BEHAVIOURAL EVIDENCE FOR THE PERCEPTION OF INDIVIDUAL IDENTITY AND GENDER VIA THE ECHOLOCATION CALLS OF A HIGH DUTY CYCLE BAT, *RHINOLOPHUS CLIVOSUS*

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DECLARATION

I, Nikita M. Finger, know the meaning of plagiarism and declare that all of the work in this dissertation, save for that which is properly acknowledged, is my own.

Signature: Signed by candidate Date: October 20, 2014

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I hope to make you proud Dad.

ABSTRACT

Different cognitive processes underlie the perception of vocalizations in many mammals, including humans. This perception now extends to a highly specialized form of sonar called echolocation. In habituation-dishabituation experiments, a high duty cycle echolocating bat, *Rhinolophus clivosus,* dishabituated significantly when echolocation calls of a different gender or individual were played to the habituation. Strong individual and gender signatures but weak geographic signatures were found in both the CF and FM components of their echolocation calls. In the individual discrimination trials reactions were more pronounced to an individual that was less acoustically similar to the habituation than to one that was more similar. Bats reacted to playbacks with a variety of social behaviours. Prior to the analysis of the experiment an ethogram was done on three groups of captive *R. clivosus* bats. This ethogram was used to categorize the behavioural responses of these bats to the acoustic stimuli in the experiments. The reactions to the habituation-dishabituation experiments show bats perceive gender and individual-specific signatures found in their conspecifics echolocation calls. This is the first study to show behavioural evidence for individual discrimination and second to show gender discrimination of echolocation calls in high duty cycle bats. This evidence supports the theory that echolocation, a system thought to have evolved solely for orientation and foraging, has been co-opted for intra-specific communication and mate recognition in bats.

Keywords: Bats · Communication · Echolocation · Individual Discrimination · Gender Discrimination · Geographic Variation · Habituation-Dishabituation Paradigm · Ethogram

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CHAPTER 1

INTRODUCTION

Vocalizations have been present in the acoustic environment of vertebrates for millions of years and were important for the survival of the ancestors of many species, including humans. These vocalizations predate the existence of language and the production of vocalizations in humans may therefore have precursors in mammals (reviewed in Sidtis and Kreiman, 2012). What acoustic cues are used to convey different information and the fitness benefits vocal communication provides has been a focus of interest in the scientific literature and cover a diversity of animal taxa. Vocalizations are used in defense (alarm calls), for reproduction (mating rituals), to manage group living (hierarchies, social bonds, territories), when foraging (conveying location of food sources), and in parent-offspring interactions (Dugatkin, 2009). In intra-specific communication, these vocalizations often convey individual identity and additional attributes of an individual such as gender. This information is important for mate recognition and group living in social species such as humans and bats (reviewed in Sidtis and Kreiman, 2012).

For many species individual-specific signatures in vocalizations are used to facilitate familial identification (stranger - neighbor), in the formation of group bonds, and in mother-offspring interactions (kin recognition). The ability to recognize other individuals via their vocalizations have been discovered in primates, chiroptera, rodents, carnivores, proboscideans, cetaceans, perissiodactly, artiodactly, anura, and aves (reviewed in Kreiman and Sidtis, 2011). Some species, such as emperor penguins (*Aptenodytes forsteri*), use acoustic signals to locate their mate in large colonies. Their syrinx produces a two voice phenomenon that generates a beat pattern unique to each individual. Penguins of this species do not have nests to use as topographical cues when switching duties during incubation or chick rearing and therefore depend on these unique acoustic cues to find their partners (Aubin et al., 2000). Individual signatures have also been used by some animals to locate and identify offspring. In herd mammals, such as sheep, the mother and baby can recognize each other by their calls alone (Searby and Jouventin, 2003). This phenomenon is also observed in subantarctic fur seals *(Arctocephalus tropicalis)* where recognizing each other's voices allows mother and pup to be reunited in a crowd (Charrier et al., 2001). Recognizing individuals is also a requirement for reciprocally altruistic behaviours (Trivers, 1971). Species who produce these behaviours, such as some monkeys (Seyfarth and Cheney, 1984), birds (Krams et al., 2008), and bats (Wilkinson, 1984), may be using vocalizations to identify individuals during these interactions. Some frogs use individual signatures in defense. Familial identification of conspecifics calls by the North American bullfrog (*Rana catesbeia*) allows males to determine the level of aggression required to protect their mating grounds. Lower levels of aggression were displayed to calls from a familiar territorial neighbor compared to an unfamiliar individual (Bee and Gerhardt, 2002). In addition to recognizing a specific individual, additional attributes of this individual such as its gender can be communicated.

Communicating gender can be important for mate choice and same sex associations. Differences in vocalizations between males and females of a species can take the form of distinct calls, different acoustic structures within the calls, or unique call patterns (reviewed in Kreiman and Sidtis, 2011). For example, sex differences in the rate of social vocalizations were found in rhesus macaques (*Macaca mulatta*). Females call at higher rates than males and these vocalizations occurred more in intra- rather than inter-sexual interactions making them important in same sex associations (Greeno and Semple, 2009). In many songbirds and some bats, males but not females produce distinct calls to defend their territories and during courtship displays (reviewed in Hauser, 1996; Davidson and Wilkinson, 2002). In Greater White-lined bats (*Saccopteryx bilineata*), male vocalizations are used by females in mate choice. Males with complex song repertoires containing unique composite syllables attracted more females to their territories than those with smaller syllable repertoires (Davidson and Wilkinson, 2002). In other species such as humans, males and females produce the same type of vocalizations but the acoustic structure of these vocalizations differ. This is mainly attributed to sexual dimorphism in the vocal production anatomy of both genders (reviewed in Lieberman, 1986). Like humans, male and female baboons produce the same types of vocalizations known as grunts. Playback experiments showed that both males and females can discriminate between grunts belonging to different genders because of differences in acoustic structure (Rendall, 2003). Elephants can even recognize the difference between genders of other species. Playback recordings of female voices to elephants evoked significantly less behavioural responses associated with threat than did recordings of male voices (McComb et al., 2014). The examples so far have reviewed vocalizations which have likely evolved in a social communication context to allow for the transfer of information from one individual to another. However some systems have evolved for purposes outside of communication but have been co-opted for communicative purposes. When a system has evolved for one function but can be used to serve another it is known as an exaptation (Gould and Vrba, 1982). Echolocation is an example of such a system because it evolved for foraging and navigation but recent evidence suggests it has been co-opted for communication (reviewed in Arch and Narins, 2008; Jones and Siemers, 2011).

When animals echolocate they determine the size, distance, and texture of objects in their environment by emitting high frequency sounds and listening to their returning echoes (Griffin, 1958; Neuweiler et al., 1980; Neuweiler, 1990). Echolocation has evolved independently in several groups of animals (bats, toothed whales, some birds, and shrews) and is an example of convergent evolution (reviewed in Davies et al., 2012). These animals have evolved special anatomical structures that allow them to both emit and hear high frequency sounds (Neuweiler et al, 1980; Hutterer, 1985; Ketten, 1992; Tyack and Miller, 2002). In addition, two types of call strategies have evolved to help these animals solve the problem of self-deafening (forward masking) associated with echolocation. Forward masking occurs when outgoing vocalizations reduce the listener's sensitivity to weaker returning echoes. These two calling strategies include the production of either low duty cycle (LDC) or high duty cycle (HDC) echolocation calls. Duty cycle refers to the ratio of signal duration to the inter-pulse interval (time between the start of one call and the end of another). Most echolocating animals (some bats, birds, and odontocete cetaceans) use LDC echolocation and separate their calls from echoes in time. LDC calls consist of a short downward frequency modulated (FM) signal which can be steeply or shallowly modulated and the duration between calls is longer than the duration of the call itself (reviewed in Fenton et al., 2012). Some bats (*Rhinolophidae* and *Hipposideridae*) on the other hand use HDC echolocation calls and separate their calls from their returning echoes in frequency rather than time. Their calls are long duration, narrowband calls dominated by a constant frequency component (CF) with short time intervals between calls (Neuweiler, 1984). During flight, these bats lower the frequency of their emitted calls to compensate for Doppler shift effects. This strategy ensures the frequency of their returning echoes stays within the frequency range of an area in the cochlear region known as the acoustic fovea. The acoustic fovea is a specialized patch of the basilar membrane sensitive to a unique frequency range called the reference frequency. The reference frequency is usually higher than the frequency these bats produce when stationary (resting frequency) (Neuweiler et al., 1980). As a result of the acoustic fovea and Doppler shift compensation, HDC bats are excellent at detecting small changes in frequency such as the beating of an insects wings (Schuller and Pollak, 1979; Bruns and Schmieszek, 1980). In addition to echolocation types, acoustic parameters of the calls vary in intensity, frequency (i.e. call frequency, bandwidth), time (i.e. call duration, inter-pulse interval), and rate among different bat species and these variations are often related to how animals exploit their particular ecological niches (reviewed in Jones and Holderied, 2007).

Despite echolocation call design being shaped by function and phylogeny (reviewed in Jones and Teeling, 2006), intra- and inter-specific signatures (age, body condition, individual, sex, group affiliation, geographic variation, and species) have been found in the echolocation calls of both LDC and HDC bats (reviewed in Jones and Siemers, 2011). If bats can perceive these signatures then they can unintentionally (eavesdropping) or intentionally convey information to a receiver. Echolocation calls are often emitted at high intensities and repetition rates, making them good signals to use in a communication channel. However, these characteristics also makes them good targets for eavesdropping bats (Barclay, 1982) and potential prey that have evolved the ability to hear high frequency sounds (Jacobs et al., 2008). Eavesdropping bats then become privy to information encoded in the calls such as location of local roosts (Jones, 2008) and foraging sites (Barclay, 1982; Balcombe and Fenton, 1988; Fenton, 2003; Dechmann et al., 2009). While some studies have provided evidence that bats can perceive such signatures (Kazial and Masters, 2004; Kazial et al., 2008; Yovel et al., 2009; Schuchmann and Siemers, 2010; Voigt-Heucke et al., 2010; Knörnschild et al., 2012; Schuchmann et al., 2012; Puechmaille et al., 2014, Bastian and Jacobs, 2014 (under review)) more information is needed to discern the role this perception plays in communication and evolution. If echolocation is used in intra-specific communication to reveal the identity and gender of an individual, it could be relevant to mediating social interactions and for mate choice.

Individual-specific signatures in bats echolocation calls help reduce jamming effects associated with echoes (Suga et al., 1987) but may also facilitate social interactions within groups. These individual-specific signatures may result from either learning or from morphological and genetic differences in vocal structure between individuals (Jones and Ransome, 1993). Individual-specific signatures have been found in the echolocation calls of many LDC bat species (Brigham et al., 1989; Kazial et al., 2001; Fenton et al., 2004; Masters et al., 1995; Obrist, 1995; Siemers and Kerth, 2006; Yovel et al., 2009, Knörnschild et al., 2012). Some species of LDC bats such as *Tadarida teniotis* enhance the differences among individual's echolocation calls by temporarily shifting their dominant frequencies when flying together. This makes it easier for an individual to identify its own echoes from those of its conspecifics (Ulanovsky et al., 2004). Only two studies on LDC bats provide evidence for the perception of individual difference in echolocation calls. Using playback experiments Kazial et al. (2008) and Yovel et al. (2009), showed that *Myotis lucifugus* and *Myotis myotis* can discriminate between playback calls belonging to different individuals. Yovels' et al. (2009) study was innovative because it used both live bats and computer modeling to demonstrate that greater mouse eared bats, *M. myotis*, could classify echolocation pulses as belonging to different individuals. The recordings played to the bats were of individuals in flight indicating that bats discriminated between individuals calls regardless of task specificity. To discriminate subjects would classify individuals by learning the average call characteristics and using this as a reference. Whether these signatures can be reliably encoded among many individuals (i.e. in large colonies) still needs to be determined. The first person to suggest a HDC bat could classify individuals via their echolocation calls was Möhres (1967) after observing a captive colony of *Rhinolophus ferrumequinum*. Since then additional studies have shown individual-specific signatures in some HDC bat species (Suga et al., 1987; Hiryu et al., 2006; Siemers et al., 2005) but no study has tested if bats can perceive these signatures and use them to discriminate between the echolocation calls of different individuals. If bats can use echolocation for individual recognition they may be able to identify other attributes of an individual, such as their gender, age, group affiliation, or species identity.

The recognition of the gender of a conspecific by its echolocation call may have fitness benefits such as finding a suitable mate, facilitating same sex roosting (maternity colonies/ bachelor colonies), or forming same sex foraging associations. Gender-specific signatures are found in both HDC and LDC bats. The mean frequency of echolocation calls have been observed to differ between males and females of some HDC species (Neuweiler et al., 1987; Suga et a., 1987; Jones, 1995; Guillén et al., 2000; Siemers et al., 2005; Yoshino et al., 2008; Chen et al., 2009; Knörnschild et al., 2012; Odendaal et al., 2014). However, only one species of HDC bats, *Rhinolophus rouxi,* is recorded to have no overlap in call frequency between males and females (Neuweiler et al., 1987). In addition to frequency, Knörnschild et al. (2012) recorded a difference in the duration of calls of *Saccopteryx bilineata*. Females had higher frequency and shorter duration calls than males. These differences were shown to be used by male *S. bilineata* to greet incoming females into the roost while warding off rival males. Only two other studies have provided behavioural evidence that show bats can perceive gender-specific signatures in echolocation calls. Using playback experiments, Kazial and Masters (2004) showed female big brown bats, *Eptesicus fuscus*, could recognize sex from a callers echolocation signal. Female vocalization rates changed significantly depending on whether the bats were listening to male or female calls. However, which acoustic parameters the bats were using to distinguish sex were not clarified. Later Grilliot et al. (2009) discovered that in *Eptesicus fuscus,* acoustic parameters related to time, shape, and frequency of the calls were sexually dimorphic when roosting (social context) but not flying. Schuchmann et al. (2012) was the first to show that HDC bats, *Rhinolophus mehelyi* and *Rhinolophus euryale,* could discriminate between male and female echolocation calls. Which acoustic parameters they used to discriminate were never identified. Puechmaille et al.

(2014) showed that female *R. mehelyi* use gender-specific signatures in mate choice and preferentially selected males with higher call frequency. Call frequency in these bats was found to be an honest signal of body size and males with higher frequency calls tended to sire more offspring. However, this positive relationship between body size/condition and the frequency of echolocation calls within a species is only found in few bat species such as *Hipposideros fulvus* (Jones et al., 1994), *H. ruber (*Guillén et al., 2000), and *R. mehelyi* (Siemers et al., 2005). In other species, such as *Asellia tridens* (Jones et al., 1993) and *Myotis adverus* (Cooper et al., 2001), body size is inversely related to dominant frequency with some species showing no relationship between body size, body condition, and call frequency (Jones, 1995; Siemers et al., 2005). Other information that could be communicated in an individual's echolocation calls include its age, social group, and species identity (reviewed in Jones and Siemers, 2011).

Communicating age via acoustic signals might hold fitness benefits and costs for the signaler. Benefits include the potential to reduce conflict, illicit care, or signal the presence or absence of reproductive viability. Alternatively, signaling competitive potential may be a cost and therefore selection would work to disguise rather than signal age. While there are no behavioural studies showing bats can discriminate a callers age from their echolocation calls, several studies show age related differences in the call frequencies of *A. tridens* (Jones et al., 1993), *R. hipposideros* (Jones et al., 1992), *R. ferrumequinum* (Jones and Ransome, 1993), *Myotis daubentonii* (Jones and Kokurewicz, 1994), *M. lucifugus* (Jones, 1995), *R. euryale, R. mehelyi* (Russo et al., 2001), *R. blasii* (Siemers et al., 2005), *R. pumilus* (Yoshino et al., 2008), and *R. monoceros* (Chen et al., 2009). Juvenile bats echolocate at lower frequencies than adults in these species. In *R. ferrumequinum,* call frequency increases from one to two years of life, plateaus in years two to three, and declines in bats older than three years. This decline is especially marked between 10 and 23 years of life (Jones and Ransome, 1993).

In addition to age-specific signatures, bats may have social group-specific signatures encoded in their echolocation calls. Hiryu et al. (2006) documented changes in the resting frequency of a captive colony of *Hipposideros terasensis*. When new bats were added to the colony they changed their resting frequencies to match those of the existing members. The idea of group-specific signatures was further supported by a study on *Noctilio albiventris* who used different combinations of constant frequency and frequency modulated signals in their echolocation calls. With the use of playback experiments they showed that bats could discriminate between familiar and unfamiliar conspecifics (Voigt-Heucke et al., 2010). The strongest behavioural reactions were shown towards playbacks of unfamiliar conspecifics. This ability to discriminate between echolocation calls of familiar and unfamiliar conspecifics (groups) may benefit a gregarious bats species such *N. albiventris* by enhancing social bonds between group members. This is supported by the fact that group members caught emerging from roosts together were seen foraging together (Dechmann et al., 2009). In addition to discriminating between groups, *N. albiventris* was also shown to discriminate between calls of conspecifics and heterospecifics (Voigt-Heucke et al., 2010).

Echolocation calls are often good indicators of species identity and are sometimes used by both researchers and bats to discriminate between different species (reviewed in Jones and Siemers, 2011). Species identification is a prerequisite for mate recognition and territorial defense (reviewed in Dugatkin, 2009). Schuchmann and Siemers (2010) showed some rhinolophid bat species could distinguish between calls of their own species from those of closely related conspecifics and heterospecifics. *Rhinolophus euryale*, whose call frequencies completely overlap with *R. mehelyi*, could discriminate between calls but this ability was diminished. Thus echolocation frequency appears to be important for species discrimination, in addition to suggesting that bats could be using other acoustic parameters such FM components. Their study lends some support to the Acoustic Communication

Hypothesis which suggests that horseshoe bats separate their CF frequency bands to allow for within species communication and inter-specific discrimination (Heller and Von Helversen, 1989; Jacobs et al., 2007; Russo et al., 2007).

Species echolocation calls are shaped by their environment in which they forage and therefore vary geographically (reviewed in Jones and Holderied, 2007). Studying geographic variation in echolocation can be challenging. Echolocation call design is often shaped by different elements such as genetic differences between populations, geographic differences of habitat structure (i.e. temperature, humidity, foliage density), and potentially by their use in communication. Several studies on horseshoe bats that have constant frequencies are showing significant geographic variation of species' echolocation call design. Geographic variation in echolocation call frequency have been noted for *Rhinonicteris aurantia* (Armstrong and Coles, 2007), *Rhinolophus capensis* (Odendaal et al., 2014), *R. ferrumequinum* (Rossiter et al., 2007; Flanders et al., 2009), *Rhinolophus monocerus* (Chen et al., 2009), *R. pumilus* (Yoshino et al., 2008), and *Tadarida brasiliensis* (Gillam and McCracken, 2007). In *R. capensis,* a clinal increase in resting frequency was observed as a result of increasing vegetation cover across its distribution range in South Africa. It increased from 75.7 kHz in the west to 86.5 kHz in the east and was significantly different among the populations measured (Odendaal et al., 2014). In playback experiments, *R. capensis* was able to discriminate between two different populations of *R. capensis* echolocating at different frequencies (Bastian and Jacobs, 2014 (under review)). *R. monoceros* also showed clinal variation with frequency decreasing with latitude in Taiwan (Chen et al., 2009). If these differences are used in communication they could have potential fitness benefits. For example, identifying individuals from the same species but from different localities might help reduce the chances of inbreeding because individuals from different areas would be selected as preferred mates. Geographic signatures (dialects) would also allow bats to recognize familiar conspecifics and potentially ward off strangers who might be seen as a threat (stranger – neighbor conflict) (Temeles, 1994). These regional dialects of populations could allow for the formation of social interactions with local individuals during foraging. Besides using cues already present in echolocation bats may be modifying their calls for communication (Fenton, 2003).

Modified echolocation calls have been documented in some bat species (Suthers, 1965; Brown, 1976; Barclay et al., 1979; Andrews and Andrews, 2003; Andrews et al., 2006; Jahelkova, 2011; Clement and Kanwal, 2012). *Noctilio leporinus* warn or "honk" at other conspecifics that they are at risk of colliding with during flight by lowering the terminal frequencies of their echolocation calls (Suthers, 1965). Modified echolocation calls have also been associated with warning behaviours in *Pteronotus parnellii,* a HDC bat, who modifies the FM component of its echolocation calls when producing quick movements during fly-bys (Clement and Kanwal, 2012). Young and adult *Myotis lucifugus* avoid in-flight collisions by modifying the frequency at the end of the call (Barclay et al., 1979). In addition, *M. lucifugus* and *Antrozous pallidus* adults were recorded producing similar modified echolocation calls during agonistic encounters with conspecifics (Brown, 1976; Barclay et al., 1979). Andrews et al. (2006) discovered modified echolocation calls in a high duty species, *R. ferrumequinum,* whose calls were modified by prolonging the FM terminal sweep portion of the call. However, the context in which these calls were produced was not described. Further investigations into if these modified calls are present in other bat species and how they are used in various behavioural contexts would help to better understand the role they play in communication.

Within the context of intra-specific communication, this study investigated the existence of individual-specific signatures in the echolocation calls of *R. clivosus* (a HDC bat) and whether these bats use such signatures to recognize different individuals and attributes of individuals such as gender. Playbacks of recorded acoustic stimuli within a classical habituation-dishabituation experimental framework were used to do so. In pursuit of this, a captive colony of bats was established to generate an ethogram for this species that could be used to categorize the behavioural responses of these bats to the acoustic stimuli in the experiments. In addition, acoustic and morphometric parameters from individuals of different locations in South Africa were compared. On a more general level these experiments addressed whether echolocation, a system that has evolved for one purpose (foraging and navigation,) has been co-opted for another (intra-specific communication).

CHAPTER 2

MATERIALS AND METHODS

Study Animals

Rhinolophus clivosus (Geoffroys horseshoe bat) is a highly gregarious bat species with a widespread geographic range across Africa and Arabia (Csorba et al., 2003). Five genetically supported groups exist in South Africa (Stoffberg et al., 2012). These groups are found in different habitats including arid savanna woodland, deserts, and forest fringes (Csorba et al., 2003). Climates in these regions range from Mediterranean climates with winter rainfall to more arid, dry climates (Stoffberg et al., 2012). *Rhinolophus clivosus* roosts in caves and mine adits (Smithers, 1983). At Guano Cave in De Hoop Nature Reserve bats can form large colonies which can consist of thousands of individuals (Laycock, 1983; McDonald et al., 1990). In this cave *R. clivosus* roosts with four other bat species: *Miniopterus natalensis, Myotis tricolor, Nycterus thebaica*, and *R. capensis*. An estimated 300,000 individual are said to reside in Guano Cave (Mcdonald et al., 1990). *R. clivosus* is a medium sized bat (around 17 g) with medium wing loading (the ratio of wing area to body mass) of 9.1 N.m^{-2} and a low aspect ratio (area of wing length to width) of 5.5 (Schoeman and Jacobs, 2003; Jacobs et al., 2007). They fly at low altitudes foraging between shrubs, praying on moths and small beetles (Rautenbach, 1982). They are HDC echolocaters and their calls have high resting frequencies (91.7±1 kHz) (Jacobs et al., 2007).

Capture and Husbandry of Bats

Rhinolophus clivosus bats were captured throughout the year 2013 (March, April, May, June, September, November, December) and during summer 2014 (January, February, May) at De Hoop Nature Reserve on the southern coast of South Africa where *R. clivosus* is known to roost (Laycock, 1983; McDonald et al., 1990; Jacobs et al., 2007). Bats were caught inside the cave during the daytime using hand nets. Sex was determined visually and age was determined by the presence of cartilaginous epiphyseal plates in the finger bones of juveniles (Anthony, 1988). Forearm length was measured (to the nearest 0.1 mm) using dial calipers. It was used as a measure of body size instead of mass because mass varies diurnally and seasonally. All pregnant, lactating, or juvenile bats were released immediately.

For playback experiments at De Hoop, bats were kept in a two-person tent (105 cm X 105 cm X 20 cm) at a field station nearby with free access to water. To ensure they were hydrated they were given water with a pipette. Bats were hand fed 45 to 55 mealworms a night in order for them to maintain their capture weight. The food source *Tenebrio molitor* larvae (mealworms) were raised on a combination of dry dog food, mineral powder (Life-Gain), fresh fruit, vegetables, and wheat bran. This ensured the mealworms contained the essential nutrients bats require based on previous experience by A. Bastian where three different bat species where kept for >1 year in captivity on mealworms fed on this diet. Daily measurements of weight, health, and eating behaviours were recorded to monitor the health of the bats. Bats were kept for no longer than five nights in the field.

To select which bats were to be part of the captive colonies, up to ten bats were caught each time at Guano Cave and closely monitored at the research house for three days. Four or five of the bats with the highest weight, best body condition, and that ate well when hand fed were transported in soft cotton bags to an animal housing facility at University of

Cape Town (UCT). The other five bats were released back into the cave. In total, three groups of bats were housed in captivity at UCT in September (four males), January (four males), and February (two males and three females). At UCT bats were kept in a netted enclosure inside an observation room where they were able to fly freely without incurring injuries. Material hung from the netting was used as roosts for the bats (Figure 1). The bats were examined by a registered veterinarian upon arrival to UCT and before they were returned to the cave after this study. Entrance to the netted enclosure was sealed with magnets and weighted at the bottom using sandbags. Disposable shoe covers, gloves, and lab coats were worn at all times while in the room to protect the bats against outside pathogens. To prevent the drying of wing membranes, the humidity was kept between 50% and 80%. The temperature was kept between 24°C and 25°C within the temperature ranges of the cave (19-31ºC) (Laycock, 1983; personal records). Bats had free access to water and food at all times via a water trough, three ice cube trays, and small food bowls placed throughout the room (Figure 1). They were also occasionally given black soldier flies as a dietary supplement and environmental enrichment. Day and night cycles were shifted using a timer switch. Night was simulated to occur at 7h00 in the morning and sunrise was set to 19h00 at night to allow observations to be done during the day. Everyday bats were hand fed at 15h00 with a stable diet of mealworms. Health monitoring sheets were used to record the bats daily weight, body condition, behaviour, and any stress occurred during the day. All capturing, handling, housing, and treatment of bats followed the guidelines recommended by the American Society of Mammalogists (Animal Care and Use Committee, 1998) and were in compliance with both the UCT Animal Ethics Committee (2013/v13/DJ) and Cape Nature (AAA007-00009-056).

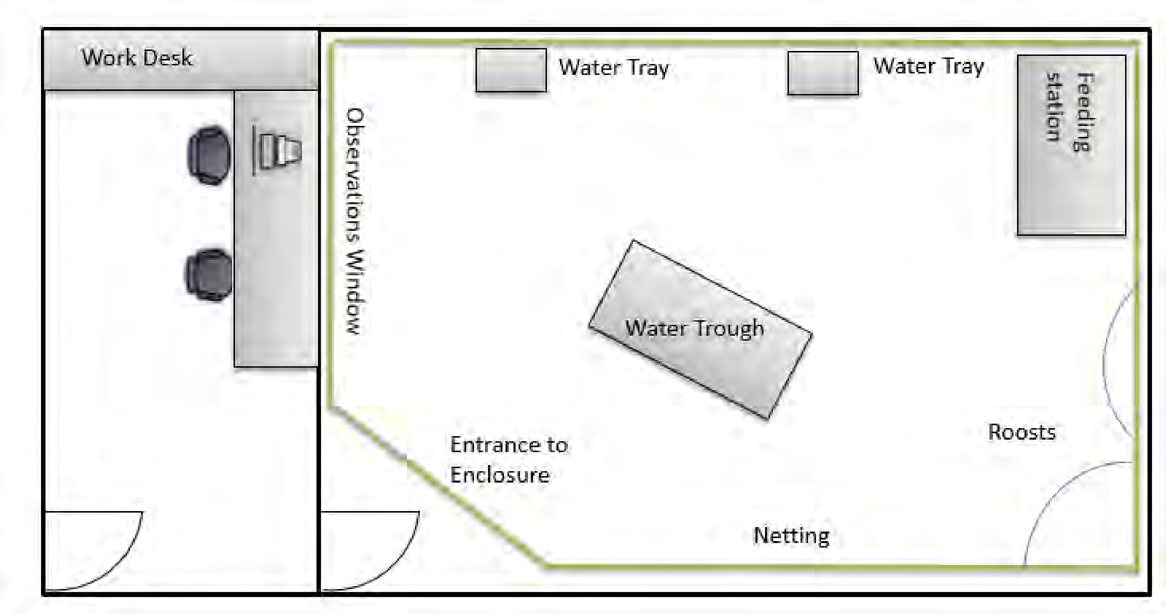

Figure 1. Layout of the bat housing and observation room (3.3 m X 3 m X 5 m) showing the roosting area, water trays and trough, feeding station, and work desk (figure generated with Floorplanner.com, Rotterdam, The Netherlands).

Determining the Gender or Individual Specificity of Echolocation Calls

A total of 122 *R. clivosus* individuals were recorded from across the geographic range of *R. clivosus* in South Africa (Figure 2). The recordings from 48 of these bats were obtained during previous experiments by other members of the UCT Animal Evolution and Systematics Group. Echolocation recordings were taken 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 sampling frequency of 384 kHz. Multiple acoustic parameters from 10 calls from each of the 122 bats were analyzed using Avisoft SASLab Pro (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany) automatic measurement function to determine if there was variation between different individuals, sexes, and locations. All 10 calls were used to test for individual variation. When testing for sex and location effects the average of the 10 calls for each individual was used. To test if 10 calls provided sufficient coverage of call variation, the variances of the acoustic parameters of 10 random calls from an individual were compared to 20 random calls of the same individual (Levenes test). This was done for three separate individuals. The first 10 calls of a recording were never used because horseshoe bats have been shown to tune into their resting frequencies from lower frequencies after periods of silence (Schuller and Suga, 1976; Siemers et al., 2005). The acoustic parameters measured were resting frequency (peak frequency of the dominant harmonic of the bat when at rest; kHz), call duration (duration of the call from beginning to end; ms), inter-pulse interval (time between the end of one call and the beginning of the next; ms), duty cycle (pulse duration / (pulse duration + inter-pulse interval) * 100, expressed in %), distance from the start frequency to the maximum amplitude of the call (distomax; ms), as well as minimum frequency (kHz), bandwidth (resting frequency - minimum frequency; kHz), and sweep rate (bandwidth / duration) of the initial and terminal FM sweeps.

Measurements were taken from the second harmonic because in HDC bats most energy is placed in this harmonic (Pye and Roberts, 1970).

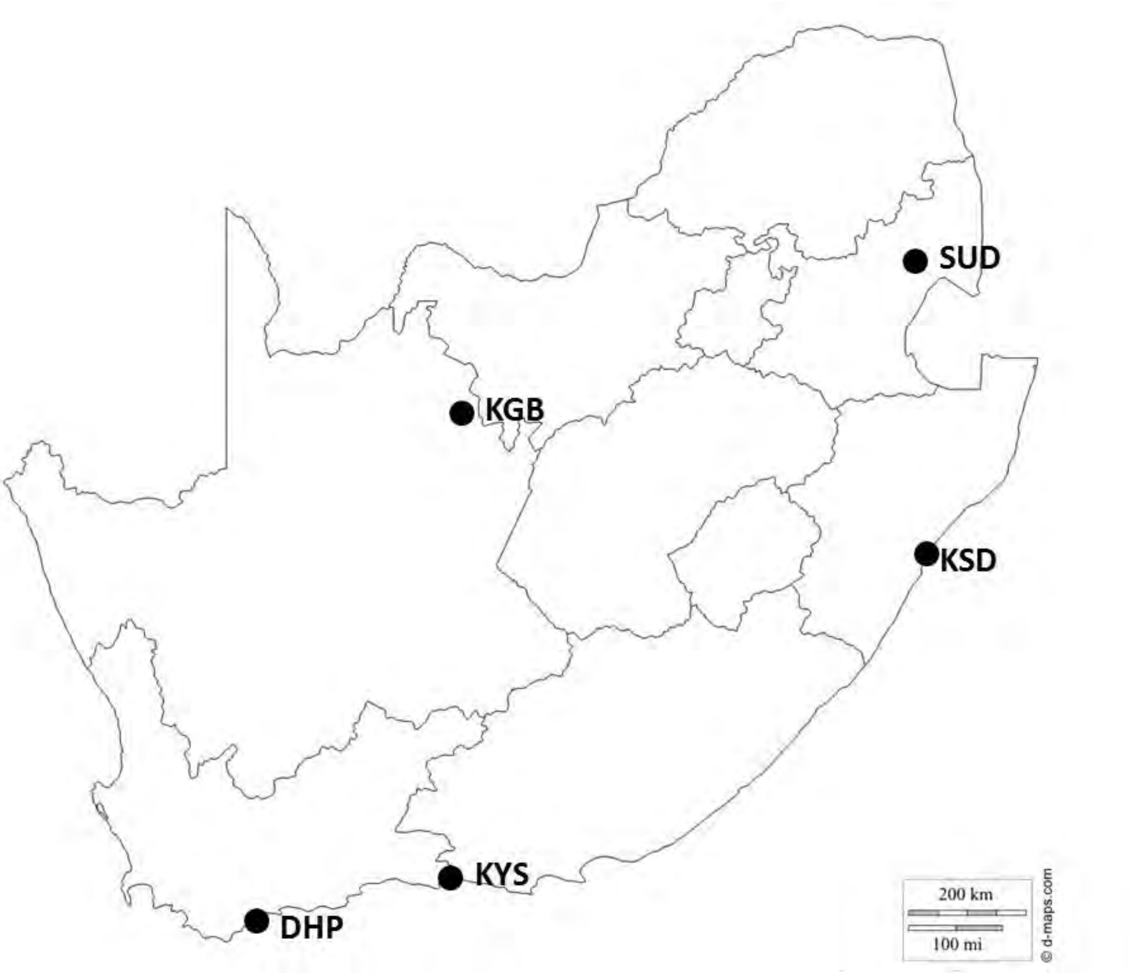

Figure 2. Localities in South Africa from where echolocation recordings were obtained. DHP = De Hoop Nature Reserve (34.43° S, 20.55° E), KYS = Knysna (34.04° S, 23.05° E), KGB = Koegelbeen Caves (28. 39º S, 23.91ºE), KSD = Kokstad (30.55° S, 29.43° E), and SUD = Sudwala (25.37° S, 30.70° E).

Compiling an Ethogram for R. clivosus

Before habituation–dishabituation experiments were conducted an ethogram of *R. clivosus* was established to better categorize the responses of bats to the experimental stimulus. An ethogram is a catalogue of an animal's behavioural repertoire and the context in which they occur. Establishing an ethogram on the study species before analyzing behavioural response data is important because the same behaviour observed in one species, such as tail wagging in dogs, may mean something completely different in another species, such as cats. Ethograms are also important for the implantation of effective species specific conservation strategies. Animals react to sudden changes in their environment by changing their behaviour. These behavioural changes are usually an indication of environmental stress as the animal aims to reduce the negative effects of unfavorable conditions (Morse, 1980). Basic behavioural data allows conservationists to monitor populations and when necessary develop appropriate management strategies.

Ethograms of bats have focused on both captive and wild colonies. Behavioural data from bats in the wild gives information on how bats interact with both other bats within their environment and with the environment itself. Captive studies allow researchers to get data on the behaviour of individual bats and interactions between the same individuals that would otherwise be difficult to get from colonies in the wild. Some of these behaviours observed in both captive and wild bats include agonistic interactions (i.e. Bastian and Schmidt, 2008; Singaravelan and Marimuthu, 2008), foraging behaviour (i.e. Fleming, 1982; Hickey and Fenton, 1990), roosting behaviours (reviewed in Kunz et al., 1982), mother infant bonding (i.e. Matsumara, 1979; Matsumara, 1981), group bonding (i.e. Boughman and Wilkinson, 1998; Voigt-Heucke et al., 2010), mating rituals (reviewed in McCraken and Wilkinson, 2000), and the production of social calls to mediate such social interactions (i.e Matsumara, 1979; Matsumara, 1981; Andrews and Andrews, 2003; Ma et al., 2006; Clement and Kanwal, 2012).

To establish an ethogram, video and audio recordings were taken of three groups of captive bats. The ethogram was used to better interpret the behaviours produced by the bats during the trials in context of the experiment. As part of a separate study to match contexts, behaviours and social calls, simultaneous sound recordings were also taken (D1000X detector, Pettersson Elektronik AB, Uppsala, Sweden). A combination of ad libitium, continuous recording, and behavioural sampling were therefore used. This entailed recording all visible behaviours for a set duration of time. Behavioural sampling was used to record social calls. Bats were videotaped using the Sony DCR-SR42E infrared-sensitive camera (Minato, Tokyo, Japan) and an IR-spotlight (Ecoline, Security-Center, Germany) for additional illumination. During behavioural sampling the group of bats was observed and each occurrence of a behaviour and the individuals involved was recorded. Bats wore necklaces with different letters for identification purposes. The microphone, camera, and spotlight were aligned on the same tripod to ensure all three devices captured the same behaviours. Before recordings began, bats were given a week or more to habituate to the environment after transportation to the housing facility at UCT. Recordings were made for two hours a day over a period of up to two weeks for each group. This took place at random times of the day between 7h00 and 19h00 when night was simulated. All video recordings were analyzed on Solomon coder (copyright András Péter, Version beta 14.03.10) and a list of behaviours was created (Table 1). Bats in the study also produced a variety of social calls. Recordings of these calls will form the contextual basis of future analyses of the social calls recorded from the captive colony of bats (not included here).

Table 1. Ethogram of *R. clivosus* from Captive Colonies

Behaviours	**Description**
Solitary behaviours	
Grooming	Cleaning of fur, wings, ears, and feet with tongue and feet
Yawn	Opening of the mouth and inhaling air
Look Up	Raising head away from the body, either fully or partially; accompanied by ear twitching
Scanning	Lifting the head and looking around the room; accompanied by ear twitching
Swivel	Rotation of body by at least 180° degrees; usually accompanied by scanning
Ear Twitching	Movement of the entire ear(s), or parts of the ear, either singly or in succession and either rapidly or slowly.
Leg Contraction	Contraction of leg(s) towards the body by bending the knee(s), either fully or partially contracted
Vibrating Wings	Vibration or small flapping of the wings against the body
Partial Wing Stretch	Partial extension of wing(s) from shoulder to fingers
Full Wing Stretch	Full extension of wing(s) from shoulder to fingers
Body Swaying	A movement of the body from side to side
Head Swaying	Movement of head from left to right without looking up
Body Shuffle	Shaking and repositioning of the body usually ending in an inactive position
Crawl	Moving from one location to another by placing one foot in front of the other
Repositioning	Picking one or both legs up and placing it back down to get comfortable; often ending in a sleeping position
Solo Flight	One bat in flight
Hovering	A bat remaining in one place in the air; usually next to a roosting conspecific
Drink from Trough	Drinking from the trough on the ground while in flight
Drink from Tray	Hanging from net and drinking from the tray

Eat Worms	Eating worms from a tray
Eat Fly	Catching a fly in flight
Chew	A repeated biting; usually occurs with eating or after grooming
Spit	Ejection of saliva from mouth; often after eating or grooming
Defecate	Discharging faeces or urine from the body
Interactive behaviours	
Tandem Flight	More than one bat in flight
Aerial Chase	The pursuit of one bat on another during flight
Aerial Collision	Two bats colliding in air during a tandem flight; often occurring after an aerial chase
Approach	During flight a bat approaching a roosting conspecific; sometimes accompanied by hovering
Land Next to	A bat landing in close proximity to a roosting conspecific
Landing on	A bat landing on a roosting conspecific
Crawl towards	Moving towards a conspecific
Crawl away	Moving away from a conspecific
Look at	Looking at a conspecific; accompanied by ear twitching
Lean towards	Body inclined and head towards the direction of conspecific while feet remain stationary
Lean away	Body and head pulled back away from direction of conspecific while feet remain stationary
Huddle	Two or more bats nestled closely to each other
Shift Position in Huddle	Repositioning of body while remaining in a huddle with one or more bats
Reach	Folded arm(s) or wrist(s) make contact with or are extended towards a conspecific
Wing Swat	Rapid swiping of arm(s) or wrist(s) in the direction of another bat
Bite	A bat using its teeth on another bat

Nuzzle	Rub or push against another bat with the nose or mouth

Playback habituation-dishabituation experiments

Playback 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. The experiments tested if bats could discriminate between sexes ("sex discrimination trials") or between individuals ("individual discrimination trials") based solely on echolocation calls. Experiments followed the habituation-dishabituation paradigm. Playback experiments following this paradigm compare the responses of animals when listening to audio recordings of different vocalizations (stimuli). These experiments are ideal when testing if subjects can discriminate between two sets of stimuli and therefore perceive these stimuli as belonging to different classes (i.e. one gender or one individual) (reviewed in McGregor, 2000).

Before each set of habituation-dishabituation trials bats were given water and fed five meal worms to motivate them. At the start of each trial a bat was placed on a perch (a small straw basket) in a sound proof, anechoic experimental box (Figure 3) which were set up following Schuchmann and Siemers (2010). 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) was placed at the opposite end of the box, all pointed at the perch (Figure 3). The camera was connected to a video monitor outside the box allowing the researcher to observe the behaviours of the bats during the trials. After a bat had become calm on the perch a sequence of calls from the same class (one individual or one gender) (habituation stimulus) was played through the loudspeaker to habituate the bats to this class (Table 2). Habituation was defined as the bat hanging calmly on the perch and remaining motionless for 20 seconds. After the bat became habituated, the stimulus was changed to the test stimulus (dishabituation stimulus) followed by a rehabituation stimulus, white noise, and a low frequency beep

(Figure 4). Any resumption of attentive behaviours such as ear or head movements or echolocation activity after the change of stimuli indicated the bat had discriminated between the stimuli. The rehabituation stimulus consisted of calls from the same class as the habituation stimulus. This was a control for false positive reactions to make sure that any response to the dishabituation trial was not due to spontaneous recovery from habituation (Rendall et al., 1996). White noise at the end of each trial was used as a 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 (Balcombe and Fenton, 1988). The low frequency sound allowed time on video and sound recordings to be synchronized by matching the audio line on both recordings. Each bat listened to five trials. A trial is a combination of one class of calls used as the habituation stimulus and the playback class used as the test stimulus followed by the rehabituation stimulus. The control trials (trial one and three in Table 2) were also used as controls for false positive reactions. The trial each bat listened to (Table 3) and the order in which the bats were tested (Table 4) was randomized each night to ensure that every night a bat listened to a different test at a different time of the night to previous nights. Later tests could then reveal if the order a bat listened to a trial had an effect on the strength of its response. Experiments occurred at night in the field and during the day in the captive colony when night was simulated.

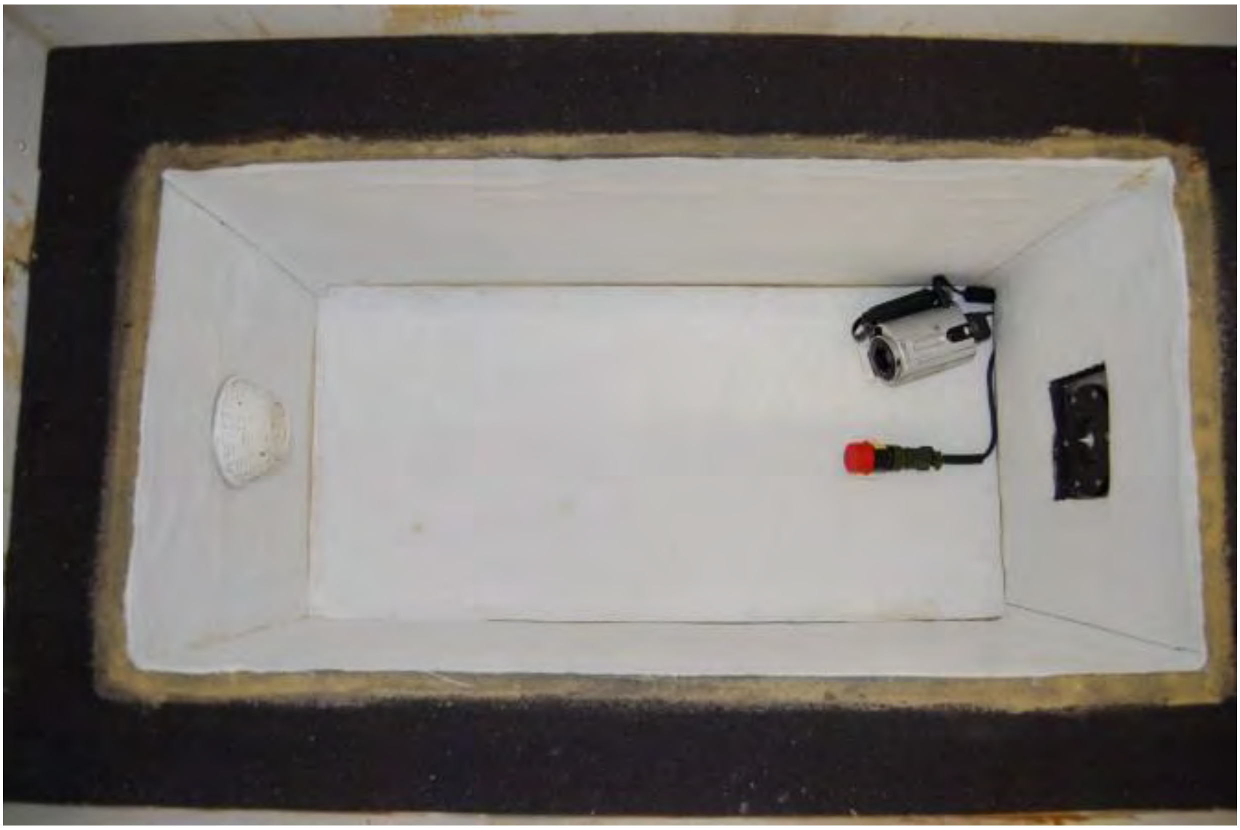

Figure 3. The sound proof, anechoic testing box (77.5 cm x 38 cm x 38 cm) containing a perch, infrared-sensitive camera, loudspeaker, and microphone (cover of the box not shown).

Table 2. Combinations of playback classes for both sex (trials 1 and 2) and individual (trials 3, 4, and 5) discrimination trials presented to each bat.

	Trial 1 *Control*	**Trial 2** *Sex test*	**Trial 3** *Control*	**Trial 4** *Indv test1*	**Trial 5** *Indv test2*
Females listening to:					
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
Males listening to:					
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

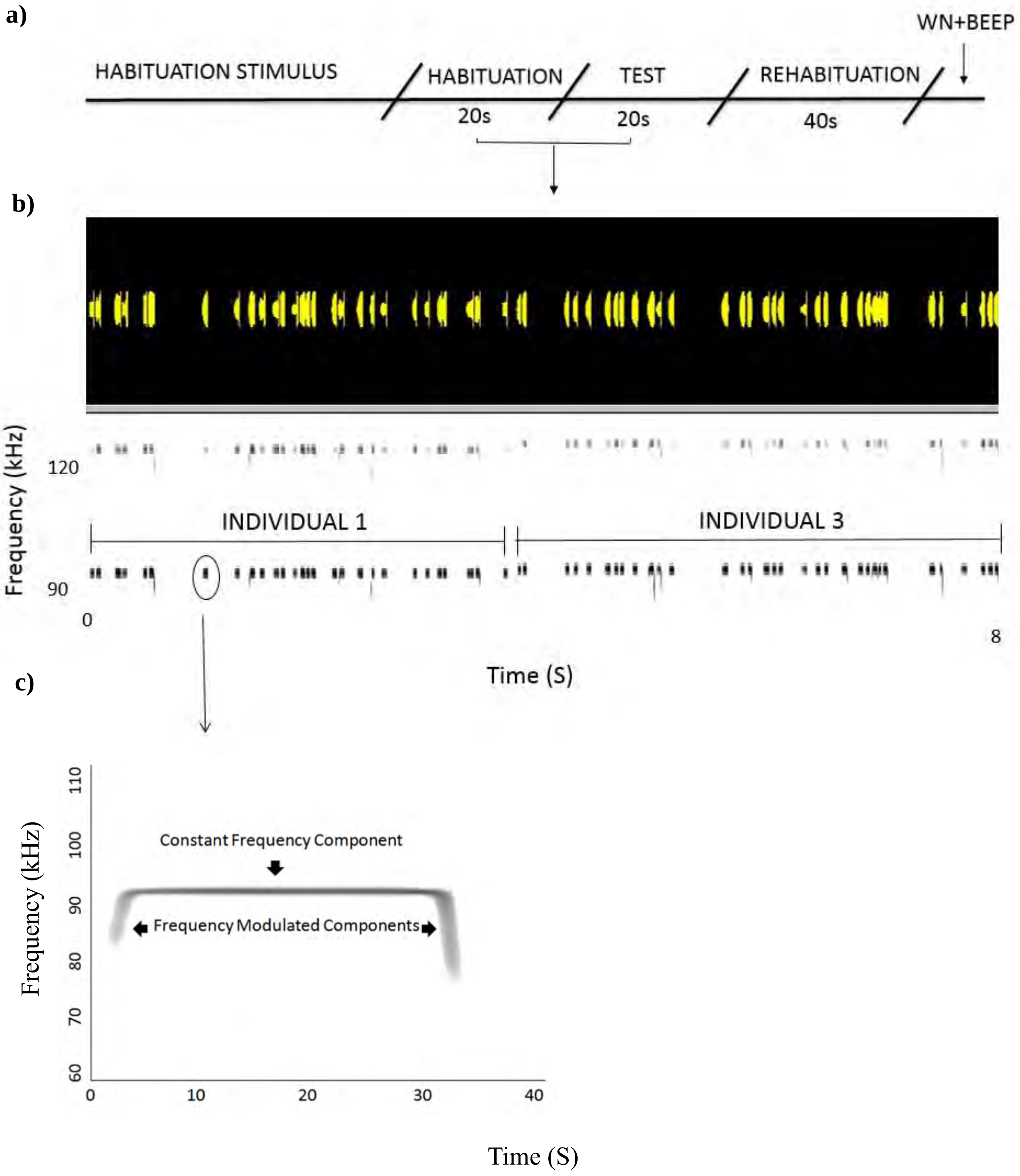
a)
HABITUATION STIMULUS
HABITUATION
TEST
REHABITUATION
WN+BEEP
20s
20s
40s
b)
Frequency (kHz)
120
90
INDIVIDUAL 1
INDIVIDUAL 3
0
8
Time (S)
c)
Frequency (kHz)
110
100
90
80
70
60
Constant Frequency Component
Frequency Modulated Components
0
10
20
30
40
Time (S)

Figure 4. Schematic of a) playback sequence, b) oscillogram and sonogram from a portion of an individual playback file (second and third harmonics shown), and c) a typical echolocation call. The slight difference in call frequency between Individual 1 and 3's calls can be observed in the sonogram.

Table 3. Randomized order of trials presented to each bat per night (trials are described in Table 1).

	Night 1	**Night 2**	**Night 3**	**Night 4**	**Night 5**
Bat A	Trial 5	Trial 2	Trial 4	Trial 1	Trial 3
Bat B	Trial 2	Trial 4	Trial 3	Trial 5	Trial 1
Bat C	Trial 3	Trial 1	Trial 5	Trial 4	Trial 2
Bat D	Trial 4	Trial 3	Trial 1	Trial 2	Trial 5
Bat E	Trial 1	Trial 5	Trial 2	Trial 3	Trial 4

Table 4. Randomized order of bats used in the experiment each night.

	Night 1	**Night 2**	**Night 3**	**Night 4**	**Night 5**
Order 1	Bat C	Bat A	Bat B	Bat D	Bat E
Order 2	Bat D	Bat B	Bat E	Bat A	Bat C
Order 3	Bat E	Bat C	Bat D	Bat B	Bat A
Order 4	Bat B	Bat E	Bat A	Bat C	Bat D
Order 5	Bat A	Bat D	Bat C	Bat E	Bat B

Playback Stimuli

Echolocation recordings from 41 bats (22 males and 19 female) at De Hoop were used. Playback files were created using Avisoft Bioacoustics SasLab Pro (Avisoft Bioacoustics, Version 4.2, Glienicke, Germany). Calls with good signal to noise ratio were selected. These natural calls were used as a template to create semi-synthetic calls to exclude noise or recording artefacts from the playbacks. These semi-synthetic calls were used to construct either sex discrimination or individual discrimination playbacks.

Sex discrimination playbacks were created using echolocation calls from either male or female individuals. To decrease the chances that bats were memorizing a specific individual or set of calls instead of characterizing the calls as belonging to a specific gender, 380 calls (10 calls per individual) from 38 different individuals (19 males and 19 females) were used to construct the playback stimuli used to test bats' ability to distinguish between genders. Two 10 minute long habituation playback files, one containing male and one containing female calls, were made by randomly mixing calls from these 19 males and 19 females, respectively. Two 20 second long dishabituation stimuli were made by using the same calls from the individuals used in habitation stimuli but compiled in a different order. Arranging calls in a different order and using a high number of calls in the playbacks (all playbacks: 690 calls: gender discrimination playbacks: 390 calls, individual discrimination playbacks: 300 calls) ensured bats were not memorizing a specific set or order of calls. The last 40 seconds of the playback sequence (rehabituation phase) contained calls from the same gender as used in the habituation. The playback sequence ended with white noise (three seconds) and the low frequency sound (five kHz, one second).

Stimuli for individual discrimination trials were made from 300 calls from three male individuals (100 calls per individual) and their respective natural inter-pulse intervals. One 10 minute long habituation playback file was made by randomly mixing 100 calls from Individual 1. A dishabituation playback file was created for each of the three individuals. The 20 second long dishabituation stimuli consisted of 100 calls from Individual 1, 2, or 3 compiled in a random order. Individual 1's dishabituation stimuli had the same calls as the habituation stimuli but presented in a different order. It served as a control test playback (trial three, Individual 1) to control for false positive reactions. The last 40 seconds of the playback (rehabituation phase) was constructed from calls from Individual 1 (habituation). The playback sequence ended with white noise and a low frequency sound.

All stimuli were normalized to the same intensity level to exclude the possibility that bats reacted to differences in intensity between calls (Figure 3). To determine the intensity level the intensity of the stimuli was compared to those of a recording taken of a bat on the perch inside the box with the microphone at the opposite end at a medium gain (microphone intensity). The intensity of the stimulus was then adjusted to two thirds that of the bats echolocation calls. The first call in all playback files was faded in and out as well as was the white noise and low frequency beep. This prevented a noise often produced by the loudspeaker when starting a playback with sudden onset of high intensity sounds.

Video Recordings

Videos of the bats' responses to playback experiments were analyzed by one observer (N. Finger) on Solomon Coder (copyright András Péter, Version beta 14.03.10) using a frame-by-frame analysis with 25 frames per second. To test for observer bias 10 randomly selected videos were coded by one other observer and tested for inter-observer agreement. Intra-observer reliability was tested when the main observer re-analyzed the same 10 videos at the end of the study. All audio and video recordings were renamed with random numbers so the videos were coded with the observer unaware of the stimulus presented to the bat. This prevented the coder from being biased by the test stimulus. Whether or not the experimental bats reacted to the habituation, test, rehabituation, and motivational stimulus were recorded as categorical data (yes or no answers). The frequency and duration (quantitative data) of all behaviours were coded for the first 20 seconds of the habituation stimuli (STARTHAB), the last 20 seconds of the habituation stimuli (ENDHAB), 20 seconds of the test stimuli (DISHAB), and the first 20 seconds of the rehabituation stimuli (REHAB). 20 second timeframes were coded because the duration of the tests stimuli was 20 seconds long. The timeframes were then comparable because the duration of the behaviours coded was the same for all four timeframes. Audio recordings were used to calculate the exact time point the habituation was played in the video files using the synchronization beep recorded on both the video and the audio device. Large audio files were split using Wave Splitter (ClaudioSoft 2000 software, Version 2.10, Ile-de-France, France) and Audacity (Audacity Team, Version 2.0.3, SourceForge.net) was used to recover corrupted files. Behaviours were then grouped into three categories attentive, active, and inactive behaviours (Table 5). Inactive behaviours were described as the bat not engaging in any movement. Attentive behaviours differentiated from active behaviours in that they indicate a response to the stimulus and were therefore considered a reaction. These behaviours include orientation behaviours (look up, scan), listening behaviours (slow and rapid ear twitching), startled behaviour (leg contraction), and the emission of echolocation calls. Echolocation was also considered a response to the stimuli. The number of bats that emitted echolocation calls to the test (dishab) stimuli of each trial was counted in BatSound Pro (Pettersson Elektronik AB, Version 3.31a, Uppsala, Sweden) with a sampling rate of 384 kHz (16 bits, mono) and slowed down 10 times. Most active behaviours with the exception of grooming usually occurred as a result of the bat settling down (followed by inactive behaviours) or were behaviours that occurred throughout the trials (single ear twitch, tip of ear twitch). Attentive behaviours were further classified into weak, medium, or strong reactions. Strong reactions included strong orientation movements and startled behaviours including full look up, scan, and full leg contraction. Weak orientation (partial look up) and startled (partial leg contraction) behaviours as well as obvious listening behaviours (rapid ear twitch) were considered medium reactions. Weak listening behaviours (slow ear twitch) were considered weak reactions. Trials where bats did not react to the white noise (motivational control) were excluded from the analysis.

Table 5. Behaviours displayed by bats during habituation-dishabituation experiments

Behaviours	**Description**	**Strength class**
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)	
Single Ear Twitch	Movement of an entire ear	
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	
Body Swaying	A movement of the body from side to side	
Grooming	Cleaning of the coat, wings, ears, and feet with tongue and feet	
Attentive		
Slow Ear Twitching	Movement of the entire ear(s) in a slow succession	Weak
Rapid Ear Twitching	Movements of the ears in a rapid succession unable to count in real time	Medium

Partial Look Up	Raising head 45° or less away from the body, sometimes accompanied by ear twitching	Medium
Full Look Up	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
Partial Leg Contraction	Contraction of leg(s) towards the body by bending the knees slightly	Medium
Full Leg Contraction	Contraction of leg(s) towards the body by bending the knees fully	Strong

ANALYSIS

Test for Perception of Acoustic Cues of Individuality and Gender

All statistical tests were done in Statistica (StatSoft, Version 12, Tulsa, USA). Two tailed non-parametric tests were used. Kappa Coefficients and Wilcoxon matched pairs tests were used to test for intra- and inter-observer reliability of the frequency and duration of behaviours coded for 10 videos. To test if bats were completely habituated to the habituation stimuli the duration of attentive behaviours at the beginning (first 20 s) and end of the habituation stimuli (last 20 s) were compared using a Wilcoxon matched pairs test across all trials. The effect of sensory habituation from 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 at the beginning of habituation (first 20 s) per order (1st, 2nd, 3rd, 4th, 5th) across individuals and nights. This determined if the order bats listened to trials (effect of sensory habituation) had an effect on the responsiveness to stimuli. To test for false positive responses to the test playbacks the duration of attentive behaviours during endhab and rehab were compared for each trial using a Wilcoxon matched pairs test. False positive reactions were tested using a Wilcoxon matched pairs test to compare the duration of attentive behaviours for endhab vs. dishab for the control test playbacks that had calls from the same gender or individual as the habituation. Comparing the duration of attentive behaviours for endhab vs. dishab for the test trials was also used to determine if the bats reacted to the test stimulus. The number of bats that emitted echolocation calls to the dishab of the test trials was compared to the control trials for both the sex discrimination (Wilcoxon matched pairs) and individual discrimination trials (Friedman Anova and post hoc Wilcoxon matched pairs with a Bonferonni correction). Yes and No reactions (the bat reacted or did not react) to both the dishab and rehab portions of the trials was also tested for statistical significance using Pearson Chi-squared tests.

Acoustic Characterization of R. clivosus Echolocation Calls

Rhinolophus clivosus echolocation calls were characterized by multiple acoustic parameters and the effect of body size (forearm length) was assessed (tested). General linear models (GLMs) tested for geographic variation (within sexes) across their distribution range in South Africa (Figure 4) as well as gender and individual differences from bats from De Hoop. The categorical predictor variables were sex, location, and individual. The dependent variables were the acoustic parameters of the echolocation calls and forearm length. When testing for location differences, stratified sampling was applied to data from De Hoop because of the large difference in sample sizes between locations. A total of 16 individuals were randomly selected (8 male and 8 female) from the De Hoop data set. All data were log transformed because of the different measurement scales of the parameters. To ensure the subset of calls used to compile the playbacks was not a bias sample and represented calls taken from the broader population of bats from De Hoop (others), acoustic parameters between the two sets of calls were compared using a GLM. Averages of 10 calls per individual were used. The categorical variables were type (playback male (n=22), other male (n=23), playback female (n=19), other female (n=24)) and the dependent variables were the acoustic parameters of the calls. A discriminant function analyses (DFA) determined if and which individual/gender-specific signatures existed in the playback calls that allowed them to perceive differences between call types (male or female, different individuals). Stimuli tested in the DFA's were calls used in the test portion of the individual (three individuals; 300 calls) and sex discrimination trials (39 individuals; 390 calls). These DFA's also indicated which acoustic parameters attributed most to classifying calls to a particular individual or gender. To reduce the number of correlated acoustic parameters, the DFA was run on components derived from a principle component analysis (PCA). Squared Malalanobis distances indicated the degree of acoustic similarity between both genders and the three individuals used in the playbacks. The shorter the Malalanobis distances to each other the more acoustically similar they are in the defined DFA's signal space. A DFA on principle components of acoustic call parameters was also used to test for location-specific signatures (500 calls from 51 individuals belonging to five different locations) and individual-specific signatures from the broader population of individuals from De Hoop (880 calls from all 88 individuals).

CHAPTER 3

RESULTS

Test for Perception of Acoustic Cues

A total of 37 (22 males and 15 females) bats listened to all sex and individual discrimination trials. For these 37 bats, there were both categorical (yes and no) and strength of response data for all trials. Data on the duration of attentive behaviours to the dishab and rehab were collected for 20 (11 males and 9 females) of the 37 bats for the sex discrimination trials and for all 37 bats for the individual discrimination trials.

Intra- and inter-observer bias was discounted for both count (Kappas Coefficient: K=0.85, N=10, P<0.05; K=0.87, N=10, P<0.05) and duration (Wilcoxons matched pairs test: T=230, Z=0.64, N=10, P=0.5249; T=151, Z=0.622, N=10, P=0.534) data. Male and female data were pooled because no differences were found in the duration of attentive behaviours between genders when listening to starthab, endhab, dishab, and rehab of individual discrimination (Mann Whitney U test: U=1291, N2=22, N1=15 P=0.273; U=1463, P=0.949; U=1452, P=0.897; U=1452, P=0.897) or sex discrimination trials (Mann Whitney U test: U=221.5, N2=22, N1=15, P=0.073; U=315, P=0.751; U=196, N2=11, N1=9, P=0.968; U=194, P=0.925). The repeated exposure to the habituation playbacks over five nights had an effect on the responsiveness of bats as the duration of attentive behaviours declined significantly (Friedman Anova: $\chi2$= 12.37, N=37, DF=4, P=0.0148). A comparison of the attentive behaviours at the beginning and end of habituation showed bats were fully habituated before listening to the test stimuli (Wilcoxon matched pairs test: T=0, Z=11.67, N=185, P < 0.001). The average duration for attentive behaviours at the beginning of habituation was 10.18 s compared to 0.006 s at the end of habituation.

The positive controls were not found be significant so it is highly unlikely that reactions to the test were a result of spontaneous recovery of prehabituated levels of response (Rendall et al., 1996). For both sex (Wilcoxon matched pairs test: T=0, Z=1.34, N=20, P=0.179) and individual (Wilcoxon matched pairs test: T=0, Z=1.34, N=37, P=0.179) control trials, no difference was found in the duration of attentive behaviours for endhab vs dishab. For the sex discrimination trials, 17 bats (85%) did not react to the rehab stimuli of the control (Pearson Chi Squared: $\chi 2$ (1, N=20) =5.58, P=0.0181) and 18 bats (90%) did not react to the rehab stimuli of the test (Pearson Chi Squared: $\chi 2$ (1, N=20) =7.62, P=0.005). In the individual discrimination trials, 35 (94%) bats did not react to the rehab stimuli of the control (Pearson Chi Squared: $\chi 2$ (1, N=37) =18.49, P=0.000) and 32 (91%) bats did not react to rehab stimuli of test individual trials (Pearson Chi Squared: $\chi 2$ (1, N=37) =18.49, P= 0.000; (1,N=37)=11.47, P=0.001) (Figure 5). The analysis of duration of attentive behaviours showed these results were non-significant. There was no significant difference in the duration of attentive behaviours of endhab vs. rehab of the control or test sex discrimination (Wilcoxon matched pairs test: T=0, Z=1.60, N=20, P=0.109; T=0, Z=1.34, N=20, P=0.179) or individual discrimination (Wilcoxon matched pairs test: T=0, Z=1.82, N=37, P=0.0679; T=0, Z=1.60, N=37, P=0.109; T=1, Z=1.75, N=37, P=0.080) trials. No false negative reactions were found. All 37 bats reacted to the white noise.

Bats were able to discriminate between genders (Wilcoxon matched pairs test: T=0, Z=3.30, N=20, P=0.001) and individuals (Wilcoxon matched pairs test: T=0, Z=1.60, N=37, P<0.01; T=0, Z=4.70, N=37, P<0.001). The duration of attentive behaviours significantly increased when playing calls from a different individual or gender than those used during habituation. In the sex discrimination trials, 27 (73%) bats reacted to the dishab portion of the test discrimination (Pearson Chi Squared: $\chi2$ (1, N=37) =18.49, P=0.000) and only two bats reacted to the control (Pearson Chi Squared: $\chi2$ (1, N=37) =4.17, P=0.0411). In the individual discrimination trials, 29 (78%) bats reacted to Individual 2 and 30 bats (81%) reacted to Individual 3 (81%) (Pearson Chi Squared: $\chi2$ (1, N=37) =8.00, P=.004; $\chi2$ (1, N=37) =6.55, P=.011). Only two bats reacted to the control (Pearson Chi Squared: $\chi2$ (1, N=37) =18.49, P=0.000) (Figure 6). Bats had the strongest reactions to calls from Individual 3 and the greatest number of medium reactions to calls from Individual 2 (Figure 7).

The number of calls emitted during the test portion of the sex discrimination trials was not significantly different between the control and test (Wilcoxon matched pairs test: T=3, Z=1.22, N=20, P=0.225). Only four bats emitted calls during the sex discrimination test compared to zero bats that emitted calls during the control. The number of calls emitted during the test of the individual discrimination trials (Friedman Anova: $\chi2$= 9.56, N=37, DF=2, P=0.00842) was significantly different to the control (Wilcoxon matched pairs test: T=0, Z=2.20, N=37, P=0.027; T=0, Z=2.36, N=37, P=0.018; αbonf=0.017). Seven and eight bats emitted calls to the two individual discrimination tests and only one bat emitted calls to the control.

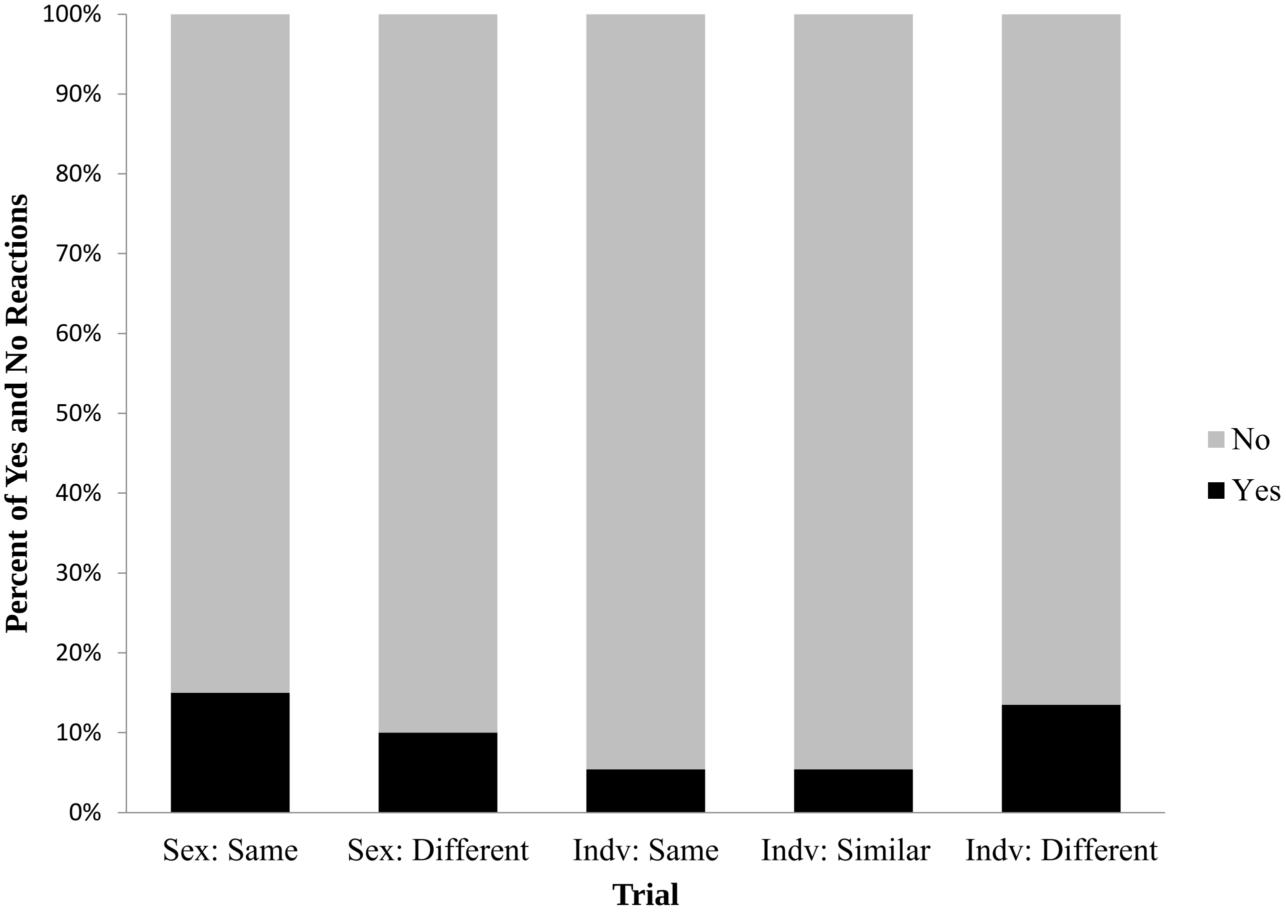

Figure 5. The number of Yes/No reactions to the rehabituation stimulus (sex discrimination trials: N=20, individual discrimination trials: N=37).

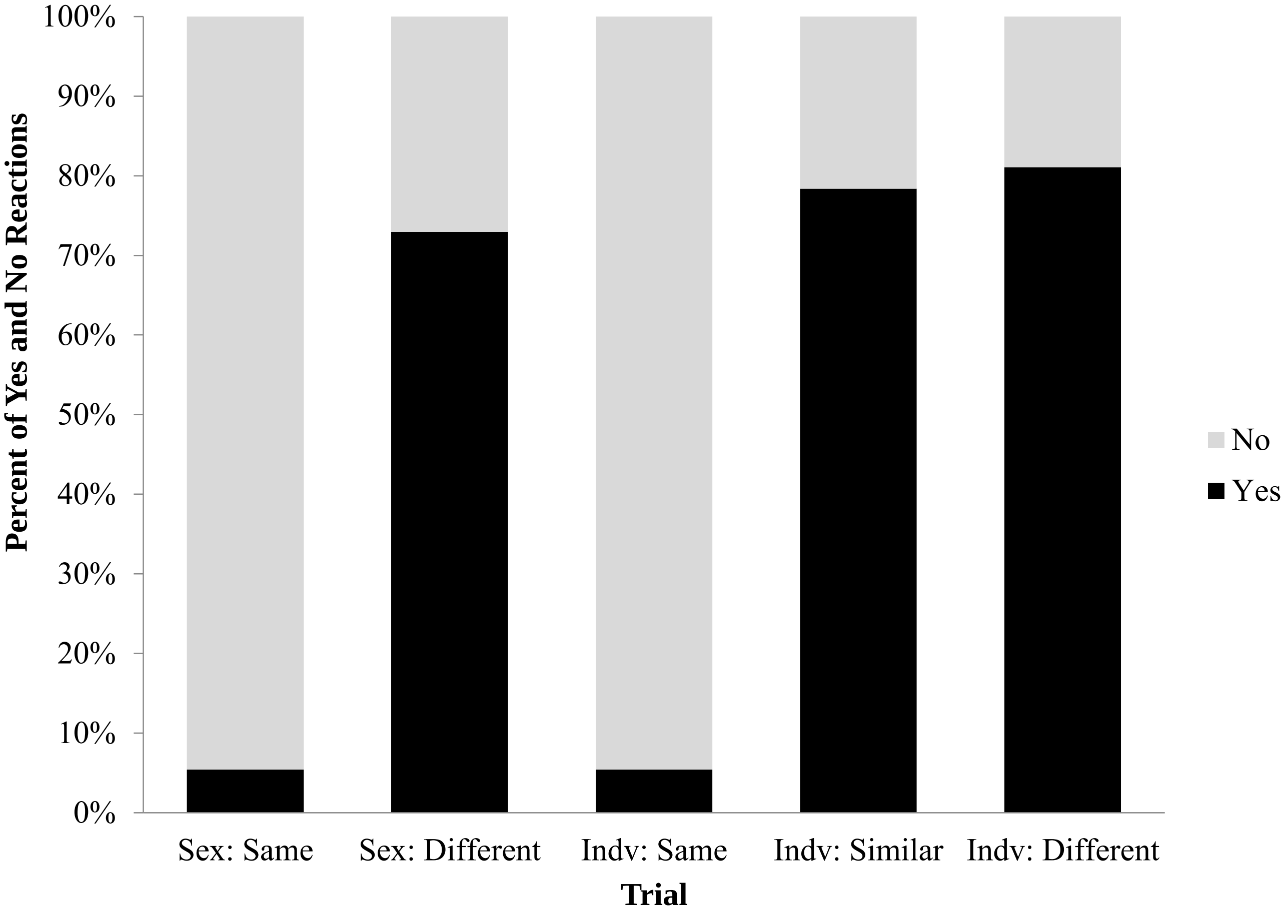

Figure 6. The number of Yes/No reactions to the test stimulus (N=37).

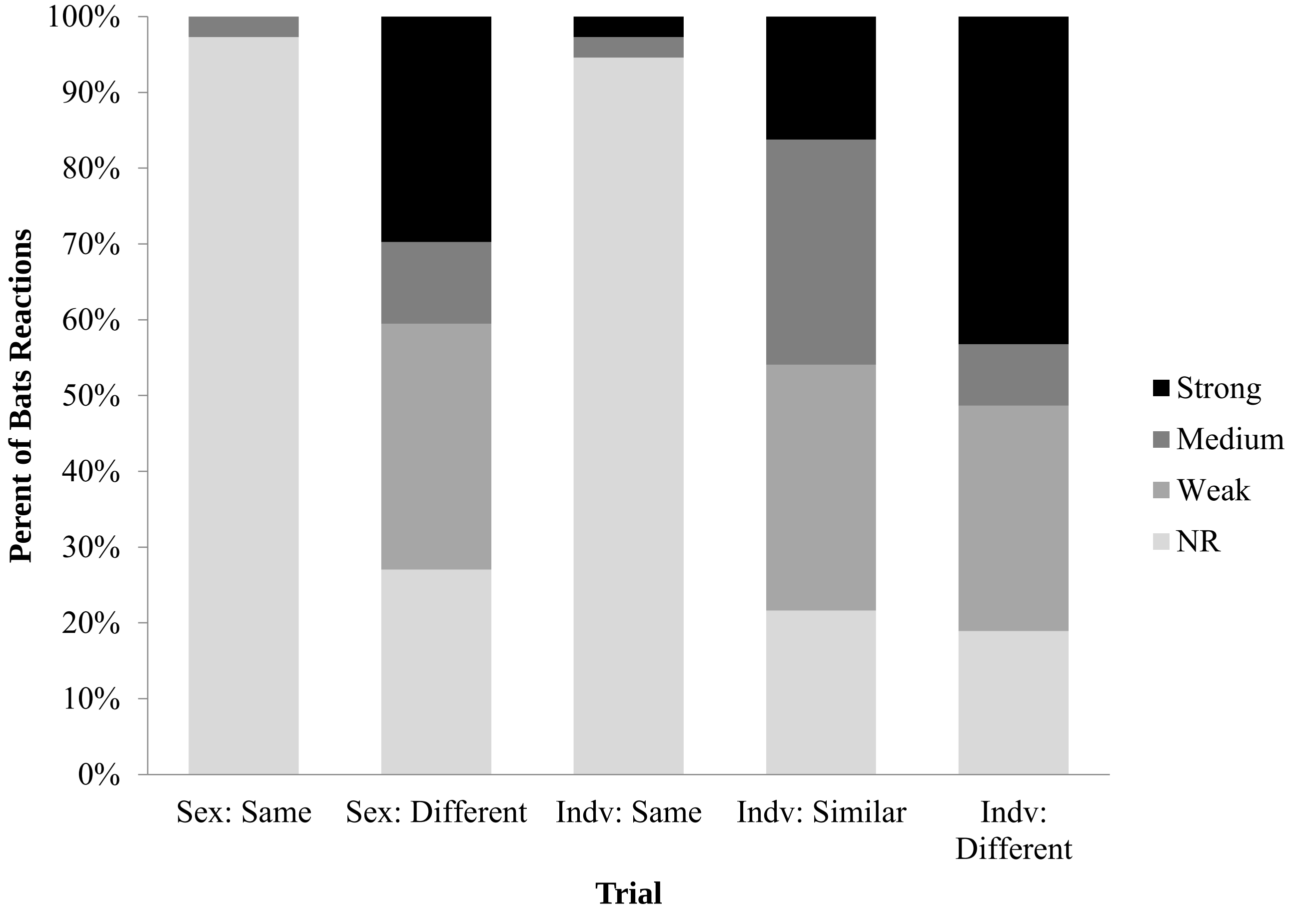

Figure 7. Percent of strong, medium, weak, and no reactions (NR) to the test portion of each of the five trials (N=37).

Acoustic Characterization of R. clivosus Echolocation Calls

Individuals

Individuals from De Hoop differed acoustically from each other in all call parameters measured (GLM: F=10.81, N=88, P=0.000). A PCA on the acoustic parameters from the calls of individuals used in the test portion of the individual discrimination trials revealed five principle components made up 84% of the variation between the three individuals calls (Table 6). A DFA on these components correctly classified calls (λ=0.60, F=42.389, P=0.000) as belonging to the correct individual 72% (Indv1: 83%, Indv2: 64%, Indv3: 69%) of the time. A canonical analysis revealed that root one accounted for 78% of the variance and is largely made up of principle component five (resting frequency). The standard deviations of resting frequency were small for the three individuals (Indv1: 0.05, Indv 2: 0.18, Indv 3: 0.27). Root two (duration and frequency parameters of terminal FM components and call duration) made up 22% of the variance (Table 7). Squared Mahalanobis Distances revealed Individual 1 (resting frequency: μ= 91.9) and 2 (μ= 91.4) were more acoustically similar (Squared Mahalanobis Distance: 3.88, F=38.265, P=0.000) than Individual 1 and 3 (μ= 90.4) (Squared Mahalanobis Distance: 6.129, F=60.469, P=0.000). Individual 2 and 3's calls were the least acoustically different (Squared Mahalanobis Distance: 2.27, F=22.4, P=0.000) (Figure 8). A DFA also compared the acoustic similarity of the calls used in the experiment to calls from individuals that listened to the experiment (listeners). Listeners were more closely clustered with Individual 2 (Squared Mahalanobis Distance: 3.96, F=25.41, P=0.000) and 1 (Squared Mahalanobis Distance: 5.18, F=32.36, $P < 0.001$) than Individual 3 (Squared Mahalanobis Distance: 11.28, F=70.96, P=0.000) (Figure 9). To determine if the acoustic parameters measured could be used to classify calls in the presence of many individuals, a DFA on PCA components was done on the acoustic parameters of calls from individuals from the broader population of bats from De Hoop (N=88) (λ=0.012, F=11.68, P<0.001).This analysis had a very low call classification success (35 %) (data not shown in tables).

Gender

A significant difference (GLM: F=4, N=88, P<0.01) was found in forearm length (GLM unequal N HSD, P<0.001), duration of the initial FM component (P=0.031), duration of the terminal FM component (P=0.009), and sweep rate of the terminal FM component (P=0.027) between males and females from De Hoop (45 males and 43 females). Females are bigger than males and have longer initial and terminal FM components with a smaller terminal sweep rate (Table 8). Males and females did not have significantly different resting frequencies (P>0.05). Acoustic parameters of calls used in the playbacks represented typical male and female echolocation calls used by bats in De Hoop. While the GLM was significant (GLM: F=3, N=88, P=0.000), a post hoc test showed acoustic parameters differed only between sex types (i.e. male playbacks differed to female playbacks; male playbacks differed to female others) (GLM unequal sample size HSD, P<0.05) but not between same sex calls (i.e. male playbacks and male others; female playbacks and female others) (GLM unequal sample size HSD, P>0.05). Therefore calls used in the playbacks were not a biased sample and represented calls taken from the broader population of De Hoop (others). A PCA on calls from 39 individuals used in the sex discrimination test playbacks revealed that five components accounted for 83% of the variance in the data (Table 6). A DFA on these components correctly classified calls (λ =0.592, F=66.460, P<0.001) as either male or female 78% (76% male and 80% female) of the time. Principle components composed of the initial and terminal FM components accounted for most of the variation (Figure 7). The distance between male and female calls was also significantly different (Squared Mahalanobis Distance: 2.75, F=42.389, P=0.000).

Locations

There was a significant effect of location (GLM: F=3.8, N=50, P=0.000) but not sex (GLM: F=1.8, N=50, P=0.081) on forearm length, resting frequency, duty cycle, duration (terminal), sweep rate (terminal), and minimum frequency, bandwidth, and sweep rate of the initial FM component. Bats from Sudwala had a lower duty cycle (P=0.017) and minimum frequency (initial) (P<0.001), but larger bandwidth (initial) (P=0.002) than those from De Hoop (Table 8). Their terminal sweep rate was significantly lower than Knysna (P=0.023). Bats from De Hoop had lower initial sweep rates than Kokstad (P=0.045) and Sudwala (P<0.001). The duration of their terminal FM component was significantly shorter than Sudwala (P=0.003) but longer then Koegelbeen (P=0.028). Bats from Koegelbeen who had the largest forearm length and lowest resting frequency were significantly bigger than those from all locations except De Hoop. Their resting frequency was significantly different to all other locations (P<0.05). A DFA on principle components derived from 500 calls from individuals belonging to these locations showed weak location-specific signatures (λ=0 .343, F=31.00, P=0.000). Four canonical roots were extracted. Root one and two accounted for 93% of the variation (Figure 7). A classification matrix correctly classified calls as belonging to the correct location 65% of the time with considerable differences in the success rate between the locations (Knysna: 23%, Kogelbeen: 80%, Kokstad: 0%, Sudwala: 92%, De Hoop: 64%).

Table 6. Contributions of acoustic parameters to Principle Components of individuals used in both sex and individual discrimination trials and for individuals from different locations. Acoustic parameters with factor loadings of >0.50 are presented. The total variance (cumulative percentage) explained by each component is represented in parenthesis. The order of parameters (1^{st}, 2^{nd}, etc.) reflects the order of importance of the parameter for each component.

Principle Components	**Acoustic Parameters**
Individual	
Component 1 (26%)	Minimum Frequency (initial), Bandwidth (initial), Duration (initial)
Component 2 (47%)	Sweep Rate (terminal), Bandwidth (terminal), Minimum Frequency (terminal), Call Duration
Component 3 (63%)	Duty Cycle, IPI
Component 4 (75%)	Duration (terminal), Minimum Frequency (terminal)
Component 5 (84%)	Resting Frequency
Individual + Listeners	
Component 1 (26%)	Minimum Frequency (initial), Bandwidth (initial), Duration (initial)
Component 2 (47%)	Sweep Rate (terminal), Bandwidth (terminal), Minimum Frequency (terminal), Call Duration
Component 3 (63%)	Duty Cycle, IPI
Component 4 (75%)	Duration (terminal), Minimum Frequency (terminal)
Component 5 (84%)	Resting Frequency
Sex	
Component 1 (24%)	Sweep Rate (terminal), Minimum Frequency (terminal), Bandwidth (terminal), Call Duration
Component 2 (47%)	Minimum Frequency (initial), Bandwidth (initial), Duration (initial), Call Duration
Component 3 (61%)	Sweep Rate (initial), Minimum Frequency (terminal), Bandwidth (terminal)
Component 4 (74%)	Duty Cycle, IPI
Component 5 (83%)	Distomax, Resting Frequency

Location

Component 1 (29%)	IPI, Call Duration, Duty Cycle, Minimum Frequency(terminal)
Component 2 (49%)	Minimum Frequency (initial), Bandwidth (initial)
Component 3 (62%)	Bandwidth (terminal), Minimum Frequency (terminal)
Component 4 (74%)	Duration (initial)
Component 5 (83%)	Resting Frequency, Distomax

Table 7. Contributions of Standardized Coefficients for canonical variables of a DFA for individuals used in both sex and individual discrimination trials, individuals who listened to the trials (listeners), and individuals from different locations. Numerical values show the importance of each predictor in predicting group assignment for each function.

	Root 1	**Root 2**	**Root 3**	**Root 4**
Individual				
Component 1	-0.121	-0.175		
Component 2	-0.602	0.545		
Component 3	0.456	-0.292		
Component 4	-0.146	-0.789		
Component 5	0.934	0.265		
Eigenvalue	1.067	0.313		
Cumulative Proportion	0.773	1.000		
Individuals + Listeners				
Component 1	-0.222	0.480	-0.135	
Component 2	-0.677	0.218	0.756	
Component 3	0.594	-0.091	0.270	
Component 4	0.249	-0.769	0.329	
Component 5	0.769	0.532	0.272	
Eigenvalue	0.906	0.429	0.016	
Cumulative Proportion	0.770	0.992	1.000	
Sex				
Component 1	-0.599			
Component 2	0.289			
Component 3	-0.733			
Component 4	-0.428			
Component 5	-0.588			
Eigenvalue	0.690			
Cumulative Proportion	1.000			

Location				
Component 1	-0.569	-0.635	-0.292	-0.350
Component 2	-0.229	-0.465	0.859	-0.036
Component 3	-0.568	0.671	0.226	0.093
Component 4	-0.489	0.413	-0.056	-0.529
Component 5	0.639	0.191	0.208	-0.670
Eigenvalue	0.651	0.613	0.090	0.003
Cumulative Proportion	0.479	0.931	0.998	1.000

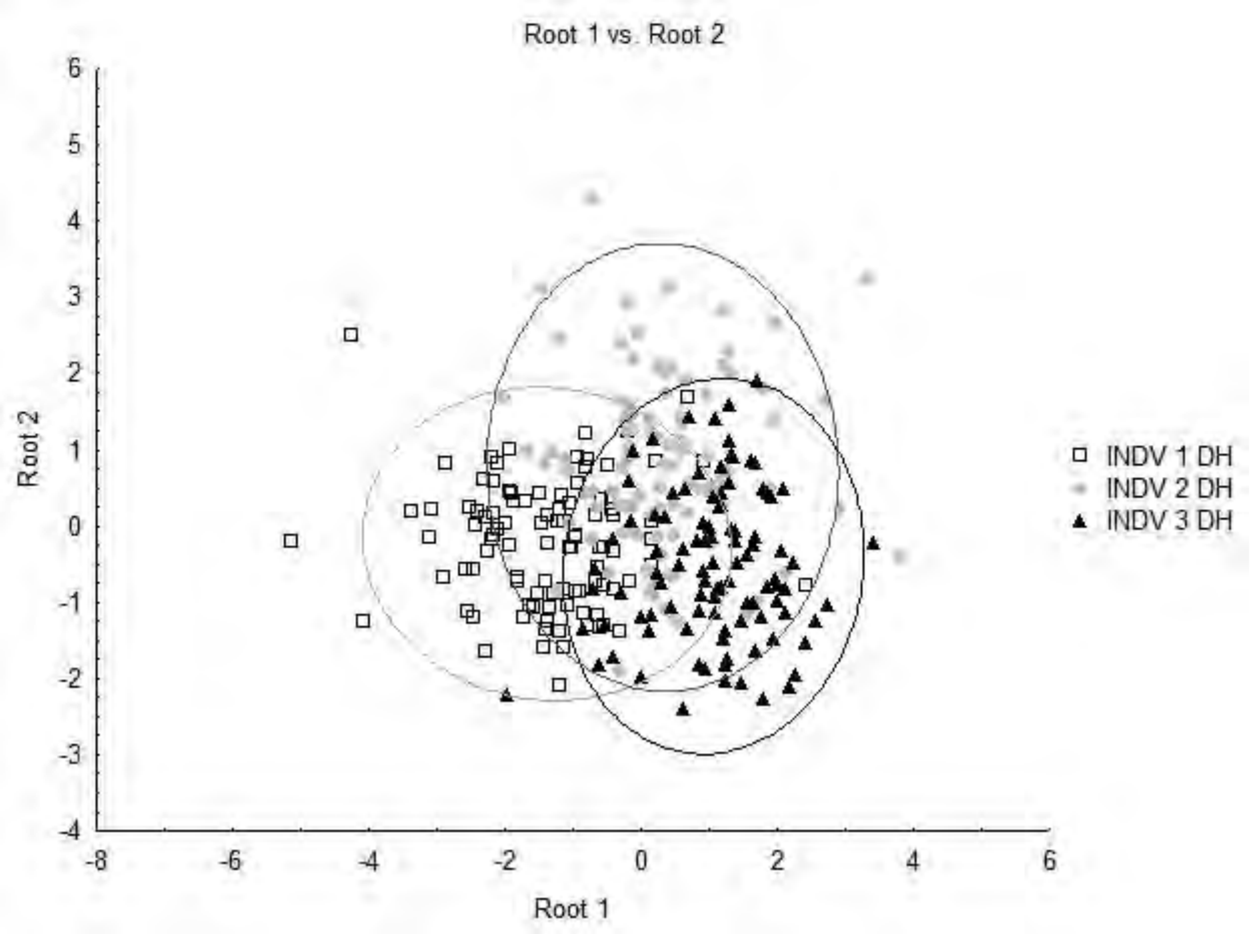

Figure 8. Canonical Discriminant Analysis on multiple acoustic parameters of echolocation calls in the individual discrimination playbacks. Ellipses fitted to each individual.

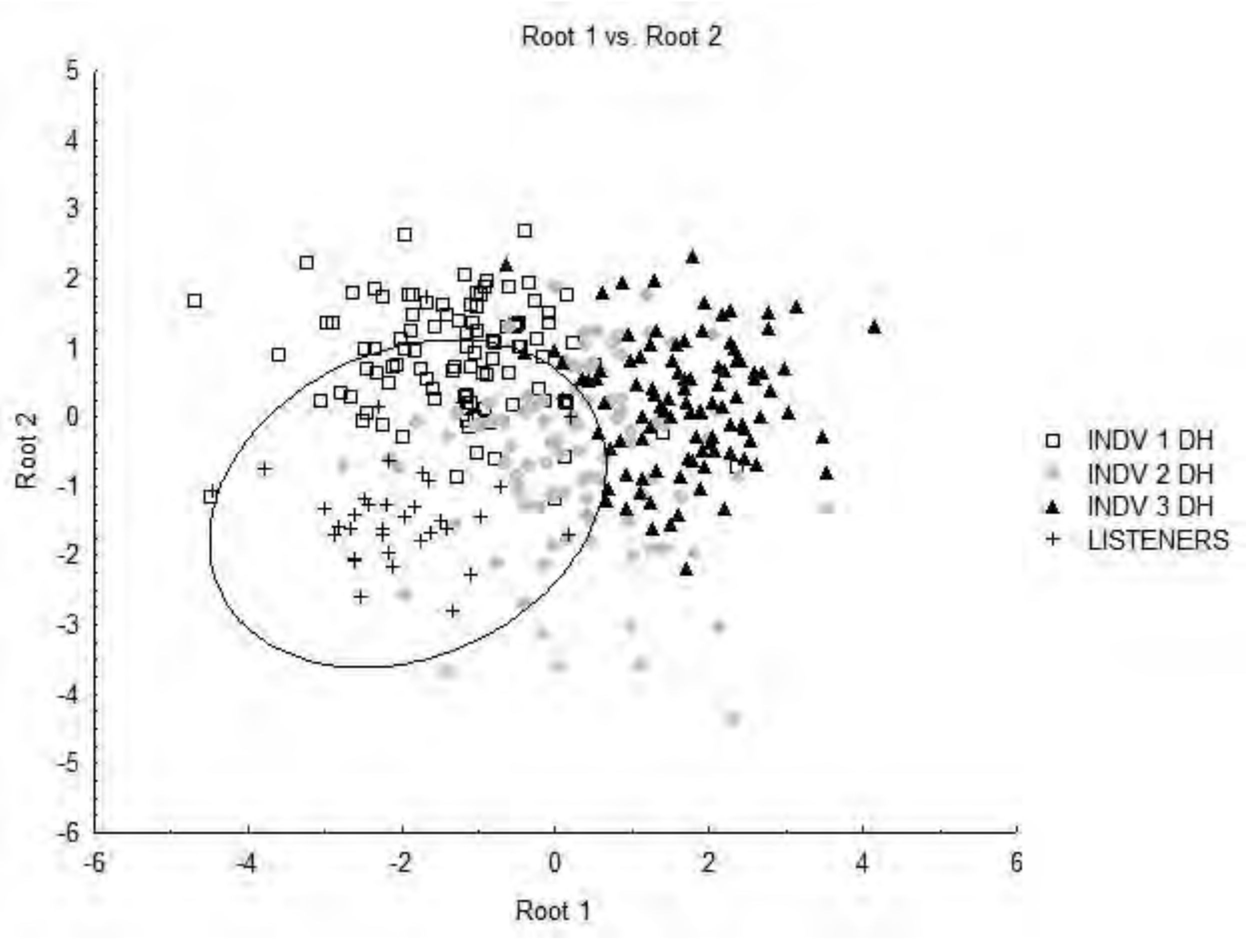

Figure 9. Canonical Discriminant Analysis on average acoustic parameters of calls from individuals who listened to experiments (ellipse) and calls used in individual discrimination playbacks.

Table 8. Acoustic measurements of echolocation calls and forearm length (mean ±SD) of *R. clivosus* for males and females from De Hoop and from different locations in South Africa. Sample sizes are given in parenthesis after sex or location as males, females. Abbreviations: FAL=forearm length, RF=resting frequency, DUR=duration, IPI=inter-pulse interval, DISTOMAX=distance from start to maximum amplitude, INT=initial FM component, TM=terminal FM component, MF=minimum frequency, BW=bandwidth, SR=sweep rate.

							FM COMPONENTS							
	FAL (mm)	**RF (kHz)**	**CALL DUR (ms)**	**IPI (ms)**	**DUTY CYCLE (%)**	**DISTO-MAX (ms)**	**MF (INT) (kHz)**	**MF (TM) (kHz)**	**BW (INT) (kHz)**	**BW (TM) (kHz)**	**DUR (INT) (ms)**	**DUR (TM) (ms)**	**SR (INT) (kHz/ms)**	**SR (TM) (kHz/ms)**
Sex														
Females (43)	55.4 ±1.5	91.7 ±0.5	36.4 ±9.3	81.4 ±65.2	48.1 ±12.1	28.6 ±8.0	80.4 ±5.2	68.6 ±3.5	11.3 ±5.3	23.0 ±3.7	2.7 ±1.7	3.9 ±3.6	5.1 ±1.8	8.0 ±3.3
Males (45)	53.9 ±1.2	91.6± 0.4	35.7 ±8.3	84.1 ±87.3	51.5 ±12.6	28.2 ±7.6	81.6 ±3.7	68.7 ±3.0	10.1 ±3.6	22.9 ±2.9	2.0 ±0.9	2.8 ±1.1	5.8 ±1.7	9.6 ±3.4
Location														
De Hoop (8,8)	55.2 ±1.7	91.6 ±0.6	33.1 ±8.4	126.7.8 ±124.4	48.5 ±13.2	25.5 ±5.6	80.2 ±3.9	68.6 ±3.6	11.4 ±3.9	22.9 ±3.6	2.7 ±0.7	3.7 ±0.9	4.4 ±1.8	6.8 ±2.7
Knysna (5,2)	52.7 ±1.1	91.9 ±0.7	31.5 ±4.4	65.8 ±36.1	46.4 ±9.3	20.9 ±6.4	76.9 ±2.6	65.9 ±2.6	15.0 ±2.7	25.9 ±2.8	2.8 ±0.6	2.9 ±0.5	5.8 ±1.3	9.4 ±2.5
Koegelbeen (1,4)	57.2 ±1.0	89.9 ±0.1	29.4 ±4.8	43.3 ±21.5	53.4 ±9.3	20.1 ±3.7	79.8 ±3.9	69.3 ±2.6	10.1 ±3.8	20.5 ±2.6	1.6 ±0.8	2.3 ±0.4	6.5 ±0.7	9.3 ±1.2
Kokstad (2,2)	53.9 ±1.0	91.5 ±0.5	33.9 ±3.9	138.4 ±107.9	46.0 ±7.4	24.2 ±5.2	77.4 ±2.4	68.5 ±3.7	14.1 ±2.7	23.0 ±4.0	2.1 ±0.5	3.2± 0.9	7.2 ±0.6	8.3 ±2.4
Sudwala (7,11)	54.3 ±1.6	91.5 ±1.0	39.3 ±5.5	93.4 ±29.6	36.7 ±7.2	26.8 ±3.7	74.8 ±3.1	70.5 ±3.7	16.7 ±2.5	21.0 ±2.6	2.8 ±1.9	4.3 ±1.3	7.7 ±1.5	5.7 ±2.1

CHAPTER 4

DISCUSSION

Perception of Acoustic Cues

The results of the individual and sex discrimination experiments show that *R. clivosus* can discriminate gender and individual identity when listening to conspecifics echolocation calls. Bats showed renewed interest after listening to calls from a different gender or individual to those they were habituated. This indicated the perception of acoustic differences in calls of different individuals and genders. In contrast when listening to calls from the same gender or same individual as in the habituation most bats stayed habituated. The majority of bats also resumed habituation when calls from the same individual or gender were played after the test (rehabituation). Therefore it is highly unlikely observed reactions to the tests of both individual and test discrimination trials were a result of spontaneous recovery of prehabituated levels of response (Rendall et al., 1996). It is important to note that in a few trials where bats showed very strong reactions to the test stimulus, they remained in this alert state even after the rehabituation stimulus was played. A similar reaction was observed in red deer where a test stimulus of a harsh roar (attention grabbing stimulus) resulted in increased levels of response to repeated playbacks of common roars from the same male that they would have typically habituated to (Reby and Charlton, 2012). In the current study the test stimulus was only 20 seconds long which may have not given some bats enough time to assimilate to the test stimulus. Bats also showed decreased levels of response the more trials they listened to. This did not have a statistical effect on the results due to the balanced experimental design. This is the first study to show high duty cycle bats are able to discriminate between echolocation calls belonging to different individuals, as opposed to groups, such as familiar or unfamiliar conspecifics (Voigt-Heucke et al., 2010), or congenerics (Schuchmann and Siemers 2010).

Only two other studies (Kazial et al., 2008; Yovel et al., 2009) have experimentally shown this ability, but in low duty cycle bats (*Myotis lucifugus* and *M. myotis*). After habituation to one bat, *M. lucifugus* responded with increased call rates to the calls from a different bat (Kazial et al., 2008). *M. myotis,* trained to discriminate calls belonging to two different individuals in an alternative food choice experiment, were able to discriminate between new individuals they were not trained to recognize (Yovel et al. 2009). Besides the current study, there is only one other study that has shown that HDC bats can identify the gender of an individual from its echolocation calls alone (Schuchmann et al., 2012). However, the acoustic parameters used by the bats to discriminate between genders in their study were not identified. Two other studies experimentally show this ability in LDC bats (Kazial and Masters, 2004; Knörnschild et al., 2012). Acoustic characterization of the echolocation calls bats listened to in the experiments in the current study revealed that bats used both individual and gender-specific signatures to discriminate between conspecifics echolocation calls.

Potential Gender- and Individual-Specific Cues in Echolocation calls.

Strong individual-specific signatures were found in the 300 calls of the three individuals used to compile the playbacks in the individual discrimination experiments. Resting frequency was identified as the strongest predictor of individual identity between these individuals. Call duration and the duration and frequency of the FM components also accounted for a small portion of the variation between individuals. This suggests that in addition to resting frequency, bats could also be using the average set of call parameters for individual recognition such as was found in the LDC bat, *Myotis myotis* (Yovel et al., 2009).

In the current study, bats had stronger reactions to calls from an individual whose resting frequency was less similar to both their own (Individual 3) and that of the individual with whose calls they were habituated (Individual 1). Weaker reactions were shown to an individual who had a similar call frequency (Individual 2) to the habituation individual. Individual 2 also had the lowest call classification rate and some of its calls were misclassified as Individuals 1's. Individual 1 had the highest classification rate. In comparison to Individual 2 and 3, Individual 1's calls had the lowest intra-individual variation in resting frequency, which could explain its higher call classification rate. The intra-individual variation in resting frequency was also small for the broader sample of individuals from De Hoop. This supports Jacobs et al. (2007) findings of relatively stable individual resting frequencies for both *R. clivosus* and *R. capensis.* Differences between individuals in the frequency of the constant portion of the calls is congruent with findings from other studies on HDC bats such as *P. parnellii* (Suga et al., 1987), *R. euryale, R. hipposideros, and R. blasii* (Siemers et al., 2005). These differences in resting frequency may help bats discriminate between calls of different individuals when small numbers of bats are around. This could be beneficial when avoiding acoustic interference (jamming) between different individual's calls. Some studies show bats even change their acoustic parameters in the presence of others to avoiding jamming (i.e. Habersetzer, 1981; Orbrist, 1995; Ulanovsky et al., 2004). The ability to discriminate between calls of different individuals (discrimination) could be used for individual recognition.

Individual recognition may help mediate social interactions in a gregarious bat species such as *R. clivosus*. Most bats have long life spans (reviewed in Wilkinson and South, 2002) and some bats such as *M. lucifugus* return to their maternity roost each year (Barclay, 1982). In such colonies, bats often form social groups with certain individuals. In the current study, certain captive *R. clivosus* individuals were observed huddled together with the same individuals more often than not. If the same is true for wild populations of *R. clivosus*, it is possible individual acoustic signatures help members of a group locate each other during roosting or foraging. Recognition of individuals may play a large role in mother offspring interactions and therefore offspring survival. The ability to recognize each other's echolocation calls would be beneficial during foraging when mothers are teaching their young how to forage and locate roosts (Gaudet and Fenton, 1984; Brigham and Brigham, 1989). However, when large numbers of bats are present, using acoustic parameters such as resting frequency to avoid jamming or individual recognition could prove problematic.

In Guano Cave at De Hoop, bats roost in large numbers (Laycock 1983; McDonald et al., 1990). A DFA on the acoustic parameters of individuals from the broader population of De Hoop (88 individuals) had a low classification success. This is not surprising given that the range of frequencies used by *R. clivosus* at De Hoop is small (three kHz), resulting in a large overlap of frequencies between individuals. Call frequency would therefore not be a reliable acoustic parameters for individual recognition or to avoid acoustic interference in the presence of many bats. In small numbers, a combination of acoustic parameters can lead to high call classification. In the experiment, resting frequency was the acoustic parameter that accounted for the majority of the variation between the three individual's calls and therefore was most likely used by the bats to discriminate between the individuals in the experiment. Low intra-individual variation in resting frequency was also found. However, the consistency of an individual's frequency over time was not determined and therefore its use for individual recognition not assessed.

Resting frequency has been shown to vary in different bat species over time and in different contexts. Lui et al. (2013) observed differences in resting frequency from day to day (sometimes more than 0.9 kHz) in horseshoe bats echolocation calls but not in the short constant frequency communication calls they produced. Perhaps these communication calls, incorporating the stable CF component of normal echolocation calls, play a more important role in individual recognition. Changes in resting frequency and changes in the fine tuning of the acoustic fovea in the cochlea region in the mustached bat were associated with body temperature changes (Huffman and Henson, 1993) In *R. ferrumequinum* changes in frequency occurred over a lifetime and mother - offspring call frequencies were positively correlated (Jones and Ransome, 1993). Differences in their resting frequencies could therefore be the result of learned, genetic, or physiological differences. More studies are needed to investigate how call frequency changes within an individual, in the presence of different numbers of individuals, and under different behavioural contexts (i.e. when foraging, in the roost, and in mother - infant interactions). In addition, echolocation call frequency has also shown to be important for inter-specific discrimination (Schuchmann and Siemers, 2010; Bastian and Jacobs, 2014 (under review)). When there were large differences in species frequencies, *R. capensis* used frequency to discriminate between conspecifics and heterospecifics calls. However bats were still able to discriminate between calls with small frequency differences suggesting other cues could be used for discrimination (Bastian and Jacobs, 2014 (under review)). The current study shows that in addition to frequency, other acoustic parameters are important in intra-specific communication for individual and gender discrimination. Studies manipulating different components of the calls while keeping other components the same might provide insight into which acoustic parameters bats are using most for discrimination.

Most studies investigating gender differences in echolocation calls of HDC bats focus on resting frequency alone and do not incorporate additional acoustic components of calls. Gender differences have been documented in the resting frequency of some species *(H. ruber, R. blasii, R. clivosus, R. hipposideros, R. monoceros, P. parnellii)* (Suga et al., 1987; Jones, 1995; Guillén et al., 2000; Siemers et al., 2005; Yoshino et al., 2008; Chen et al., 2009;

Knörnschild et al., 2012; Odendaal et al., 2014) but the overlap was still large. *R. rouxi* is the only species documented to have no overlap in frequency despite the lack of size difference between genders (Neuweiler et al., 1987). Larger HDC bats are usually known to call at lower frequencies than smaller bats (Heller and von Helversen, 1989; Barclay and Brigham, 1991; Jones, 1996). Species where no difference in resting frequency were found include *R. mehelyi, R. euryale,* and *R. hipposideros* (Russo et al., 2001; Siemers et al., 2005). *R. mehelyi* and *R. euryale* were still able to discriminate between calls of conspecifics suggesting gender information is carried in other call components (Schuchmann et al., 2012). Like these species, *R. clivosus* calls did not have sexually dimorphic resting frequencies (despite males being smaller than females) but bats were still able to discriminate between calls from different genders of the same species. Analyses of call parameters revealed that gender is encoded in the FM components of the calls. Females have longer initial and terminal FM components with a lower terminal sweep rate (Table 8). Their initial sweep rates were also lower, although not statistically significant. A DFA revealed strong statistical evidence for gender-specific signatures. Duration, rate, and frequency parameters of the FM components, as well as call duration made up the principle components with the highest standardized coefficients. This included initial and terminal sweep rates, minimum frequency and bandwidth.

The discovery of gender-specific signatures in echolocation calls and of the ability of conspecifics to perceive these signatures has important research impacts. Some behavioural evidence in bats suggests that these signatures provide fitness benefits in the form of mate recognition and choice. Male *S. bilineata* used gender-specific signatures in echolocation calls (females had shorter calls than males) to greet incoming females into the roost and ward off rival males (Knörnschild et al., 2012). *R. mehelyi* also used gender-specific signatures in mate choice. Resting frequency was found to be an honest signal of body size in males. Females selected males with higher frequency calls (Puechmaille et al., 2014). *R. mehelyi* could discriminate between different gendered conspecifics echolocation calls but no difference in call frequency between genders was found. This suggests that *R. mehelyi* could be using other call components (such as the FM components) for discrimination. *R. clivosus* also showed no sexual dimorphism in resting frequency, instead differences in the FM components of calls were found. In addition, individuals were shown to have unique resting frequencies. *R. clivosus* could be using these individual and gender-specific signatures for mate choice. Gender-specific signatures could help when forming same sex roosting (maternity colonies/bachelor colonies) or foraging associations. Little is known about *R. clivosus* roosting and foraging behaviour in the wild. To understand the fitness benefits of this adaptation, more behavioural studies are needed to test for potential benefits.

In addition to individual-specific and gender-specific signatures, weak location signatures were found in the echolocation calls of *R.clivosus* from five different regions in South Africa. Calls from Knysna, Kokstad, and De Hoop had low classification success even though geographical variation does exist between these locations. Bats from these locations represent each of the five genetically supported geographical groups of *R. clivosus* that exist in Southern Africa (Group 1: De Hoop; 2: Knysna; 3: Koegelbeen; 4: Kokstad and Sudwala; 5: Sudwala) (Stoffberg et al., 2012). Koegelbeen and De Hoop differed the most from the other locations. Bats from De Hoop differed from bats in the other locations in their FM components (duration (terminal), sweep rate (terminal), and minimum frequency, bandwidth, and sweep rate of the initial FM component). While no significant interaction was found between gender and population, within the population of De Hoop, gender significantly affected the FM components of echolocation calls. This could be connected to the large difference in the FM component observed between De Hoop and the other populations. Bats from Sudwala differed from those from Knysna in their terminal sweep rate. When analysing acoustic and morphometric data from Sudwala caution should be taken because bats from

Sudwala compromised both genetic groups four and five. In addition group four could possibly consist of two separate genetic lineages, a northern and southern. Koegelbeen was the only location where bats were found to be significantly larger with smaller call frequencies (-1.5 kHz). Stoffberg et al. (2012) found that bats from group three (Koegelbeen and Postmasburg) had the lowest resting frequency. Unlike the other areas Koegelbeen is located in a very arid region on the central plateau of South Africa. According to James Rule, animals in arid environments tend to be larger (James 1970). Longer vocal cords with larger cavities are likely to produce lower frequency sounds (Guillen et al., 2000). With the exception of Koegelbeen, very little geographic variation was found in resting frequency in *R. clivosus.* This finding is contrary to what has been found in other HDC bats species (Yoshino et al., 2008; Armstrong and Coles, 2007; Rossiter et al., 2007; Chen et al., 2009; Flanders et al., 2009) including sympatric *R. capensis* (Odendaal et al., 2014) where large variation in call frequency was found.

Ethogram of R. clivosus

A variety of solitary and interactive behaviours was observed in captive *R. clivosus* bats. Bats engaged in roosting, locomotive, ingestive, eliminative, grooming, resting, and social behaviours. The majority of behaviours produced appear to be similar to those observed in other species but the context in which some behaviours where exhibited differed. Behaviours like wing stretching, which often accompanied behaviours such after waking up, grooming, or settling down to rest in *R. clivosus,* have shown to accompany completely different behaviours in other species. In *Cynopterus sphinx,* wing displays were observed during feeding as an aggressive behaviour to ward off intruders (Singaravelan and Marimuthu, 2008). In *N. albiventris* and *N. Lepornius,* wing stretches are used in olfactory communication. These species possess glands in the subaxillary region underneath their wings that produce an oily, strong scented secretion (Brooke and Decker, 1996; Voigt-

Heucke et al., 2010). Other behaviours such as leaning towards or away from an individual have been described as approach or avoidance behaviours in other bat species. Briefly leaning towards new arrivals in roosts was observed in *Pteropus poliocephalus* (Nelson, 1965) and *Pteropus giganteus* (Neuweiler, 1969) and was speculated to occur to obtain olfactory cues for identification. This behaviour was observed in the current study in *R. clivosus* when one bat would land next to or approach another bat. Both bats would often lean towards and sometimes nuzzle (potentially sniff) each other. In *M. lyra,* bats were shown to have a similar greeting ceremony where they would approach each other while hanging upside down and then maintain body contact for several minutes. During these interactions, a series of soft social calls were emitted. These greeting behaviours are thought to support cohesion between group members (Schmidt, 2013). Agonistic behaviours were frequently observed in *R.clivosus* in the current study, especially in the male only groups. These behaviours included biting, wing swatting, and sometimes aerial chases and collisions. Social calls accompanied many occurrences of behaviours in *R. clivosus*. Social calls could be used to express intention and/or emotional state such as in *M. lyra* where systematic changes in call structure were observed for interactions of different intensities (Bastian and Schmidt, 2008). The compiled ethogram in the current study will provide contexts for these recorded social calls to be analysed in the future.

Behavioural Responses to Playback Experiments

Since little was known of the behaviours of *R. clivosus,* this ethogram was essential to better understand the bats reactions to the playbacks. When reacting to the test portion of the trials most bats displayed orientation and listening behaviours (look up, scan, and ear twitching). Similar reactions to playbacks were observed in *R. mehelyi* and R. *euryale* (Schuchmann and Siemers, 2010; Schuchmann et al., 2012). In addition to these behaviours, some species have reacted to echolocation playbacks by increasing their echolocation call rates (Kazial et al., 2008; Schuchmann and Siermers, 2010; Schuchmann et al., 2012). In the current study only a few individuals reacted to playbacks with echolocation calls. This was found to be significant for individual discrimination but not sex discrimination trials. Voigt-Heucke et al. (2010) observed no difference in the number of echolocation calls between playbacks but individuals did respond with honk calls (type of social call). It was suggested that these calls communicated individuality. In the current study, no social calls were recorded during the experiments even though social calls were observed during social interactions in the captive colonies. Other behaviours, such as wing stretching, body shuffling, or grooming were considered to be displacement or transition behaviours in *R.clivosus* and several other animal species (reviewed in Broom, 1981). In the video recordings from both the playback experiments and captive colonies, these behaviours usually occurred after slowly waking up, when shifting positions, or before settling down to rest. They were therefore considered active and not attentive behaviours. When displayed in the experiment, they were not considered as a response to the stimuli. As previously mentioned, some behaviours such as wing stretching in other bat species may signal a reaction (Voigt-Heucke et al., 2010). Ethograms are therefore essential to help provide a better understanding of the different behaviours produced by a species before using them to interpret experimental results.

Vocalizations in Bats

R. clivosus produces two types of auditory vocalizations, social calls and echolocation. Social calls evolved in bats to specifically facilitate communication and therefore mediate social interactions. Social calls have been described in detail for several bat species (i.e. Aldrige et al., 1990; Andrews and Andrews, 2003; Bohn et al., 2008; Bastian and Schmidt, 2008) and were observed in video and audio recordings in the current study in captive *R. clivosus.* In contrast, echolocation was thought to evolve for foraging and navigation but has now been shown to be adapted for communication. Studies have shown that bats can discriminate between echolocation calls of different individuals (HDC: the current study; LDC: Kazial et al., 2008; Yovel et al., 2009), sexes (HDC: Schuchmann et al., 2010, the current study; LDC: Kazial and Masters, 2004; Knörnschild et al., 2012), groups (FM and CF signals: Voigt-Heuke et al., 2010), and species (HDC: Schuchmann et al., 2010). It is likely that bats use these two systems to solve different communicative tasks. Social calls are usually used in direct social interactions. Since social calls are not limited by any other function (i.e. foraging and navigation), social call design can be as complex as needed to carry the necessary information. Social calls can be low in frequency to maximize the distance over which the signal can be heard while reducing the effects of attenuation (Lawrence and Simmons, 1982). In contrast, echolocation is limited in the context of communication by its function of prey detection and obstacle avoidance. For example, the range over which echolocation calls are transmitted is usually short as a result of atmospheric attenuation. In addition, the frequencies of many species echolocation calls have to be high for increased resolution of small objects. Using echolocation to communicate does also have fitness benefits. Most echolocation calls are outside the hearing range (ultrasonic range) of other animals so bats can communicate without the danger of being heard by predators such as birds, baboons, and even humans. In addition, bats can both navigate and communicate at the same time using one signal thereby reducing energy costs. Echolocation is therefore unique because two functions coexist in one system.

Conclusion

In this study the perception of vocalizations in the context of intraspecific communication and mate recognition were documented in the HDC bat species, *R. clivosus.* Echolocation calls carried intra-specific signatures, individual and gender identity, and bats were able to perceive these signatures in playback experiments. Resting frequency accounted for the greatest variation between individuals' echolocation calls and therefore was most likely used by the listening bats to discriminate between individuals in the experiment. Other acoustic parameters accounted for some of the variation between individuals, suggesting bats could be using a combination of call parameters to increase discrimination ability. In addition, attributes of an individual, such as its gender, are conveyed mainly in the FM components of their calls. The ability to perceive this information suggests bats are likely using echolocation calls in intraspecific communication to mediate social interactions and for mate recognition. These results also highlight some of the limitations of using echolocation as communication channel. In large colonies, echolocation may not be the best strategy to use for individual discrimination and recognition because of the large overlap between call parameters. To better understand the sensory constraints of using echolocation for auditory communication in comparison to social calls, further research should document the different contexts in which each are produced (i.e. studies matching behaviours to social and echolocation calls). Revealing the evolutionary drivers of these two sensory systems will give us an understanding of how they have diverged in their functional properties. It is hoped that these findings will encourage future research into auditory perception in bats and into the evolution and functionality of signal design.

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