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Acoustic parameters of bat echolocation calls in Zambia: a collaborative effort to develop a call library for non-invasive research and monitoring

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Passive acoustic monitoring (PAM) of bats enables non-invasive research that improves monitoring efficiency, and can be used for species identification, documenting occurrence and measuring activity levels. However, equipment costs and a dearth of experienced personnel, as well as a lack of local open access reference datasets (call libraries), have limited the study of African bat communities using PAM. This study compiles the first publicly available call library of this scale from Zambia. Echolocation calls were recorded upon release of captured bats during various projects from 2015 to 2023, using full spectrum ultrasound detectors. Acoustic calls from 238 individuals of 22 species were collated. We aimed to determine whether Zambian bat species could be accurately distinguished using acoustic measures. We predicted that some species (or species groups) would be easily identifiable, while other species would have substantial similarities in their calls, which would hinder identification. After considering multicollinearity, we selected five acoustic parameters to analyse the recordings: 'Frequency of Maximum Power', 'Preceding Interval', 'Start Slope', 'End Slope' and 'Ledge Duration'. Principal Component Analysis was conducted to identify parameters that were best able to separate the calls of different functional groups (identified by sonotype) or species. Discriminant Function Analysis was then used to determine the accuracy with which the parameters may be used to acoustically distinguish species or sonotypes. The parameters 'Start Slope' and 'Frequency of Maximum Power' were the most useful for separating the species considered. It was possible to separate some sonotypes and species with relatively high accuracy. Many species, however, could not be identified with certainty, underscoring the importance of other identification techniques, such as morphological measures or genetic sampling.

Key words: bat survey, ultrasonic acoustic detector, South-Central Africa, passive acoustic monitoring

Introduction

During this time of accelerated anthropogenic change, impacts on biodiversity are of urgent concern. Monitoring species distributions and populations remains a priority for researchers and conservationists, as we aim to collect baseline data with which to record and track ecological changes over time. For enigmatic species the task becomes particularly difficult, however improvements in technology have allowed for data to be collected non-invasively and remotely (such as visually using cameras and acoustic recorders), vastly increasing our understanding of the behaviour and ecology of many species across taxon (Stephenson, 2020). Passive acoustic monitoring (PAM) with recorders has been widely demonstrated to be a particularly useful method for studying vocalising species

(Britzke *et al*., 2013; Gibb *et al*., 2019; Stephenson, 2020).

One such enigmatic group of vocalising species are bats (Chiroptera), which represent the second largest order of mammals and a large component of biodiversity in most ecoregions. Bats occupy diverse niches and perform important ecosystem functions, such as suppression of pests in agro-ecosystems (Cleveland *et al*., 2006; Boyles *et al*., 2011; Riccucci *et al*., 2014; Taylor *et al*., 2018), plant pollination, and seed dispersal (Seltzer *et al*., 2013; Aziz *et al*., 2021). As bats are good bioindicators in a variety of landscapes, they are an important target taxon for conservation monitoring (Jones *et al*., 2009; Park, 2015; Russo *et al*., 2015).

The nocturnal and volant habits of bats has meant that their research requires specialist biologists, often working with invasive techniques and expensive equipment (Kunz *et al*., 2009). A significant contribution to improved research on bats has been the development of ultrasound detectors, which enables identification of many echolocating species without capture (Britzke *et al*., 2013; Gibb *et al*., 2019). Technological advances have led to greatly reduced size and cost of ultrasonic detectors, which facilitates non-invasive and remote monitoring of bat occurrence and activity over larger spatial scales (Gibb *et al*., 2019). In bat research, there is huge potential in the use of PAM to collect large amounts of behavioural and monitoring data (Gibb *et al*., 2019).

The specific characteristics of bat echolocation calls are a reflection of the species' morphology and the niche it occupies; acoustic parameters such as frequency attributes, bandwidth, duration, inter-pulse interval and amplitude, can determine how the bat navigates different aerosphere habitats and targets specific prey (Aldridge *et al*., 1987; Norberg and Rayner, 1987; Denny, 2004; Kalko *et al*., 2007). Hence there is a close association between the call parameters of a species and its functional group (Schnitzler *et al*., 2001; Jones *et al*., 2006, 2007).

It should be noted that there are distinct limitations of using PAM. Many bat species do not emit laryngeal ultrasonic calls (e.g., pteropodid fruit bats) and there are also differences in detection distances for many echolocating species, which will favour the recording of some over others by bat detectors (Monadjem *et al*., 2017; Dekker *et al*., 2022). Additionally, identification of bat calls is complicated by plasticity (Odendaal *et al*., 2014; Russo *et al*., 2018; Montauban *et al*., 2021) and regional variation (Aspetsberger *et al*., 2003; Taylor *et al*., 2005, 2013*b*). The difficulty in distinguishing some co-existing bat species on echolocation calls alone can limit our ability to use bat detectors as effective monitoring tools (Gibb *et al*., 2019; Görföl *et al*., 2022). In addition to specialist knowledge and equipment, familiarity with local species assemblages and accurate reference call recordings, are required for the analysis of acoustic data (Kunz *et al*., 2009; Stephenson, 2020). There are also other considerations when using ultrasound detectors, such the choice of detector model, microphones and analysis software to ensure comparability (Adams *et al*., 2012; Gibb *et al*., 2019). Although the increased use of bat detector technology has contributed to improving the study of bats, the limitations of PAM in terms of accurate species identifications are often not fully appreciated and so this, in addition to inadequate reference material and insufficient surveyor skills and experience, may result in species assemblages and activity levels being mis-represented; meaning that assessments of anthropogenic impacts may also be inaccurate (Clement *et al*., 2014; Russo *et al*., 2018).

Reference acoustic datasets (call libraries) for confirmed species, collected across their distribution ranges, are essential to improve the accuracy of species identification from acoustic data. Efforts are needed to collate sufficient samples with which to obtain parameter ranges, explore regional variation and to better determine the parameter combinations for separating species with similar echolocation calls (Karine *et al*., 2001; Walters *et al*., 2013; Clement *et al*., 2014; Görföl *et al*., 2022). In some parts of the world, collections of acoustic data are managed in open access databases, e.g., the Asian Bat Call Database (ABCD), ChiroVox (Görföl *et al*., 2022) and The Sonozotz project (Zamora-Gutierrez *et al*., 2020). However, acoustic focused research on African bats has lagged behind (Racey, 2013; Monadjem *et al*., 2020) creating obstacles to their effective study. Despite a surge in interest in bat ecology across the continent in the past ten years (Racey, 2013; Cooper-Bohannon *et al*., 2016; Monadjem *et al*., 2020), access to acoustic technology remains a challenge for African researchers. As a result, few acoustic studies have been conducted in tropical Africa, with call libraries published only for a handful of countries and a small proportion of species across the continent (Monadjem, 2005; Taylor *et al*., 2005, 2013*a*, 2013*b*; Monadjem *et al*., 2007, 2020; Parker *et al*., 2018; Kearney *et al*., 2019; Moir *et al*., 2021).

This paper compiles and utilises the first large-scale collection of bat acoustic data for Zambia, a country situated at an ecological transition zone between southern and central Africa and representing

a diverse bat fauna of approximately two-thirds of the 125+ bat species known to occur in the southern African region (Monadjem *et al*., 2020; Benda *et al*., 2022).

In this study we set out to determine whether Zambian bat species could be accurately distinguished acoustically. Our aims are to: (i) provide reference bat call data with which academics, professional ecologists and amateur naturalists can compare acoustic recordings; (ii) explore whether using different full spectrum acoustic detectors results in differences in call parameters and examine this variation as a potential issue when combining data from multiple sources, as we have done here; and (iii) investigate the potential for distinguishing Zambian bat species from acoustic recordings by identifying call parameters contributing the most to call variation and then testing the use of these parameters to distinguish between functional groups and species acoustically. We expected that separation by functional group is possible due to distinct sonotype characteristics (Schnitzler *et al*., 2001) but that accurate identification to species level may not always be possible.

Materials and Methods

Field Surveys

Zambia is located in south-central Africa, and comprises a highly diverse landscape, encompassing nine ecoregions (Dinerstein *et al*., 2017). Bats were captured with the use of monofilament mist nets, varying from 3–18 m in length and assembled at varying heights on single and triple high poles. Captures were conducted mostly during the rainy season (November to April), between 2015 and 2023, predominantly in Miombo woodland dominated landscapes at multiple locations in 16 study sites (Fig. 1). Nets were checked at maximum intervals of 15 min and all bats were released after processing at the site of capture on the same night. Surveys were conducted as part of multiple projects, including the Bats in Zambia project (Taylor-Boyd *et al*., 2019) and the Social and Environmental Trade-Offs in African Agriculture project (Devenish *et al*., 2024).

Taxonomy and Identification

Species identifications were based on morphological features, following reference field guides for the region (Monadjem *et al*., 2020). Morphometric measurements (forearm length and taxon specific features such as noseleaf horseshoe width) were taken using dialMax Vernier Dial Callipers (error ± 0.1 mm) and mass was calculated using Pesola Spring Scales (accuracy ± 0.3%).

In addition, species identification was confirmed for some individuals of cryptic species via genetic analysis of faecal or non-lethal wing tissue samples, following lab procedures outlined in Mata *et al*. (2021). Polymerase chain reactions were performed using the SFF primer set (Walker *et al*., 2016) to amplify a 202 bp region of the mitochondrial Cytochrome Oxidase I gene (COI), or primers LGL 765F and LGL 766R (Bickham *et al*., 1995, 2004) to amplify the full mitochondrial cytochrome *b* gene. Sequencing was done on a MiSeq platform (Illumina, USA) and with a 3730xl DNA Analyzer (Thermofisher, USA). Sequences were compared to BOLD (Ratnasingham *et al*., 2007) and NCBI GenBank databases (Benson *et al*., 2013) for species identification.

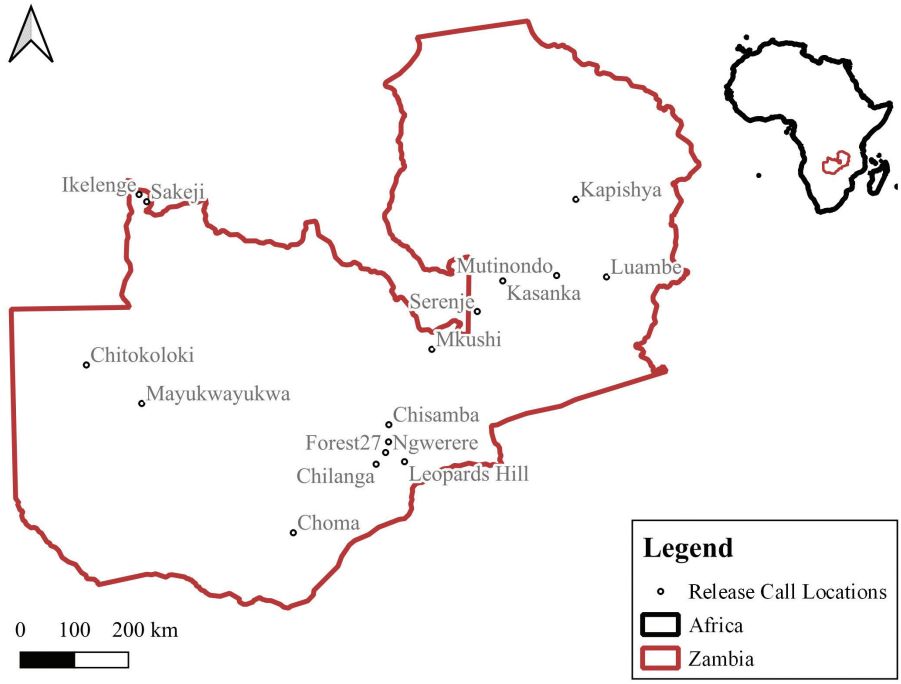

Fig. 1. Locations of the 16 study sites across Zambia. Bat mist-netting was conducted at multiple locations within these sites

We follow the taxonomy of Simmons *et al*. (2024). Further information on the current known distributions of the species has been recently published by Monadjem *et al*. (2024). *Laephotis botswanae* has recently been shown to be genetically and morphologically identical to *Laephotis angolensis* (Taylor *et al*., 2022) and the two are now considered conspecific as *L. angolensis* (Taylor *et al*., 2024). The *Miniopterus* complex remains unresolved in the region (Monadjem *et al*., 2013) and hence we refer to our specimens as *Miniopterus* sp. The single *Nycteris* captured is referred to as *Nycteris* sp. as a conservative identification due to inconclusive genetic results and the need for taxonomic resolution within the genus in Africa (Demos *et al*., 2019). We categorised species into one of five functional groups based on habitat type and foraging mode: open aerial, edge aerial, edge trawling, narrow flutter and narrow passive (Schnitzler *et al*., 2001).

Echolocation Calls

We used full spectrum bat detectors to take acoustic recordings of the echolocation calls of bats during release (sensu Monadjem *et al*., 2017). These calls were collated in a call library held by Bats without Borders (www.batswithoutborders.org), and can be accessed via email request to info@batswithoutborders.org. The following detector models were used across multiple expeditions, depending on availability at the time: BatBox Baton XD (Batbox Ltd, UK) with R05 recorder (Roland Europe Group Ltd, UK), Batlogger M (Elekon AG, Switzerland), Petterson D240x (Pettersson Elektronik AB, Sweden), Anabat Walkabout (Titley Scientific, UK), Echo Meter Touch 2 (Wildlife Acoustics, USA), and Echo Meter Touch 2 Pro (Wildlife Acoustics, USA). For consistency, recordings were made during release rather than in the hand as this works for all echolocating species, and the differences between these calls and those taken in the hand for high duty cycle echolocating bats have been shown to be very small (e.g., Soisook *et al*., 2008). The detector was held approximately two metres from the bat and recording initiated upon release, continuing whilst the bat was followed as far as possible until it was no longer visible or audible as indicated by the detector sound or sonogram. Although release was avoided when other free-flying bats were heard or seen on the detector sonogram, occasionally there were recordings of free-flying bat passes at the time of release, and these were noted and removed prior to analysis. SonoBat software (version 30 Universal) was used to measure call parameters for individual pulses, and to rate their quality (a synthesized measure 'based on the total points of the sonogram above a threshold value') to avoid inclusion of uncharacteristic calls. Only calls with quality ratings over 0.8 were included in the dataset. To reduce deviation from search phase call parameters, only sequences with 10 or more pulses (up to a maximum of 32) were included in further statistical analysis. Parameters for sequences with < 10 pulses are also reported but were not included in analyses.

Statistical Analysis

We calculated the mean of each call parameter extracted by SonoBat software for a sequence from each bat. Of the 102 parameters given by the software, measurements based on amplitude (which can show significant variation with distance from the microphone) were removed, as well as those derived by additional computation (e.g., ratio or percentage calculations), reducing the number of parameters down to 20. All statistical analyses were performed using R 4.2.2 (R Core Team, 2023) through RStudio 2023.06.1+524 (Posit team, 2023). Multicollinearity between the remaining parameters was assessed by the variance inflation factor (VIF) using the 'vifcor' function of the 'usdm' package Version 1.1-18 (Naimi *et al*., 2014). We applied a correlation threshold of 0.4, which resulted in the selection of five uncorrelated variables for further analysis (Table 1). Descriptive statistics for all 20 parameters are given for the call library calls as a reference (Supplementary Tables S1–S4), including for species not used for further analysis due to small sample sizes.

To assess the likelihood of erroneous morphological identifications, comparisons between call sequences of individuals with and without genetic identifications were made using a *t*-test, where sample sizes allowed. The selected call parameters did not differ significantly — with the exception of Frequency of Maximum Power for *Laephotis capensis* ($t_{15.78}$ = -2.20, P = 0.043), Start Slope for *L. capensis* ($t_{19.51}$ = 3.46, P = 0.003) and *Scotophilus dinganii* ($t_{13.23}$ = 2.97, P = 0.011) and End Slope for *S. dinganii* ($t_{13.94}$ = 3.02, P = 0.009). Given that there is no indication of differences in the other parameters, and that distributions of these parameters overlap, it is likely that these differences are due to factors not considered in this study, such as release environment.

We tested for systematic differences in the call parameters from the different full spectrum detectors used to record echolocation calls. The influence of different detectors on parameter variation was investigated using a randomized block ANOVA with species as the blocking factor, followed by Tukey multiple comparison tests, using the 'aov' and 'TukeyHSD' functions of the 'stats' package Version 3.6.2 (R Core Team, 2023).

To investigate the relative contribution of each acoustic parameter to the variation in bat calls among functional groups and species, a Principal Component Analysis (PCA) was carried out using scaled parameter variables for species with a sample size greater than the number of variables using the 'prcomp' function of the 'stats' package Version 3.6.2 (R Core Team, 2023) (Table 1).

To assess how well bat echolocation calls could be assigned to functional groups using the selected acoustic parameters,

TABLE 1. Definitions of five echolocation call parameters selected based on VIF analysis and used in further analysis (www.sonobat.com)

Parameter	Definition
Frequency of Maximum Power (kHz)	The frequency of the maximum amplitude of the call
Preceding Interval (ms)	Time between the current call and the previous call
Start Slope (kHz/ms)	Slope at the start of the call, calculated from the first 5% of the call duration
End Slope (kHz/ms)	Slope at the end of the call, calculated from the final 5% of the call duration
Ledge Duration (ms)	Duration of the ledge, i.e., the most extended flattest slope section of the body of the call preceding the characteristic frequency

Discriminant Function Analyses (DFA) were carried out for species with a minimum sample size to variable ratio of 3:1 (Williams *et al*., 1988). Firstly we used Quadratic Discriminant Function Analysis (QDA) as the assumption of common covariance was not true for some of the parameters, as indicated by the 'leveneTest' function of the 'car' package Version 3.1-2 (Fox and Weisberg, 2019). However, Linear Discriminant Function Analysis (LDA) has been shown to be robust for bat echolocation data, so is additionally reported here as this method generates discriminant function coefficients, which highlight the most important parameters for group assignment (Papadatou *et al*., 2008). The data were split into a training (70%) and testing dataset (30%) randomly within groups using the 'caret' package Version 6.0.94 (Kuhn, 2008) 'createDataPartition' function and then normalized using 'preProcess' and 'predict' functions. DFA were carried out using the 'MASS' package Version 7.8-53.1 (Venables *et al*., 2002) (Table 1). DFA was then repeated to assess how well bat echolocation calls can be assigned to species using the parameters with the highest LDA coefficients separating functional groups.

Ethical Standards

Capturing and handling was carried out by trained individuals in line with internationally recognised best practice guidance outlined in Kunz *et al*. (2009). All of the work conducted in this study was approved by the University of Stirling's Animal Welfare and Ethical Review Body (Ref: AWERB 2023 15052 10438).

Results

A total of 238 individual bats of 22 species (Table 2), captured at 16 widely distributed sites across Zambia (Fig. 1), contributed to this acoustic dataset, representing around a quarter of the bat species currently recognised to occur in Zambia. In addition to the five bat call parameters for the 22 species investigated in this study (Table 3), the full dataset for all 20 parameters is included in Supplementary Tables S1–S4.

Detector Variation

For the 18 species with release calls from multiple detectors, of all five call parameters, only End Slope indicated a significant difference between detectors ($F_{4, 211} = 5.78$, $P < 0.001$); differences were found between the Echo Meter Touch 2 and both the Batbox Baton ($P = 0.004$, 95% C.I. = [-5.16, -0.68]) and the Anabat Walkabout ($P = 0.046$, 95% C.I. = [0.03, 5.31]) according to the post hoc test. These differences between detectors were not consistently higher or lower across species.

Principal Component Analyses (PCA)

To identify the parameters contributing most to the variation in call parameters, a PCA was conducted for 17 species in four functional groups (Table 2). The first two PCs combined explain 59.6% of the variation, rising to 76% with PC3, demonstrating that there is relatively good separation into functional groups (Fig. 2). In PC1, loadings

Table 2. The number of individuals of each species with release calls used in the analyses (PCA = Principle Component Analysis, DFA = Discriminant Function Analysis) and the functional group that they are allocated to based on Schnitzler and Kalko (2001)

Functional group	Family	Species	Total number of bats	Genetically identified bats	PCA	DFA — by functional group	DFA — by species
Open Aerial	Molossidae	*Mops nigeriae*	9	2	9	9	9
	Molossidae	*M. pumilus*	6	4	6	6	6
Edge Aerial	Miniopteridae	*Miniopterus* sp.	12	0	12	12	12
	Vespertilionidae	*Afronycteris nana*	12	0	12	12	12
	Vespertilionidae	*Glauconycteris variegata*	8	0	8	8	8
	Vespertilionidae	*Laephotis angolensis*	11	7	11	11	11
	Vespertilionidae	*L. capensis*	46	35	46	46	46
	Vespertilionidae	*Neoromicia anchietae*	16	16	16	16	16
	Vespertilionidae	*N. zuluensis*	12	11	12	12	12
	Vespertilionidae	*Nycticeinops schlieffeni*	8	1	8	8	8
	Vespertilionidae	*Pipistrellus rusticus*	18	11	18	18	18
	Vespertilionidae	*Scotophilus dinganii*	16	9	16	16	16
	Vespertilionidae	*S. viridis*	10	9	10	10	10
	Vespertilionidae	*Vansonia rueppellii*	8	0	8	8	8
Edge Trawl	Vespertilionidae	*Myotis bocagii*	3	2	0	0	0
	Vespertilionidae	*M. welwitschii*	9	0	9	0	9
Narrow Flutter	Hipposideridae	*Hipposideros caffer*	1	0	0	1	0
	Hipposideridae	*H. ruber*	1	0	0	1	0
	Hipposideridae	*Macronycteris vittatus*	19	0	19	19	19
	Rhinolophidae	*Rhinolophus mossambicus*	2	0	0	2	0
	Rhinolophidae	*R. simulator*	9	0	9	9	9
Narrow Passive	Nycteridae	*Nycteris* sp.	1	0	0	0	0

TABLE 3. Mean, standard deviation (SD), and min and max values of the five parameters explored in this study for all 22 species. Lines separate species of different families. *n* — sample size

Species	*n*	Preceding Interval (ms)			Frequency of Max Power (kHz)			Start Slope (kHz/ms)			End Slope (kHz/ms)			Ledge Duration (ms)		
		Mean (SD)	Min	Max	Mean (SD)	Min	Max	Mean (SD)	Min	Max	Mean (SD)	Min	Max	Mean (SD)	Min	Max
M. nigeriae	9	136.0 (128.2)	37.5	465.0	24.6 (3.6)	20.7	30.3	-4.0 (1.8)	-1.7	-6.5	-2.7 (1.9)	0.0	-5.7	0.4 (0.0)	0.4	0.5
M. pumilus	6	118.9 (27.4)	87.9	160.0	25.7 (4.0)	23.0	33.6	-3.1 (1.3)	-2.1	-5.5	-3.8 (1.3)	-2.1	-5.3	0.4 (0.1)	0.3	0.5
Miniopterus sp.	12	63.8 (14.1)	35.2	90.7	59.5 (2.3)	54.1	63.0	-24.0 (5.8)	-13.1	-38.2	-8.8 (4.0)	-10.1	-5.6	0.5 (0.1)	0.3	0.7
A. nana	12	99.2 (56.7)	53.8	216.0	70.1 (3.1)	65.7	76.9	-27.7 (5.1)	-19.1	-37.5	-8.2 (4.0)	-11.6	-8.4	0.6 (0.1)	0.4	0.8
G. variegata	8	89.9 (36.1)	48.2	148.0	37.6 (2.7)	34.6	42.6	-17.7 (7.3)	-12.1	-5.9	-6.4 (4.6)	-0.1	-4.7	0.6 (0.1)	0.4	0.8
L. angolensis	11	70.8 (16.7)	44.3	94.9	40.8 (3.4)	36.9	46.4	-16.0 (3.3)	-11.0	-21.6	-7.9 (2.6)	-11.0	-9.3	0.4 (0.1)	0.3	0.6
L. capensis	46	71.3 (17.6)	29.4	132.0	46.0 (4.3)	41.1	58.7	-23.0 (3.9)	-15.4	-31.5	-8.2 (3.3)	-1.4	-9.9	0.4 (0.1)	0.3	0.6
M. bocagii	3	91.6 (25.1)	62.6	107.0	47.6 (3.4)	44.9	51.4	-14.2 (2.9)	-10.8	-16.0	-20.2 (6.6)	-13.3	-26.4	0.3 (0.0)	0.3	0.3
M. welwitschii	9	71.4 (32.3)	32.3	120.0	39.7 (5.0)	34.7	49.9	-19.2 (3.7)	-14.1	-24.8	-18.8 (4.5)	-13.6	-25.9	0.4 (0.1)	0.3	0.6
N. anchietae	16	82.0 (20.5)	59.2	131.0	48.8 (3.1)	44.6	56.2	-22.6 (3.3)	-16.3	-27.6	-5.7 (2.3)	-0.9	-9.5	0.5 (0.1)	0.4	0.6
N. zuluensis	12	83.6 (24.3)	46.3	137.0	52.8 (4.0)	45.3	57.9	-29.1 (12.7)	-18.0	-8.2	-2.7 (2.3)	-0.6	0.9	0.5 (0.1)	0.3	0.8
N. schlieffeni	8	80.8 (39.0)	50.5	162.0	48.5 (4.5)	43.4	57.2	-29.1 (9.8)	-24.1	-7.7	-5.7 (3.4)	-1.1	-7.1	0.5 (0.1)	0.3	0.6
P. rusticus	18	69.9 (13.5)	44.2	98.7	61.5 (4.5)	57.3	72.1	-23.9 (4.3)	-12.0	-29.3	-7.2 (3.8)	-1.7	-9.8	0.5 (0.1)	0.3	0.6
S. dinganii	16	73.8 (20.4)	43.1	112.0	37.7 (2.6)	32.2	43.3	-15.1 (2.8)	-10.4	-21.0	-6.7 (3.2)	-10.5	-9.8	0.4 (0.1)	0.3	0.5
S. viridis	10	58.3 (12.0)	39.3	77.3	48.3 (4.8)	42.2	58.3	-16.7 (6.7)	-13.5	-9.7	-6.4 (3.0)	-1.2	-9.6	0.4 (0.0)	0.4	0.5
V. rueppellii	8	80.8 (36.7)	55.5	168.9	49.9 (2.2)	46.5	53.7	-23.1 (4.0)	-19.0	-28.2	-7.4 (5.1)	-10.7	0.4	0.5 (0.1)	0.4	0.5
H. caffer	1	96.4 (NA)	–	–	148.1 (NA)	–	–	-0.1 (NA)	–	–	-36.9 (NA)	–	–	0.4 (NA)	–	–
H. ruber	1	44.3 (NA)	–	–	133.4 (NA)	–	–	-0.1 (NA)	–	–	-12.7 (NA)	–	–	0.4 (NA)	–	–
M. vittatus	19	66.1 (21.3)	37.9	101.0	64.4 (1.9)	60.5	66.8	-0.1 (0.0)	-0.1	-0.2	-1.7 (1.1)	-0.6	-4.7	1.3 (0.9)	0.3	3.3
R. mossambicus	2	97.9 (8.6)	91.8	104.0	38.5 (2.1)	37.0	40.0	-0.1 (0.0)	-0.1	-0.1	-3.0 (1.3)	-2.0	-3.9	0.4 (0.0)	0.3	0.4
R. simulator	9	98.9 (13.8)	81.2	122.0	77.9 (1.6)	75.7	79.9	-0.1 (0.0)	-0.1	-0.2	-2.4 (1.2)	-1.1	-4.5	0.7 (0.9)	0.3	3.1
Nycteris sp.	1	68.5 (NA)	–	–	71.3 (NA)	–	–	-17.7 (NA)	–	–	-16.5 (NA)	–	–	0.5 (NA)	–	–

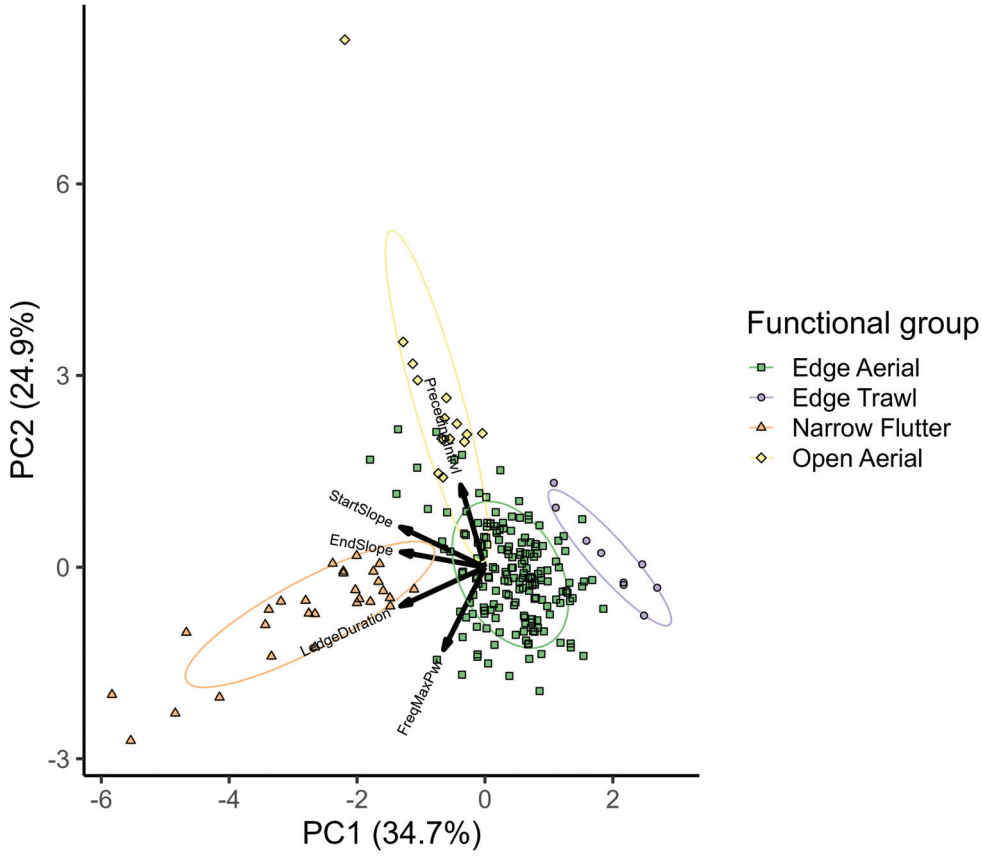

FIG. 2. Plot of the first two principal components 1 (PC1) and 2 (PC2), which combined explain 59.6% of the variation. Each point represents an individual bat call sequence, with shape and colour indicating functional group. Ellipses represent 68% confidence intervals. Arrows show the direction and strength of the correlation of a parameter with the principal components

are highest on a combination of slope and duration measures, whilst for PC2, Frequency of Maximum Power and Preceding Interval are emphasised (Table 4).

Narrow flutter species tend to have calls with a longer Ledge Duration and higher End Slope, whilst those of open aerial species have a lower Frequency of Maximum Power and longer Preceding Intervals (Fig. 2). Calls of edge trawling species tend to have lower values of all parameters compared to other species but seem to be separated best by lower Ledge Duration (Fig. 2). Species within functional groups, however, show considerable overlap based on the first principal components (Fig. 3).

Discriminant Function Analyses (DFAs)

To explore the extent to which functional groups can be separated using the five echolocation call parameters, DFA were carried out for 19 species belonging to three functional groups; open aerial, edge aerial and narrow flutter, with most being edge aerial species (Table 2). Both the QDA ($P < 0.001$) and LDA ($P = 0.001$) analyses resulted in similar mean model accuracies of 98.5% and 97.0% respectively for assigning calls to functional group level. LDA coefficients for LD1 indicate that Start Slope (coefficient = 1.691) Frequency of Maximum Power (coefficient = 1.097) contribute most to this separation (Supplementary Table S5).

Species separation, using Frequency of Maximum Power and Start Slope, resulted in similar QDA and LDA accuracy rates of 52.5% ($P < 0.001$) and 55.9% ($P < 0.001$) respectively, with LD1 placing most emphasis on Frequency of Maximum Power (coefficient = -3.5455) (Supplementary Table S6). Of the 17 species evaluated, nine had an accuracy level of 50% or lower, with the remaining eight at 75% or higher (Supplementary Table S7). Four species were identified to a relatively high accuracy: *Macronycteris vittatus* (100%), *Rhinolophus simulator* (100%), *Mops nigeriae* (99.1%), and *Afronycteris nana* (98.2%). The relatively low accuracy for the majority, however, does demonstrate significant overlap between two or more species, in values of one or both parameters, which we find in the dataset (Table 3).

TABLE 4. PCA loadings for each of the principal components (PC) with most positive or negative values indicating the greatest contribution to that component. The percentage of variance explained by each PC is shown in parentheses

Parameter	PC1 (34.7)	PC2 (24.9)	PC3 (16.4)	PC4 (13.9)	PC5 (10.1)
Preceding Interval	-0.158	0.632	-0.696	-0.282	0.107
Frequency of Maximum Power	-0.269	-0.634	-0.587	0.018	-0.426
Start Slope	-0.550	0.309	0.383	-0.187	-0.648
End Slope	-0.546	0.118	-0.046	0.784	0.266
Ledge Duration	-0.549	-0.299	0.150	-0.520	0.563

DISCUSSION

With the increasing use of PAM techniques to study bats, the development of open access reference call libraries is vital (Kunz *et al*., 2009; Walters *et al*., 2013). We present the first large scale bat call reference library and description of acoustic parameters collated for Zambia, providing an important resource for future acoustic research and monitoring of the 22 species presented here, which constitute a quarter of the bat species known to occur in the country (Monadjem *et al*., 2020). We showed that calls collected using a variety of acoustic detector equipment are comparable for this dataset. Finally, we have demonstrated that reliable separation of bat sonograms into functional groups is possible using only five parameters of full spectrum recordings, with Start Slope and Frequency of Maximum Power being the most suitable for this purpose. As predicted, despite a good level of accuracy for some species, identification to species level within functional groups using these parameters can be unreliable given the considerable overlap in call parameters for even this subset of species. Additional parameters may be needed to aid in the identification of species where there is overlap, and further research is needed to explore more subtle acoustic differences in more detail. The study emphasizes the potential for PAM for research on Zambian bats.

There is a need to consider detector technology, as well as software used when presenting or using reference datasets from multiple sources, for example community science projects (e.g., Challéat *et al*., 2024). For this study, calls recorded using different detector equipment were used but this did not result in substantive differences in most acoustic parameters. Although differences in End Slope were found between some detectors, this was not identified as one of the key parameters to assign bat calls to functional groups or species. Our analyses suggested that data collected by different types of detectors can be combined for projects involving recordings from multiple sources, e.g., community science projects, as long as subsequent call analysis is conducted using parameters known to be unaffected by this variability. Further studies, using multiple detectors for recording the same bat and considering larger sample sizes (DFA differentiating between species using the five variables included in this study requires a minimum sample size of 15), are required to provide a better understanding of this potential issue. Despite the lack of detector variation in call parameters shown here, we still recommend caution when making direct comparisons between recordings using different detectors and also for comparison with call libraries and published parameters. The acoustic equipment used by data contributors should be recorded and explored as a potential variable.

Of the five parameters selected in this study, only two parameters: Frequency of Maximum Power (or the equivalent Peak Frequency or Frequency of Maximum Energy) and Preceding Interval (or the equivalent inter-pulse interval) have been commonly used to distinguish between bat species (e.g., Walters *et al*., 2012; Monadjem *et al*., 2020). The Frequency of Maximum Power values in this study differ somewhat compared with the equivalent Frequency of Maximum Energy values presented by Monadjem *et al.* (2020), with slightly higher mean values but overlapping distributions for most species (see Supplementary Table S6). The consistently higher Frequency of Maximum Power values for all species, and not only narrow flutter bats with high duty cycle calls, indicates that this is unlikely to be due to Doppler shifts. The difference may be due to the measurement methods; SonoBat computes measures for each call pulse, whilst BatSound used by Monadjem *et al.* (2020) requires a manual approach. Release calls are also known to be generally higher frequency and often shorter duration than free flying calls and release habitat may also have been an influential factor, despite efforts made to obviate uncharacteristic calls by following the released bat for as long as possible, as well as selecting longer call sequences and using the software quality measures. However, for some species there

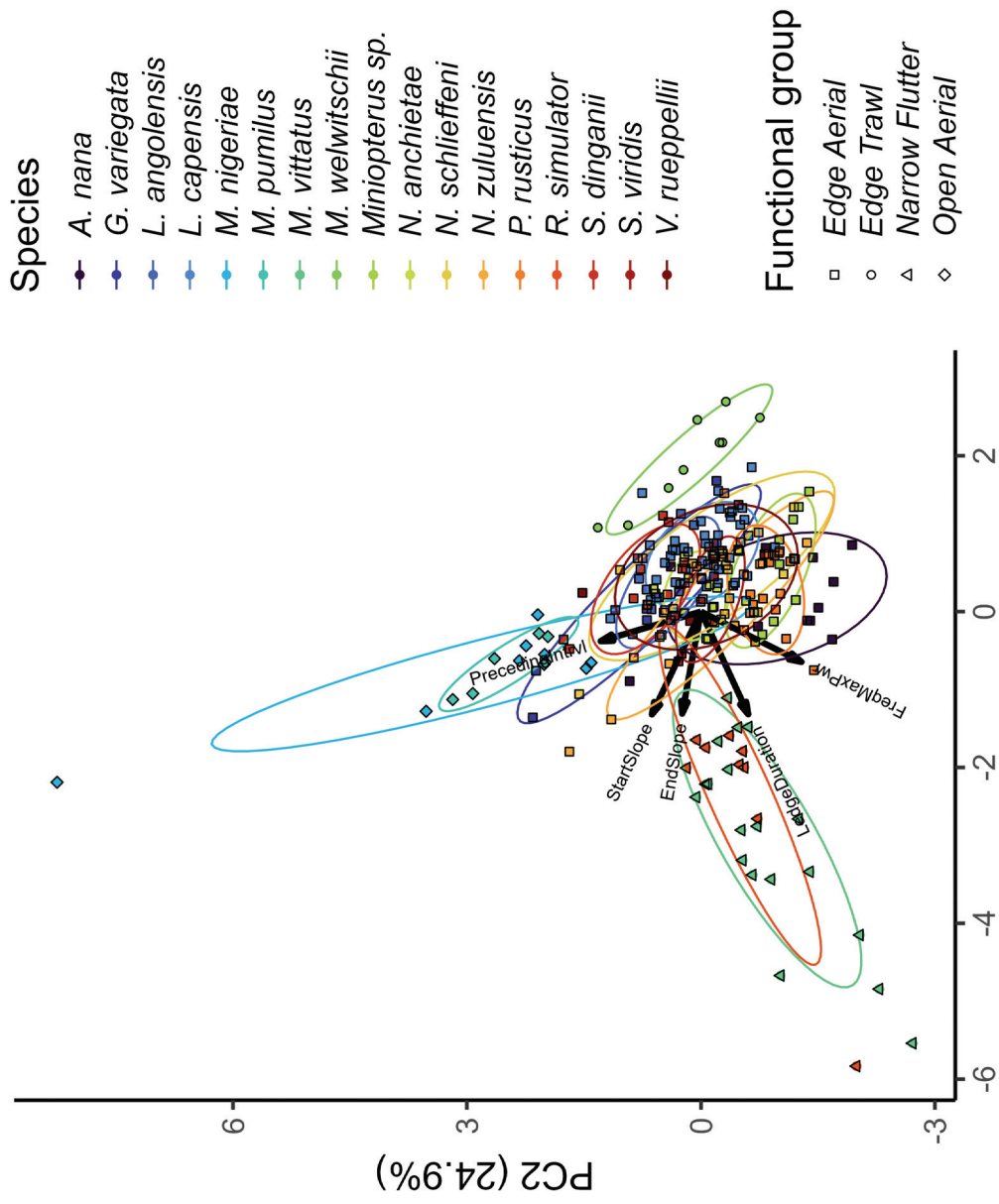

FIG. 3. Plot of the first two principal components 1 (PC1) and 2 (PC2), which combined explain 59.6% of the variation. Each point represents an individual bat call sequence, with shape indicating functional group and colour indicating species. Ellipses represent 68% confidence intervals. Arrows indicate the direction and strength of the correlation of a parameter with the principal components

are larger differences which could indicate regional variation or the need for taxonomic clarification. *Vansonia rueppellii* for example is over 10 kHz higher in this study than Monadjem *et al.* (2020), with no distribution overlap. This is also the case when compared to the Frequency of Maximum Energy measured manually for the species at a site in the Kafue National Park, Zambia (Kearney *et al*., 2010). The inter-pulse interval is also lower in this study (M = 80.8) compared to Kearney *et al*. (2010) (M = 131.7) although there is an overlap in values. For other species with no overlap, the difference is less than 10 kHz and there are small sample sizes in one or both of the datasets. The *Miniopterus* species Frequency of Maximum Power is closer to *M. mossambicus* than *M. natalensis*, however, further genetic analysis is needed, especially since both species are now known to occur in Zambia (Benda *et al*., 2022). There are currently no published data on the Start Slope, End Slope and Ledge Duration of the species in this study with which to make direct comparisons.

The use of commonly reported parameters only can be limiting in the quest for more accurate species identifications using PAM and exploring a wider range can help to tailor analyses to species assemblages (Walters *et al*., 2013). Start Slope for example, though not commonly reported, was identified as an important variable by Walters *et al*. (2012). The slope and duration characteristics of a call, which are also less commonly reported, may be more variable due to influences of habitat characteristics (Murray *et al*., 2001). By reducing multicollinearity, we were better able to focus on variables which best reflect the main characteristics of a call. This also allowed us to reduce the number of variables in an objective way to allow for the inclusion of more species, due to limited sample sizes of some species in this dataset. However, the inclusion of other more commonly used parameters in Supplementary Tables S1–S4 allow for these to be compared with other datasets.

In addition to presenting acoustic parameters for 22 African bat species recorded in Zambia, in this study we show that separation of echolocation calls into functional groups, based on sonotype categories, is highly accurate (98.5%), in agreement with suggestions that an automatic classifier by broader sonotype categories is possible (Roemer *et al*., 2021). Our study also highlights the need for caution when attempting species level identifications, in agreement with Russo *et al*. (2018). Despite a large sampling effort and spatial coverage, this dataset includes only 25% of the species known to occur in Zambia but shows overlaps in acoustic parameters between several species. Some functional groups are also absent from the analyses (such as narrow passive bats) or include less species (such as open aerial bats). More information on the distributions and ecological niches as well as comparisons with recordings of the full suite of co-occurring species in the country are required (Zamora-Gutierrez *et al*., 2020). Unresolved taxonomy can also present an issue, as was encountered here, when identifying cryptic species and those which are difficult to distinguish by morphology. A better collection of barcoded specimens from the country is required to aid future non-lethal genetic confirmation of individuals and in future studies, genetic material should be collected wherever possible (e.g., Benda *et al.*, 2022).

Passive acoustic monitoring of bats informed by good quality call libraries can be invaluable in monitoring populations and to determine the impacts of anthropogenic activities (Russo *et al*., 2015; Gibb *et al*., 2019). The move towards the use of automatic classifiers in PAM emphasises the importance of gathering good baseline bat calls, whether classification is based on call measures (e.g., Walters *et al*., 2012) or more complex machine-learning (e.g., Aodha *et al*., 2018, 2022). Increasing sample sizes not only allows for more robust analyses but also allows for more acoustic parameters to be explored as useful variables for species identification. As an ongoing collaborative project, we call on other bat researchers and ecologists working in Zambia and across the species ranges to add species and increase sample sizes for this call library.

Supplementary Information

Contents: Supplementary Tables: Table S1. Mean, standard deviation (SD), minimum (Min) and maximum (Max) measures of five frequency parameters at key structural features of the call for all 22 species regardless of sample size. Lines separate species of different families; Table S2. Mean, standard deviation (SD), minimum (Min) and maximum (Max) measures of six frequency parameters along the call sweep for all 22 species regardless of sample size. Lines separate species of different families; Table S3. Mean, standard deviation (SD), minimum (Min) and maximum (Max) measures of three time-based parameters for all 22 species regardless of sample size. Lines separate species of different families; Table S4. Mean, standard deviation (SD), minimum (Min) and maximum (Max) measures of six slope-based parameters for all 22 species regardless of sample size. Lines separate species of different families; Table S5. Coefficients of linear discriminants for five call parameters; the most positive or negative values indicating greatest contribution to functional group separation in each linear discriminant (LD) function; Table S6. Coefficients of linear discriminants for the two call parameters with most contribution to functional

group separation (Table 1); the most positive or negative values indicating greatest contribution to species separation in each linear discriminant; Table S7. LDA prediction summary of 17 species with sample sizes of more than six individuals with predicted identifications of test individuals (Predicted) compared to the true identifications (Observed). Supplementary Information is available exclusively on BioOne.

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Author Contribution Statement

HTB: Research concept and design, collection and/or assembly of data, data analysis and interpretation, writing, critical revision, and final approval of the article; EFM: research concept and design, data analysis and interpretation, critical revision, and final approval of the article; AM: research concept and design, data analysis and interpretation, critical revision, and final approval of the article; RCB: research concept and design, collection and/or assembly of data, critical revision, and final approval of the article; CM: collection and/or assembly of data, and critical revision of the article; VAM: research concept and design, collection and/or assembly of data, and critical revision of the article; HR: research concept and design, collection and/or assembly of data, and critical revision of the article; BK: collection and/or assembly of data; CM: collection and/or assembly of data; KP: research concept and design, data analysis and interpretation, critical revision, and final approval of the article.

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