To seek or speak? Dual function of an acoustic signal limits its versatility in communication

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The perception of different attributes of conspecifics is an integral part of intraspecific communication. It can facilitate the recognition of interaction partners or the assessment of potential mates. Acoustic signals can encode fine-scaled information through the interplay of acoustic variability and specificity. A reliable vocal signature is both unique within a class and variable between classes. Therefore, acoustic complexity might be associated with the number of classes to be discriminated. We investigated the assumption that limitations to signal design may affect the communicative functionality of a signal. To do so, we chose a signal with potentially dual functionality which may therefore display such limitations. In bats, echolocation is used primarily for foraging and orientation but there is increasing support for its additional role in communication. An acoustic analysis of echolocation pulses of the bat *Rhinolophus clivosus* confirmed sex and individual vocal signatures in echolocation pulses. A habituation—dishabituation playback experiment suggested that bats perceived these signatures because listening bats clearly discriminated between the sexes (two classes) and between individuals (representatives of a multiclass category), although to different degrees. The simple acoustic structure of these vocalizations provides sufficient specificity for sex discrimination but has limitations for individual discrimination because pulse parameters of individuals increasingly overlapped with increasing group size. We conclude that selection for the primary function of echolocation restricts the acoustic space available for communication. However, we frequently observed echolocation pulses with conspicuous structural modifications. Statistical analyses revealed that these vocalizations yielded increased individual distinctiveness. Such added systematic variation may indicate a communicative function and perhaps a signalling intent of the emitter, although the latter has yet to be tested. The findings suggest that the required specificity for effective communication could be obtained through modification of echolocation variants when adaptations for orientation and foraging constrain the evolution of complex communication signatures.

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Acoustic signals are important vectors in animal communication systems and are used by many group-living taxa to mediate social interactions. A functional communication system relies on unambiguous and specific signals whose coding system is shared between communication partners (e.g. Bradbury & Vehrencamp, 2011). Such communicative specificity is often achieved through the acoustic structure of the signal. Distinct features of the acoustic signal may encode vocal signatures (e.g. species identity),

contextual cues (e.g. courtship calls) or arousal states (e.g. levels of urgency). Therefore, an effective communication signal should feature both specificity and structural flexibility to allow the communication of more complex information. This idea has been incorporated in the 'social complexity hypothesis' (see Freeberg, Dunbar, & Ord, 2012; Oller & Griebel, 2008). A corollary to this is that limitations in signal flexibility may limit the complexity of information conveyed. Such limitations may arise, for example, as a result of trade-offs in signals that have more than one function. Multiple functions can arise from the co-option of traits that had evolved in the context of one function being used to serve an additional function.

Co-opted traits that are used in communication are often visual or olfactory signals, for example bird plumage (Cowen, 2005;

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Gluckman & Cardoso, 2010) or urine scent marking (Gosling & Roberts, 2001), respectively. Acoustic signals, on the other hand, have almost exclusively evolved for communication and therefore co-option of vocalizations for additional functions are rare. Two exceptions to this are sounds emitted by some insects and echolocation pulses emitted by bats. For example, the ultrasonic clicks of some moths in the subfamily Arctiinae evolved in response to bat predation (e.g. Dunning, Acharya, Merriman, & Ferro, 1992; Jacobs & Bastian, 2017; Miller, 1991) but have apparently been co-opted for use in intraspecific communication (e.g. Sanderford & Conner, 1990). Bats and dolphins (and a few bird species and shrews) use acoustic signals for orientation and foraging in the form of echolocation (Brinkløv, Fenton, & Ratcliffe, 2013; Siemers, Schauermann, Turni, & von Merten, 2009; Thomas, Moss, & Vater, 2004) and recent research has suggested that bat and dolphin echolocation may also be used in communication (e.g. Gregg, Dudzinski, & Smith, 2007; Jones & Siemers, 2011); thus, it might be a trait with two functions. In multifunctional traits, one or more functions may be limited from exhibiting an optimum adaptive response (Hansen, 2015). That is, optimization of the trait for one function may limit optimization of that trait for the other function/s, resulting in a trade-off (Garland, 2014). In the case of bat echolocation, acoustic signals that have primarily been selected in the context of orientation and foraging may not have the requisite structural flexibility of communication signals. Echolocation therefore provides an excellent opportunity to study signal design in general and the association between structural complexity and communicative versatility.

Among the different types of echolocation systems that evolved in bats, high duty cycle echolocation (HDC; relatively long pulse durations compared to silent periods between pulses) is a specialized form of echolocation that enables bats to detect small flying insects even within dense vegetation using only acoustic cues (Schnitzler & Denzinger, 2011). These echolocation pulses are characterized by a long constant frequency component (CF) of narrow bandwidth which is flanked by frequency modulated sweeps (FM) of broad bandwidth. The FM components allow precise range determination and exact target localization through neuronal processing of the time elapsed between the emission of the pulse and the return of the echo (Simmons & Stein, 1980). Echoes of the CF component carry information about the target via acoustic glints. When the CF component reflects off the fluttering wings of an insect, the returning echo carries distinctive peaks in frequency and intensity, the so called 'acoustic glints'. These glints are caused by Doppler shifts in frequency as a result of the motion of the wings and changes in the effective reflective area of the wings when they are at different angles to the impinging echolocation pulses, respectively (Neuweiler, 2003). Although these glints can be minute, down to ±20 Hz modulation depth (Ostwald, Schnitzler, & Schuller, 1988), bats obtain detailed information about the target velocity and direction of movement (Neuweiler, 1990). The long duration of the CF component enables these bats to discriminate between different types of insects by analysing the pattern of sequential glints caused by distinct wing beat cycles (Nachtigall & Moore, 1988). Furthermore, the frequency of the CF component is tightly linked to the specialized auditory pathway of these bats which contains an 'auditory fovea'. The fovea is an area of increased sensitivity and high resolution of a narrow range of frequencies that can detect and integrate the minute modulations of the glints (Neuweiler, 1990). Thus, the relatively simple acoustic structure of FM-CF-FM echolocation pulses is adapted to the central perceptual tasks associated with habitat and foraging mode (Schnitzler & Denzinger, 2011). This structural optimization may

constrain the encoding of additional vocal cues and thus the communicative function of these vocalizations.

Nevertheless, echolocation is consistently discussed in a communication context (e.g. Barclay, 1982; Heller & von Helversen, 1989; Siemers, Beedholm, Dietz, Dietz, & Ivanova, 2005). The assumption of a dual function is supported by (1) the ability of bats to eavesdrop on echolocation pulses of other bats to extract information about e.g. feeding sites, (2) the occurrence of private frequency bands between sympatric species and (3) the existence of self-reporting signatures in echolocation pulses (see Jones & Siemers, 2011 for an overview). The fact that echolocation pulses of some species carry intra- and interspecific signatures encoding attributes of the emitter such as age or species affiliation (reviewed in Jones & Siemers, 2011) makes them candidates for communication signals. Recent playback experiments provide evidence that HDC bats can perceive these vocal signatures by discriminating between species, populations, the sexes and body condition on the basis of echolocation alone (Bastian & Jacobs, 2015; Knörnschild, Jung, Nagy, Metz, & Kalko, 2012; Lin, Liu, Chang, Lu, & Feng, 2016; Puechmaille et al., 2014; Schuchmann, Puechmaille, & Siemers, 2012; Voigt-Heucke, Taborsky, & Dechmann, 2010).

Our aim in this study was to test the limitations that multifunctionality and the potential resultant trade-offs impose on acoustic signals, particularly in a communication context. We proceeded by selecting categories of information (such as sex and individual identity) that are likely to be informative for bats in a communication context and likely to be represented in echolocation pulses by vocal signatures. Vocal signatures can represent different levels of recognition entities, ranging from e.g. species to group and down to individual recognition. Each level contains a different number of classes that need to be discriminated if signatures are to be reliable identity cues. We chose sex and individual as categories as they differ in the number of classes they contain. We experimentally tested the perception of the signatures of sex (male versus female, two-class category) and individual identity (pairwise tests of three random individuals from a pool of many individuals, representative of a multiclass category) by listening bats. We then compared the distinctiveness of the two-class category of sex with the multiclass category of individual identity based on the acoustic structure of the emitted vocalization.

Our model species, Geoffroy's horseshoe bat, *Rhinolophus clivosus*, like other species of horseshoe bats (Rhinolophidae), uses high duty cycle FM-CF-FM echolocation pulses. It roosts in groups of variable numbers of conspecifics comprising tens to hundreds of individuals with seasonally fluctuating sex ratios (McDonald, Rautenbach, & Nel, 1990; Monadjem, Taylor, Cotterill, & Schoeman, 2010). It also occurs sympatrically and syntopically with other species of horseshoe bats (Monadjem et al., 2010; Schoeman & Jacobs, 2011). Accordingly, it has been repeatedly shown that horseshoe bats can discriminate between their own species and heterospecifics based on echolocation pulses (Bastian & Jacobs, 2015; Li et al., 2013; Schuchmann & Siemers, 2010). Therefore, species-specific communication channels might be important in this species making it well suited for investigating limitations imposed on a communicative signal as a result of multifunctionality. Recent research (Raw, 2016) indicates that species discrimination is based on a single-class category level, i.e. the recognition of its own species. A potentially meaningful two-class category is sex, where differences in acoustic structure exists between two states, male and female. Acoustic sex identification is found in various vocalizing taxa including insects (von Helversen & von Helversen, 1997), birds (Cure, Aubin, & Mathevon, 2011; Stirnemann, Potter, Butler, & Minot, 2015) and

mammals (Honorof & Whalen, 2010; Rendall, Owren, Weerts, & Hienz, 2004). Individual identity is a much finer-scaled category which requires higher specificity. Although little is known about the social structure of most horseshoe bats, many bat species form stable social groups including individual-based relationships (Kerth, 2008; Kulzer, 2005; Möhres, 1967; Ortega, 2016). Finding interaction partners after foraging separately can be one of the functions making individual discrimination relevant.

We hypothesized that in HDC bats, such as *R. clivosus*, echolocation parameters may be used to discriminate between conspecifics but higher levels of variability in echolocation required for the communication of complex information may be limited by its adaptation to foraging and orientation. To test our hypothesis, echolocation pulses of *R. clivosus* were used in habituation–dishabituation playback experiments to test whether bats could discriminate between males and females (two-class category) as well as between individuals (three pairwise tasks of three different individuals as representatives of a multiclass category) based on echolocation pulses. We included a multiparametric analysis of echolocation pulses to identify acoustic parameters that potentially encode vocal signatures. There are strong indications that the FM components are the carriers of vocal signatures in horseshoe bats (Bastian & Jacobs, 2015; Jones & Siemers, 2011; Raw, 2016; Schuchmann et al., 2012); therefore, we specifically analysed these FM components. We frequently observed echolocation pulses with conspicuous upward sweeps at the terminal FM component which we analysed separately to test whether this added acoustic complexity may lead to increased individual specificity of echolocation pulses.

METHODS

Study Animal and Study Site

Rhinolophus clivosus has a widespread geographical range in Africa and Arabia (Csorba, Ujhelyi, & Thomas, 2003). Like most horseshoe bats, *R. clivosus* is a perch hunter emitting FM-CF-FM echolocation pulses (Neuweiler, 1989; Voigt, Schuller, Greif, & Siemers, 2010) with frequencies of the CF component ranging between 90 and 93 kHz when at rest (i.e. resting frequency, RF) in South Africa (Jacobs, Barclay, & Walker, 2007; Fig. 1).

At the study site, Guano Cave in De Hoop Nature Reserve in the south of the Western Cape Province, South Africa (−34.4540°. 20.3994°), *R. clivosus* occurs in groups of variable numbers of individuals (McDonald et al., 1990) and with four other bat species of which one is another horseshoe bat, *Rhinolophus capensis* (Jacobs et al., 2007; McDonald et al., 1990).

Capture and Husbandry of Bats

Rhinolophus clivosus bats were captured with hand nets throughout the year in 2013 (March to June, September, November, December) and during the austral summer of 2014 (January, February, May). Sex and age were determined visually on site (Kunz, 1990). All pregnant, lactating or juvenile bats were released immediately. Forearm length is a proxy of body size in bats (Guillén, Juste, & Ibañez, 2000; Safi, Meiri, & Jones, 2013) and was measured instead of mass because mass varies diurnally and seasonally (Neuweiler, 2000; dial callipers to the nearest 0.1 mm).

Bats used in playback experiments in the field at De Hoop were housed at the De Hoop Nature Reserve in a nylon tent (105 × 105 and 200 cm high) placed in a dark room. Bats had free access to water but were also given water twice daily using a pipette and hand fed 45–55 mealworms (*Tenebrio molitor* larvae) a night. The mealworms were raised on a combination of dry dog food, mineral powder (Life-Gain), fresh fruit, vegetables and wheat bran,
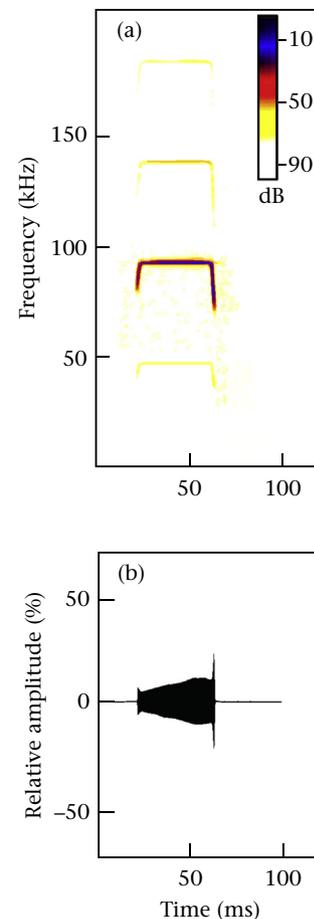

Figure 1. Visual presentation of an echolocation pulse in the form of (a) a sonogram and (b) an oscillogram of a pulse emitted by an individual *Rhinolophus clivosus*.

ensuring that bats obtained the essential nutrients they require (S. Schmidt, personal communication; Kunz, 1990; Wilson, 1990). Nevertheless, the health of the bats was monitored daily. Each bat was kept in captivity for no longer than 5 nights and then released back into the cave.

Playback experiments were also done at the University of Cape Town (UCT) on two sets of captive colonies. Here, bats were kept in captivity for about a month as part of a separate study. They were housed in a netted enclosure (3.3 × 3.0 and 5.0 m high) inside an observation room where they were able to fly freely. The bats were examined by a registered veterinarian upon arrival and before they were returned to the cave to ensure that they were healthy. Observers wore protective clothing while in the room to prevent the introduction of pathogens. Controlled temperature and humidity conditions (24–25 °C and 50–80% relative humidity) were kept within the ranges of the cave (Laycock, 1983; personal records) and day and night cycles of light were shifted (night: 0700–1900) to allow observation of active bats during the daytime cycle of observers. Bats had free access to water and food (mealworms) but were also hand fed daily. The diet was occasionally supplemented with black soldier flies, *Hermetia illucens*.

Ethical Note

All capturing, handling, housing and treatment of bats followed the guidelines recommended by the American Society of Mammalogists (Gannon & Sikes, 2007), adhered to the ASAB/ABS Guidelines (ASAB/ABS, 1997) and were in compliance with both the

Animal Ethics Committee of UCT (2013/v13/DJ) and Cape Nature (AAA007-00009-056). In addition, researchers were trained in laboratory animal ethics (A.B. FELASA-B certificate; N.F. UCTs internal course on laboratory animal science).

Recording and Analysis of Echolocation Pulses

Echolocation pulses of *R. clivosus* individuals from De Hoop Nature Reserve were recorded from bats held 30 cm in front of the microphone of an ultrasound D1000X detector (Pettersson Elektronik AB, Uppsala, Sweden) with a medium gain and a sampling frequency of 384 kHz. The acoustic measurements were taken from the dominant second harmonic of the pulses because it has the best signal-to-noise ratio (Möhres, 1953). The first 10 pulses of a recording were never used because horseshoe bats tune into their resting frequencies from lower frequencies after periods of silence (Schuller & Suga, 1976; Siemers et al., 2005). Pulses were selected from the remaining sequence, at any position in the recording, based on their quality (good quality: high signal-to-noise ratio). We selected 10 good-quality pulses per individual. To test whether 10 pulses provided sufficient coverage of pulse variation, the variances of the acoustic parameters of 10 good-quality pulses from an individual were compared to 20 good-quality pulses of the same individual (Levene's test) and repeated for three different randomly selected individuals. Furthermore, previous work on echolocation pulses showed low within-individual variation between pulses (see Jacobs et al., 2007; also reviewed in Jones & Siemers, 2011).

Multiple acoustic parameters were measured using the automatic measurement function in Avisoft SASLab Pro (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). We measured resting frequency (RF; frequency of maximum amplitude of the CF component; kHz), pulse duration (duration of the pulse from beginning to end; ms), interpulse interval (time between the end of one pulse and the beginning of the next; ms) and time between the start of the pulse to the maximum amplitude of the pulse (distomax; ms). On the initial and terminal FM components, we measured minimum frequency (kHz), maximum frequency (kHz) and duration (time from the beginning of the FM component until the beginning of CF component, time from the end of the CF component to the end of the FM component; ms). From these measurements, we calculated duty cycle (pulse duration/(pulse duration + interpulse interval) × 100, expressed in %), bandwidth (maximum frequency − minimum frequency; kHz) and sweep rate (bandwidth/duration; kHz/s) of the initial and terminal FM components. Since the FM components may be more flexible and variable across sex and individuals than the CF component, and therefore more likely to contribute to communication functions, we calculated the coefficients of variation ((standard deviation/mean) × 100) for the acoustic parameters associated with the FM and CF components. We also analysed modified echolocation pulses from a subsample of the same 88 individuals and the same set of hand-held recordings. In these modified echolocation pulses the terminal FM component ends in an ascending appendage (Fig. 2). We observed this type of modified pulse frequently in hand-held recordings of different *Rhinolophus* species including *R. clivosus*. We confirmed that modified echolocation pulses are indeed part of the natural repertoire and not artefacts of the hand-held recording situation, by locating them in emissions of groups of free-flying bats in captive colonies (data collected from a separate study) as well as in recordings of bats inside the experimental box used in the study. In addition to the parameters measured for normal echolocation pulses, we also measured the sweep rate, maximum frequency and bandwidth of the ascending appendage of the modified component.

To identify sex and/or individual signatures encoded in echolocation pulses we carried out a multivariate general linear model
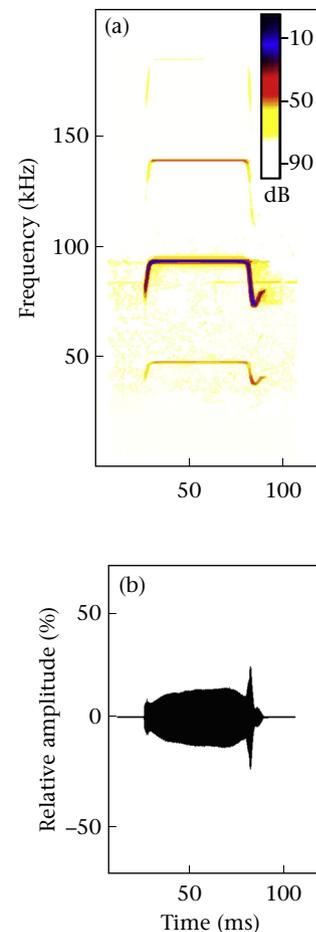

Figure 2. Visual presentation of a typical modified echolocation pulse of *Rhinolophus clivosus* in the form (a) a sonogram and (b) an oscillogram of a pulse emitted by the same individual that emitted the echolocation pulse in Fig. 1.

(GLM) on the acoustic parameters. In the case of sex-specific signatures, the mean values of the 10 pulses for each individual were used. Sex, individual identity and body size were used as categorical variables (independent) and the acoustic parameters as the dependent variables. We included body size to consider potential allometric effects on acoustic features (Fitch, 2006; Titze, 1994).

Habituation−Dishabituation Playback Experiment

Using a habituation−dishabituation playback experiment (Eimas, Siqueland, Jusczyk, & Vigorito, 1971; McGregor, 2000) we tested whether bats can discriminate between the sexes and/or between different individuals based solely on echolocation pulses. During this experiment a test subject was habituated to one stimulus class (for example to pulses of males) until it no longer showed a behavioural reaction towards the stimulus, i.e. it became habituated. The stimulus class was then changed (for example to pulses of females) and the behaviour of the bat during and after this switch quantified. Resumption of activity (i.e. dishabituation) such as ear or head movements or echolocation activity after the change of stimulus class shows the bat discriminated between the stimuli classes by perceiving differences in acoustic properties.

Procedure and set-up

Experiments were done in the field at De Hoop in November, December and May 2013 and at the University of Cape Town in

January and February 2014. Experiments occurred at night at De Hoop and during the day, when night was simulated, in the captive colony at UCT.

Experiments took place in an anechoic experimental box (77.5 × 38 cm and 38 cm high) following Schuchmann and Siemers (2010) and Bastian and Jacobs (2015). It contained a perch (small straw basket) for the bat to hang on and, at the opposite end of the box, there was a speaker (USG Player Light, Avisoft, Glienicke, Germany), an ultrasound detector (D1000X, Pettersson Elektronik AB, Uppsala, Sweden), and an infrared-sensitive camera (Sony DCR-SR42E Minato, Tokyo, Japan) all pointing at the perch. The camera was connected to a monitor outside the box allowing the researcher to observe the behaviour of the bats.

Each bat listened to five different trials in consecutive nights. A trial was one experimental session in which a bat was exposed to one unique combination of stimuli as explained below (Table 1). The randomization of stimuli combinations across nights and test subjects ensured that a bat listened to a different test stimulus every night and entered the experiment at a different time each night.

The experimental procedure followed that established by Bastian and Jacobs (2015). Once the bat was calm on the perch (i.e. inactive) for at least 20 s, a playback of echolocation pulses from the same class (one individual or one sex) was played back to habituate the bats to this stimuli class (habituation stimulus; Table 1). Complete habituation was defined as the bat hanging calmly on the perch and remaining motionless for 20 s during habituation playback. After the bat became habituated, the stimulus class was changed (dishabituation stimulus) to either a test stimulus (different individual or sex) or control stimulus (same individual or sex). The playback of the dishabituation stimulus was followed by a rehabituation stimulus (pulses from the same class as the habituation stimulus), then white noise and finally a low-frequency sound (beep; see Fig. 3 and 'Controls' below).

Generation and design of playback stimuli

The playback stimuli were generated following Bastian and Jacobs (2015) using echolocation pulses from 41 bats from De Hoop (22 males and 19 females) recorded prior to the experiments. As for the acoustic measurements described previously, only pulses with good signal-to-noise ratio, which occurred after the tuning-in phase (Schuller & Suga, 1976; Siemers et al., 2005), were selected. These natural pulses were used as templates to create semisynthetic pulse copies (Avisoft SASLab Pro) that excluded any noise or recording artefacts. Each semisynthetic pulse was used with its natural interpulse interval when compiling the different playbacks. To account for simple pseudoreplication (Hurlbert, 1984; McGregor, 2000) where a listening bat memorizes a specific individual instead of forming a template for 'female' or 'male' or memorizes a specific set of pulses instead of forming a template for the sex or individual

in general, the maximum number of individuals (i.e. 38 individuals for the sex discrimination trials) or pulses per individuals (i.e. 100 pulses for the individual discrimination trials) was used. The order of pulses in the playback files was randomized by assigning a random number to each pulse, then sorting via this random number and using the resultant sequence to order the pulses in the playback files.

Playbacks for sex discrimination trials were created using echolocation pulses from either 19 males or 19 females with 10 pulses per individual. Two 10 min habituation playback files were made by randomly mixing pulses from these males or females, respectively. The dishabituation stimuli were 20 s long and made by using the same pulses used in the habituation stimuli but compiled in a different random order. The last 40 s of the playback sequence (rehabituation phase) also contained pulses from the same individuals used in the habituation but again compiled in a different random order.

The four different playback stimuli for individual discrimination trials contained pulses from three different male individuals (100 pulses per individual). One 10 min habituation playback file was made by randomly mixing 100 pulses from Individual 1. A 20 s dishabituation playback file was created for each of the three individuals using 100 pulses from each of Individual 1 (as the control), Individual 2 and Individual 3 (as the tests). The rehabituation phase was constructed from the same pulses from Individual 1 (habituation) but compiled in a different random order.

All playbacks were normalized to the same intensity level. To determine an adequate intensity level, the intensity was adjusted to two-thirds that of a recording taken of a bat echolocating spontaneously on the perch inside the box with the microphone at the opposite end at medium gain. The first pulse in each playback file was faded in and out as was the white noise and low-frequency beep to prevent a noise burst often produced by the loudspeaker when starting a playback with a sudden onset of sound.

Both sex and individual rehabituation playbacks ended with white noise (3 s) and a low-frequency sound (5 kHz, 1 s; Fig. 3). The low-frequency sound (beep) allowed synchronization of time on video and sound recordings by matching the audio line on both recordings. The white noise and the rehabituation sequence served as a controls for false negative responses (see next section).

Controls

In the analysis of playback experiments, the behaviour of animals can be misclassified due to false positive responses (i.e. a regain in activity of the bat is misinterpreted as a discriminatory response to a stimulus) and/or false negative responses (i.e. a lack of activity is misinterpreted as no discrimination). Control trials (Table 1) and the rehabituation stimulus (Rendall, Rodman, & Emond, 1996) controlled for false positive reactions to make sure that any reaction to the dishabituation stimulus was not due to spontaneous recovery from habituation. The white noise at the end

Table 1
Combinations of playback classes presented to each bat in the habituation–dishabituation experiments

	Trial 1 Control	Trial 2 Sex test	Trial 3 Control	Trial 4 Individual test 1	Trial 4 Individual test 2
Female listeners					
Habituation	Female	Female	Individual 1	Individual 1	Individual 1
Dishabituation	Female	Male	Individual 1	Individual 2	Individual 3
Rehabituation	Female	Female	Individual 1	Individual 1	Individual 1
Male listeners					
Habituation	Male	Male	Individual 1	Individual 1	Individual 1
Dishabituation	Male	Female	Individual 1	Individual 2	Individual 3
Rehabituation	Male	Male	Individual 1	Individual 1	Individual 1

Trials 1 and 2 are sex discrimination trials and trials 3, 4 and 5 are individual discrimination trials. Each trial contained three playback phases (habituation, dishabituation and rehabituation) with either the same (control) or different playback classes (test).
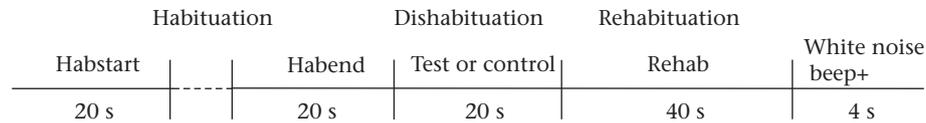
Habituation		Dishabituation	Rehabituation	
Habstart	Habend	Test or control	Rehab	White noise beep+
20 s	20 s	20 s	40 s	4 s

Figure 3. Scheme of the habituation–dishabituation experiment. Depicted is the general schemata of a playback sequence and the different time phases that were analysed.

of each rehabituation playback was used to control for false negatives. This ensured that any lack of reaction to the test or rehabituation stimulus was not due to experimental fatigue or sensory habituation (Pancratz & Cohen, 1970). Trials in which bats did not react to the habituation stimulus or white noise were repeated on a different night.

Acoustic characterization of playbacks

We tested whether the subsample of pulses used to compile the male and female playbacks were indeed representative pulses of the population of *R. clivosus* bats from De Hoop. To gauge the position of the playback pulses within the acoustic space of the pulses of bats from the sample site, we used the a priori classification method of discriminant function analyses (DFAs). In addition to the classification of pulses based on their acoustic structure, the DFAs indicate which acoustic parameters discriminated between the pulses of individuals or sexes the most, i.e. which pulse parameters carried vocal signatures that could allow bats to discriminate between the stimuli. Because the set of acoustic parameters may result in multicollinearity, we first did principal components analyses (PCAs). The extracted factor scores of uncorrelated components were entered into the DFAs as variables. Only principal components with eigenvalues >1 (Kaiser criterion) were used in the DFA.

To analyse the sex discrimination playbacks, all pulses used from the 38 individuals in the dishabituation playback were entered into a DFA (males: $N = 149$; females: $N = 165$). Similarly, all 300 pulses of the three individuals used for the individual discrimination playbacks were used. The degree of acoustic similarity/dissimilarity between the sexes or the three individuals was assessed with the test of significance of the squared Mahalanobis distances from the DFAs. DFAs were also used to test the distinctiveness of individual-specific signatures in different group sizes, namely 6, 12, 18, 24, 30, 36, 42 and all 88 individuals sampled from De Hoop. We used a regression with replication to assess whether the group size can predict classification success rates of echolocation pulses. In this analysis, a regression line is fitted using least-squares regression for replicated y-values on transformed data (Zar, 2007). We ran nine replicates for each of the group sizes besides the 88-individual group by randomly subsampling individuals (R package 'MASS'; R Core Team, 2012; Rstudio Team, 2015; Venables & Ripley, 2002) to account for a possible sampling uncertainty, i.e. the bias caused by the set of individuals selected.

To test whether modified echolocation pulses provide stronger individual signatures then the regular echolocation pulses due to their increased acoustic complexity of the FM modification, the classification success of five echolocation pulses from 19 individuals (14 males and five females) was compared to the classification success of five modified echolocation pulses from the same individuals. Classification success of individuals' pulses of both types (echolocation and modified echolocation) were compared using a Wilcoxon matched-pairs test. This analysis was done on the maximum number of individuals from which we had good-quality pulses of both echolocation and modified echolocation pulses.

Analysis of Experiments

Videos of the behavioural responses of the bats during playback experiments were coded by one observer on SolomonCoder (András Péter, http://solomoncoder.com; v14.03.10) using a frame-by-frame analysis with 25 fps. We analysed four time sections in each trial, the first 20 s of the habituation stimuli (habstart), the last 20 s of the habituation stimuli (habend), 20 s of the test stimuli (dishab) and the first 20 s of the rehabituation stimuli (rehab) (Fig. 3).

Validation procedures

Prior to analyses, all audio and video recordings were renamed with random numbers to ensure the observer was unaware of the stimulus presented to avoid observer bias during coding. To test for interobserver bias, 10 randomly selected videos were coded by a second observer and frequency and duration of behaviours were tested (Kappa coefficients or Wilcoxon matched-pairs test). Within-observer reliability was tested when the main observer reanalysed the same 10 videos at the end of the study (Kappa coefficient and Wilcoxon matched-pairs test). The effect of sensory habituation on the reactions of bats participating in multiple trials over multiple nights (repeated measures) was tested with a Friedman ANOVA. This was done by comparing the duration of attentive behaviours during habstart per order (first, second, third, fourth and fifth trial a bat listened to) across individuals and nights. A Mann–Whitney U test was used to test whether female and male bats differed in the duration of reactions during the four time sections (habstart, habend, dishab, rehab) to determine whether the data could be pooled. Besides the visual observation during the experiments, we verified that the bats were habituated, by comparing the duration of attentive behaviours at the beginning (habstart) and end of the habituation playback (habend) using a Wilcoxon matched-pairs test across all trials. We tested for false positive reactions by comparing the duration of attentive behaviours during habend and rehab for each trial using a Wilcoxon matched-pairs test. False positive reactions were tested for with the control trials using a Wilcoxon matched-pairs test comparing the duration of attentive behaviours during habend and dishab.

Discrimination tests

Quantitative data, i.e. frequencies and durations of behaviours, were coded for the 20 s of each of the four sections in the trials: habstart, habend, dishab and rehab (Fig. 3). Prior to analysis, an ethogram of the behaviours observed in captive groups of *R. clivosus* bats was compiled (Table A1 in the Appendix). To classify the behavioural reactions of the bats to the acoustic stimuli we grouped behaviours into three categories: attentive, active and inactive. Inactive behaviours were described as the bat not engaging in any movement. Active behaviours included tip of ear twitch, single ear twitch, head swaying, crawl, partial wing stretch, full wing stretch, vibrating wings, body shuffle, body swaying and grooming. Attentive behaviours were differentiated from active behaviours in that they indicated a direct reaction to the stimulus and were therefore considered an attentive reaction. These behaviours include orientation behaviours (head lift, scan, echolocate), listening behaviours (slow and rapid ear twitching) and startle behaviour (leg contraction). Attentive behaviours were further subdivided into weak, medium or strong reaction categories to describe the strength of the reaction of an individual to the test stimulus (Table A1 in the Appendix).

Whether or not bats reacted to the habituation, dishabituation, rehabituation and motivational stimulus was recorded as categorical binary yes or no answers. We also recorded whether bats emitted echolocation pulses in response to the playback (BatSound, Pro Pettersson Elektronik AB, Version 3.31a, Uppsala, Sweden; sampling rate of 384 kHz, 16 bits mono, slowed down 10 times).

The number of bats that emitted echolocation pulses to the dishab of the test trials was compared to the control trials for both the sex discrimination (Wilcoxon matched-pairs test) and individual discrimination trials (Friedman ANOVA and post hoc Wilcoxon matched-pairs test with a Bonferroni correction (α-Bonferroni)). To test whether bats discriminated the pulses in the test stimulus from the pulses in the habituation stimulus, the durations of attentive behaviours for habend versus dishab for the test trials were compared. Yes and no reactions to both the dishab and rehab parts of the trials were used to compare test and control trials using Pearson chi-square tests.

General Data Analysis

All statistical tests were two tailed with a 5% level of significance and done in Statistica (Statsoft Inc., Tulsa, OK, U.S.A.), SPSS (IBM Corp., Armonk, NY, U.S.A.) or in R (Rstudio Team, 2015). Nonparametric tests were used when parametric tests were not suitable. For GLM, PCA and DFA statistics, the acoustic parameters were log-transformed to account for the different measurement scales and orders of magnitude difference in the range of the data. For regression analysis on percentages, we used the arcsine transformation (Zar, 2007) of the square root of the y-values.

RESULTS

Acoustic Characterization of Echolocation Pulses

In addition to the 37 individuals tested in the experiments, we analysed echolocation pulses of the 41 individuals used to generate the playbacks and an additional 10 individuals from our database resulting in a total of 88 *R. clivosus* (45 males and 43 females) from De Hoop (see Table A2 in the Appendix). The echolocation pulses had a mean peak frequency of 91.7 ± 0.5 kHz with durations of 36.1 ± 8.7 ms. The terminal FM part was usually present but the initial FM was sometimes absent (see Table A2 in the Appendix). An analysis on the coefficient of variation for the FM versus CF components showed that the resting frequency of the CF component had a much lower coefficient of variation (0.55%) than the parameters of the FM components (range 6.41–190.54%).

Sex signatures

Males and females differed in the acoustic structure of their echolocation pulses and in body size (GLM: $F_{13,74} = 4$, $N = 88$, $P < 0.01$; Tukey post hoc HSD for unequal N: $P < 0.001$). The mean length of the forearms of females were 1.5 mm longer than that of males (Tukey HSD: $P < 0.01$) and the mean durations of the initial (INT) and terminal (TM) FM components were 0.7 and 1.1 ms longer, respectively (INT: Tukey HSD: $P = 0.031$; TM: Tukey HSD: $P = 0.009$) with 1.6 Hz lower terminal sweep rates (Tukey HSD: $P = 0.027$; Table A2 in the Appendix). Despite the size difference, males and females did not differ significantly in their resting frequencies (Tukey HSD: $P = 0.852$) or durations of their echolocation pulses (Tukey HSD: $P = 0.705$; Table A2 in the Appendix).

A PCA on pulses from 38 individuals used to compile the sex discrimination playbacks yielded five principal components which accounted for 83% of the variance in the pulses (Table A3 in the Appendix). Despite males and females having similar resting frequencies and CF pulse durations (Table A2 in the Appendix), a DFA

on the five PCs correctly classified pulses as male 76% of the time and as female 80% of the time (DFA: Wilks's $\lambda = 0.592$, $F_{5,307} = 66.460$, $P < 0.001$; test of significance of the squared Mahalanobis distance: 2.75, $F = 42.389$, $P < 0.001$). The results of the DFA showed that principal components 3 and 1 (composed of acoustic parameters on the initial and terminal FM components) accounted for most of the variation in echolocation pulses (Table A4 in the Appendix).

The playbacks of male and female echolocation pulses used as stimuli in this study were typical representatives of males and females. There was no significant difference between the acoustic parameters of the pulses used in the playbacks and pulses from the larger population of individuals from De Hoop (GLM: $F_{11,33} = 3$, $N = 88$, $P < 0.05$; Tukey HSD for unequal N: $P < 0.001$).

Individual-specific signatures

Individual bats ($N = 88$) from De Hoop differed acoustically from each other (GLM: $F_{11,957} = 10.81$, $P = 0.000$). However, the classification success of 35% for all 88 individuals was lower than the 72% for the three individuals used in the experiment (DFA: Wilks's $\lambda = 0.012$, $F_{87,785} = 11.68$, $N = 88$, $P < 0.001$; Tables A5 and A6 in the Appendix).

Five principal components from a PCA on the acoustic parameters of echolocation pulses used to compile the playbacks for the individual discrimination trials ($N = 3$ bats, $n = 300$ pulses) accounted for 84% of the variation between the pulses of these three individuals (Table A7 in the Appendix). A DFA on these five principal components correctly classified 72% of the pulses (DFA: Wilks's $\lambda = 0.60$, $F_{10,1346} = 42.389$, $P < 0.001$; Table A8 in the Appendix). A canonical analysis revealed that Root 1 accounted for 77% of the explained variance in the data and is largely made up of principal component 5 which is mainly associated with resting frequency (Tables A7 and A8 in the Appendix). The standard deviations of resting frequency were small but variable for each of the three individuals (Individual 1: mean $\pm$ SD = 91.9 ± 0.05 kHz; Individual 2: 91.4 ± 0.18 kHz; Individual 3: 90.4 ± 0.27 kHz). Root 2, which is largely made up of principal components 4 and 2 and therefore acoustic parameters of the terminal FM components (Table A7 in the Appendix), accounted for 22% of the variance in the data (Table A8 in the Appendix). Squared Mahalanobis distances indicated that Individuals 2 and 3 were acoustically more similar (test of significance of the squared Mahalanobis distance: 2.27, $F_{5,293} = 22.4$, $P < 0.001$) than individuals 1 and 2 (test of significance of the squared Mahalanobis distance: 3.88, $F_{5,293} = 38.265$, $P < 0.001$). Individuals 1 and 3 were the most acoustically different individuals (test of significance of the squared Mahalanobis distance: 6.129, $F_{5,293} = 60.469$, $P < 0.001$). This difference is also reflected in the different classification success of pulses for each individual (Individual 1: 83%; Individual 2: 64%; Individual 3: 69%) with most misclassifications occurring between Individuals 2 and 3. As a result of the increased misclassifications between the acoustically more similar representatives of the multiclass category, the overall classification success was lower for individual classification (72%) than for sex classification (78%).

In acoustic space the echolocation pulses of the 37 bats that listened to the playbacks, clustered more closely with those of Individual 2 (test of significance of the squared Mahalanobis distance: 3.96, $F_{5,329} = 25.41$, $P < 0.001$) and 1 (test of significance of the squared Mahalanobis distance: 5.18, $F_{5,329} = 32.36$, $P < 0.001$) than with those of Individual 3 (test of significance of the squared Mahalanobis distance: 11.28, $F_{5,329} = 70.96$, $P < 0.001$; Fig. 4, Tables A9 and A10 in the Appendix).

A DFA on echolocation pulses from different numbers of individuals subsampled randomly from the total of 88 individuals forming groups of 6, 12, 18, 24, 30, 36 and 42 individuals showed

that classification success of pulses to the correct caller decreased as group size increased (Fig. 5). The subsequent regression analysis confirmed that group size can predict classification success rates (regression with replication: $R^2 = 0.808$; $y = 93.06 - 1.17x$; $F_{1,62} = 257.50$; $P < 0.001$). In addition, classification success also depended on which individuals were included in the analysis and the similarity of these individuals' pulses.

The comparison of the occurrence of modified echolocation pulses from the three recording situations showed that on average 9% of the pulses were modified echolocation pulses when hand-held, 6% when free-flying and 5% when in the experimental box. These calls were embedded within a series of echolocation calls that did not have the modification or occurred as second calls in the typical doublet echolocation pattern suggesting that modified pulses were not recording artefacts. Of the hand-held recordings from the 88 *R. clivosus* individuals, 33 individuals emitted modified echolocation pulses. However, only 19 of the 33 recorded individuals had at least five pulses with good signal-to-noise ratios that we could use in the analysis. These structurally more complex echolocation pulses with a modified terminal frequency component (Fig. 2) had considerably higher classification success rates than regular echolocation pulses emitted by the same individuals in the same set of recordings (Wilcoxon matched-pairs test: $Z = 3.07$, $N = 19$, $P = 0.002$) increasing from 41% to 76% (Fig. 6, Table A11 in the Appendix). Thus, when acoustic complexity increased classification success also increased. The acoustic analysis of modified echolocation pulses identified maximum frequency of the modified component as well as a combination of the parameters: resting frequency of the CF component, the duration of the modified appendage and to a lesser extent of the minimum frequency of the initial FM component and the sweep rate of the modified component as main contributors to individual specificity (Tables A12 and A13 in the Appendix).

Acoustic Discrimination Experiments

Data basis

A total of 37 bats (22 males and 15 females) participated in all sex and individual discrimination experiments (185 trials).
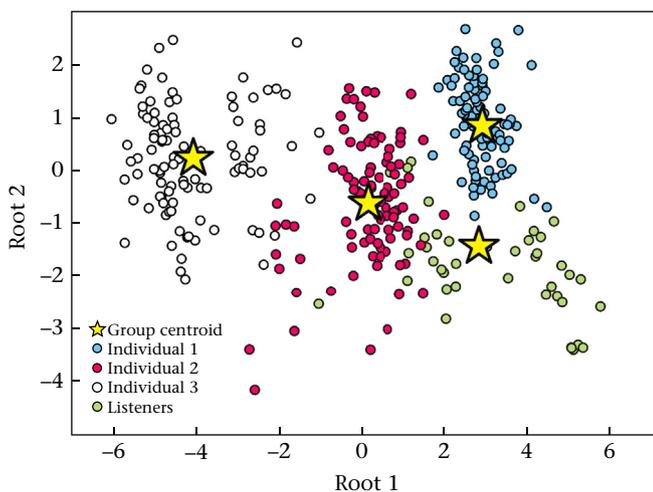

Figure 4. Acoustic space of the different playbacks in relation to each other and to pulses of the listening bats. The pulses of the three individuals used to generate the playbacks and the averaged pulses of the bats listening to the playbacks are shown. The acoustic space is defined through the results of a discriminant function analysis which was based on multiple acoustic parameters. Canonical scores for Roots 1 and 2 are plotted. Each playback individual is colour-coded (see key), whereas the averaged pulses of the listening bats are shown as white circles. The asterisks are the group centroids.
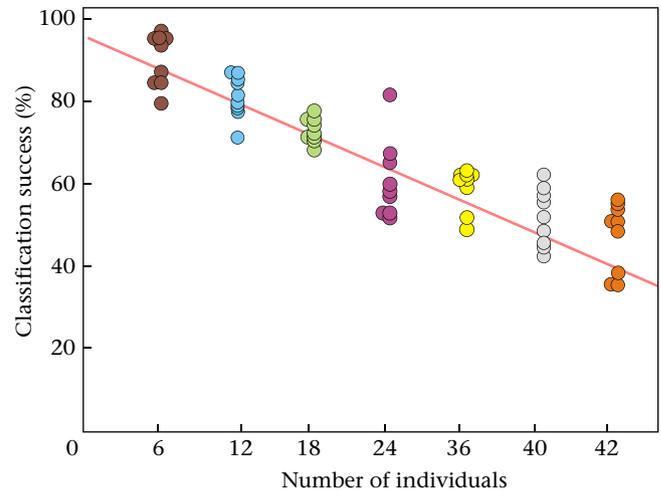

Figure 5. The effect of group size on individual classification success rates of echolocation pulses. The classification success rates from discriminant function analyses of echolocation pulses are shown for different numbers of individuals. Classification success rates were calculated for nine random subsamples per group size. Each group size is colour coded. A regression analysis was done on arcsine transformed data ($y = 93.06 - 1.17x$; $P < 0.001$; $R^2 = 0.808$).
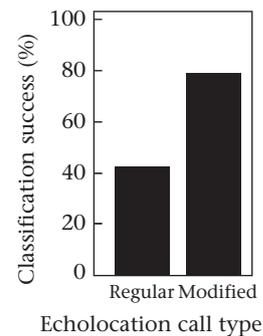

Figure 6. The effect of acoustic modifications of echolocation pulses on classification success. Classification success rates are shown for regular echolocation and modified echolocation pulses from the same 19 individuals (five pulses each and per individual) based on a discriminant function analysis of multiple acoustic parameters.

Analysable data on categorical data (yes and no reactions) and strength of reaction (weak, medium, or strong reaction) to dishab stimuli, as well as the duration of attentive behaviours during habstart and habend, were collected for all 37 bats and all stimuli combinations. However, data on the duration of behaviours in reaction to dishab and rehab stimuli, the number of echolocation pulses emitted in reaction to the dishab stimuli and categorical data in reaction to the rehab stimuli were analysed for 20 bats (11 males and nine females). In the sex discrimination trials, only initial reactions (occurring in the first 5 s) to the dishab stimuli were used to gather categorical, count and strength of reaction data due to technical issues during some of the trials. As only a few bats reacted to the playbacks by emitting echolocation pulses we decided to exclude this response from further analysis. We pooled the data sets for males and females because there were no differences between the sexes in the duration of attentive behaviours for the four analysed time phases habstart, habend, dishab, rehab in the sex discrimination trials (Mann–Whitney U test: $U = 221.5$, $N_1 = 15$, $N_2 = 22$, $P = 0.073$; $U = 315$, $P = 0.751$; $U = 196$, $N_1 = 9$, $N_2 = 11$, $P = 0.968$; $U = 194$, $P = 0.925$, respectively) and individual discrimination trials (Mann–Whitney U test: $U = 1291$, $N_1 = 15$, $N_2 = 22$, $P = 0.273$; $U = 1463$, $P = 0.949$; $U = 1452$, $P = 0.897$; $U = 1452$, $P = 0.897$, respectively).

Validation

Intra- and interobserver bias was discounted for both frequency and duration data (Kappa's coefficient: $K = 0.85$, $N = 10$, $P < 0.05$; $K = 0.87$, $N = 10$, $P < 0.05$, respectively; Wilcoxon matched-pairs test: $Z = 0.64$, $N = 10$, $P = 0.525$; $Z = 0.622$, $N = 10$, $P = 0.534$, respectively).

Repeated exposure to the experiment over 5 consecutive nights had an effect on the responsiveness of bats: the duration of attentive behaviours elicited initially by the habituation stimuli declined significantly (16.5 s in the first night versus 6.5 s in the last night; Friedman ANOVA: $\chi^2_4 = 12.37$, $N = 37$, $P = 0.015$). However, the balanced experimental design, i.e. randomized playback combinations and order of bats across all nights accounted for the statistical effect of this sensory habituation.

A comparison of the duration of attentive behaviours at the beginning and end of habituation showed a significant decline from 10.175 to 0.001 ms indicating that bats were fully habituated before exposure to dishabituation stimuli (Wilcoxon matched-pairs test: $Z = 11.67$, $N = 185$, $P < 0.001$).

The control trials were significantly different to the test trials in both the sex and individual discrimination tasks (Pearson chi-square test: $\chi^2_1 = 4.17$, $N = 37$, $P = 0.041$; $\chi^2_1 = 18.49$, $N = 37$, $P < 0.001$, respectively) indicating very low levels of spontaneous recovery from habituation which means few or no false positive reactions. Only two bats regained attentive activity during the playback of the control stimulus in both sex and individual discrimination trials. There was no significant difference in the duration of attentive behaviours between habend and dishab in these control trials for sex discrimination (Wilcoxon matched-pairs test: $Z = 1.34$, $N = 20$, $P = 0.179$) or for individual discrimination (Wilcoxon matched-pairs test: $Z = 1.34$, $N = 37$, $P = 0.179$). There was also no significant difference in the duration of attentive behaviours between habend and rehab in control and test trials (the second method to control for false positive reactions) for both the sex discrimination trials (Wilcoxon matched-pairs test: $Z = 1.60$, $N = 20$, $P = 0.109$; $Z = 1.34$, $N = 20$, $P = 0.179$, respectively) and the individual discrimination trials (Wilcoxon matched-pairs test: $Z = 1.82$, $N = 37$, $P = 0.068$; $Z = 1.60$, $N = 37$, $P = 0.109$; $Z = 1.75$, $N = 37$, $P = 0.080$, respectively).

The same method of controlling for false positive responses was applied to the categorical data (yes and no answers). In the sex discrimination trials, 17 bats (85%) did not show any attentive activity to the rehab stimuli (pulses from the same sex as the habituation) in the control trial (same sex as the habituation; Pearson chi-square test: $\chi^2_1 = 5.58$, $N = 20$, $P = 0.018$) and 18 bats (90%) did not react to the rehab stimuli in the test trial (different sex as the habituation; Pearson chi-square test $\chi^2_1 = 7.62$, $N = 20$, $P = 0.005$). In the individual discrimination control trial, 35 (94%) bats did not react to the rehab stimuli (Pearson chi-square test: $\chi^2_1 = 18.49$, $N = 37$, $P < 0.001$). In the individual test trials, 35 (94%) and 32 (91%) bats did not react to the rehab stimuli (Pearson chi-square test: $\chi^2_1 = 18.49$, $N = 37$, $P < 0.001$; $\chi^2_1 = 11.47$, $N = 37$, $P = 0.001$, respectively). It is therefore unlikely that reactions to the test were a result of spontaneous recovery of prehabituated levels of reaction (Rendall et al., 1996). Similarly, all 37 bats reacted to the white noise indicating no false negative reactions.

Discrimination

The video analysis revealed that bats are able to discriminate echolocation pulses of males and females as well as of the three different individuals. By regaining activity after habituation when listening to echolocation pulses of a different stimuli class, the bats behaviour indicated the perception of acoustic differences between the classes.

In the two-class categorization task of sex discrimination, 27 (73%) bats reacted significantly more frequently to the test then expected by chance (Pearson chi-square test: $\chi^2_1 = 18.49$, $N = 37$, $P < 0.001$). Similarly, the duration of attentive behaviours significantly increased when playing back pulses from a different sex from 0.03 to 2.70 ms (Wilcoxon matched-pairs test: $Z = 3.30$, $N = 20$, $P = 0.001$).

In the individual discrimination task bats listened to playbacks of two individuals at a time. This resulted in the recordings of responses of listening bats to the acoustic stimuli of all possible combinations of pairs of individuals compiled from three different individuals as representative of a multiclass category of stimuli. In this discrimination test, there was only a slight difference in the number of bats that responded to the two different test individuals: 29 (78%) bats reacted to the test (different individual) when the test individual had a similar pulse frequency to the individual used to habituate the listener and 30 (81%) bats reacted when the test individual had a different pulse frequency to the individual used to habituate the listener (Pearson chi-square test: $\chi^2_1 = 8.00$, $N = 37$, $P = 0.004$; $\chi^2_1 = 6.55$, $N = 37$, $P = 0.011$).

The duration of attentive behaviours increased significantly when playing pulses from a test individual (Individual 2 or Individual 3) compared to the control (Individual 1; Wilcoxon matched-pairs test: $Z = 4.70$, $N = 37$, $P < 0.001$; $Z = 1.60$, $N = 37$, $P < 0.01$, respectively). However, a more detailed analysis of the strength of the responses revealed a more comprehensive pattern. Across the acoustic space of all five playbacks (three individuals and two sexes), the general pattern was that the more similar test and habituation stimuli were, the weaker the responses of the listening bats (Fig. 7). The strongest responses reflect a direct orientation towards the sound and were observed in the sex task and the individual task where the two individuals were acoustically more dissimilar (Fig. 7). Responses towards the individual (Individual 3) that was acoustically more different to the habituation individual (i.e. Individual 1) were more pronounced than towards the individual (Individual 2) that was acoustically more similar to the habituation individual (i.e. Individual 1; Wilcoxon matched-pairs test: $Z = 4.70$, $N = 37$, $P < 0.001$; $Z = 1.60$, $N = 37$, $P < 0.01$, respectively).

DISCUSSION

Our results supported the hypothesis that bats can discriminate between conspecifics using vocal signatures in echolocation calls. The results of the playback experiments show that *R. clivosus* discriminated between individuals and sex by their echolocation pulses alone. Such perceptions of vocal signatures are mandatory precursors if echolocation is to function in communication. The two-class task of sex discrimination showed that sex signatures are present and are readily perceived by bats. They appear to be encoded in the frequency modulated components of the pulses. Pulses emitted by females had longer initial and terminal FM components with lower terminal sweep rates. This minute acoustic difference in the signal resulted in high classification success rates (even in large groups) allowing bats to discriminate between males and females. However, in accordance with our hypothesis, variability in echolocation required for the communication of complex information may be limited by its function as a sonar signal. Unlike sex signatures, individual-specific cues became more ambiguous (i.e. classification success decreased) in larger groups as overlap in acoustic attributes increased and individual signals became less distinct. In general, echolocation pulses had sufficient systematic variation to encode the two classes of female and male allowing bats to discriminate between different sexes or between one individual and another, provided the individuals were not acoustically
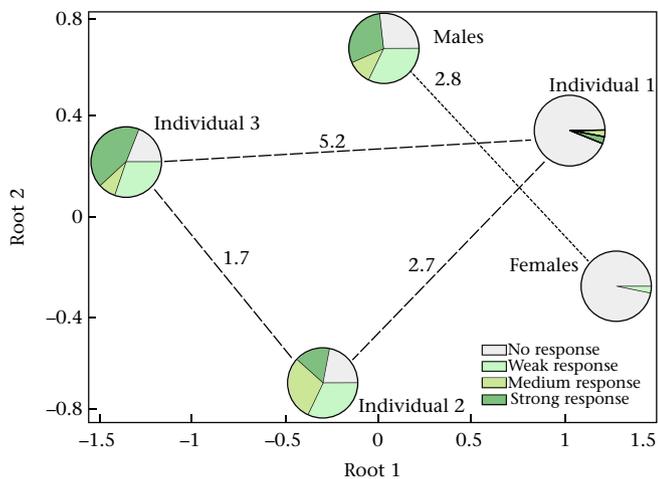
Figure 7. Acoustic space of the five playbacks (three individuals and two sexes) and the associated strength of response (no, weak, medium and strong response) of the listening bats ($N = 37$). Lines and numbers represent the squared Mahalanobis distances and pie charts reflect the respective response strength categories for each playback.

too similar. The latter result revealed limitations in the structural complexity of echolocation pulses, rendering the signal ambiguous and therefore unsuitable for reliable individual identification in large groups. Modified echolocation pulses, on the other hand, which have pronounced upward sweeps at the end of the terminal FM component, added complexity to the signal and increased individual distinctiveness even in larger groups.

Encoding detailed information requires an acoustic signal to be diverse and specific at the same time. This can be achieved by context specificity in which specific acoustic signal designs are exclusively used in a specific context or by adding unique components to otherwise similar call types. For receivers to perceive and discriminate different attributes of the sender, signals need to be distinct between the different classes. This distinction occurs when there is greater between- than within-class variability (Bee, Kozich, Blackwell, & Gerhardt, 2001) or when a signal attribute is present in one class and absent in the other (e.g. Beecher, 1989; Rendall et al., 1996). Correspondingly, acoustic signals often diverge between classes, for example between the sexes, producing distinct acoustic features that provide sex signatures in vocalizations. The use of acoustic signals in sex and individual recognition has been well established for birds, anurans and mammals (e.g. Amézquita, Flechas, Lima, Gasser, & Hödl, 2011; Bee et al., 2001; Charlton et al., 2011; Jouventin, Aubin, & Lengagne, 1999; Searby & Jouventin, 2003). Observed differences can range from differences in call structure, frequency or time (e.g. Norcross & Newman, 1993).

Drivers of acoustic sex divergence include courtship or mate choice as well as same-sex associations (female–female and male–male interactions). For these interactions to occur it is important for individuals to be able to discriminate between males and females. Although echolocation pulses differ between males and females in some horseshoe bat species, acoustic space of the two sexes can also overlap (Chen, Jones, & Rossiter, 2009; Guillén et al., 2000; Siemers et al., 2005; Suga, Niwa, Taniguchi, & Margoliash, 1987; but see Neuweiler et al., 1987). While several studies have shown sex (Kazial & Masters, 2004; Knörnschild et al., 2012) and individual or group discrimination in bats (Kazial, Pacheco, & Zielinski, 2008; Voigt-Heucke et al., 2010; Yovel, Melcon, Franz, Denzinger, & Schnitzler, 2009), only one other study has demonstrated the ability of HDC bats to perceive sex signatures in echolocation pulses (Schuchmann et al., 2012). All other studies were done on bats using low duty cycle echolocation (LDC; the pulse

duration is short relative to the silent periods between pulses). This LDC system is very different to HDC echolocation. In contrast to the FM-CF-FM structure of pulses emitted by HDC bats, LDC bats emit FM pulses with downward frequency modulated sweeps of short duration and broad bandwidth. Knörnschild et al. (2012) showed that males of bat species using LDC FM echolocation respond differently towards playbacks of echolocation pulses from females and males. The males emit courtship calls after hearing female echolocation pulses and aggressive calls after hearing male ones (see also Kazial & Masters, 2004). Applying a different approach, Puechmaille et al. (2014) studied the potential role of echolocation in mating in horseshoe bats. They provided indirect support for female mate choice based on echolocation in *Rhinolophus mehelyi* using a two-choice experiment in which females showed a preference for high-frequency pulses of males which in turn correlated with good body condition. They combined the experiment with an analysis of genetic relatedness and found that males with higher frequency echolocation pulses sired more offspring, linking echolocation frequency to reproductive success in their interpretation. However, all studies thus far that have investigated sex differences in echolocation pulses of HDC bats have focused on resting frequency alone (e.g. Jones & Siemers, 2011) and did not incorporate additional acoustic components of pulses. In contrast to these studies, we did not find sexually dimorphic resting frequencies (despite males being smaller than females) in *R. clivosus* echolocation pulses. Despite this, bats were still able to discriminate between pulses from different sexes. Similar results were found in the only other study (Schuchmann et al., 2012) to test the ability of bats (*R. mehelyi* and *Rhinolophus euryale*) to discriminate between males and females. Although the authors suggested that other pulse components facilitated discrimination they did not attempt to identify which components these were. Despite considering many more parameters than just resting frequency (as did Schuchmann et al., 2012; Schuchmann & Siemers, 2010), we still have not identified reliable cues for sex discrimination. However, our results suggest that parameters of the initial and terminal FM components (Table A2) appear to be good candidates. Perhaps bats use these in combination with the distribution of emitted intensities over the spectral parameters of these components to form distinct templates that allow sex discrimination (Schuchmann et al., 2012; Yovel et al., 2009).

In contrast to sex signatures, individual signatures require high variation between classes (i.e. individuals) with extremely low within-class variation. Requirements for communicative specificity are especially high in large groups comprising many individuals. For example, highly specific signals are found in king penguins, *Aptenodytes patagonicus*, where young are able to identify the calls of their parents from among hundreds of individuals because of the high variability between individual penguin calls; each penguin has a unique frequency modulation shape to the syllables of their calls (Jouventin et al., 1999). In bats, individual signatures have been found in echolocation pulses by measuring frequency parameters of recorded pulses (Hiryu et al., 2006; Siemers et al., 2005; Suga et al., 1987; Voigt-Heucke et al., 2010). In some HDC bats, differences between individuals of the same species have been reported in the resting frequency of the pulses (Jacobs et al., 2007; Siemers et al., 2005; Suga et al., 1987). Resting frequency was identified as the strongest predictor of individual identity between the individuals used to compile the playbacks (Table A8 in the Appendix) in our experiment and therefore most likely to be used by *R. clivosus* to discriminate. For pulse frequency to successfully encode individual identity it must have a high specificity. In HDC bats, each individual has an individualized acoustic fovea in the cochlear and subsequent auditory pathways. This is very narrow and highly sensitive frequency band of about ±8 kHz where the greatest

number of neurons are tuned around the individual-specific centre frequency, making resting frequency a good candidate for individual acoustic badges (Neuweiler, 1990; Schuller & Pollak, 1979). In the current study, bats showed stronger reactions to pulses from an individual whose acoustic parameters (including resting frequency) were less similar to both their own (Individual 3) and those of the individual whose pulses they were habituated to (Individual 1). In addition, in a few trials where bats showed very strong reactions to pulses from Individual 3 they remained in this alert state even after the rehabituation stimulus was played. A similar reaction was observed in red deer, *Cervus elaphus*, where a test stimulus of a harsh roar (an attention grabbing stimulus) resulted in increased levels of reaction of females to repeated playbacks of common roars from the same male that they would have typically been habituated to (Reby & Charlton, 2012). Pulses of Individual 3 had much lower frequencies which could be biologically meaningful as they could indicate age and sexual maturity (Jones & Ransome, 1993).

In addition to high interindividual variability, low within-individual variability of the signal is important for listening animals to be able to reliably discriminate individuals. In our study, classification success was highest for Individual 1 which had the lowest within-individual variation in resting frequency. Therefore, acoustic dissimilarity between individuals combined with lack of variability within an individual's pulses may determine whether a signal can be used for individual discrimination. This assumption was supported by the discrimination analysis of increasing group sizes; the more individuals were included, the lower the classification success became because of increasing overlap between pulses of individuals (Fig. 5). This suggests that there is a limit to interindividual variability of pulse parameters, such as, for example, resting frequency, probably as a result of selection in the context of the foraging and orientation function of echolocation.

The range of resting frequencies used by *R. clivosus* at De Hoop was small, around 3 kHz across 88 individuals and is probably limited by the optimal detection range for the habitat. The range at which bats can detect objects is influenced by the frequency, duration, intensity and directionality of its echolocation pulses, as well as the habitat and body size of the bat (Jakobsen, Brinkløv, & Surlykke, 2013). Furthermore, habitat influences detection range through a complex interaction between pulse frequency, humidity and temperature which determines the degree of atmospheric attenuation of the echolocation pulses (Luo, Koselj, Zsebők, Siemers, & Goerlitz, 2014) and has a direct impact on detection range. There is evidence that atmospheric attenuation exerts a selection pressure on frequency in horseshoe bats. In two different African horseshoe bat species, *Rhinolophus simulator* and *Rhinolophus swinnyi*, both with very low interindividual variation at any one site in its range, climatic factors including temperature and humidity were good predictors of geographical variation in echolocation pulse frequencies (Mutumi, Jacobs, & Winker, 2016). So, although signal frequency-based characteristics in signals of other taxa have been shown to be an important feature for signal divergence and as carriers of vocal signatures (e.g. green frog, *Rana clamitans*: Bee et al., 2001; sheep, *Ovis aries*: Searby & Jouventin, 2003; yellow-bellied marmot, *Marmota flaviventris*: Blumstein & Munos, 2005; Houbara bustard, *Chlamydotis undulata*: Cornec, Hingrat, & Rybak, 2014), in HDC bats the foraging function of echolocation can place limitations on this parameter, limiting its between-class variability and therefore its versatility in communication. In addition, frequency parameters of echolocating bats on the wing change frequently with different environments and as a result of Doppler shift compensation which further impairs identity signatures in flying bats (e.g. Knörnschild et al., 2012; Siemers et al., 2005).

In addition to resting frequency, pulse duration and the duration and minimum frequency of the terminal FM component also

accounted for some variation between individuals. The results of the discrimination experiment confirm that *R. clivosus* perceives these other acoustic parameters in the pulses of conspecifics.

Sex and, to a lesser extent, individual signatures are mainly encoded in the FM components of the pulses. The pulses emitted by females had longer initial and terminal FM components with a lower terminal sweep rate. Their initial sweep rates were also lower although this difference was not statistically significant. In HDC bats, FM components of the pulses (especially the terminal FM component) are thought to be used as time markers for orientation allowing precise distance perception for obstacle avoidance and prey capture (Fawcett, Jacobs, Surlykke, & Ratcliffe, 2015; Neuweiler et al., 1987; Simmons, 1973; Tian & Schnitzler, 1997; Vogler & Neuweiler, 1983). However, our findings suggest that the FM component may have more systematic variation than the CF component. The coefficient of variation for the FM components was much larger than that for the CF component. The small differences necessary for discrimination between the sexes (two states) may have minimal functional consequences on the ranging abilities of the sonar system. However, the transmission of more complex information may require greater flexibility and this could be limited by the ranging function of the FM components. The role of FM components in communication reported here supports findings of other studies which indicate that, like LDC bats (Yovel et al., 2009), HDC bats use the entire acoustic space available to them (Bastian & Jacobs, 2015; Raw, 2016) for discrimination tasks. It is therefore possible that bats are able to use signal components, normally used in orientation, for sex discrimination without compromising either function due to the low variation needed to encode sex. However, even with additional acoustic parameters individual signatures were weak when many individuals were tested. The components of FM-CF-FM echolocation pulses are tightly connected to their function in foraging and orientation. This function is a key innovation in the evolution of bats and its performance and its structural integrity is therefore likely to be highly preserved.

In other animal taxa, acoustic signals that are not constrained in the way that echolocation is diversify through variability between signal attributes to improve uniqueness (Braune, Schmidt, & Zimmermann, 2008; Schul & Bush, 2002). For example, eagle owls, *Bubo bubo*, vary in several acoustic parameters, such as start frequency, maximum frequency and duration of certain call components to increase the distinctiveness of their calls, facilitating individual identification solely on the acoustic structure of the signal (Grava, Mathevon, Place, & Balluet, 2008). Furthermore, many other animals modify their signals to increase call versatility in a communication context. For example, during times of poor visibility and in the presence of neighbours, female Diana monkeys, *Cercopithecus diana*, increase acoustic distinctiveness to facilitate individual identification by varying the fundamental frequency contours of their calls (Candiotti, Zuberbühler, & Lemasson, 2012). However, such changes in signals may not be available when the signal has a dual function and the structural requirements for the primary function are strict and complexity is constrained. As a result, information that requires a larger amount of signal space, such as individual signatures, is limited in bats and other animals and they may compensate for such limitations in different ways. For example, some horseshoe bat populations of *R. capensis* exhibit an increased sensitivity to minute acoustic differences in circumstances when signal divergence is restricted and classes overlap in acoustic space (Bastian & Jacobs, 2015); thus the constraint can be alleviated to a certain extent through increased perceptual acuity of the perceiver. In swamp sparrows, *Melospiza georgiana*, requirements of feeding ecology for bigger bills, in some environments, constrain the broadband trills of songs used in mate choice. These birds compensate for a decrease in song performance by

increasing song complexity (Ballentine & Pfennig, 2006). Similarly, there are indications that modifications of echolocation pulses can increase their distinctiveness as vocal signatures and thus their potential communicative function by adding systematic variation (Andrews & Andrews, 2003; Andrews, Andrews, Wills, & Bevis, 2006; Barclay, Fenton, & Thomas, 1979; Brown, 1976; Clement & Kanwal, 2012; Jahelková, 2011; Schmidt, 2013; Suthers, 1965). In this study, *R. clivosus* pulses had a modified terminal FM component in the form of an additional upward sweep (Fig. 2). This increase in systematic variation could be used to facilitate communication. If so, these modified pulses would epitomize the dual function of echolocation within a single pulse. However, no information is available that would allow an assessment of whether these added modifications to the FM part have any effect on the ranging function. Transitional calls, previously described in other bat species, are calls that gradually change from typical echolocation pulses into social calls (Andrews & Andrews, 2003; Barclay et al., 1979; Fenton, 2003; Schmidt, 2013). The bat *Megaderma lyra*, for example, uses echolocation pulses that are short broadband frequency, downward modulated sweeps. During landing, these echolocation pulses gradually change into the landing strophe through a lowering of frequency and attaching an upward sweep at the end (Schmidt, 2013). Like other mammals, bats have social calls that have evolved to transmit different types of information (Fenton, 1985, 2003). Social calls have been described in detail for several bat species (e.g. Aldridge, Obrist, Merriam, & Fenton, 1990; Andrews et al., 2006; Bastian & Schmidt, 2008) and were also recorded in captive *R. clivosus* for a separate study. Since social calls are not limited by any other function they can be complex and flexible to carry more detailed information. Thus, complex songs are part of the repertoire of many different bat species (e.g. Barlow & Jones, 1997; Behr & von Helversen, 2004; Knörnschild, Glöckner, & Helversen, 2010; Ma, Kobayasi, Zhang, & Metzner, 2006; Sachteleben & von Helversen, 2006; Smarsh & Smotherman, 2015). However, bats may use echolocation for communication in contexts where it would be feasible and advantageous to do so. For example, while foraging, the use of echolocation pulses to communicate allows the bats to orient and communicate at the same time without having to switch from one system to another to do so. In this context, using echolocation as a communication signal is energetically advantageous because the sender does not need to invest additional energy in emitting an additional signal (Voigt & Lewanzik, 2012). In addition, for a true communication system to evolve, both participants should benefit (Bradbury & Vehrencamp, 2011; Maynard Smith & Harper, 2003). The benefits for the perceiver are apparent. Echolocation pulses are emitted frequently and inevitably by orienting or foraging bats. Furthermore, most bats emit echolocation pulses with high intensities, making it easy for the listener to hear these vocalizations (e.g. Siemers et al., 2005). Extracting information from echolocation pulses of a conspecific can, for example, aid in locating feeding grounds or suitable roosts (Jones & Siemers, 2011) or provide general spatial information (Chiu & Moss, 2008). But the perception of vocal signatures can also allow a quick assessment of the individuals in the vicinity of the receiver, facilitating adjustments of the receiver's behaviour to enable an adequate response to the situation. Such situations may involve the recognition of conspecifics (Gillam, 2007; Ruczynski, Kalko, & Siemers, 2007), the discrimination between familiar and unfamiliar individuals by interaction partners (Voigt-Heucke et al., 2010) or localization of potential mates for reproduction (Knörnschild et al., 2012). The evolution of distinctive signatures in the sender and the recognition of these by the perceiver can have a selective advantage for both partners in the interaction. This recognition can be beneficial through decreased conflicts with neighbours, increased altruism

from familiar kin, decreased risk of inbreeding, decreased aggressive competition, increased stability in reciprocal interactions and whenever being recognizable improves mating success, depending on the biology of the species (Tibbetts & Dale, 2007). Nevertheless, benefits for signallers and receivers and thus the driving forces shaping a communication system are not always the same for both sender and receiver. Information extraction as described above can be achieved via eavesdropping by the receiver alone. Eavesdropping as gleaning information from unintentionally or inevitably emitted cues is not usually considered true communication because the perceived cue is not intentionally emitted by the sender for a communicative purpose (e.g. Bradbury & Vehrencamp, 2011; Danchin, Giraldeau, Valone, & Wagner, 2004; Hauser, 1996). Our study cannot provide insight into the intention of the sender because it was designed to characterize vocal signatures and assess their perception. Vocal signatures, in general, are only relevant for communication if they can be perceived by the intended receiver whether the sender intended to communicate or not (Freeberg et al., 2012; Mumm, Urrutia, & Knörnschild, 2014; Townsend, Hollen, & Manser, 2010). Although the selective responsiveness we found in the listening bats can be interpreted as support for the communicative potential of echolocation, there is to our knowledge no evidence that bats intentionally echolocate to communicate. In addition to the previously mentioned potential benefits of information acquisition via echolocation, most echolocation pulses are outside the hearing range of other animals so bats can communicate without the danger of being heard by predators (e.g. predatory birds, snakes and even humans; Sparks, Roberts, & Jones, 2000) and some prey (e.g. insects: Fullard, 1987; Jacobs, 2000; frogs: Gerhardt & Schwartz, 2001; Loftus-Hills & Johnstone, 1970).

Conclusion and Research Outlook

Vocal signatures of sex and individual identity are encoded in echolocation pulses and perceived by conspecifics in the bat *R. clivosus*. The number of distinct character states or attributes of the sender appears to be restricted by the primary function of echolocation which is adapted to the specific foraging niche and habitat. We conclude that signal complexity is crucial to signal specificity and the orientation function of echolocation constrains its operational versatility as a communication signal.

Until recently it was unknown whether echolocation has enough vocal plasticity to be used in communication (Bastian & Jacobs, 2015; Raw, 2016). In our experimental approach, we expanded the one-class categorization system of previous studies on other horseshoe bat species (species discrimination), to a two-class (sex) and representative multiple-class categorization system (individual) and furthermore emphasized the multiparametric acoustic space rather than considering frequency alone. Although pulse frequency is an important parameter, the FM components play a central role in encoding communication cues. However, as a result of the dual function of echolocation it may only partially serve the function of a communication cue in contexts in which a low-class categorization system is sufficient e.g. while foraging in small groups (Dechmann et al., 2009) or when localizing maternity colonies (Barclay, 1982). Its versatility can be increased through slight modifications of echolocation signals without compromising the primary function of echolocation. We thus propose that in HDC bats, increases in the variability of echolocation pulses have evolved by an addition of an upward sweep to the FM component of the pulses. This interpretation assumes signalling is intentional (emission of a signal in response to the existence of a perceiver and its response) which has yet to be tested. Generally, research on the sender's side is sorely missing. It is crucial to tackle the question of whether echolocation is a form of unintentional signalling of

signatures which arose as a by-product of, for example, vocal tract morphology (i.e. echolocation as a cue for eavesdropping conspecifics) or intentional signalling (echolocation as a communication signal). This answer has crucial implications for the nature of information conveyance via echolocation: eavesdropping or true communication (e.g. Boughman & Moss, 2003; Bradbury & Vehrencamp, 2011). Seyfarth and Cheney (2003) called for experimental research on birdsong which can be usefully applied to echolocation. They proposed experiments in which a particular vocalization is treated as the dependent variable and the vocalization-eliciting stimuli or situations are manipulated. Unless such research on echolocation is conducted and provides support for intentional signalling by the echolocator for communication purposes, we advocate that the communication function of echolocation be applied cautiously because gleaning information is as likely as true communication to explain most published results (e.g. Knörnschild et al., 2012; Schuchmann et al., 2012; Siemers et al., 2005; Voigt-Heucke et al., 2010)). As emphasized in this context by Dechmann, Wikelski, Van Noordwijk, Voigt, and Voigt-Heucke (2013), a key difference between 'cue' and 'signal' lies in the selective force shaping it: for echolocation to be a true communication signal, it must have evolved at least partially in response to a result it had on the perceiver being beneficial to the sender (Bradbury & Vehrencamp, 2011; Danchin et al., 2004; Maynard Smith & Harper, 2003).

If the minute modifications of echolocation pulses found in our study species are indeed communication signals and perceived by bats, adding systematic acoustic complexity to echolocation pulses represents an effective way to encode communicative signatures. Research exploring and testing the multifunctionality of acoustic signals will improve our understanding of how vocalizations have diverged in their functional properties and give greater insight into the evolution and functionality of signal design. To test the assumption that modified pulses evolved to increase the communicative function of echolocation pulses, the specific contexts in which each type of pulse is produced should be documented (e.g. studies matching behaviours and contexts to social calls and echolocation pulses). An important consideration is how the ranging performance of the bat is affected by alteration to the FM component of its pulse. The FM component is crucial for accurate determination of distances to objects (Fawcett et al., 2015). The upsweeps added to the FM components of echolocation pulses could interfere with the temporal processing of the bat's echolocation system. If it does, then the use of these modified pulses would be limited to times when the bat is not tracking an object. This is worthy of further investigation. More detailed knowledge of the acoustic parameters used by bats to extract information could improve our understanding of the evolution of the dual functional of echolocation pulses. It would also give us insight into the extent to which acoustic discrimination is achieved by minute differences in a sender's pulses potentially in conjunction with increased perception abilities of receivers (Bastian & Jacobs, 2015; Tobias, Planqué, Cram, & Seddon, 2014).

More urgently though, we need experimental approaches that reveal the biological relevance of the extracted communicative information to the perceiver. From the perspective of the perceiver, this is the crucial test of the proposal that echolocation signals may serve a communicative function. Such studies could start with field experiments based on playbacks that further explore sex-specific behaviour towards male and female echolocation pulses (Knörnschild et al., 2012). A suitable approach would be choice experiments investigating the cognitive categorization by bats through testing the ability to extract and evaluate information for decision making (as one study has already shown: Yovel et al., 2009), all crucial components of communication.

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Appendix

Table A1
Behaviours displayed by bats during habituation—dishabituation experiments and coded on videos recordings of the playback experiment

Behaviour	Description	Response strength
Inactive		
No movement	Not engaging in any movement and in a sleeping position with its head down	
Active		
Tip of ear twitch	Movement of the top portion of the ear(s)	
Head swaying	Movement of head from left to right without looking up	
Crawl	Movement along the perch by placing one foot or hand next to another	
Partial wing stretch	Partial extension of wings from shoulder and fingers from wrist	
Full wing stretch	Full extension of wings from shoulders and fingers from wrist	
Vibrating wings	Vibration or small flapping of the wings against the body	
Body shuffle	Shaking and repositioning of the body usually ending in an inactive position	
Swaying	A movement of the body from side to side	
Grooming	Cleaning of the coat, wings, ears and feet with tongue and feet	
Attentive		
Single ear twitch	Movement of an entire ear	Weak
Slow ear twitching	Movement of the entire ear(s) in slow succession	Weak
Rapid ear twitching	Movement of the ears in rapid succession; unable to count in real time	Medium
Look up partial	Raising head 45° or less away from the body, sometimes accompanied by ear twitching	Medium
Look up full	Raising head 90° away from the body; often accompanied by ear twitching	Strong
Scan	Head up, looking around the environment; always accompanied by ear twitching.	Strong
Leg contraction partial	Contraction of leg(s) towards the body by bending the knees slightly	Medium
Leg contraction full	Contraction of leg(s) towards the body by bending the knees fully	Strong

Table A2
Descriptive statistics of acoustic measurements of echolocation pulses and forearm length of male and female *R. clivosus* from De Hoop, South Africa

Acoustic parameter	Males (45)		Females (43)		All (88)	
	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
FA (mm)	53.9±1.2	51.4–56.9	55.4±1.5	50.5–58.9	54.7±1.5	50.6–58.9
RF (kHz)	91.6±0.4	90.0–92.2	91.7±0.5	90.2–93.0	91.7±0.5	90.0–93.0
DUR (ms)	35.7±8.3	18.0–54.2	36.4±9.3	18.2–61.0	36.1±8.7	18.0–61.0
IPI (ms)	84.1±87.3	9.8–463.2	81.4±65.2	10.4–354.0	82.8±76.9	9.8–463.2
DC (%)	51.5±12.6	23.4–86.6	48.1±12.1	15.2–74.6	49.8±12.4	15.2–86.6
Distomax (ms)	28.2±7.6	8.5–44.8	28.6±8.0	13.1–52.9	28.4±7.8	8.5–52.9
MF (INT) (kHz)	81.6±3.7	73.0–88.0	80.4±5.2	69.9–89.0	81.0±4.5	69.9–89.0
MF (TM) (kHz)	68.7±3.0	59.5–73.4	68.6±3.5	60.5–79.3	68.7±3.3	59.5–79.3
BW (INT) (kHz)	10.1±3.6	3.5–18.2	11.3±5.3	1.8–21.5	10.7±4.5	1.8–21.5
BW (TM) (kHz)	22.9±2.9	18.1–32.0	23.0±3.7	11.1–31.2	23.0±3.3	11.1–32.0
DUR (INT) (ms)	2.0±0.9	0.9–4.3	2.7±1.7	0.5–10.6	2.3±1.4	0.6–10.6
DUR (TM) (ms)	2.8±1.1	1.6–5.4	3.9±3.6	1.8–25.4	3.4±2.7	1.6–25.4
SR (INT) (kHz/ms)	5.8±1.7	1.8–8.9	5.1±1.8	2.4–9.1	5.4±1.7	1.8–9.1
SR (TM) (kHz/ms)	9.6±3.4	4.1–16.0	8.0±3.3	1.7–16.7	8.8±3.4	1.7–16.7

Sample sizes are given in parenthesis and measurements as mean ± SD, ranges as minimum and maximum values. FA = forearm length, RF = resting frequency, DUR = duration, DC = duty cycle, IPI = interpulse interval, distomax = distance from pulse start to maximum amplitude, INT = initial frequency modulated component, TM = terminal frequency modulated component, MF = minimum frequency, BW = bandwidth, SR = sweep rate.

Table A3
Principal components (PC) extracted from acoustic parameters of echolocation pulses used in sex playbacks

PC	Acoustic parameters	Eigenvalues	Cumulative %
1	Sweep rate (terminal FM), minimum frequency (terminal FM), bandwidth (terminal FM), pulse duration	3.183	24
2	Minimum frequency (initial FM), bandwidth (initial FM), duration (initial FM), pulse duration	2.925	47
3	Sweep rate (initial FM), minimum frequency (terminal FM), bandwidth (terminal FM)	1.864	61
4	Duty cycle, interpulse interval	1.655	74
5	Distomax, resting frequency	1.186	83

Acoustic parameters with factor loadings of >0.50 are presented and listed in order of importance (i.e. factor loadings) to each component. Only principal components with eigenvalues >1 are shown and were used in the DFA. Cumulative percentage is the total variance explained by each principal component. The analysis was based on N = 38 bats.

Table A4
Relative contributions of acoustic parameters to the factors extracted from the DFA on the echolocation pulses of male and female bats

Factor	Acoustic parameters	Root 1
3	Sweep rate (initial FM), minimum frequency (terminal FM), bandwidth (terminal FM)	−0.733
1	Sweep rate (terminal FM), minimum frequency (terminal FM), bandwidth (terminal FM), pulse duration	−0.599
5	Distomax, resting frequency	−0.588
4	Duty cycle, interpulse interval	−0.428
2	Minimum frequency (initial FM), bandwidth (initial FM), duration (initial FM), pulse duration	0.289
Eigenvalue		0.690
Cumulative %		100

Numerical values are standardized coefficients of canonical variables and indicate the importance of each factor (listed from highest to least importance). Factors consist of and correspond to principal components (e.g. Factor 1 = PC 1) from the PCA (Table A3). Only roots that added significantly to the difference between groups are displayed. The analysis was based on N = 38 bats.

Table A5
Principal components (PC) extracted from acoustic parameters of echolocation pulses used of all sampled individuals

PC	Acoustic parameters	Eigenvalues	Cumulative %
1	Sweep rate (terminal FM), duration (terminal FM), interpulse interval, pulse duration, duration (initial FM), duty cycle	3.816	29
2	Bandwidth (initial FM), minimum frequency (initial FM), minimum frequency (terminal FM), duration (initial FM), bandwidth (terminal FM)	2.921	51
3	Duty cycle, interpulse interval	1.580	63
4	Sweep rate (initial FM)	1.484	75
5	Resting frequency, distomax	1.122	84

Acoustic parameters with factor loadings of >0.50 are presented and are listed in order of importance (i.e. factor loadings) to each component. Only principal components with eigenvalues >1 are shown and were used in the DFA. Cumulative percentage is the total variance explained by each principal component. The analysis was based on $N = 88$ bats.

Table A6
Relative contributions of acoustic parameters to the factors extracted from a DFA of echolocation pulses of all individuals

Factor	Acoustic parameters	Root 1	Root 2	Root 3
1	Sweep rate (terminal FM), duration (terminal FM), interpulse interval, pulse duration, duration (initial FM), duty cycle	−0.858	0.545	0.316
2	Bandwidth (initial FM), minimum frequency (initial FM), minimum frequency (terminal FM), duration (initial FM), bandwidth (terminal FM)	−0.765	−0.328	−0.656
4	Sweep rate (initial FM)	0.579	0.363	−0.101
3	Duty cycle, interpulse interval	0.450	−0.047	0.156
5	Resting frequency, distomax	0.227	0.796	−0.553
Eigenvalue		4.196	2.185	1.411
Cumulative %		48	74	90

Numerical values are standardized coefficients of canonical variables and indicate the importance of each factor for each root (listed from highest to least importance for Root 1). Factors consist of and correspond to principal components (e.g. Factor 1 = PC 1) from the PCA (Table A5). Only roots that added significantly to the difference between groups are displayed. The analysis was based on $N = 88$ bats.

Table A7
Principal components (PC) extracted from acoustic parameters of echolocation pulses used in individual playbacks

PC	Acoustic parameters	Eigenvalues	Cumulative %
1	Minimum frequency (initial FM), bandwidth (initial FM), duration (initial FM)	3.308	26
2	Sweep rate (terminal FM), bandwidth (terminal FM), minimum frequency (terminal FM), pulse duration	2.83	47
3	Duty cycle, interpulse interval	1.999	63
4	Duration (terminal FM), Minimum Frequency (terminal FM)	1.459	75
5	Resting frequency	1.231	84

Acoustic parameters with factor loadings of >0.50 are presented and listed in order of importance (i.e. factor loadings) to each component. Only principal components with eigenvalues >1 are shown and were used in the DFA. Cumulative percentage is the total variance explained by each principal component. The analysis was based on $N = 3$ bats.

Table A8
Relative contributions of acoustic parameters to the discrimination of echolocation pulse of individuals used in the playbacks

Factor	Acoustic parameters	Root 1	Root 2
5	Resting frequency	0.934	0.265
2	Sweep rate (terminal FM), bandwidth (terminal FM), minimum frequency (terminal FM), pulse duration	−0.602	0.545
3	Duty cycle, interpulse interval	0.456	−0.292
4	Duration (terminal FM), minimum frequency (terminal FM)	−0.146	−0.789
1	Minimum frequency (initial FM), bandwidth (initial FM), duration (initial FM)	−0.121	−0.175
Eigenvalue		1.067	0.313
Cumulative %		77	99

Numerical values are standardized coefficients of canonical variables and indicate the importance of each factor for each root (listed from highest to least importance for Root 1). Factors consist of and correspond to principal components (e.g. Factor 1 = PC 1) from the PCA (Table A7). Only roots that added significantly to the difference between groups are displayed. The analysis was based on $N = 3$ bats.

Table A9
Principal components (PC) extracted from acoustic parameters of echolocation pulses used in individual playbacks and the average of the pulses of the bats that listened to the trials

PC	Acoustic parameters	Eigenvalues	Cumulative %
1	Minimum frequency (initial FM), bandwidth (initial FM), duration (initial FM)	3.418	26
2	Sweep rate (terminal FM), bandwidth (terminal FM), minimum frequency (terminal FM), pulse duration	2.897	47
3	Duty cycle, interpulse interval	1.744	63
4	Duration (terminal FM), minimum frequency (terminal FM)	1.583	75
5	Resting frequency	1.085	84

Acoustic parameters with factor loadings of >0.50 are presented and listed in order of importance (i.e. factor loadings) to each component. Only principal components with eigenvalues >1 are shown and were used in the DFA. Cumulative percentage is the total variance explained by each principal component. The analysis was based on $N = 40$ bats.

Table A10
Relative contributions of acoustic parameters to the discrimination of echolocation pulses used in individual playbacks and the average of the pulses of the bats that listened to the trials

Factor	Acoustic parameters	Root 1	Root 2
5	Resting frequency	0.769	0.532
2	Sweep rate (terminal FM), bandwidth (terminal FM), minimum frequency (terminal FM), pulse duration	−0.677	0.218
3	Duty cycle, interpulse interval	0.594	−0.091
4	Duration (terminal FM), minimum frequency (terminal FM)	0.249	−0.769
1	Minimum frequency (initial FM), bandwidth (initial FM), duration (initial FM)	−0.222	0.480
Eigenvalue		0.906	0.429
Cumulative %		77	22

Numerical values are standardized coefficients of canonical variables and indicate the importance of each factor for each root (listed from highest to least importance to Root 1). Factors consist of and correspond to principal components (Factor 1 = PC 1) from the PCA (Table A9). Only roots that added significantly to the difference between groups are displayed. The analysis was based on $N = 40$ bats.

Table A11
Descriptive statistics of acoustic measurements of modified echolocation pulses of *R. clivosus* individuals from De Hoop, South Africa

Acoustic parameter	Mean±SD	Range
RF (kHz)	91.5±0.9	90.0−93.0
DUR (ms)	43.5±10.1	25.9−80.0
IPI (ms)	208.6±283.0	2.1−2056.0
DC (%)	45.3±25.0	1.9−95.0
Distomax (ms)	41.6±4.5	7.0−223.3
MF (INT) (kHz)	81.4±5.3	67.0−89.6
MF (TM) (kHz)	71.0±4.3	57.0−82.0
BW (INT) (kHz)	10.1±5.4	1.4−26.0
BW (TM) (kHz)	20.5±4.6	9.0−36.0
DUR (INT) (ms)	2.6±1.5	0.3−7.3
DUR (TM) (ms)	4.1±1.4	1.3−8.3
SR (INT) (kHz/ms)	4.8±3.7	0.5−26.7
SR (TM) (kHz/ms)	5.5±2.1	1.6−15.0
DUR (TOT) (ms)	48.3±10.8	29.2−83.0
DUR (MOD) (ms)	4.8±2.9	1.1−11.6
MAX (MOD) (kHz)	83.5±4.5	75.0−97.0
BW (MOD) (kHz)	12.5±5.9	0.0−30.0
SR (MOD) (kHz/ms)	3.4±2.3	0.0−11.0

Measurements are shown as mean ± SD and ranges as minimum and maximum values. The analysis was based on $N = 19$ bats and $n = 5$ pulses per bat. RF = resting frequency, DUR = duration, DC = duty cycle, IPI = interpulse interval, distomax = distance from pulse start to maximum amplitude, MF = minimum frequency, MAX = maximum frequency, BW = bandwidth, SR = sweep rate, INT = initial frequency modulated component, TM = terminal frequency modulated component, MOD = modified component, TOT = total, including modified component.

Table A12
Principal components (PC) extracted from acoustic parameters of modified echolocation pulses used for the comparison with echolocation calls of the same individuals

PC	Acoustic parameters	Eigenvalues	Cumulative %
1	Bandwidth (initial FM), minimum frequency (initial FM), sweep rate (modified component), bandwidth (terminal FM)	4.174	23
2	Total duration, pulse duration (excluding modification)	8.064	45
3	Duty cycle, interpulse interval	10.124	56
4	Sweep rate (initial FM), minimum frequency (terminal FM), pulse duration (excluding modification), duration (modified component)	12.058	67
5	Maximum frequency (modified component)	13.405	75
6	Duration (initial FM), duration (terminal FM), sweep rate (terminal FM)	14.649	81
7	Resting frequency, duration (modified component), minimum frequency (initial FM), sweep rate (modified component)	15.760	88

Acoustic parameters with factor loadings of >0.50 are presented and listed in order of importance (i.e. factor loadings) to each component. Only principal components with eigenvalues >1 are shown and were used in the DFA. Cumulative percentage is the total variance explained by each principal component. The analysis was based on $N = 19$ bats and $n = 5$ modified pulses per bat.

Table A13
Relative contributions of acoustic parameters to the discrimination of modified echolocation pulses used for the comparison with echolocation calls of the same individuals

Factor	Acoustic parameters	Root 1	Root 2	Root 2
5	Maximum frequency (modified component)	1.278	0.548	0.433
7	Resting frequency, duration (modified component), minimum frequency (initial FM), sweep rate (modified component)	−1.273	−0.201	0.615
2	Total duration, pulse duration (excluding modification)	1.177	−0.186	−0.073
3	Duty cycle, interpulse interval	−1.124	0.259	−0.209
6	Duration (initial FM), duration (terminal FM), sweep rate (terminal FM)	−0.826	0.272	0.310
4	Sweep rate (initial FM), minimum frequency (terminal FM), pulse duration (excluding modification), duration (modified component)	−0.501	0.687	−0.152
1	Bandwidth (initial FM), minimum frequency (initial FM), sweep rate (modified component), bandwidth (terminal FM)	0.492	−0.487	0.284
Eigenvalue		8.503	0.595	0.121
Cumulative %		92	99	100

Numerical values are standardized coefficients of canonical variables and indicate the importance of each factor for each root (listed from highest to least importance to Root 1). Factors consist of and correspond to principal components (Factor 1 = PC 1) from the PCA (Table A12). Only roots that added significantly to the difference between groups are displayed. The analysis was based on $N = 19$ bats and $n = 5$ modified pulses per bat.