may have evolved for increased detection distances, this would likely not apply to *R. fumigatus_East,* which inhabits savannah woodland biomes, a relatively high clutter habitat.

Allopatric divergence may explain the deviation from the general allometric relationship of *R. fumigatus_East*. Extended periods of geographic isolation of two populations may enforce different selective pressures due to the differences in environmental conditions (i.e., competitors, climate, predators, and resources) experienced by each isolated population. The environmental differences may lead to selection of divergent traits that are adapted specifically to the local environment that population inhabits (Jacobs, *et al*., 2017). These traits can manifest morphologically, physiologically, and behaviourally (i.e., variation in acoustic signals) (Jiang, *et al*., 2015). The sensory drive hypothesis (describing the action of both natural and sexual selection on signalling systems (Endler, 1992) posits that signals that are easy to detect are likely to be favoured and thus selected for, and that due to reproductive isolation of allopatric populations, speciation by sensory drive may occur as a by-product of adaptation to different habitats (Boughman, 2002). Signals are directly influenced by natural selection via habitat transmission and on perception via perceptual tuning. Alternatively, signals are influenced indirectly by sexual selection via perceptual tuning (Boughman, 2002). Habitat transmission can lead to divergence of signal properties of allopatric populations (Schluter & Price, 1993), as a signal that transmits well in one habitat may be degraded in another (Ryan & Wilczynski, 1991), due to the structural features of that specific habitat (Bradley & Vehrencamp, 1998). These differences in habitat structure can also drive divergence in perception (Boughman, 2002). Perceptual tuning (the local adaptation in perception) results in females becoming more sensitive

to certain signals than they are to others. This may lead to mate preferences and as these preferences and signal adaptations coevolve, allopatric populations can diverge in mating traits. Divergent signals and preferences can reduce the probability of inter-population mating, leading to sexual isolation, ultimately driving early divergence of lineages (Boughman, 2002). Selection on specific traits is likely to remain divergent if reproductive isolation of allopatric populations is maintained, thus no longer depending on sensory drive. These traits may continue to contribute to reproductive isolation and ultimately allow speciation to continue to completion as the result of other process, such as resource competition or divergence in ecological and morphological traits (Boughman, 2002). This may be the case with the eastern and western populations of *R. fumigatus*, where extended periods of isolation and local adaptation to their environments lead to the speciation of *R. fumigatus_East*, with differences in signal properties and morphological traits.

An assumption that the FHH makes is that acoustic variation is driven by ecological selection (e.g., phenotypic plasticity or genetic variation). The deviation of *R. fumigatus_East* from the general allometric relationship between frequency and body size may be the result of phenotypic plasticity, which is the ability of one genotype to produce more than one phenotype under different environmental conditions (Scheiner, 1993; Price, *et al*., 2003; Finger, 2021). Pfenning *et al.,* (2010) suggests that phenotypic plasticity may drive acoustic variation if it promotes both successful dispersal and survival in different environments, and that it would be favoured over genetic divergence as it would take fewer generations for new adaptations to arise. Only a few studies have reported significant differences in the relationship between acoustic signals and geographic distance between populations. Investigating factors such as phenotypic

traits is extremely complicated due to many confounding variables at play and can result in inaccurately associating variation of those traits as being due to the effects of selection. For example, one confounding factor in phenotypic traits investigations may be body size. Selection could be acting directly on body size and in turn this may result in correlated changes of other phenotypic traits that are correlated with body size. This is a great example of correlation not being causation, as it would not be possible to conclude that these changes in phenotypic traits are due to selection. This is something to keep in mind when investigating echolocation as although echolocation and body size are inversely correlated (Heller & von Helversen, 1989; Barclay & Brigham, 1991; Jones, 1996; Jacobs, *et al*., 2007; Finger, 2021) it is important to exclude the effects of body size on echolocation to understand the processes that result in variation in this trait. In Rhinolophidae, research suggests that variation in echolocation frequency precedes changes in body size, and thus that variation in echolocation frequency may be the result of processes acting directly on echolocation and not due to changes in body size (Stoffberg, *et al*., 2011), where continued geographic and/or reproductive isolation (Nosil, 2012) lead to local adaptation and eventually phenotypic divergence of these populations (Schluter, 2000; Shafer & Wolf, 2013).

The results of the intra- and inter-lineage and species analyses suggests that selection may have acted on echolocation frequency independently of body size in *R. fumigatus_East*, and further suggests that factors other than increased prey detection and the ability to enter dense vegetation are responsible for *R. fumigatus_East* having a lower echolocation frequency (55.1±1.5 kHz) relative to their body size (12.7± 0.9 g). For example, *R. clivosus* sensu lato (also known as *R. clivosus* geoffroyi (Stoffberg, *et al*., 2012) or *R. clivosus* augur (Brenda & Vallo,

2012)) also deviates from the general allometric relationship in horseshoe bats, emitting a higher peak echolocation frequency than expected for its body size (Wu, *et al*., 2015). Jacobs *et al.* (2007) and Russo *et al.* (2007) attributes this exception to the acoustic communication hypothesis, where acoustic communication during social interactions is facilitated by the deviation in peak echolocation frequency. The use of echolocation in social communication has been well described in several studies (Dechmann & Safi, 2005; Dechmann, *et al*., 2009; Jones & Siemers, 2010; Gillam & Fenton, 2016) including species such as *Rhinolophus ferrumequinum* (Ma, *et al*., 2006), *Tadarida brasiliensis* (Bohn, *et al*., 2008), *Saccopteryx bilineata* (Knornschild, *et al*., 2012), *Rhinolophus Euryale* and *Rhinolophus hippposideros* (Russo, *et al*., 2007), *Rhinolophus clivosus* (Jacobs, *et al*., 2007), *Pteronotus parnellii* (Kanwal, *et al*., 1994), *Myotis myotis* (Yovel, *et al*., 2009), *Megaderma lyra* (Bastian & Schmidt, 2008; Janssen & Schmidt, 2009) and *Eptesicus fuscus* (Kazial & Masters, 2004) to name a few. The use of body size (as predicted by forearm length) as a predictor of echolocation frequency has been well documented in African Rhinolophidae (Jacobs, *et al*., 2007), where a significant inverse correlation was found. However, there is some support that apart from the negative correlates between echolocation frequency and body size, call frequency also correlates with various other morphological traits (Armstrong & Coles, 2007), such as nose-leaf width (Robinson, 1996), pinna size (Guppy & Coles, 1988), and cochlear size (Francis & Habersetzer, 1998). Wu *et al.*, (2015) hypothesized that morphological traits such as nasal capsule size and pinna size may be stronger predictors of echolocation frequency than body size. They found that among Asian horseshoe bats, morphological traits such as ear length, ear width and nasal size were significantly correlated with echolocation frequency. Similarly, Odendaal and Jacobs (2011) found that nasal capsule length was the best predictor of echolocation frequency, and this trait

was not correlated with body mass in three geographically isolated populations of *Rhinolophus capensis*. Furthermore, in *Rhinonicteris aurantia,* Armstrong and Coles (2007) found that nasal chamber size showed significant geographic variation, and this was linked to variation of echolocation frequency in two geographically isolated populations. Moreover, Stoffberg (2007) found that in rhinolophids, rostrum size (structure housing the nasal chambers) was the best predictor of echolocation frequency, where nose leaf width specifically was the best predictor of echolocation frequency in horseshoe bats. Here, a significant negative correlation between echolocation frequency and nose leaf width exists in horseshoe bats (Robinson, 1996). It has therefore been suggested that there may have been a decoupling of echolocation apparatus from body size and that selection may have altered skull parameters independently of body size, thus directly influencing echolocation frequency (Odendaal & Jacobs, 2011).

Comparing these morphological traits between *R. fumigatus* and *R. capensis*, the average ear width of *R. capensis* (males: 23.6 mm; females: 23.2 mm) is smaller than that of *R. fumigatus* (males: 26.1 mm; females: 24.0 mm) (Monadjem, *et al*., 2010), while the average noseleaf width of *R. capensis* is less than 9 mm (De Hoop: 8.50 ± 0.16 mm; Table Farm: 8.44 ± 0.21 mm; Steenkampskraal: 8.22 ± 0.23 mm) and *R. fumigatus* is greater than 9 mm with prominent nasal inflations. Both morphological traits are smaller in *R. capensis* than in *R. fumigatus.* However, Odendaal (2009) found that of three *R. capensis* populations, one population (Steenkampskraal) with larger body size and skull parameters have lower echolocation frequency calls, whereas another population (De Hoop) used higher echolocation frequencies with smaller skull parameters. Although the skull parameters of *R. capensis* show considerable differentiation, Odendaal (2009) found no correlation between echolocation frequency and noseleaf width and

cochlea width. Other studies have produced varied results, where some have only found a moderate relationship between echolocation frequency and nose leaf width and no significant difference in the cochlea width between population of *Rhinonicteris aurantia* (Armstrong & Coles, 2007). Francis and Habersetzer (1998) found similar cochlea widths between populations of *H. cervinus* even when they had differences in echolocation frequency. Although the decoupling of echolocation apparatuses from body size may be plausible, based on this study it not only inaccurate but also impossible to draw conclusion as to whether this applies to *R. fumigatus.*

In conclusion, although this study would have benefited with the inclusion of field studies that used live bats in flight room and bats in the field, the results of this study preliminarily indicate that the variation in echolocation frequency of *Rhinolophus fumigatus_East* cannot be explained by the variation in body size as proposed by the foraging habitat hypothesis. That is, the lower echolocation frequency of *Rhinolophus fumigatus_East* relative to its body size does not enable this species to better detect fluttering targets in dense vegetation than *Rhinolophus capensis* and subsequently enter this vegetation better than its larger sister lineage *Rhinolophus fumigatus_West*. Being clutter foraging specialists and already possessing the ability to acquire greater resolution images and distinguish fluttering targets from background vegetation, altering the duty cycle and pulse durations of echolocation pulses did not significantly increase target strengths of bat prey.

Directions for future research

To better understand the deviation from the general allometric relationship between echolocation frequency and body size in Rhinolophus fumigatus_East, a comparison between different populations should be done in the field to understand the effect that geographic and/or reproductive isolation may have on this relationship. This should also be done at different scales (i.e., not only on the laboratory with semi-synthesized pulses but also in a flight room with live bats, as well as in the field (natural habitat)) to understand the influence that scale may have had on the results. Furthermore, other than the foraging habitat hypothesis, allometric divergence by way of the sensory drive hypothesis as well as the communicative hypothesis may explain the deviation in echolocation frequency and presents avenues for future research in Rhinolophus fumigatus lineages.

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Appendix

Table 5: The difference between the High duty cycle and Low duty cycle signal pulse and the echo pulse (dB) (|Psignal – Pecho|) of three *Rhinolophus* species in three degrees of vegetation density.

Species	HDC			LDC		
	Sparse	Moderate	Dense	Sparse	Moderate	Dense
R. fumigatus_East	29,12867	29,14779	29,21494	26,32082	26,17343	26,24574
R. fumigatus_West	28,3954383	28,38098	28,40676	23,73077	23,91961	23,88386
R. capensis	17,8708583	17,98376	17,97079	13,41979	13,50049	13,67153
R. fumigatus_West	28,3729325	28,47126	28,40724	22,38011	22,21057	22,52243
R. capensis	23,8744801	23,75311	23,70925	12,89326	12,93485	13,02465
R. fumigatus_East	29,027761	29,03693	28,90832	25,41846	25,53177	25,4009
R. capensis	20,95254	20,9193	20,88036	5,506309	5,507156	5,530809
R. fumigatus_East	28,8429944	29,0458	28,84628	25,97422	25,7806	26,00571
R. fumigatus_West	28,6356718	28,70075	28,79404	23,13593	23,29162	23,32142
R. fumigatus_East	29,110214	28,81823	28,96177	25,70612	25,73468	25,69788

R. capensis	20,2681534	20,32544	20,08655	11,80033	11,68872	11,83284
R. fumigatus_West	28,4513956	28,4329	28,4543	23,52578	23,67012	23,52474
R. fumigatus_West	28,5741249	28,36698	28,42384	22,75512	22,93119	22,948
R. fumigatus_East	28,9868972	29,20222	29,00258	25,37293	25,50048	25,63193
R. capensis	22,9733443	22,88182	23,16299	7,890621	8,035175	8,127288
R. capensis	22,3922144	22,50817	22,28702	8,293579	7,974395	8,238345
R. fumigatus_West	28,3611789	28,41479	28,43284	15,43226	15,40454	15,3831
R. fumigatus_East	28,9476691	28,88267	28,96768	25,46623	25,52464	25,49811

Table 6: The difference between the signal pulse and the echo pulse (dB) (|Psignal – Pecho|) of three Rhinolophus species in three degrees of vegetation density. Table shows data from the class variation tests (Tests 3-5).

		R. fumigatus_East			R. fumigatus_West			R. capensis		
		Sparse	Moderate	Dense	Sparse	Moderate	Dense	Sparse	Moderate	Dense
High Duty Cycle	1	29,17731	29,29566	29,24202	28,10293	28,0751	28,23131	22,80859	22,80907	22,84127
	2	29,31877	29,14799	29,35517	28,3243	28,19021	28,36453	21,84317	21,83913	22,03103
	3	29,15022	29,11394	29,22617	28,16522	28,08646	28,02992	23,46077	23,66333	23,48927
	4	29,26739	29,11731	29,19345	28,30846	28,20971	28,26021	22,96819	23,04276	22,96411
	5	29,23773	29,20922	29,32718	28,14166	28,11467	28,28909	23,77336	23,54969	23,50129
	6	29,34624	29,12617	29,20231	28,21647	28,21871	28,28641	23,25819	23,38362	23,41741
	7	29,28303	29,3227	29,25649	28,04839	28,24898	28,10727	22,33561	22,3568	22,36923
	8	29,0913	29,05775	29,22869	28,1914	28,19325	28,11793	22,01666	21,99635	22,13784
	9	29,21919	29,26437	29,09259	28,29594	28,3014	28,36335	23,10731	23,23354	23,225
	10	28,86957	28,78064	28,79416	28,45657	28,44257	28,31286	18,46572	18,479	18,28211
Low Duty Cycle	1	27,07138	26,92841	27,01809	23,99674	23,92015	23,86304	9,405573	9,386859	9,452412
	2	26,07428	25,84224	26,12917	24,11662	24,14147	24,06119	14,29261	14,21369	14,18404
	3	26,33277	26,3082	26,0823	24,29028	24,11865	23,99749	11,09988	11,09619	11,22518
	4	25,89289	25,83947	25,90318	22,61205	22,54607	22,50259	7,817713	7,652767	7,689539
	5	25,70834	25,61935	25,62262	20,02266	19,98307	19,97359	8,024367	7,930886	8,063786
	6	26,71767	26,61166	26,79326	22,38061	22,35006	22,48629	6,404103	6,380075	6,482511
	7	25,27569	25,35002	25,21831	23,23811	23,38586	23,33294	4,776741	4,740145	4,929135
	8	25,93256	25,97771	25,90038	21,71443	21,6093	21,71022	2,50301	2,749118	2,769901
	9	25,60799	25,57005	25,45888	24,15459	24,0637	24,03546	6,831413	6,89498	6,71238

	10	26,26151	26,11904	26,22283	22,58799	22,38202	22,77602	10,44086	10,53002	10,51731
Natural Variation	1	28,8567	28,91061	28,95348	23,45824	23,479	23,40666	6,646071	6,673772	6,489149
	2	26,85508	26,47144	26,85534	19,48203	19,56603	19,51703	3,855528	3,950041	4,038234
	3	29,09708	29,11035	28,93199	28,17264	28,2422	28,07833	22,30894	22,19388	22,28727
	4	26,49278	26,47102	26,433	28,40448	28,70686	28,56387	9,148884	9,223483	9,001575
	5	26,22797	26,05624	26,25162	27,95718	28,05985	28,0046	11,50042	11,48067	11,35652
	6	29,20564	28,98932	28,92728	22,44598	22,6275	22,37876	14,5208	14,88858	14,57613
	7	26,42833	26,50024	26,3372	28,08835	28,09102	28,04326	23,07012	22,98253	22,95307
	8	26,6232	26,66995	26,66142	22,08087	22,01643	22,06957	22,34769	22,10569	22,07001
	9	25,94365	25,84976	25,83183	22,16727	22,26617	22,33955	6,416705	6,133387	6,423257
	10	29,08426	28,98022	28,99824	28,39328	28,23869	28,33928	12,20019	12,12821	12,11702