The function of modified echolocation pulses in horseshoe bats (Family: Rhinolophidae)

A dissertation at UCT presented by

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ABSTRACT

Recent studies suggest that echolocation pulses may be implicated in communication. In one of these studies, the horseshoe bat, *Rhinolophus clivosus*, was found to emit echolocation pulses with an unusual and pronounced upsweep at the end of the echolocation pulse (at the FM component). It was hypothesized that these “modified echolocation pulses” may serve a communication function, enabling horseshoe bats to echolocate and communicate at the same time. This study investigated the function of these modified echolocation pulses. Hand-held recordings from several species of horseshoe bats were surveyed to determine how widespread modified pulses are within the family of Rhinolophidae. Modified echolocation pulses were found to be emitted by 11 out of 12 horseshoe bat species considered in this study. These species are distributed across several genetic subgroups of rhinolophid species. Four of them are in the capensis clade, *R. capensis, R. simulator, R. swinnyi* and *R. denti*, five of them *R. damarensis, R. eloquens R. hildebrandtii, R. fumigatus and R. darlingi*, in the fumigatus clade, one in the landeri clade (*R. landeri*), and two in the ferrumequinum clade (*R. clivosus and R. ferrumequinum*). These results suggest that only *R. maendeleo* from the Adami clade did not emit modified echolocation pulses. However, the absence of modified echolocation pulses in *R. maendeleo* is more likely than not due to the small sample size for this species (three individuals, Fig. 6). Gathered from these results is that modified pulses are not randomly emitted because they are not emitted in specific clades but across the clades and species. I tested the prediction that modified echolocation pulses are emitted in the presence of other bats by comparing emission rates across conditions that present different social valences, for example, solitary bats during foraging and bats flying in a flight cage in the presence of conspecifics. I surveyed different conditions namely: foraging, emerging from a roost, bats kept in a captive group and hand-held. I did experiments in which bats were exposed to different social valences in a controlled environment. There was a significant difference in the number of modified pulses across species, N=1309, H= 907.02, 4 d.f, $p<0.05$ (Fig.9). In flight- room experiments, the social contexts in which these modified pulses were emitted were further investigated, using *R. capensis,* as a model species. In the first experiment, *R. capensis* individuals were flown under different conditions, either alone, while sequences of standard and modified pulses of conspecifics were played back, or with conspecifics or with heterospecifics present in the flight- room. *R. capensis* emitted modified echolocation modified pulses in all conditions including when they flew alone. However, there were significant differences in the number of modified pulses emitted amongst the different conditions (N=19, χ^2=6.00, 2 d.f, p=0.04). In standard echolocation pulses (i.e. without the modification), the FM component plays a role in distance measuring (ranging) and the modification of the FM component may affect this very important function. A second experiment was therefore conducted during which bats were flown on an obstacle course to determine if modified pulses adversely affected the ranging ability of bats. If so, bats should not emit modified echolocation pulses in conditions, like an obstacle course, that require precise ranging information. *R. capensis* individuals (N=19) were held through four conditions each, namely: flying alone, flying with a conspecific, flying with a conspecific put in a small cage, and flying while conspecific pulses were being played back. There were no modified echolocation pulses emitted throughout all the conditions. Thus modified echolocation pulses were not emitted when precise ranging was required which suggests that the modification may have impaired the ranging function of the FM component. In conclusion, modified echolocation pulses were emitted more often when the bat was in the presence of other bats, which suggests that it may function as a communication signal. However, it appears that modified echolocation pulses may adversely impact the ranging function of the FM component so that bats were unable to use the modification when precise ranging is required.

MANWELEDZO

Miungo i shumiswa ho nga nyamilemalema u wana zwiliwa. Miungo heyo ina khonadzelo ya u shumiswa nga nyamilemalema u davhidzana. Kha tsedzuluso ntswa, ho waniwa muungo wo shandukaho zwituku. U vho fhambana na wo doweleaho kha minyamulemalema ya dzi horseshoe. Muungo uyu ho pfi u tendela nyamulemalema uri u ambedzane na minwe, u dovhe u wane zwiliwa nga mungo muthihi. Tsedzuluso yashu i khou toda u engedza ndivho kha heyi miungo yo shandukiswaho. Ri khou sedzesa kha uri heyi miungo yo shandukiswaho, i shuma mini. Ro shumisa miungo yo rekhodiwaho musi nyamilemalema yo fariwa nga tshanda. Ri tshi toda u wanulusa uri heyi miungo yo shandukiswaho yo phadalala u swika ngafhi kha zwipisisi zwa nyamilemalema wa dzi horseshoe. Ro wanulusa uri fumi na nthihi kha fumi na mbili ya zwipisisi zwa horseshoe zwi khou shumisa miungo yo shandukiswaho. Zwipisisi zwi no i shumisesa ndi zwa nyamilemalema ya kha tshigwada tsha capensis. He tshi tshigwada tsho itiwa nga *Rhinolophus capensis*, *R. simulator*, *R. swinnyi* na *R. denti*. Zwinwe zwi gwada na zwone zwi a shumisa hei miungo, ndi tshi gwada tsha fumigatus (*R. damarensis*, *R. eloquens*, *R. hildebrantii*, *R. fumigatus* na *R. darlingi*), tshi gwada tsha landeri (*R. landeri*) na tshi gwada tsha ferrumequinum (*R. clivosus* na *R .ferrumequinum*). Tshipisisi *R. eloquens* a tshi ngo kona u vheiwa kha hezwi zwi gwada, fhedzi tshi a shumisa hei miungo. Tshipisisi tsha *R. maendeleo* khatsho miungo yo shandukiswaho a I ngo wanala; hezwi zwi nga divha fhungo lauri ho rekodiwa minyamulelema miraru fhedzi. Zwino nomboro ya nyamilemalema I dovha I thukhusa kha u kona u vhona uri miungo yo shandikiswaho I a wana naa kha hetsho tshipisisi. Rekhodo dzo dzhiiwaho fhethu ho fhambanana dzo shumiswaho u sedzulusa uri iyi miungo i shumisiwa ngafhi. Fhethu afha hu katela musi nyamulemalema i tshi khou toda zwiliwa, i tshi bva bakoni, yo vheiwa kha haya li si ladzo, musi dzo fariwa nga tshanda tsha muthu na musi yo pangwa lufherani hu khou itiwa tshipirimende. Ho vha na phambano kha ku shumisele kwa miungo, N=1309, H= 907.02, 4 d.f, $p<0.05$ (Fig.9). Miungo iyi yo shumisesiwa musi nyamulemalema, wo fariwa nga tshanda tsha muthu. Zwa sumba u nyamulemalema u ngavha u sa khou farea zwavhudi, kana u khou pfa u vhavha. Datha ya dakani I dina phambano kha nomboro ya nyamulemalema I rikhodiwaho, u fhenya izwi, ro ita zwipirimende ro tou bata nomboro nkene ya nyamilemalema. Ro dovha ra wana uri i shumisiwa na musi nyamilemalema u tshi khou fhufha hu re na minwe nyamilemalema, (N=19, χ^2=6.00, 2 d.f, p=0.04); zwa sumbedza uri i shumisiwa u ambedzana. Ro do sedzulusa vhudavhidzani hu iteaho nga u shumisa uyu mungo. Ro dovha ra I panga kha lufhera, ra vheya na labi la u i

thivhela ndila, ri tshi khou toda u sedzulusa uri i a khakhisa ku shumele kwa nyamulemalema i tshi fhufha na u toda zwiliwa. Ro wana uri miungo yo shandukiswaho i a khakhisa ku shumele kwa nyamulemalema musi I tshi toda zwiliwa; kha ho lwu lufhera ayi ngo shumisiwa heyi miungo. Hezwi zwi amba uri musi nyamulemalema u tshi toda u vhanyuludza, ayi shumisi miungo hei yo shandukiswaho. Tsedzuluso heyi, yo sumbedza uri , miungo yo shandukiswaho i nga divha i tshi shumisiwa u ambedzana nga nyamulemalema. Fhedzi ha, a I koni u amba na u vhanyludza nga tshifhinga tshithihi .

AUTHOR’S DECLARATION

I hereby declare that the project entitled “The function of modified echolocation pulses in horseshoe bats (Family: Rhinolophidae)”, is an original work done by me under the supervision of Associate Professor David S. Jacobs and Dr Anna Bastian. All external sources incorporated to aid in the understanding of this work, have been referenced and acknowledged.

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CF – Constant Frequency

DSC – Doppler shift compensation

FM – Frequency modulated

HDC – High duty cycle

MOD – Modification

No - number

1. INTRODUCTION

Most animals use vocalization exclusively for communication e.g. bird songs (Kaplan 2000). This communication in animals requires the sender to attempt to put out a message and for the receiver to interpret its meaning (Marler 1961). An example of this is the warning call of the galah (*Eolophus roseicapilla*) (Kaplan 2000) which is emitted by the sender upon spotting a threat. The call is perceived by conspecifics that interpret the call and respond accordingly by seeking shelter. The sender and perceiver need to use the same code for information to be understood (Barataud 2015) similar to human languages. This is seen in calls used in the marking of a territory and in courtship. Courtship calls are involved in mate choice and they give information about the species' identity and the individual's quality (fitness) (Pedroso et al. 2013). An example of courtship calls in bats is displayed by sac-winged bats (*Saccopteryx bilineata*). They emit whistles which are heard by females. Upon hearing these calls, females respond by emitting a screech sound which signals to the males that the females are ready to breed (Wilkins et al. 2013). The result of successful communication is signalled by a change in the receiver's behaviour based on the information extracted from the sender's calls (Bradbury and Vehrencamp 1998; Wyatt 2003).

This study has focused on acoustic signals. Acoustic signals have evolved mainly for communication. These are sounds produced by animals in different social contexts, for example, courtship calls during mating (Truax 2001). Such calls can be generated by a syrinx or larynx such as in birds and mammals, respectively. They can also take the form of substrate borne vibrations e.g. in crickets (*Grylloidea*), katydids (*Pterophylla camellifolia*), grasshoppers (*Caelifera*), cicadas (*Cicadoidea*) (Alexander 1957) and some mammals (Comstock 2010). The cicada (*Cicadoidea*) produces sound by a rapid buckling and unbuckling of drum-like tymbals (Bennet-Clark 1997; Fonseca and Clark 1998).

In bats, acoustic signals are used in the form of social calls and echolocation pulses (Fenton 2003). Social calls serve a communication function (Brown and Grinnell 1980). The social calls of bats are vocalizations with a lower frequency than their echolocation pulses (Belwood and Fullard 1984) and are often audible to people and other mammals. The lower frequency minimizes the impact of attenuation and hence increases the range of the sound

(Fenton 2003) so that even individuals far away can perceive the signal. Bats also use acoustic signals of generally higher frequency than their social calls for orientation and foraging through echolocation (Jacobs 2016; Schnitzler and Denzinger 2011; Schnitzler et al. 2003). Echolocation is an adaptation for orientation in which an echolocating animal emits ultrasonic signals and receives echoes when those signals are reflected off objects in the bat's environment (Fawcett et al. 2015; Griffin 1958; Griffin et al. 1960; Grinnell and Griffin 1958; Jacobs and Bastian 2016). Bats produce sound to get information about their surroundings when the echoes are reflected. The two types of bat vocalizations (social calls and echolocation pulses) differ in the combination of duration, frequency, and the pattern of frequency change over time (Fenton 2003), with social calls being emitted occasionally during flight in different situations, while echolocation pulses are emitted continuously by a bat in flight (Pfalzer and Kusch 2003).

Since echolocation has evolved for foraging and orientation, its acoustic design is adapted to the specific tasks and habitat conditions of the niche in which it forages (Neuweiler 1990; Schnitzler and Kalko 2001; Simmons and Stein 1980). These tasks include a) orientation, which involves the ability of the bat to find, learn, and return to a specific place, and b) prey acquisition. Echolocation design for foraging is influenced firstly by the bats foraging habitat space: a) open, when bats hunt for prey far from the vegetation or the ground, b) edge, the bat seeks prey flying near vegetation edges, in gaps or above flat water surfaces; and c) narrow, bats only find food that is positioned on or near vegetation. Secondly, it is influenced by the foraging mode including, gleaning (taking food from surfaces), aerial hawking (catching prey in the air with or without background echoes), and clutter rejection foraging (these bats can distinguish echoes from the wings of fluttering insects from echoes from the background (Neuweiler 1989; Schnitzler 2003). Despite these selection pressures shaping the signal, there is a continuous discussion around the potential functional extension of echolocation as a communication signal (Finger et al. 2017; Jacobs and Bastian 2018; Jones and Siemers 2011; Knörnschild et al. 2012; Raw et al. 2018; Voigt-Heucke 2010). There is evidence that echolocation can be used in a communication context by bats, at least in the form of eavesdropping to glean information (Barclay 1982; Jones et al. 2011; Peake 2005) about the echolocating bat or the potential foraging grounds. The information is gathered through feeding buzzes emitted in the pulses of the echolocating bat (Barclay 1982; Fenton 2003; Knörnschild et al. 2012). For example, bats can discriminate between the pulses of conspecifics and those of heterospecifics based on the conspecifics' echolocation pulses only (Bastian and Jacobs 2015; Raw et al. 2018; Schuchmann and Siemers 2010). Furthermore, bats are also able to discriminate between species, sex, and individuals (Bastian and Jacobs 2015; Finger et al. 2017; Kazial et al. 2008; Puechmaille 2012; Raw et al. 2018) and determine age and family affiliations through echolocation pulses (Masters et al. 1995). Echolocation may also play a role in reproduction (Grilliot et al. 2009), for example through mate choice (Puechmaille et al. 2014).

The family of horseshoe bats (Rhinolophidae) has echolocation pulses that are dominated by a long constant frequency component (CF) with shorter frequency modulated (FM) components at the beginning (the initial FM) and end (terminal FM) of the pulse (Griffin 1958; Griffin et al. 1960; Grinnell and Griffin 1958). The initial FM starts at a lower frequency and sweeps upward until the CF is reached, whereas the terminal FM sweeps downward from the CF to a lower frequency (Schnitzler et al. 1985). The CF component is adapted for fluttering target detection and evaluation (Kober and Schnitzler 1990; Schnitzler et al. 2003). The terminal FM component is adapted for precise localization of the target through ranging (Schnitzler and Kalko 2001; Simmons and O'Farrell 1977) by determining the distance to the target by measuring the time delay between the emitted signal and the returning echo in each of these frequencies represented in the emitted pulse (Jones and Rayner 1989; Schnitzler and Kalko 2001) and localize by analysing the time and intensity differences on the bat's ears to determine the direction of the prey (Fenton et al. 2012). Horseshoe bats are high-duty cycle echolocating bats (Griffin 1958). High-duty cycle echolocating bats (HDC), use a long duration pulses. They hunt for fluttering insects amongst the vegetation (Jones and Rayner 1989; Schnitzler et al. 1985). With each pulse they emit, they receive an excessive amount of echoes from the foliage (Vanderelst 2011). However, fluttering prey introduces frequency and amplitude modulation in the returning echoes called acoustic glints (Jones and Rayner 1989; Schnitzler et al. 1985). Acoustic glints are generated when the calls are reflected off the wing of the prey which is at a different angle to the impinging echolocation beam during the insect’s wing-beat cycle (Jones and Rayner 1989). Therefore, the echoes reflected by the bat carry spectral and temporal changes that provide information about the prey (Jones and Rayner 1989). The intensity of the echo determines the size of the object, spectral changes (Schmidt 1988) depict the texture of the object and the time elapsed between the emission of a pulse and the time taken for the echo to return determines the distance to the prey (Schoeman and Jacobs 2011). HDC echolocating bats have a region of over representation of neurons (the auditory fovea) in the auditory cortex. The auditory fovea is sensitive to a unique and narrow range of frequencies (Jacobs 2016;

Neuweiler et al. 1980). The auditory fovea is an important adaptation of receivers in bats using FM-CF-FM signals for the processing of fluttering information presented by the CF component (Neuweiler et al. 1980; Schnitzler and Denzinger 2011). The auditory fovea keeps the carrier frequency of the insects' echoes near a reference frequency (Neuweiler et al. 1980; Schnitzler and Denzinger 2011), enabling the HDC bat to process flutter information resulting in evaluation and detection of insects flying in clutter. These bats lower the frequency of their emitted pulses to compensate for the Doppler shift that echoes from their pulses undergo as a result of the relative velocity of the bat and the object reflecting the echo, hence Doppler Shift Compensation (DSC) (Schnitzler et al. 1985). DSC during flight results in the returning echo returning to the bat at a frequency within the range of the auditory fovea of the bat.

A study on *R. clivosus* by Finger et al. (2017) indicated that the species is emitting "modified echolocation pulses". These modified echolocation pulses are standard echolocation pulses but with an additional upsweep on the terminal FM component of the pulses (Fig 1). The FM component is responsible for localization of the target, it has a ranging function (Schnitzler and Kalko 2001; Simmons and O'Farrell 1977). The FM component helps the bat determine the distance to which the prey is and can further determine the direction in which the target is flying. Given the adverse effect such a modification is likely to have on the ranging performance of the bat, it has been proposed that the modified pulses may be used in communication (Finger et al. 2017). In a direct comparison between standard echolocation pulses and modified echolocation pulses done in the above-mentioned study, it was shown that pulses that had these modifications carried more distinct individual signatures. It was assumed that these modified echolocation pulses potentially represent a "hybrid" pulse, incorporating both functions of echolocation - orientation and communication in one signal.

This study investigated the function of modified echolocation pulses systematically. The hypothesis tested was that modified echolocation pulses are deliberately produced by horseshoe bats potentially as a communicative signal. These horseshoe bats receive a lot of information about the target from fluttering prey wings and that information also carries background noise from the vegetation clutter (Jones and Rayner 1989). Therefore, the modification may interfere with the clear processing of this information from the FM component when catching prey. The prediction was that horseshoe bats could modify one of the FM components of their echolocation pulse without interfering with the ranging performance of the echolocation pulse because the other FM component would still be available for ranging.

If the hypothesis is valid, modified echolocation pulses should:

a) Be widespread amongst rhinolophid species, suggesting that they are not randomly emitted and that they are not a trait that has evolved in a specific lineage. Non-random emission of such modified echolocation pulses in different social contexts, with different social valences may imply that the bats are deliberately emitting the modified echolocation pulses. The deliberate emission of modified echolocation would strengthen the communication potential of these pulses. Successful communication requires the emitter to attempt to send the message that will be perceived by the receiver.

b) Be produced in social contexts, but particularly in social contexts when the bat is in flight and both functions are needed, requiring the bat to emit modified pulses.

c) Never occur on both FM components of the same pulse because modifications to both FM components would compromise the ranging performance of the bat. A corollary to this is that bats should be able to negotiate an obstacle course while emitting pulses with only one FM component modified.

d) High-duty cycle bats with only one FM component, like the Hipposideridae, should never display this modification because they have only one FM component in their echolocation pulses.

The predictions of the hypotheses were tested by a survey of recorded echolocation sequences from multiple species of horseshoe bats in different contexts as well as by experiments in which we exposed a focal species, *R. capensis*, to several different social contexts to determine in which context these modified echolocation pulses occur most frequently. The experiments also included an obstacle course to test if modified echolocation pulses are emitted when precise ranging information is needed.

2. METHODS AND MATERIALS.

2.1 Study animals.

The family of horseshoe bats (Rhinolophidae) consists of 25 known species in Africa of which 15 occur in southern Africa. Rhinolophid bats dwell in caves, tunnels, mine adits (Csorba et al. 2003; Kunz 1982), and hollow trees (Monadjem 1982). They give birth in the middle of December, proceeding to the following year (Csorba et al. 2003; McDonald 1990). Generally, females give birth to a single young (Csorba et al. 2003). They roost singly or in small to larger groups in ceiling cavities of caves (Csorba et al. 2003; McDonald 1990). These groups are generally comprised of both sexes (McDonald 1990). They are also found in roosts mixed with heterospecifics (McDonald 1990). Horseshoe bats produce FM-CF-FM pulses which range from 33 kHz to 111 kHz for the species of horseshoe bats used in this study. Experiments were conducted on one focal species, *Rhinolophus capensis,* which is endemic to the southern and western cape regions of South Africa (McDonald 1990). Its distribution is restricted to the coastal region from the Eastern Cape Province to Cape Town and then north to the Orange River which forms the border between South Africa and Namibia, in the north-western part of South Africa (Monadjem 1982). In De Hoop Nature Reserve, the echolocation pulses of *R. capensis* have relatively high peak frequencies (83.9 ± 1.2 kHz) (Jacobs et al. 2007) and long duration (41.2 ± 3.2 ms) (Odendaal et al. 2014). Recorded echolocation pulses of *Hipposideros caffer*, family Hipposideridae (Andersen 1918) were also surveyed. This species is widely distributed throughout southern Africa; it is however absent from much of the southern and central parts of the region (Monadjem 1982). *Hipposideros caffer* roosts in a wide range of caves, sinkholes, and cavities, including anthropogenic ones such as mines and culverts (Monadjem 1982). Colonies vary in size from less than a dozen to hundreds of individuals. *Hipposideros caffer* produces FM-CF pulses with a high peak frequency (142.3 ± 0.6 kHz) and intermediate duration (8.4 ± 0.7 ms) (Monadjem 1982). These recordings were also taken in the southern region of Africa. The recordings surveyed for *Hipposideros caffer* are from Lekkersing (Northern Cape) 28°15'33.012"S, 17°1'15.996"E, Sudwala (Mpumalanga) 25°20'15"S, 30°37'57"E, Pafuri (Kruger National Park) 23.9884°S, 31.5547°E, Henkries (Goodhouse) in the Northern Cape 28.957°S, 18.105°E and Mkuzi (KwaZulu-Natal) 28°49’53’’S, 32°20’58’’E.

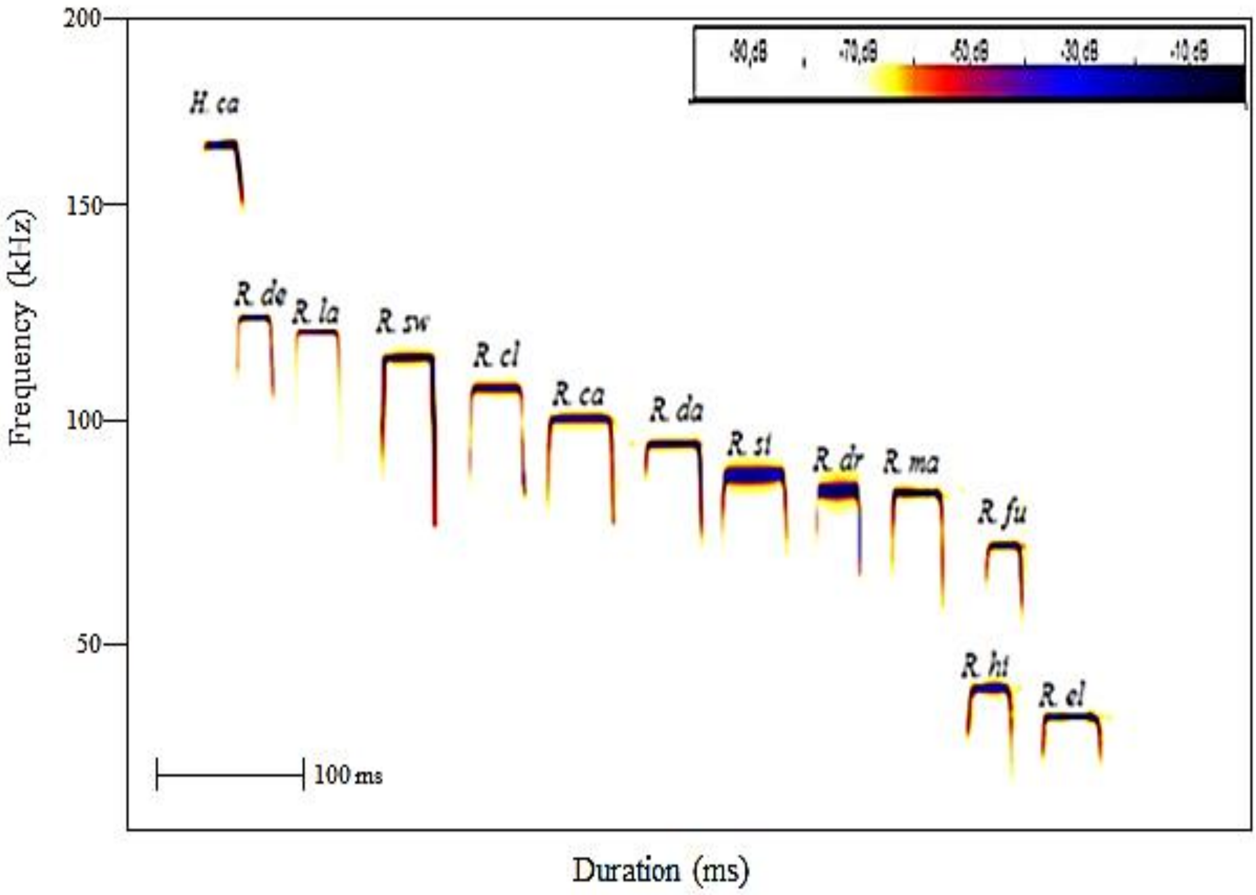

FIG 1: Sound spectrogram (sampling parameters: FFT size 1024; Hanning window-left channel, Mono, 16 bitz) of 12 FM-CF-FM echolocation pulses of Rhinolophid species and one CF-FM hipposiderid species (*Hipposideros caffer*). These pulses were recorded from hand-held bats across Southern Africa and are presented in order of decreasing CF frequencies. The specific location of the bats recorded is as follows: *Hipposideros caffer (H. ca)* from Sudwala caves in Mpumalanga, *R. landeri (R. la)* from Nhascuvo cave in Mozambique, R. *swinnyi* (*R.sw*) from Kalenda in Rwanda, *R. clivosus (R. cl)* and *Rhinolophus capensis (R. ca)* from De Hoop nature reserve in Western Cape, *R. damarensis (R. da)* from Soetfontein in the Northern Cape, *R. simulator (R. si)* from the Echo caves in Mpumalanga, *R. darlingi* (*R. da*) from Nhascuvo cave, *R. maendeleo* (*R. ma*); the location is undocumented, *R. fumigatus* (*R. fu*) from Chitake Baobabs in Zimbabwe, *R. hildebrandtii (R. hi)* from Mushanduke in Zimbabwe and lastly *R. eloquens (R. el)* from Kanandusi in Kenya.

2.2 Social contexts.

Echolocation sequences from different species and conditions were analysed, to test if conditions with a higher social valence contain more modified echolocation pulses than conditions with a lower social valence. Social valence was determined by the amount of interaction a bat has with other bats. The recordings stem from three datasets: 1) an existing database containing the conditions in which the echolocating bat(s) were hand-held, when they emerged from their roost at dusk, when they foraged, or when they freely interacted in a captive colony (group). 2) a set of recordings of *R. capensis* in a flight room in which they were exposed to four conditions with different social valence, namely flown alone, flown with played back echolocation from conspecifics, flown in the presence of conspecifics and flown in the presence of heterospecifics. 3) A set of recordings of *R. capensis* in a flight room, in which they had to negotiate their way through an obstacle course.

2.2.1 Hand-held recordings.

As stated in section 2.2, hand-held recordings of 12 rhinolophidae species and one hipposideridae species, were taken from the lab’s database. The hand-held recordings from the captured bats were taken using an ultrasound D1000X detector (Pettersson Elektronik AB, Uppsala, Sweden). The sampling frequency was at 500 kHz. During recordings, hand-held bats were 30 cm away from the microphone. The hand-held condition also enabled the investigation of how widespread modified echolocation pulses are, across species of horseshoe bats. In this instance, if they are emitted by all horseshoe bats surveyed in the study, it may suggest that they are not randomly emitted, indicating a deliberate emission of modified echolocation pulses.

2.2.2 Captive group recordings.

The recordings from captive groups of *R. clivosus* of this condition were taken during the study by Finger et al. (2017). Individuals of *R. clivosus* were captured at the Guano cave in De Hoop Nature Reserve. They were captured using hand nets during the day. The captured individuals did not represent any social group as they were caught opportunistically. These bats were housed at the University of Cape Town. They were recorded while in the flight room, hanging on the perch, The prediction tested in this condition was that, modified echolocation pulses would be emitted because; the bats were in the presence of other bats.

The conditions in sections 2.2.3 to 2.2.5 were recorded using an Avisoft Ultrasound Gate 816 H (Avisoft Bioacoustics, version 4.2 Berlin, Germany) microphone. Pulses were recorded onto a Dell laptop (Xiamen, China) with Avisoft SasLab Pro software (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). Recordings were analyzed using BatSound Pro software (Version 3.3.1.4, Pettersson Elektronik AB, Uppsala, Sweden) using a sampling rate of 500 kHz and Audacity software (version: 2.0.6.0, Audacity team, Pittsburgh, Pennsylvania).

2.2.3 Emergence bats recordings.

In De Hoop Nature Reserve, recordings started around 19h00 in March, when the bats emerged from a cave in the reserve (Hot Hole Cave). The microphones were placed 6 m away from the entrance of the cave. Recordings were made as the bats emerged from the cave to go forage. Bats emerging from roosts elsewhere in South Africa were also recorded outside and close to the entrance of the Sudwala Cave and the Kalkoenkrans Cave in Mpumalanga Province and KwaZulu Natal Province close to the entrances of Beneva and Bazley Tunnels. In all the above-mentioned roosts besides Beneva tunnel, bats roosted in mixed colonies, with conspecifics and heterospecifics.

2.2.4 Bat recordings from foraging bats.

Foraging bats (*R. clivosus and R. capensis*) were recorded at several sites within De Hoop Nature Reserve and recordings were made from 19h00 to 22h00 in March over ten nights. Other recordings were taken from foraging bats at several sites around South Africa by David Jacobs. These included Lekkersing in the Northern Cape Province and Beneva and Bazley Tunnel in KwaZulu Natal. Foraging bats were also recorded close to the Sudwala Cave and the Kalkoenkrans Cave in Mpumalanga.

The conditions in sections 2.2.5 to 2.2.6 were recorded using an Avisoft Ultrasound Gate 116H (Avisoft Bioacoustics, Berlin, Germany) microphone. Pulses were directly recorded onto a computer with Avisoft SasLab Pro software (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). Recordings were analysed using BatSound Pro software (Version 3.3.1.4, Pettersson Elektronik AB, Uppsala, Sweden) using a sampling rate of 500 kHz and Audacity software (version: 2.0.6.0, Audacity team, Pittsburgh, Pennsylvania).

2.2.5 Conditions with different social valence (Experiment1)

Experiments were conducted to further investigate if modified echolocation pulses are used in communication. This was done by exposing *R. capensis* to conditions that present different social valences. The prediction was that there are differences in the frequency of occurrence of modified echolocation pulse emissions rates across conditions. The conditions include flying the *R. capensis* alone, flying *R. capensis* with conspecifics present, and flying *R. capensis* with a heterospecific.

2.2.6 Obstacle course (Experiment 2)

The hypothesis that the modification impairs the ranging function of the FM component was directly tested using an obstacle course experiment. Here the bat (*R. capensis*) had to navigate its way through the obstacle course, see 2.3.4.2. The same as in the foraging condition, it was predicted that whenever there is a modification of the FM component, both the FM components should be present on the same pulse, with one FM component left unmodified and may compensate for the ranging function performed by the FM component.

2.3 Experimental approach

2.3.1 Ethical Note

This research was done under ethical clearance from the University of Cape Town animal ethics committee (permit number: 2017/V21/Bastian) and a permit from Cape Nature (CN44-30-2033).

2.3.2 Study site

R. capensis individuals were studied in the southern part of its range in De Hoop Nature Reserve (34º26’S, 20º25’E) (Jacobs et al. 2007) near Bredasdorp in the Western Cape, South Africa.

2.3.3 Capture and husbandry of bats

Rhinolophus capensis and *Myotis tricolor* bats were captured using Ecotone mist nets in December 2017 to test whether modified pulses are emitted in the presence of other bats. Individuals of the two species were captured again in February & March 2018, for the obstacle course, to test whether the emission of modified echolocation pulses by horseshoe bats impairs the primary function of the FM component-orientation/navigation. The Ecotone mist nets had lengths of 3, 6, and 9 m; and a mesh size of 14×14 mm. The preferred monofilament mist nets were erected using poles that were made to stand using rocks and ropes. Bats captured in mist nets were placed into small bat bags made of soft cotton. Sex and age were determined visually on-site (Haarsma 2008). All pregnant, juvenile, and lactating bats were released immediately. Most female *R. capensis* were pregnant or lactating in December 2017 and for this reason, only male *R. capensis* were therefore used in experiments. The bats were transported to the research house in the reserve, not far from the cave were the bats were captured. Bats were housed in a three-person tent (200 cm × 200 cm ×110 cm) in a quiet, dark room in the research house. The tent walls were lined with towels for the bats to crawl on and under. Below the towels were trays of water which could be reached by the bats by climbing down the towels. Live black soldier flies (*Hermetia illucens*) were placed in the tent throughout captivity so that the bats could catch and feed upon them. In addition, bats were manually given water via a plastic pipette and fed with mealworms (*Tenebrio molitor*) before being flown in the experimental conditions. Mealworms were fed to the bats using forceps, but if the bat wouldn’t eat from the forceps, mealworms were decapitated, and the semi-liquid innards were squeezed into the bat's mouth to stimulate feeding on the novel food source while being handled. The mealworms were raised on a medium comprised of dog food, pieces of fruit, vegetables, and bread to increase their nutritional value. To minimize the stress on bats while feeding, feeding was illuminated with red light. No electronic equipment or loud noises were allowed near the feeding station. Bats were kept at ambient temperatures for a maximum of three hours; this included experiment and feeding time. They were released immediately after the experiments. No bat was kept in the research house overnight.

2.3.4 Experimental design

All experiments were conducted in a flight room (3.5 m × 2.7 m × 2.2 m), erected in an outbuilding of the research house in De Hoop Nature Reserve in December 2017 and February/March 2018. Recordings were done using Avisoft Ultrasound Gate 116H (Avisoft Bioacoustics, Berlin, Germany) microphones. Pulses were recorded directly onto a computer with Avisoft SasLab Pro software (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). (USG Player Light, Avisoft, Glienicke, Germany) speakers were used for playback. After the experiments were completed, the bats were caught inside the flight room using hand nets. Each bat's fur was clipped before release to avoid repeated use in the experiments in the event of it being recaptured. Sharp dissection scissors with rounded tips were used.

2.3.4.1. Conditions of different social valence

This experiment was designed to test if modified pulses have a communicative function by investigating the context in which they are emitted. The aim was to investigate when and to whom modified pulses were emitted. *R. capensis* individuals were exposed to different conditions that represent different social valence. The prediction was that the frequency of emission of modified pulses by *R. capensis* individuals would differ across the different social valences. *Myotis tricolor* was used in conditions testing the response of *R. capensis* to heterospecifics.

The maximum number of *R. capensis* individuals captured per night was four. For each night, we kept the same number of captured *M. tricolor*. A condition took 20 minutes, making two hours the total duration of the experiment per night. It took two hours because the bats needed to be put in the flight room before the commencement of the experiment and then caught to be removed from the flight room after the experiment. The overall duration of experiments per night is 80 minutes. The additional 30 minutes are added by logistics that accompany the preparation of a condition. Four conditions were conducted on each individual, each condition presenting a different social valence.

Firstly, an individual *R. capensis* was flown alone, this also served as our control. As it has been predicted that modified pulses will be emitted in the presence of other bats, when the bat is flying alone it gives provides information about whether the modified pulses are deliberately emitted to a present bat or not. An additional prediction was that a bat flying on its own would not emit modified echolocation pulses. This is because, for communication to be successful, there should be an emitter of a pulse and a receiver of that same pulse. Secondly, individual *R. capensis* were flown while conspecific echolocation pulses were played back to it. This condition tests whether modified echolocation pulses are emitted solely to conspecifics or to all other bats. The playback pulses were a mixture of standard echolocation and modified echolocation pulses; modified echolocation pulses were added to induce the emission of modified echolocation pulses by the listening bat. In this condition, the prediction was that modified echolocation pulses would be emitted more frequently, relatively similar to when the bat is flying with a live conspecific individual in a flight room. Thirdly, the bat was flown with a heterospecific (*M. tricolor*) and the prediction tested was that modified pulses would be emitted but less frequently compared to when the bat is on a condition where it is interacting with a conspecific. Although bat species can recognize signal cues from heterospecifics, they spend most of their time with conspecifics and therefore are expected to communicate with conspecifics more than heterospecifics. Lastly, all *R. capensis* individuals caught in a night were flown together. The prediction tested was that the frequency of modified pulses emitted would be more than the frequency in which the bats emit modified pulses while flying with heterospecifics.

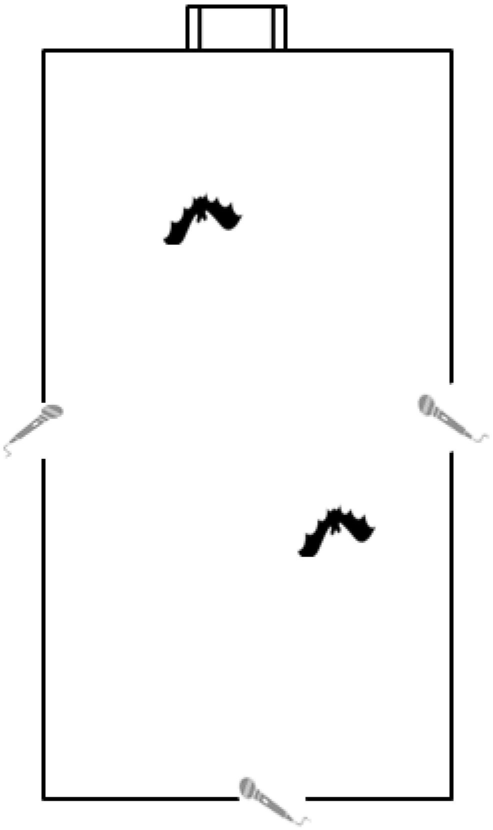

FIG. 2. A plan view schematic of the experimental setup for conditions on *Rhinolophus capensis,* to test the potential communicative function of modified echolocation pulses. The microphones in the diagram represent the microphones used to record the bats during the experiment and the box represents the entrance to the flight room.

2.3.4.2 Obstacle course

This experiment involved flying *R. capensis* individuals through an obstacle course to test whether the primary function of the FM component, which is ranging (Schnitzler and Kalko 2001), becomes impaired when there is a modification of the FM component in a pulse. The experiment requires *R. capensis* individuals to perform precise ranging since the flight room is small. The hypothesis in this condition was that the modification of the FM component does not impair the ranging function of the FM component.

Procedure and set-up

The experiment was conducted in February/March 2018. To create an obstacle, a 2 m×2 m sheet of cotton fabric was used to obstruct most of the flight room. A gap of 28 cm between the edge of the fabric and one wall of the flight room was left to allow the bat to pass through (Figure 3). This is slightly narrower than the average wingspan (28.8 ± 1.2 cm) of *R. capensis* (Jacobs et al. 2007) which it should have been able to negotiate provided ranging ability was not impaired. The fabric had bells sewn on the edges of the side towards the gap. This served as a notification to observers of the bat brushing the edge of the sheet in its attempt to negotiate the gap. The times that the bells rang were counted in each condition. This was used as a measure of the ranging challenge that the obstacle course presented to the bat during each condition. Four conditions were conducted each night with a maximum of five bats caught per night. The conditions therefore took up to two hours per night. The microphones were put in different parts of the flight room, with two of the microphones placed in the gap between the obstacle and the wall. The microphones placed on the obstacle gap were not at the same height, with one lower than the other and a space of 30 cm between them. This was to get most recordings from this gap since it is important to observe the pulses that the bats emit while passing through. Two camcorders were placed on opposite sides of the flight room to capture the activities of the bats while they negotiated the obstacle course.

The conditions started with a control condition in which *R. capensis* was flown alone, for ten minutes. The bat was allowed to negotiate its way through the obstacle course. The prediction tested was that no modified echolocation pulses would be emitted since the bat was flying alone. The bat will then manoeuvre the obstacle course with ease since not emitting modified echolocation pulses eliminates the probability of the FM component being impaired by the modification.

In the second condition, a single *R. capensis* was flown while a conspecific was placed in a small cage at the corner of the flight room. The prediction tested was that the caged bats would echolocate while the bat manoeuvres across the flight room. We made sure that the cage was in a condition that wouldn’t pose any harm to the bat. The small cage is made of timber, with soft steel nets making the walls and roofs the cage. The cage was hung on the ceiling of the flight room. The bat was released on the floor of the small cage, the surface of this floor is hard and therefore towels were lined on this floor to avoid the cage from injuring the bat. The bat used the cage wall as a perch.

In the third condition, two *R. capensis* individuals were flown together. The prediction tested was that modified echolocation pulses will be frequently emitted. Therefore, it will take time for the bat to manoeuvre through the obstacle course; provided that the modification impairs the ranging function of the FM component.

Lastly, the bat was made to fly while pulses from a conspecific were being played back. The playback speakers were placed at 1 m above ground on a table placed close to the side where the microphones were and opposite the entrance door (Fig. 2). The prediction tested was that that the modification impairs the ranging function of the FM component. If so, more modified echolocation pulses would be induced and the bat should also take more time to negotiate its way through the obstacle course.

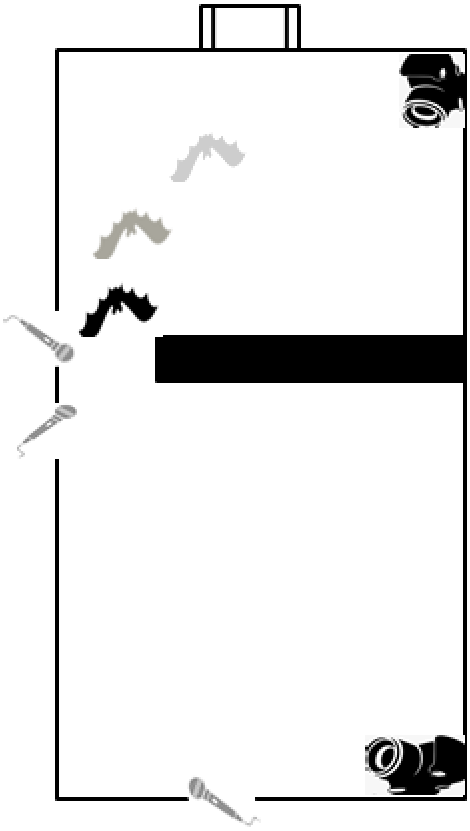

FIG.3. A plan view schematic of the obstacle course set up were *R. capensis* individuals were made to navigate their way through the obstacle course. The recording microphones were placed very close to the obstacle gap, to maximize the number of recordings obtained when the bat passes through the obstacle gap. The playback speakers were placed on a table stationed just before the flight room. Therefore, the speakers were close to the side where the microphones were and opposite the entrance door. The playback was played at 24dB at a distance of 1m from the flight room and a height of 1m from the ground.

2.3.5 Generating playback stimuli

The playback stimulus was generated using Avisoft SasLab pro software (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). Although this condition might be related to that of flying the bat with conspecifics, it eliminates the possibility of the bat seeing a conspecific and thinking it is an object. *R. capensis* pulses from different (N=12) individuals, were recorded on the first night of field work and were therefore used in the generation of the playback stimuli.

These pulses were assembled to form a 13-minute-long recording. Only pulses with a good signal-to-noise ratio were used. The pulses were used as they were, with minimal filtering. The only filtering done on these recordings was to remove the background noise from the recordings. BatSound Pro (version: 3.3.1.4; Pettersson Elektronik, AB, Uppsala, Sweden) was used to filter all the noise in the frequency of 27 kHz to 50 kHz. Whenever files were extracted for playback generation, the extraction was done using the same version of Audacity (version: 2.0.6.0, Audacity team, Pittsburgh, Pennsylvania), to avoid assembling files with different formats. The playback was played at 24dB at a distance of 1m from the flight room and a height of 1m from the ground.

2.4 The analysis of echolocation pulses

The recordings and species identity of the bats from which the recordings were taken was from the databases of David Jacobs and reported in publications emanating from his research group e.g. Dool et al (2016); Jacobs and Bastian (2018). Some hand-held recordings were obtained during this study (see 2.2). Bats recorded in the current study were classified to species by comparing the frequency of the CF component of their pulses and the locality at which they were captured with the CF frequency and distributions of known species obtained from Monadjem et al. (2010). Recording conditions in which individual bats could not be identified, such as during foraging and emergence, each pass was treated as a different individual, and bats were identified from CF frequencies and the locality. Echolocation pulses were recorded in the field in several different conditions, while the bats were emerging from roosts and while foraging. These recordings in the two conditions were done using two multiple microphone arrays each consisting of four microphones. The recordings were done for five nights, three hours per night. The microphones were connected to a Dell laptop (Latitude E7240) through an eight-channel Avisoft-Ultrasound Gate 816 recorder. In analysing the selected files, the total number of pulses that are in a selected file is summed up for the whole file. Depending on the type of microphone used, a single recording may have a different number of files of the same recording in different channels. Each microphone represents what it recorded in the form of a channel when viewed in Audacity. The settings on Audacity were set to mono and the sampling rate format was at 16-bit. The difference in the number of recordings in a file is because an eight-microphone array will have eight channels and a four-microphone array will have four channels. These are recordings of the same activity at the same time, this is why only a channel with good quality echolocation pulses was selected, saved as a wav file, and then analysed in BatSound pro software (version: 3.3.1.4; Pettersson Elektronik, AB, Uppsala, Sweden). In this case, the channels with good-quality echolocation pulses were chosen as the channels that had little to no background noise when viewed in an oscillogram format in Avisoft SasLab Pro (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). In the analyses of selected files using BatSound Pro, the echolocation pulses in the recordings were viewed in a spectrogram window, at a threshold of five and a time expansion factor of ten. The number of modified echolocation pulses was counted, as well as the number of standard echolocation pulses in a file that has countable pulses. In the modified echolocation pulses identified, it was noted whether the modification was on the initial or the terminal FM component of the pulse. From the spectrogram, two parameters were obtained viz. harmonic structure and shape of the echolocation pulse. Pulse duration was measured to the nearest 1 ms in the oscillogram on the pulse interval and pulse length command. In the command, an onscreen measuring cursor was used to measure the pulse duration using a time resolution of 1 ms; the second harmonic was used in the analyses. This is because bats in the Rhinolophidae and Hipposideridae families emit pulses that are dominated by the second harmonic (Jones, 1999). In a case where a recording has a high number of pulses, only the number of modified echolocation pulses within the sequence is counted manually and Avisoft SasLab Pro is used to count the total number of pulses in the entire recording, using the automatic measurements tool. Then the number of modified echolocation pulses was subtracted from the number of pulses in the whole sequence to obtain the number of standard echolocation pulses in a recording. To count the total number of pulses in a single recording, the noise during and in between pulses has to be cleaned; this is done by using the circle curser found on Avisoft SasLab Pro. There is noise with noticeable frequencies and they get counted as echolocation pulses in Avisoft SasLab Pro if not removed. After the cleaning process is done, the automatic measurement gives out the total number of pulses in the entire recording.

The number of pulses per timeframe was standardized to ten seconds using the formula. $\frac{\mathrm{X}}{\mathrm{Y}} \times \text{ time(s)}$. Because the different recordings had different durations, X is the total number of pulses (including all standard and modified echolocation pulses in a recording), Y is the duration of the recordings, and the time (s) used was ten seconds. To be able to perform comparisons across conditions, the recordings needed to be of the same duration. The ten-seconds timeframe is viable, as ten seconds of recording is considered a long sequence (Barataud, 2015) and sufficient.

Since the playback stimulus generated had not been edited extensively, it was then difficult to distinguish the playback pulses from those emitted by the bat in the experiment. To remedy this issue, the playback recording was studied in detail. The number of modified echolocation pulses on the playback stimulus was counted. This then meant that additional modified echolocation pulses on the trial recording were emitted by the bat in that particular trial.

In-flight room experiments, three microphones (Avisoft recorder, model 116H, supplied by Avisoft Bioacoustics, Glienicke, Germany) were used. The selection of files using Audacity had to be done for three channels. The number of modified and standard echolocation pulses was obtained in all conditions (bat flying alone; bat flying alone while echolocation pulses from a conspecific are being played back; bat flying with a heterospecific; and bat flying with a conspecific). The conditions were *R. capensis* was flown with other conspecifics led to the high number of pulses in a recording. This experiment used nineteen individuals of *R. capensis*. Due to flying *R. capensis* conspecifics caught in a night together, there were eight recordings of this condition instead of nineteen individuals compared to other conditions. Thereafter, eight recordings from each of the three conditions (flying an *R. capensis* alone, flying *R. capensis* with heterospecific, and playing back *R. capensis* conspecific pulses while an *R. capensis* individual is flying). For the obstacle course experiment, the proportion of standard echolocation pulses with only one FM component was compared with standard echolocation pulses with both FM components. This was done to determine if modified echolocation pulses follow the same pattern as that of standard echolocation pulses in terms of which FM component dominates the pulses.

Ten individuals from each condition, hand-held, forage, emergence, captive group, experiment 1, and obstacle course experiment were randomly selected. From the recordings of these individuals, the CF frequency of standard and modified echolocation pulses was measured. This was to determine if modified echolocation pulses have CF frequencies that differ from that of standard echolocation pulses.

The echolocation pulses in Fig 1 were extracted from pulses in recorded sequences using BatSound pro software, with a threshold of five. To extract these pulses, a pulse with a good signal-to-noise ratio (where the background noise is less) was selected and copied. Pulses were selected from all species surveyed in this study.

2.5 Statistical analyses

All statistical tests were done in Statistica (STATISTICA 10, 32-bit) using a 5% level of significance. To determine the statistical significance in the number of modified echolocation pulses emitted amongst rhinolophid species, a Kruskal Wallis test was used. This was followed by a post-hoc multiple comparisons test. The Kruskal-Wallis test was also used to compare the statistical significance of modified echolocation pulses emitted across the different conditions. Kruskal Wallis test was also used to test if the CF frequencies of standard pulses differed from the CF frequencies of the modified pulses; N=10 individuals from each condition were randomly selected and used for this analysis. A Mann-Whitney test followed for comparisons of the CF frequencies among the different conditions, to test if the modification of pulses caused a difference between frequencies of a standard echolocation pulse and that of modified echolocation pulses. A Friedman repeated measures ANOVA test was used to determine which condition (in conditions that presented different social valence (Experiment 1)) induces the emission of modified echolocation pulses and from these conditions, which one had the highest number of modified pulses emitted. Wilcoxon matched-pairs signed ranks test was used to determine differences between conditions.

3. RESULTS

3.1 A layout of the echolocation pulses observed

3.1.1 A structural description of the echolocation pulses observed

Three kinds of echolocation pulses were observed throughout the survey. The standard rhinolophid bat echolocation pulses (Fig 4a) and two kinds of modified echolocation pulses: pulses with modification of the terminal FM component (Fig 4b) and pulses with modification of the initial FM component (Fig 4c). The modifications consisted of an upsweep of the FM components on either the initial FM component or the terminal FM component but never on both FM components on the same pulse. Thus, modifications of the FM component were only present when both FM components were present (Fig 4b and 4c). There was no change in the CF component frequencies of *R. capensis* individuals in different conditions, regardless of the occurrence of modifications (Table 1). Differences in the CF frequencies between standard and modified echolocation pulses were not significantly different in all conditions (emergence, hand-held, captive group, forage, and experiment 1) N=10, p=1.00, emitted by 10 individuals of *R. capensis*.

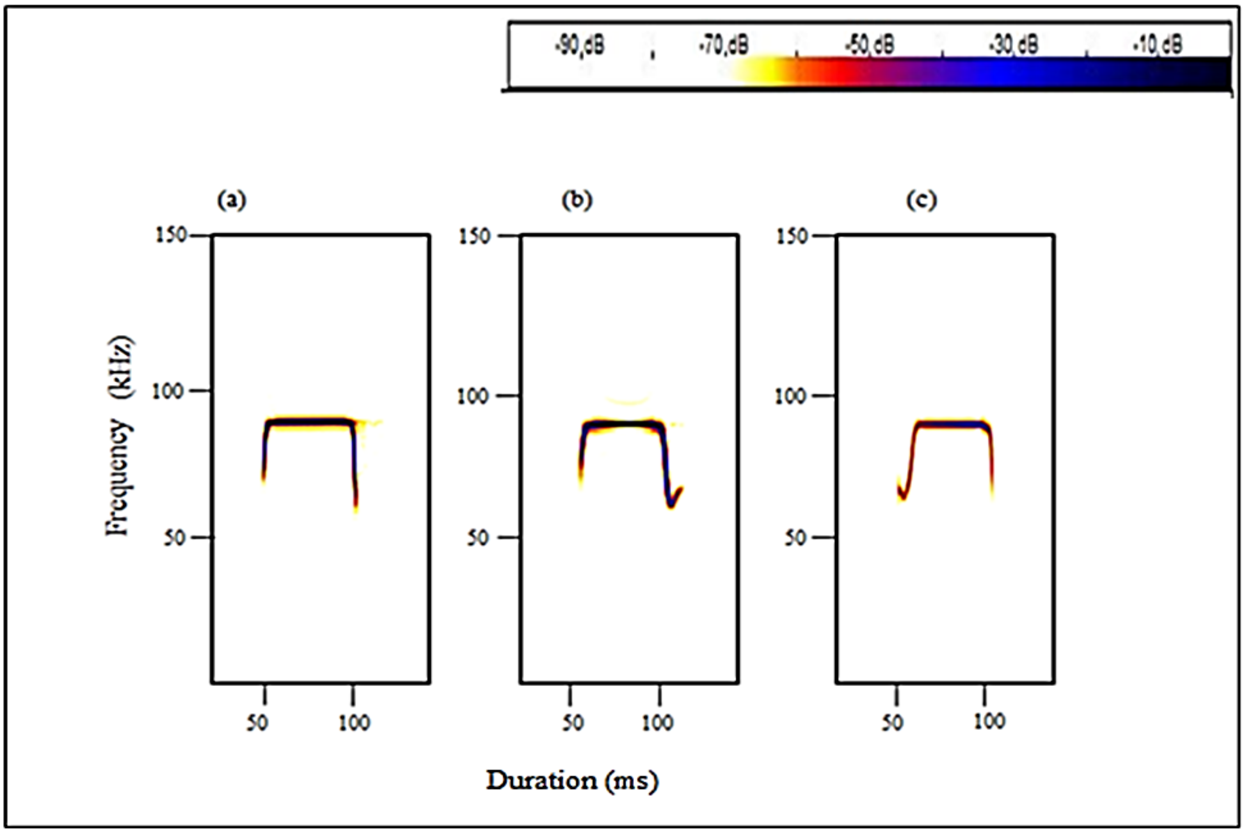

FIG. 4. Sound spectrogram (sampling parameters: threshold of five, a time expansion factor of ten, maximum frequency of 250 000 Hz FFT size 1024; Hanning window-left channel, Mono, 16 bitz at a time resolution of 1 ms) of different kinds of echolocation pulses from *Rhinolophus damarensis*, with a CF frequency of (89 ± 0.2 kHz); (a) a standard pulse, (b) a

modified echolocation pulse with the modification on the terminal FM and (c) a modified echolocation pulse with the modification on the initial FM.

TABLE 1. A comparison of Mean±SE values of the CF component, between modified and standard echolocation pulses of R. *capensis*. Individuals were randomly selected within different conditions; Colony (N= 10), emergence (N= 10), experiment 1 (N= 10), forage (N= 10), and hand-held (N= 10).

Conditions	**Modified pulse CF (kHz)**	**Standard pulse CF (kHz)**
	Mean±SE	Mean±SE
Colony	84±0.03	84±0.03
Emergence	90±0.15	90±0.17
Experiment 1	86±0.03	86±0.03
Forage	95±0.05	95±0.05
Hand-held	86±0.05	86±0.05

In a total of 1328 individuals/passes from 13 rhinolophid species, in different kinds of conditions considered in this study (hand-held, emergence, forage, captive group, conditions presenting conditions of different social valence (Experiment 1) and obstacle course, standard echolocation pulses dominated the recordings. Among modified echolocation pulses, modification of the terminal FM component was the dominant condition (Table 2). The modified echolocation pulses were either preceded by standard pulses or alternated with standard echolocation pulses or the two kinds of pulses occurred consecutively (Fig.5).

TABLE 2. A summary of the number, of different echolocation pulses emitted by individuals/passes of different rhinolophid bat species, in different conditions

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Total no of echolocation pulses	Total no of standard echolocation pulses	Total no of modified echolocation pulses	Total no of pulses with mod of the initial FM component	Total no of pulses with mod of the terminal FM component
143845	142736	1109	69	1040

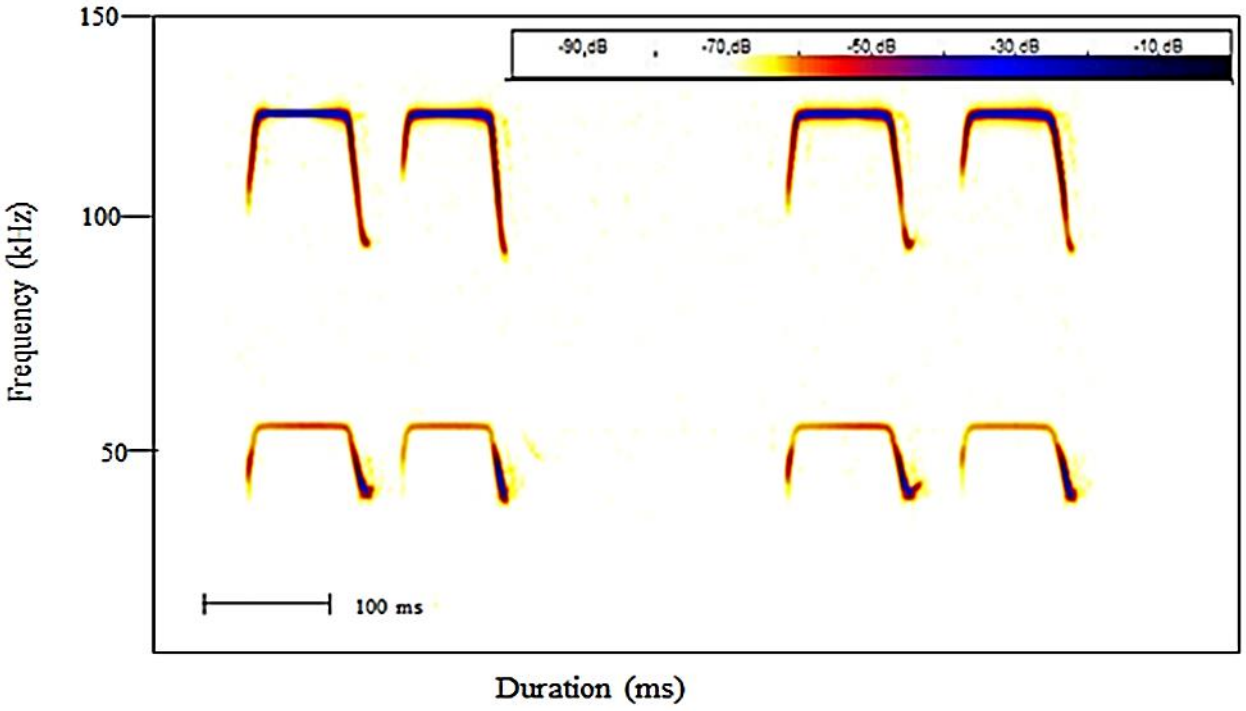

FIG. 5 Sound spectrogram (sampling parameters: threshold of five, a time expansion factor of ten, maximum frequency of 250 000 Hz FFT size 1024; Hanning window-left channel, Mono, 16 bitz at a time resolution of 1 ms) showing a sequence of four calls from the horseshoe bat, *R. denti* with a call frequency of 117 ± 0.2 kHz, in which a standard echolocation pulse is preceded and followed by a modified pulse.

3.2 The taxonomic distribution of modified echolocation pulses

3.2.1 The occurrence of modified pulses in different species of horseshoe bats and *Hipposideros caffer*

The analysis of hand-held recordings included a total of 912 individuals of known species. This included 896 individuals from 12 rhinolohid bat species and 16 *Hipposideros caffer* individuals. The modified echolocation pulses are widespread in the rhinolophid bat species, with 11 out of 12 species emitting modified echolocation pulses; *R. maendeleo* did not emit modified echolocation pulses. There were no modified echolocation pulses emitted by *Hipposideros caffer* individuals.

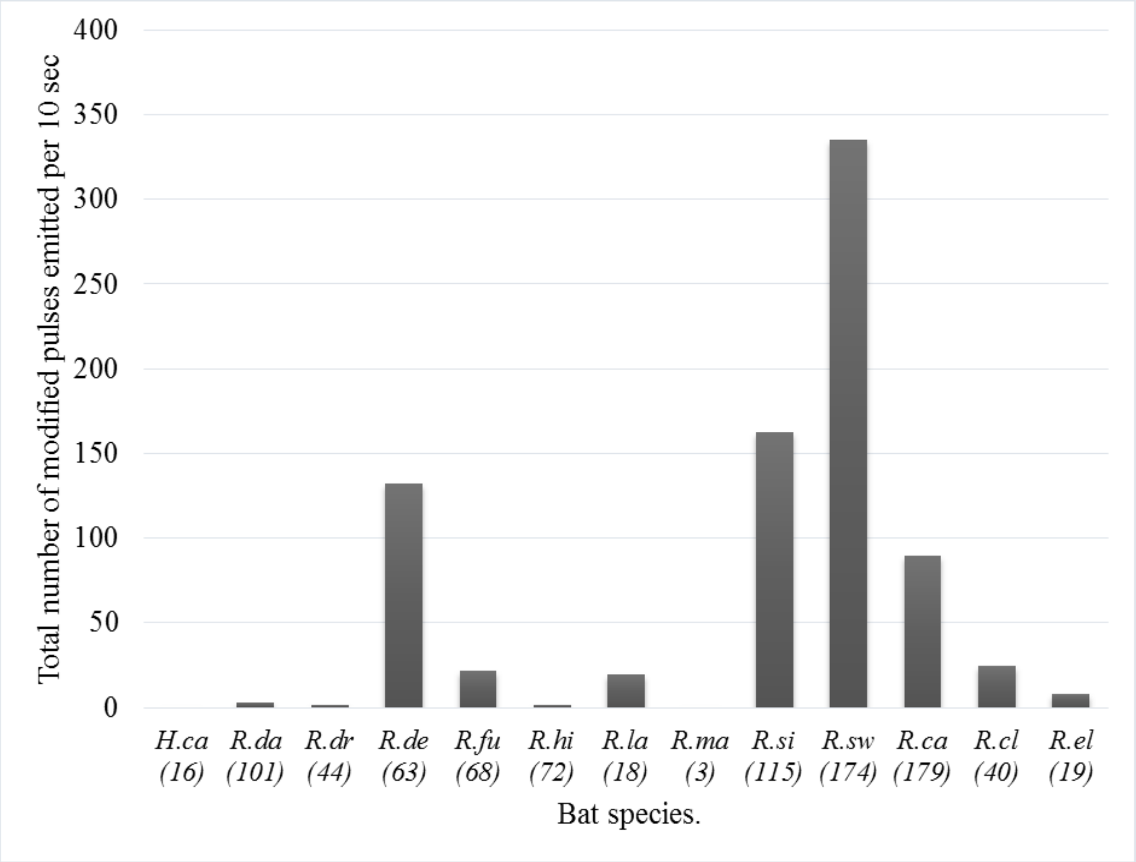

FIG. 6. The number of modified echolocation pulses in a ten seconds bin, emitted across 12 rhinolophid bat species and *Hipposideros caffer*. Sample sizes of the number of individuals surveyed are indicated in brackets, alongside each species. Abbreviations of the species are the same as in Fig. 1.

3.2.2 Phylogenetic affiliations of rhinolophid bat species emitting modified pulses

The 11 rhinolophid bat species found to emit modified echolocation pulses, were classified into different clades in the most recent phylogeny of southern African horseshoe bats (Dool et al. 2016). The species emitting modified echolocation pulses fell into four phylogenetic groups: the capensis group (*R. swinnyi, R. simulator, R. capensis* and *R. denti*), fumigatus group (*R. damarensis, R. hildebrandtii, R. darlingi, R. fumigatus* and *R. eloquens*), ferrumequinum group (R. *clivosus*) and the landeri group (*R. landeri*). Modified echolocation pulses were not emitted by the species *R. maendeleo* from the Adami group (Csorba et al. 2003).

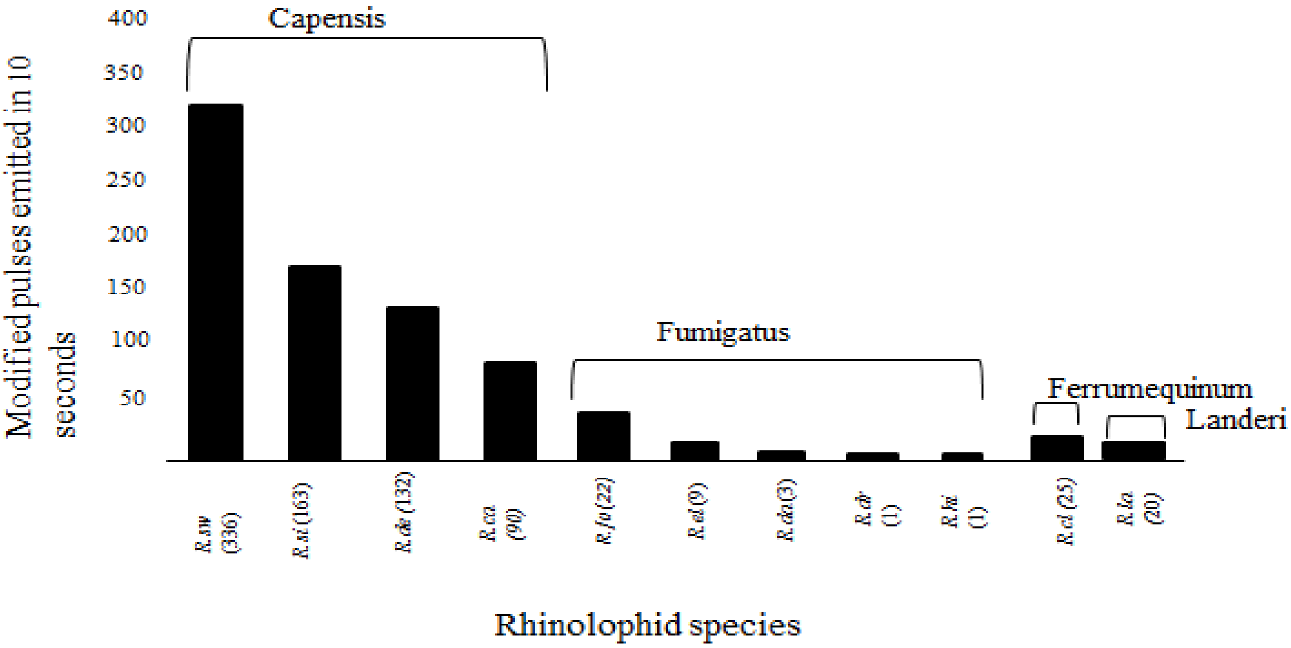

FIG. 7. The phylogenetic grouping of rhinolophid bat species that have been found to emit modified pulses; these species were classified and grouped based on Dool et al. (2016). In parentheses are numbers of modified pulses emitted by individuals of each species in 10 seconds, each block arc indicates the group in which different rhinolophid species have been classified. Abbreviations of the species are the same as in Fig. 1.

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3.2.3 The variation in the number of modified pulses emitted by rhinolophid species

There was a significant difference in the number of modified echolocation pulses emitted by hand-held individuals (N=893) of 11 rhinolophid species, H=244.40, 10 d.f., $p<0.05$ (Fig. 8).

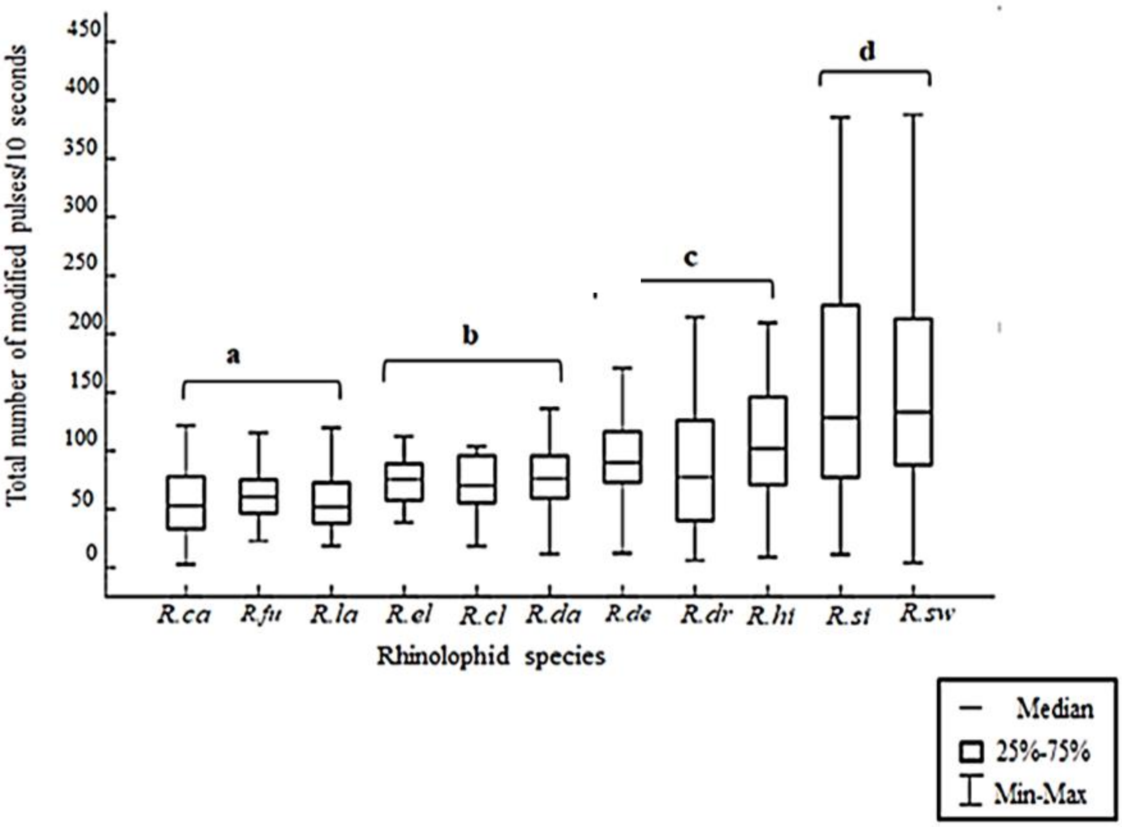

FIG. 8. A comparison of the (median/Quart/range) number of modified echolocation pulses. These pulses were emitted by 11 rhinolophid bat species. The small letters indicate the grouping of species based on multiple comparisons of the modified pulses. Species *R. ca*, *R. fu*, and *R. la* comprising group (a) are not significantly different from each other ($p>0.05$) but are significantly different from species in groups b, c, and d ($p<0.05$). Species *R. el*, *R. cl*, and *R. da* comprising group (b) are not significantly different from each other ($p>0.05$) but are

significantly different from species in groups a, c, and d ($p<0.05$). Species *R. dr*, *R. de*, and *R. hi* comprising group (c) are not significantly different from each other ($p>0.05$) but are significantly different from species in groups a, b, and d ($p<0.05$). Species *R. si* and *R. sw* comprising group (d) are not significantly different from each other ($p>0.05$) but are significantly different from species in the other groups ($p<0.05$). Abbreviations of the species are the same as in Fig. 1.

3.3 The variation in the number of modified echolocation pulses due to different conditions and sample sizes

3.3.1 Variation of modified echolocation pulses emitted by rhinolophid bat species across conditions

The number of pulses with modification of the terminal FM component was higher than that of pulses with modification of the initial FM component. This was true for all the different conditions. The hand-held condition had the largest number of modified echolocation pulses and there were no modified echolocation pulses emitted in the obstacle course experiment. The modification of the initial FM component was only emitted in the hand-held condition and not emitted in the other conditions.

TABLE 3. The number of echolocation pulses emitted by 11 rhinolophid species in six conditions; • indicates conditions where passes instead of individuals of known identities were used.

Conditions	No of individuals/ passes	Standard pulses	Modified pulses	
			Of the terminal FM	Of the initial FM
Handheld	893	96826	823	69
Emergence	225•	8167	26	0
Forage	130•	8622	187	0
Colony	23	1562	2	0
Experiment 1	19	3175	2	0
Obstacle course	19	24384	0	0

A total of 1309 individuals of rhinolophid bat species, obtained from five different conditions; there were significant differences in the number of modified echolocation pulses emitted across these conditions, N=1309, H= 907.02, 4 d.f, $p<0.05$ (Fig.9).

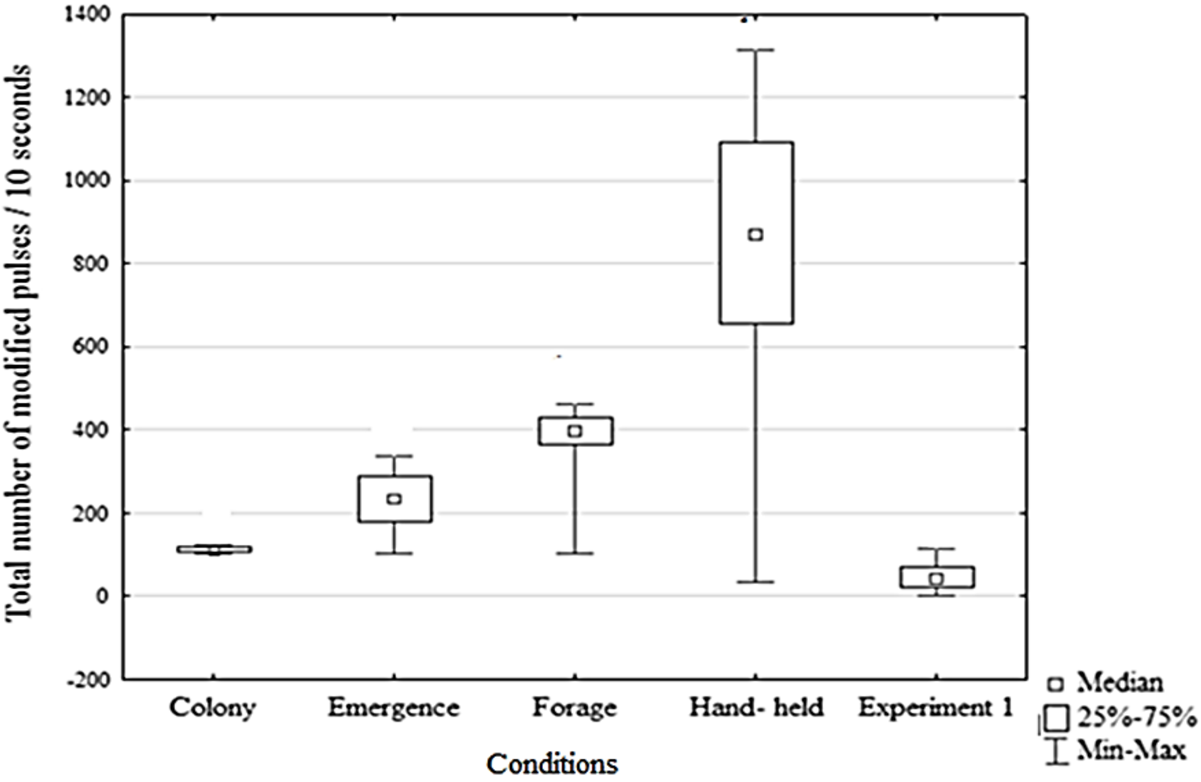

FIG. 9. The total number of modified pulses per 10s periods emitted by individuals/passes of rhinolophid species in five different conditions. With 130 passes of foraging bats, 225 passes of emerging bats, 893 hand-held bat individuals, and 19 *R. capensis* individuals from experiment 1. The modified pulses emitted in the Colony, Emergence, and in Experiment 1 are not significantly different from each other ($p<0.05$); but are significantly different from modified pulses emitted in Forage and Hand-held conditions ($p>0.05$).

3.3.2 An account for variation due to different sample sizes

To remove the effect of different sample sizes (Fig. 9), *R. capensis* individuals were randomly selected from each condition (hand-held, emergence, forage, captive group, and experiment 1). There was a significant difference in the number of modified echolocation pulses emitted in the five conditions ($H=18.09$, 4 d.f., $p =0.001$, Fig. 10).

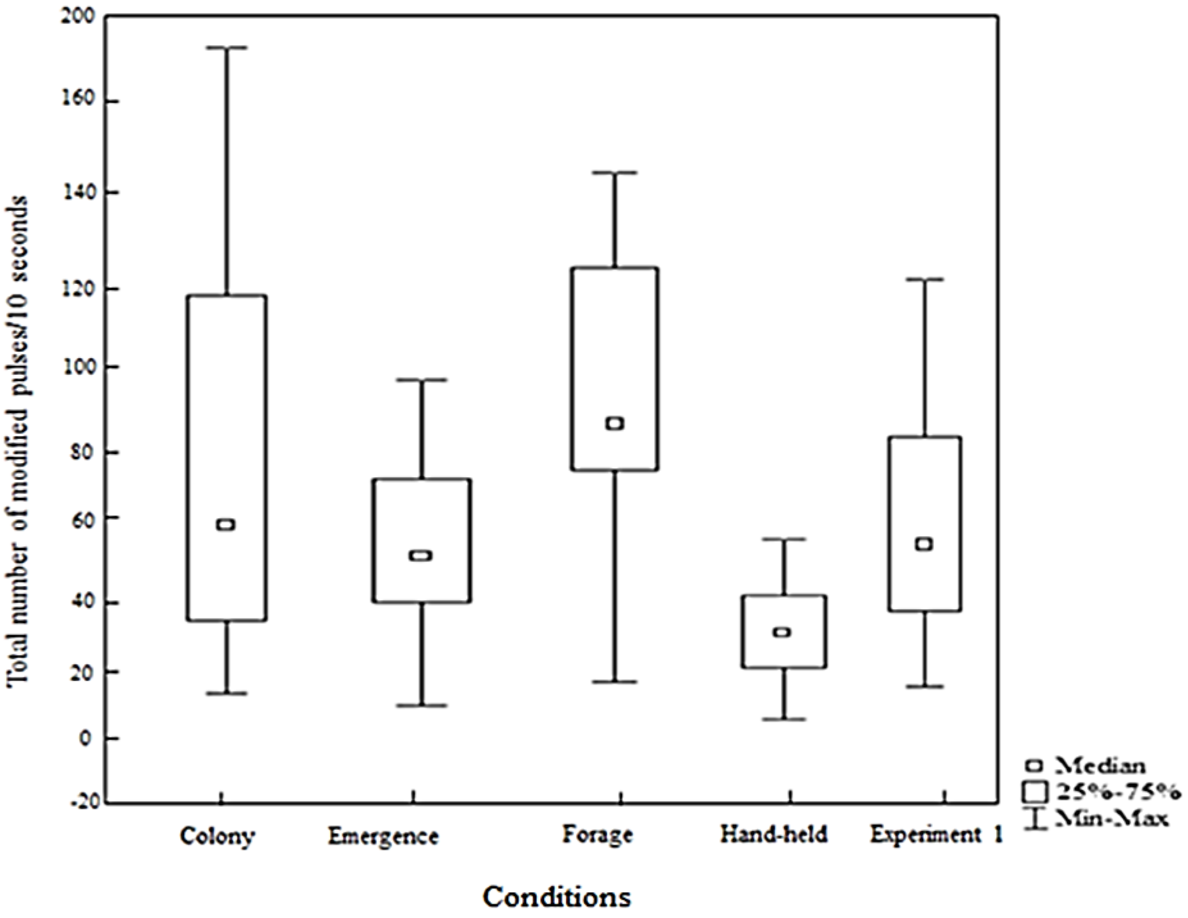

FIG. 10. A comparison of how frequently modified echolocation pulses were emitted by 19 *R. capensis* individuals in different conditions. The modified echolocation pulses emitted in the Colony, and during foraging are not significantly different from each other ($p<0.05$); but are significantly different from modified echolocation pulses emitted in Hand-held, Emergence and Experiment 1 ($p>0.05$). Additionally, modified echolocation pulses emitted in Hand-held, Emergence and Experiment 1 are not significantly different from each other ($p<0.05$).

3.4 Testing the social context in which modified echolocation pulses are emitted: Experiment 1(Conditions presenting different social valences)

In 19 individuals of *R. capensis* subjected to four different conditions, there was a significant difference in the number of modified echolocation pulses emitted by *R. capensis* across the four conditions (N=19, r=6.00, 2 d.f, p=0.04). *R. capensis* emitted modified echolocation pulses while flying alone. There was no significant difference in the number of modified echolocation pulses emitted when *R. capensis* was flying alone and flying while conspecific pulses were played back N=19, *r*=1.35, 1 d.f, p=0.25. *R. capensis* flying alone showed a significant difference with the number of pulses emitted when *R. capensis* flew with a heterospecific, N=19, *r*=0.473, 1 d.f, p= 0.04. The number of modified echolocation pulses was significantly lower when *R. capensis* flew with conspecifics than when *R. capensis* flew with a heterospecific N= 8, Z=2.52, p=0.01. There was no significant difference in the number of modified pulses emitted when *R. capensis* flew with conspecifics and conspecific pulses being playback N=8, Z= 0.140, p= 0.88 (Fig.11).

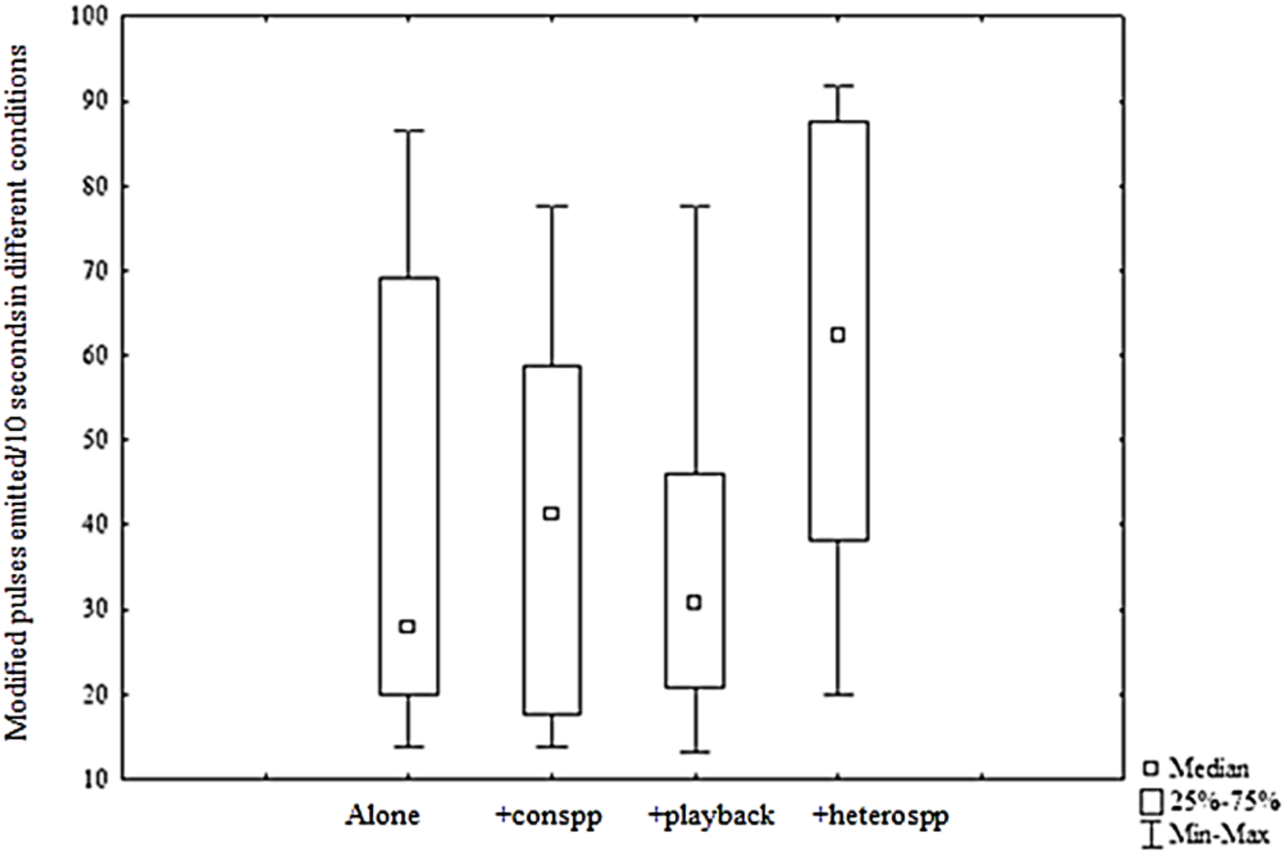

FIG.11. The number of modified echolocation pulses emitted in 10 sec when *R. capensis* was exposed to conditions that represent different social valences

3.5 Emission of modified echolocation pulses in a ranging condition: Obstacle course (Experiment 2).

In a total of 19 *R. capensis* individuals, 76 conditions (4 conditions per individual), and 24384 pulses emitted, there were no modified echolocation pulses emitted in the obstacle course. When *R. capensis* was made to fly alone, fly while there is a conspecific in a small cage, fly with a conspecific, and fly while recordings of conspecific pulses are played back; individuals reacted differently to different conditions. Although there were no modified echolocation pulses emitted by all bats on these conditions, there were instances in each condition where *R. capensis* was unsuccessful in navigating its way through the obstacle course (Table 4). The standard echolocation pulses emitted had more pulses with both FM components on the same pulse (Table 5).

TABLE 4. Illustrations of the behaviour of 19 individuals of *R. capensis* in the obstacle course experiment. A "✓" indicates that the bat navigated its way through the obstacle course. An "x" indicates that a bat failed to navigate its way through the obstacle course. "½" shows that only one bat flew through, and the "$^{2}/_{2}$" indicates that both bats flew through to the other side of the obstacle.

Individual ID	Alone	Caged conspec	Flying.Conspec	Conspec. Playback
R.ca 1	✓	✓	½ ✓	✓
R.ca 2	✓	✓	½ ✓	✓
R.ca 3	✗	✓	½ ✓	✓
R.ca 4	✓	✓	½ ✓	✓
R.ca 5	✗	✗	½ ✓	✓
R.ca 6	✗	✗	½ ✓	✗
R.ca 7	✓	✓	½ ✓	✓
R.ca 8	✓	✓	½ ✓	✓
R.ca 9	✓	✓	✗	✗
R.ca 10	✓	✓	$^{2}/_{2}$ ✓	✓
R.ca 11	✓	✓	½ ✓	✓
R.ca 12	✗	✗	$^{2}/_{2}$ ✓	✓
R.ca 13	✓	✓	$^{2}/_{2}$ ✓	✓
R.ca 14	✓	✓	$^{2}/_{2}$ ✓	✗
R.ca 15	✓	✓	$^{2}/_{2}$ ✓	✗
R.ca 16	✗	✗	✗	✗
R.ca 17	✓	✗	½ ✓	✗
R.ca 18	✗	✗	✗	✗
R.ca 19	✓	✓	$^{2}/_{2}$ ✓	✓

TABLE 5. Numbers of standard, one FM, and two FM echolocation pulses emitted by *R. capensis* flying an obstacle course exposed to four different conditions (Fig.3).

Conditions	**Standard pulses**	**Standard pulses with one FM**	**Standard pulses with two FM's**
1.*R. ca* alone	5200	2080	3120
2.*R. ca*+ caged conspecific	6990	2447	4543
3.*R. ca* + Flying conspecific	8090	2831	5259
4.*R. ca* + conspecific pulse playback	4100	1230	2870

4. DISCUSSION

The results indicate that that modified echolocation pulse may have an upsweep on the initial FM or final FM terminal, but never on both terminals of the frequency modulated components on the same pulse (Fig.4). All bats considered for this study except *R. maendelo* produced modified pulses. The absence of observed modified echolocation pulses from *R. mandeleo* may be due to the small sample size (N=3) collected for this species (Fig.6). Bats were able to suppress modified pulses when accurate distance ranging was required. This is to say, no modified pulses were emitted in the obstacle course experiment (Table 3) However, modified echolocation pulses were emitted in a social context. Moreover, these pulses were emitted when the bat was flying alone or hand-held (Fig 9; Fig.10; Fig.11).

A definition of modified echolocation pulses by Finger et al. (2017) indicated that the upsweep is on the terminal FM component. In this study, there were pulses with a modification of the initial component (Fig. 4; section 3.1.1). This then expands the definition of modified echolocation pulses, into a standard echolocation pulse with an upsweep on either the initial or terminal FM component. Modified echolocation pulses were emitted more when other bats were present, suggesting that they may be used in a social context (Fig 11; section 3.4). Results also suggest that modified echolocation pulses are not emitted in an echolocation context (section 3.5), possibly because the modification may interfere with the ranging function (Finger et al. 2017). Therefore this is to say, modified echolocation pulses may be deliberately emitted only when a horseshoe bat attempts to communicate with another bat. However, when in a flight room alone, R. *capensis* produced modified echolocation pulses (Fig 11; section 3.4); suggesting that the communication function of the modified pulses is unclear. However, the bat may have been attempting to communicate with bats flying outside the flight room. *R. capensis* had emitted modified echolocation pulses in other conditions (Fig.9; Fig. 10; Fig.11); for this reason, pulses with modifications may occur even when the bat is flying alone. From our experimental (experiment 1) results, *R. capensis* individuals emitted more modified echolocation pulses when flown with heterospecifics, than when flown with conspecifics (Fig. 11). These findings support our prediction that modified echolocation pulses will be emitted when another bat is present to enable communication. However, modified echolocation pulses being emitted more frequently in the presence of heterospecifics than with conspecifics, considering that in both cases *R. capensis* was in the presence of other bats, suggests that there may be other unknown factors for the emission of modified echolocation pulses.

4.1 Modified echolocation pulses and ranging function

Neuweiler et al. (1987) proposed that the initial and terminal FM components perform the same function and that they are used for precise ranging as in low-duty cycle bats. They are thus important parts of the signal, that allow for obstacle avoidance in flight (Neuweiler et al. 1987). Therefore, the ranging function of the FM component has to be maintained. It was hypothesized that the modifications impair the ranging function of the FM components. The prediction tested was that, if a modification occurs in a pulse, the pulse should have both the FM components present and the modification should not happen on both the FM components of the same pulse (Fig. 3). Correspondingly, species or individuals which emit pulses with one FM component only, should not emit modified echolocation pulses. Hence, the prediction that *H. caffer* should not emit modified echolocation pulses. All modified echolocation pulses with both FM components had one FM component modified and the other left unmodified. There were no simultaneous modifications of both FM components on the same pulse from our results, suggesting that the modification may interfere with the ranging performance of the bats' echolocation. This was also supported by *H. caffer* having no modifications to its single FM component pulses. Furthermore, when *R. capensis* individuals were flown in a simple obstacle course in a social context, they never emitted modified echolocation pulses, suggesting that the bats may have been trying to avoid compromising their ranging performance. The results suggest that modification of the FM component may impair the ranging function of this component. Leaving one FM component unmodified and modifying the other FM component in conditions with different social valences suggests that bats may be attempting to optimize the two functions of the FM components; ranging and communication.

The absence of modified echolocation pulses emitted in the obstacle course may imply that, when precise ranging is needed, modified echolocation pulses are not emitted. Even though there was variation in the numbers of standard echolocation pulses with one FM component in the different conditions, they all were less in number compared to standard pulses that had two FM components (Table. 5). This further indicates that there is preservation of the ranging function by horseshoe bats. Therefore when precise ranging is not needed, as opposed to an obstacle course that presents a small space to navigate; modified echolocation pulses may be emitted. This is quantified by the emission of modified echolocation pulses when bats are foraging; further strengthening the potential use of modified echolocation pulses by horseshoe bats, as a communicative signal. Some support for this is that modified pulses were predominantly emitted in the presence of other bats.

In spectrogram observations, whenever there was a missing FM component in a standard echolocation pulse, it was usually the initial FM component. This observation can therefore explain why there are fewer standard echolocation pulses with the initial FM component emitted by *R. capensis* on the obstacle course (Table 5). However, the observation is in contrast with the observation by Neuweiler et al. (1987) who observed that the initial FM component was always present in the signals emitted by horseshoe bats. Our observations are, however, in line with observations by Schnitzler et al. (1985), who also observed echolocation pulses of *Rhinolophus rouxi* that always had a terminal FM component. The initial FM component was only observed sometimes on these pulses. In addition, the presence of the initial or terminal FM component varied. Lastly, there was no regular pattern in the sequence in which standard and modified echolocation pulse were emitted. Modified echolocation pulses were found to be preceded by standard echolocation pulses, alternate with standard pulses or were emitted consecutively (Fig. 5).

4.2 The variation of modified echolocation pulses across conditions with different social valence

If modified echolocation pulses have a communication function, as we hypothesized, most of these modified echolocation pulses were predicted to be produced in a social context. The prediction tested was that modified echolocation pulses will be emitted when the horseshoe bat is in the presence of other bats and not when it is flying alone. Therefore, one would expect that these bats would not use modified echolocation pulses while at rest as it was predicted that the bat should preferably be in flight and instead use social calls for example (Peterson et al. 2018), or not use modified echolocation pulses when solitary. This is because echolocation pulses are mainly emitted by bats in flight (Griffin 1958; Griffin et al. 1960) and horseshoe bats already have a communication system that uses social calls. However, horseshoe bats also emitted modified echolocation pulses when hand-held and when flying alone such as in the experiment where the emission rates of modified echolocation pulses were tested in conditions that present different social valences. An observation from this study showed that many horseshoe bats emitted modified echolocation pulses when being hand-held by a human; for example, there was an adult female *R. capensis* that only emitted modified echolocation pulses when hand-held. Suggesting that modified echolocation pulses might also be emitted when the bat is in distress. Distress calls are vocalization of animals in conditions of extreme stress (Pfalzer and Kusch 2003; Russ et al. 2004) and have been described in various bat species (Pfalzer and Kusch 2003). Moreover, in bats, this condition involves the bat being physically constrained in any way (Pfalzer and Kusch 2003; Russ et al. 2004; Russ et al. 1998); for example, when a bat is caught in a mist net (Russ et al. 1998). Among a lot of distress call functions, they can be a call for help from conspecifics (Conover 1994; Koenig et al. 1991), give information about the predator to conspecifics (Conover 1994), and attract individuals to help scare off the predator (Neudorf and Sealy 2002) and also to startle the attacker. However, it is not clear at this stage why bats would use modified echolocation pulses while being held instead of their usual social calls (Pfalzer and Kusch

2003). It could be that when in stressful conditions, bats will use both social calls and echolocation pulses. Transitions of echolocation pulses to social calls have been observed in *R. ferrumequinum* emerging from a roost (Andrews and Andrews 2003) as well as in a captive group of *R. clivosus* while in tandem flight (Peterson et.al 2018). Hand-held pulses aside; modified echolocation was mainly produced in conditions where the bat was orientating viz. in the captive group, while bats emerged from the roost and while foraging. There could be benefits of emitting modified echolocation pulses in a condition such as foraging. The foraging bat will not have to switch from an echolocation pulse to a social call (Finger et al. 2017) while trying to communicate with other bats nearby. The modified echolocation pulse will function as a dual signal. This will then protect the bat from predators since echolocation pulses are not audible to most other mammals (Fenton 1982; Neuweiler 1984). Social calls, in contrast, are audible to other mammals (Fenton 2003). They are calls that involve social interactions (Peterson et.al 2018). The presence of other bats was observed during the analysis of the foraging dataset, where there were pulses from heterospecifics (*Miniopterus natalensis* and *Myotis tricolor*), and there were also pulses from conspecifics. (Jacobs 2015) indicated that echolocating bats fly in conspecific and heterospecific groups. Horseshoe bats also live in mixed roosts; this means they roost with heterospecifics and conspecifics (McDonald 1990). Modified echolocation pulses were also emitted when the bats were emerging from the roosts, also presenting a social context in which these pulses are emitted.

4.3 Distribution of modified echolocation pulses

Species that emitted modified echolocation pulses were widely distributed across the phylogeny of southern African rhinolophids from the more basal landeri group to the more derived capensis group, implying that the modified echolocation pulses have independently arisen in several southern African horseshoe lineages and therefore, modified echolocation pulses are not a trait that has evolved in a specific lineage. Echolocation itself may have evolved independently more than once in bats (Jones and Teeling 2006). It was essential to investigate how widespread modified echolocation pulses are in horseshoe bats. Having hypothesized that modified echolocation pulses are emitted directly for the potential use as a communication signal, the prediction surveyed was that they should be ubiquitous across species of horseshoe bats. This is to say, if these modified echolocation pulses are found in most of the species of rhinolophids, it then suggests that they are not randomly emitted. In my results, modified echolocation pulses are present in all but one species (*R. maendeleo*) of horseshoe bats that we studied. The species *R. maendeleo* was not classified by Dool et al. (2016) but was described by Csorba et al. (2003) as belonging to the Adami group. However, the absence of modified echolocation pulses in *R. maendeleo* is more likely than not due to the small sample size for this species (three individuals, Fig. 6). This is supported by my finding that sample size affects how the frequency of occurrence of modified echolocation pulses may be detected (section 3.5 on results). Determining how widespread modified echolocation pulses are in horseshoe bats shows that this modification could be emitted by all species of horseshoe bats. However this finding does not directly prove that the modified echolocation pulses are deliberately emitted but I could only speculate from knowing that modified echolocation pulses are not randomly emitted. The deliberate emission of modified pulses would have been more conclusive if there were no modified echolocation pulses emitted by hand-held bats and those flying alone. Considering that modified echolocation pulses were emitted more frequently in the presence of other bats, this gives the communication function a greater potential as the purpose of emitting modified echolocation pulses.

5. CONCLUSION

Modified echolocation pulses are widespread and emitted in social contexts suggesting that they are not randomly emitted and may be emitted while the bat attempts to communicate. The non-random emission of modified echolocation pulses strengthens the potential communication function of modified echolocation pulses. Although there were no modified echolocation pulses emitted by *H. caffer*, these modified echolocation pulses do not impair the ranging function of the FM component in horseshoe bats. It is only when precise ranging is required that they will not be emitted, such as in the case of *R. capensis* navigating the obstacle course and *H. caffer* which has a single FM component in its signal structure. The latter result may be due to *H. caffer* being unable to modify its single FM component without compromising the ranging function of its echolocation pulse. Rhinolophids appear to circumvent this problem by modifying only one FM component at a time and not modifying pulses with only one FM component. Together these results suggest a dual function for echolocation, orientation, and communication. At this stage, it is difficult to say that modified echolocation pulses have a definite communication function. The results are inconclusive since it was not clearly shown that bats deliberately emit the modified echolocation pulses to alter another bat's behaviour, which is the standard definition of a communication signal. They have indirectly implied non-random emission through results that they are widespread, however, when directly tested in the experiment that exposed *R. capensis* to social contexts with different social valences, *R. capensis* did not only emit modified echolocation pulses only when in the presence of other bats or flight but *R. capensis* also emitted modified echolocation pulses when hand-held and flying alone. The latter result renders our study inconclusive and more work needs to be done to fully understand why bats emit modified echolocation pulses. However, it is unlikely to be an artefact because bats were able to produce them in different situations and suppress them in others.I would recommend that further studies be done to give clearer and more information on whether modified echolocation pulses are deliberately emitted from the receiver to a sender. This could be done through experiments on horseshoe bats; to clearly and observe when modified echolocation pulses are emitted. However, there should be a relative distribution of data collected on different species. This is to avoid an irregular distribution of the data which may limit the findings of the study. Moreover, the observed modified pulses should be described quantitatively; this is to say, other characteristics of the modified pulses should be measured; to gather more insight about modified echolocation pulses.

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